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Current Perspectives on Viral Disease Outbreaks

Epidemiology, Detection and Control

Edited by David Claborn

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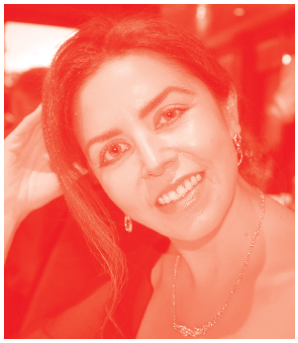

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Meet the editor

David Claborn is a Professor of Public Health at Missouri State University (MSU), USA, where he teaches courses in environmental health, infectious disease, and international health. He obtained a DrPH from the Uniformed Services University of the Health Sciences, Bethesda, Maryland, with his dissertation on the re-emergence of malaria in South Korea. He has been the director of the Master of Public Health program at MSU since 2013. Prior to his academic career, Dr. Claborn served in the US Navy for 20 years, retiring at the rank of Commander in 2008. His work as a medical entomologist has taken him to several international settings including Japan, Australia, South Korea, Italy, and, during Operation Desert Storm, Saudi Arabia.

Contents

Preface

The COVID-19 pandemic served as a reminder to the world that infectious diseases, especially those caused by viruses, are still an important part of medicine and public health. As of 6 December 2021, the World Health Organization's Global Dashboard reported 265,194,191 confirmed cases with 5,254,115 deaths (https://covid19.who.int/). The outbreak has undermined public trust in health authorities who have had to change their recommendations as new data and analyses became available. Initial expert advice that masks were not helpful has morphed into mask mandates. Politicians have criticized the public health actions of their opponents, then quickly adopted the same actions upon a change in political power. New technologies that utilize advanced biomedical procedures have provided effective vaccines and treatments in remarkably short periods of time but have also generated distrust leading to vaccine hesitancy on the part of some groups.

In the meantime, viruses and their associated diseases continue to emerge. Many people would be surprised at the number of diseases that have been linked to obscure viruses, many of which have emerged only since the development of technologies that have enabled researchers to identify the agents. **Table 1** is a partial list of viruses and viral diseases that have been listed by researchers as emerging. One surprising issue associated with this list is the number of diseases transmitted by vectors, especially ticks. This conclusion suggests that much greater emphasis should be placed on environmental sampling to determine just how many viruses present a hazard to the global population. At the same time, there is much work to be done in the detection, prevention, monitoring, and treatment of viral diseases in the human population. New technologies have allowed successful treatment of viral disease with an efficacy undreamed of just a few years ago. Better surveillance systems have allowed researchers to detect emerging diseases sooner and monitor the developing outbreak on a daily basis. Nevertheless, health authorities still find themselves in the position of using public health measures developed more than 100 years ago to deal with the Spanish influenza pandemic. Those measures include social distancing, masking, contact-tracing, and controversial issues such as closures of businesses, restaurants, and public gathering sites. Many of the measures being used are not well documented. For instance, in the United States, it is common for authorities to recommend maintaining a distance of six feet between individuals as a social distancing measure. Where did this recommendation originate? Is it actually effective or should the distance be greater? The reality is that much is still unknown about the coronavirus, other viruses, and how to interrupt their transmission. Much is unknown about effective prevention and treatment measures as well. This book provides an opportunity to address some of the gaps in information regarding viral disease outbreaks. The content is not limited to human viral disease, though most of the chapters address some element of human disease. Subjects range from detection to monitoring to treatment. Most of the chapters report some new information, though some of the chapters take a historical perspective. The science of monitoring viral diseases is one characterized by rapid change, thus this book provides a snapshot of developing information at the time of publication. It documents the rapid changes

Chittoor (vector) [2]	Mayaro fever (vector) [3]
Cytomegalovirus (direct contact) [2]	MERS (air-borne; zoonotic) [1]
Bagaza virus (vector) [2]	Nipah (human to human; foodborne) [2]
Bhanja (vector) [2]	Non-polio flaccid paralysis (fecal-oral) [2]
Buffalopox (direct contact) [2]	Noroviruses (fecal-oral) [2]
Cat Que virus (vector) [2]	Oropouche fever (vector) [3]
Chandipura virus (vector) [2]	Oya virus (vector) [2]
Chickenpox (air-borne) [2]	Parainfluenza (air-borne) [2]
Chikungunya (vector) [2]	Quaranfil viurs (vector) [2]
Chobar Gorge virus (vector) [2]	Respiratory syncytial virus (air-borne) [2]
Coxsackie virus (fecal-oral) [2]	Rhinovirus (airborne) [2]
Crimean-Congo Hemorrhagic fever (vector; human-human) [2]	Rift Valley fever (zoonotic and vector) [3]
	Rotavirus (fecal-oral) [2]
Dengue (vector) [2]	Rubella (air-borne) [2]
Equine Encephalitis virus (vector) [2]	Sapoviruses (fecal-oral) [2]
Gamjam virus (vector) [2]	SARS-CoV-2 (airborne) [1]
Hand, foot and mouth disease (fecal-oral) [2]	Severe fever thrombocytopenia syndrome virus (vector) [2]
Hepatitis B (blood) [2]	St. Louis encephalitis (vector) [1]
Human parvovirus (parenteral) [2]	Thottapalayam (rodent) [2]
Influenza (air-borne) [2]	Umbre virus (vector) [2]
Japanese encephalitis (vector) [2]	Venezuelan equine encephalitis (vector) [3]
Kaisodi virus (vector) [2]	Yellow fever (vector) [2]
Kammavanpettai (vector) [2]	Zika (vector, sexual) [2]

Table 1.
Partial list of viral diseases and viruses that have been listed as either emerging and having potential for emergence (mode of transmission) (reference).

in the sciences of epidemiology, pharmacology, vaccinology, and others. The book could be twenty times as large as it is, so new books and updates on the subject will probably be necessary.

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Section 1

Viral Diseases and Treatment

Chapter 1

Convalescent Plasma Immunotherapy - A Possible Mitigation Strategy for SARS-CoV-2 Pandemic

Rajendran Manikandan, Mithilesh Singh, Vishal Chander, Gaurav Kumar Sharma, Suresh Bindu and Murali Dinesh

Abstract

Recently, a newly emerged severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused a pandemic coronavirus disease (COVID-19). More than 10 million confirmed cases and 503,867 associated deaths of SARS-CoV-2 have been reported worldwide to WHO in the end of July 2020. According to WHO guidelines, there is no effective therapy available for treating devastating SARS-CoV-2. Consequently, lack of evidence for appropriate treatment and vaccines has led to the re-emergence of convalescent plasma (CP) immunotherapy. Herein, we discuss the historical perspectives of CP against SARS-CoV, MERS-CoV, H1N1 pandemic and mainly the clinical outcomes of COVID-19 patients with respect to neutralizing antibodies (nAbs). A brief possible clinical protocol for CP transfusion with its adverse effects and limitation were also highlighted. It is concluded that, CP transfusion with high neutralizing antibody titer administered in early course of disease significantly improved clinical outcomes in COVID-19 patients by reducing morbidity and mortality. Thus, CP immunotherapy is considered as noteworthy candidate to be further re-evaluated as a most suitable therapeutic option against SARS-CoV-2 pandemic.

Keywords: SARS-CoV-2, COVID-19, Convalescent Plasma, Neutralizing antibodies, Pandemic

1. Introduction

In December 2019, a newly emerged respiratory diseases associated with pneumonia from Wuhan, China caused by a initially named 2019 novel coronavirus (2019-nCoV) [1] and recently referred as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) producing coronavirus disease (COVID-19). Thus 2019-nCoV, caused a pandemic across the globe, the World Health Organization (WHO) declared it as a 'Public Health Emergency of International Concern (PHEIC)' on January 30th, 2020. Considering its devasting threats worldwide, WHO declared SARS-CoV-2 as a pandemic spread on March, 2020 [2]. As per WHO guidelines, no effective therapy is available to treat devastating SARS-CoV-2, the treatment is mainly symptomatic and supportive. Thus, the current lack of evidence for effective treatment and vaccines, led to the re-emergence of classical and historical intervention called convalescent

plasma (CP) immunotherapy. Since the early 20th century, a passive CP immunization strategy has been used in prevention and control of infectious disease [3]. CP seems to be the first line of defense against the SARS-CoV-2, since it has been more successfully adapted in the treatment of SARS, MERS (Middle East Respiratory Syndrome) and H1N1 pandemic with significant efficacy and safety. In order to combat current devastating pandemic, this review mainly highlights on the clinical immunotherapy studies conducted in the fields of neutralizing antibodies (nAbs) predominately present in convalescent plasma (CP) for the treatment and management of COVID-19.

2. Virology of SARS-CoV-2

Coronaviruses (CoVs) belongs to the family *Coronaviridae* and the order *Nidovirales* possessing a single-stranded, positive-sense RNA genome ranging from 26 to 32 kb in length [4]. SARS-CoV and SARS-CoV-2 comes under the genus *Betacoronavirus* of the subfamily *Orthocoronavirinae* and is further belongs to subgenus *Sarbecovirus* [5].

Based on the phylogenetic tree analysis, nucleotides of SARS-CoV-2 shares 96% sequence identity with the SARS-like (SL) virus named BatCoV-RaTG13/Bat-SL-RaTG13 [6] and 88–88.2% identity [6–8] with bat-derived SARS-like coronaviruses named Bat-SL-CoV-ZC45 and Bat-SL-CoV-ZXC21, suggesting that bats are the most likely reservoir. Interestingly, the phylogenetic study showed that MERS-CoV and SARS-CoV were about 50% and 79% similar to SARS-CoV-2, respectively [6, 7]. The Spike (S) protein of SARS-CoV-2 was found to be approximately 75% homologous to the SARS-CoV spike [6].

The organization of the CoV genome contains a 5′-leader-UTR-replicase-S(Spike)- E (Envelope)- M(Membrane)- N(Nucleocapsid)-3′ UTR-poly(A) tail with accessory genes interspersed in the structural genes on the 3′ end of the genome [9]. In 5′ terminal, 2/3rd of viral RNA primarily locates the first ORF (ORF1a/b) which translates 2 polyproteins namely, pp1a and pp1ab, and encodes 16 non-structural proteins (NSP), while the remaining ORFs encode accessory and structural proteins [10]. The structural proteins of SARS-CoV-2 contains spike (S), an envelope (E), membrane (M) and nucleocapsid (N) protein that are located at the one third 3′ terminal of the genome [11]. The critical step for SARS-CoV-2 entry is binding of trimeric spike (S) glycoprotein to host cell angiotensin-converting enzyme 2 (ACE2) receptors similar to that of SARS-CoV entry [6]. Coronavirus nAbs targets primarily surface spike glycoprotein that mediate viral entry into host cells. The symptoms of COVID-19 most commonly are fever, cough, myalgia or fatigue, dyspnoea, pneumonia and lesser common were sputum production, headache and diarrhea. The complication of SARS-CoV-2 are mostly acute respiratory distress syndrome (ARDS), followed by shock, myocardial dysfunction and acute kidney injury [12, 13]. A detailed overview of COVID-19 disease progression was discussed, with particular reference to immunopathology and immunobiology [14].

SARS and MERS are caused by zoonotic coronaviruses that belong to the genus *Betacoronavirus* within *Coronaviridae.* SARS-CoV emerged in southern China in 2003 and caused 8098 cases worldwide, including 774 related deaths with an estimated 14–15% case-fatality rate [15]. In 2012, the first case of MERS occurred in Saudi Arabia. A sum of 2,494 cases and 858 related deaths with an overall case-fatality rate up to 34.4% [16]. More than 10 million confirmed cases of SARS-CoV-2 were reported globally by WHO, including 503,867 associated deaths as on the date of compilation 30th June-2020 (**Figures 1** and **2**). A total of 566,840 confirmed cases of SARS-CoV-2 were reported in India, including 16,893 associated deaths as on the date of compilation 30th June-2020 (https://covid19.who.int/) (**Figures 3** and **4**).

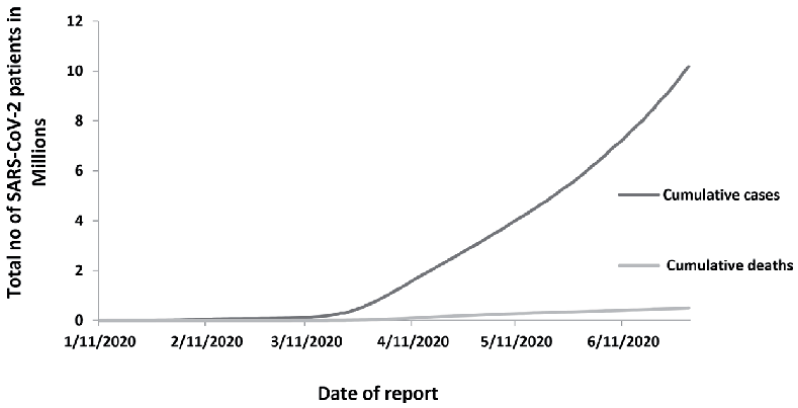

Figure 1.
Total number of cumulative SARS-CoV-2 cases and deaths across the globe during current pandemic. Source adapted from (https://covid19.who.int/). [accessed 2020-06-30].

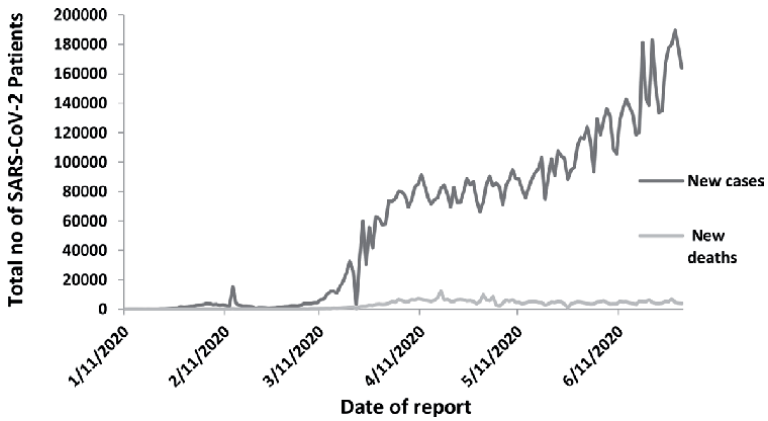

Figure 2.
Total number of daily new confirmed SARS-CoV-2 cases and deaths across the globe during current pandemic. Source adapted from (https://covid19.who.int/). [accessed 2020-06-30].

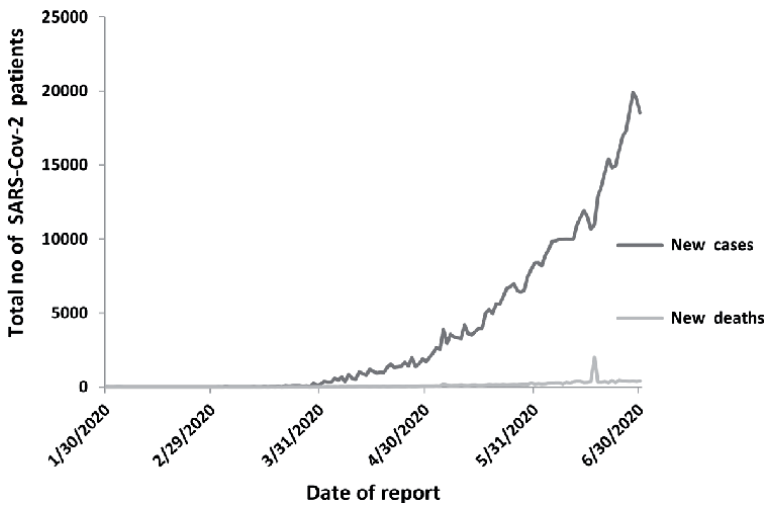

Figure 3.
Total number of daily new confirmed SARS-CoV-2 cases and deaths in India during current pandemic. Source adapted from (https://covid19.who.int/). [accessed 2020-06-30].

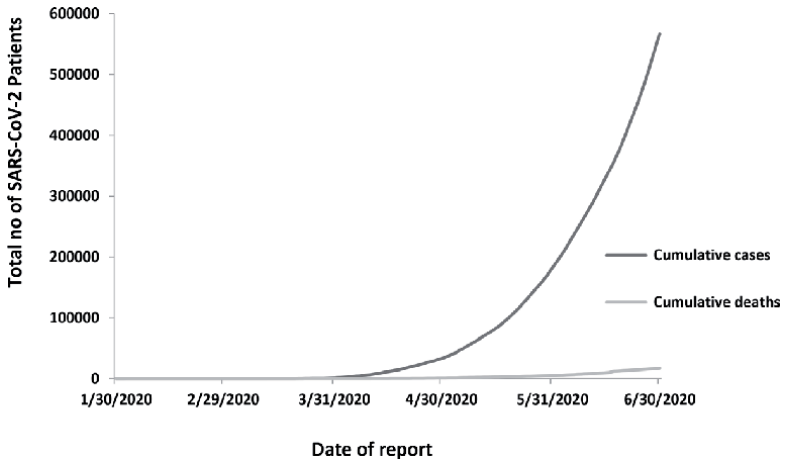

Figure 4.
Total number of SARS-CoV-2 cumulative cases and deaths in India during current pandemic. Source adapted from (https://covid19.who.int/). [accessed 2020-06-30].

3. Historical perspectives of CP

Serum therapy was the only effective treatment option for infectious diseases prior to the discovery of antibiotics. In the year 1890, serum therapy was founded by Emil von Behring. The use of serum blood therapy has been proposed for treating diphtheria. At the same time Behring and Kitasato developed an effective therapeutic serum against tetanus.

Documented reports of the Spanish flu H1N1 pandemic (1918–1920) stated that transfusion of influenza-convalescent human blood products (whole blood, plasma, or serum) reduced morbidity and mortality. Hence, convalescent plasma could be an effective therapy during viral outbreaks and pandemics [17]. Treatment of severe H1N1 infection with convalescent plasma revealed that 1 dose of convalescent plasma with >1:160 neutralizing antibody was successful in reducing mortality and the viral load of the respiratory tract decreased by $>3\log_{10}$ copies/mL within 48 h of plasma therapy [18]. H5N1-infected BALB/c mice treated with H5N1-specific $F(ab')_2$ fragments derived from horses provided proof that passive immunotherapy is effective for immunologically competent and incompetent hosts [19].

A Lyophile serum was used effectively to prevent and/or treat many diseases such as measles, chickenpox, mumps, German measles, erysipelas, hemolytic streptococcal infections and scarlet fever [20]. Argentine hemorrhagic fever (AFH) caused by Junin virus is one of the few infectious viral disease in which CP administration is a specific treatment of choice that can neutralize viremia after immune plasma transfusion [21]. CP has been used with varied outcomes in combating Lassa fever and Ebola virus [22, 23]. For most viral diseases, the first week of infection peaks with viremia. The patient typically produces a primary immune response by day 10–14 followed by virus clearance.

In the SARS-CoV-1 outbreak, CP therapy was used and found to be more effective in patients who received transfusions within 14 days of the onset of symptoms [24]. The analysis further revealed that virus was cleared 1 day after CP transfusion, preceded by fever subsidence and pulmonary infiltrate resolution. CP transfusion may be considered as alternative treatment in cases where SARS-CoV patients experience severe deterioration and fail to respond to the available treatment such as ribavirin or methylprednisone [25].

Meta-analysis by Nottingham University-World Health Organization Collaborating Center showed that CP containing MERS-CoV-specific antibodies from recovered patients could be the most promising near-term therapy for infected individuals. In MERS-CoV patients, treatment with CP was restricted by a limited pool of donors with adequate antibody levels [26] and the usage of CP in three critically ill respiratory failure MERS-CoV patients in South Korea resulted in significant clinical improvements [27].

4. Convalescent plasma (CP)

The first and foremost important criteria for the convalescent plasma immunotherapy is that recovered SARS-CoV-2 donor should have high neutralizing antibody (nAb) titer and specific to the virus [28]. It is proposed that SARS-CoV-2 specific nAbs may reduce the viral load, severity of diseases and further also increase the nAb titer level of COVID-19 patients. CP may be a potentially effective strategy and first line of defense against the current wreaked havoc SARS-CoV-2 viral pandemic.

4.1 Clinical outcomes of CP

A Single dose of CP with a high concentration of nAbs rapidly reduced the viral load and eventually increased clinical outcomes of ten severe adult SARS-CoV-2 patient from china by successful use of the CP immunotherapy. Single dose of 200 mL of inactivated CP with high neutralization titer of >1:640 was transfused according to the WHO blood transfusion protocol. Interestingly this recent study showed significant increase in nAb titer level, disappearance of SARS-CoV-2 RNA at an undetectable level, reduction of pulmonary lesions and amelioration of laboratory parameters in all patients after CP transfusion. The clinical symptoms were improved within 3 days and the viraemia was also disappeared on 7 days of CP immunotherapy (**Figure 5**) [28].

The recent study from China over 175 patients recovered from SARS-CoV-2 clearly showed the production of SARS-CoV-2-specific neutralizing antibodies after 10–15 days of infection. Interestingly this study had several findings that convalescent plasma recovered from SARS-CoV-2 patients specifically inhibited SARS-CoV-2 alone, but not the SARS-CoV infection, the peak of neutralizing antibodies were detected in all patients after 10 days of infection and variations in nAb titers

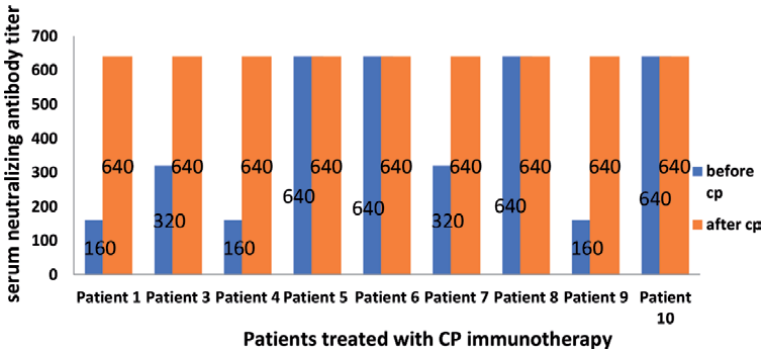

Figure 5.
Comparison of serum neutralizing antibody titer before and after CP immunotherapy. [CP, Convalescent plasma]. Source adapted from Duan et al. [28].

were observed as 30% of infected patients corresponds to low nAb titer (< 500), 17% to medium-low titers (500–999), 39% to medium-high titers (1000–2500) and 14% to high nAb titers (>2500). Moreover, the elderly and middle-aged patients had substantially higher plasma nAb titers and spike-binding antibodies than young aged SARS-CoV-2 infected patients [29]. Further this study showed the noteworthy findings of CP immunotherapy can be specifically used for the prevention and treatment of SARS-CoV-2.

Five critical patients from china with SARS-CoV-2 and acute respiratory distress syndrome (ARDS) had eventually recovered after CP immunotherapy. Clinically significant improvement of four out of five were reported promptly. This recent study showed increase in nAb titer level (**Figure 6**) and titers of IgG and IgM in the sera got increased in a time-dependent manner after convalescent plasma transfusion. It was observed that the Ct value of all patients seems to be negative after 12 days of transfer of CP immunotherapy [30].

Ye et al. reported an increase in anti-SARS-CoV-2 nAb titers and further increase in IgG and IgM antibodies. CP transfusion resulted in the resolution of ground glass opacities and consolidation in patients lung. The clinically significant outcome of this study is that all the six SARS-CoV-2 infected patients were found negative in throat swab analysis by real-time PCR assay due to reduction in the viral load after CP immunotherapy [31].

SARS-CoV-2 infected patients over the age of sixty years received CP treatment had a significantly prolonged recovery time estimated by viral clearance (10 to 29 days) compared to younger patients, who recovered from the disease in less than a week after receiving CP immunotherapy [32].

The level of specific neutralizing antibody against SARS-CoV peaked at 4 months and gradually disappearing to an undetectable level of 25.8% (IgG) and 16.1% (nAbs) in serum after 3 years of recovery [33]. Similarly, MERS-CoV infected patients showed a low prevalence of 2.7% IgG seroreactivity and the antibodies titer dropped rapidly within 3 months of recovery [26].

Even though the level of specific nAb titers in sera were decreasing gradually, it is also possible to isolate potent neutralizing human monoclonal antibodies (mAbs) from memory B cell repertoire of convalescent patients against SARS-CoV [34] and SARS-CoV-2 [35]. The detailed possible FDA approved protocol for convalescent plasma immunotherapy transfusion was discussed in this recent studies for SARS-CoV-2 [36] and Ebola virus [37]. The possible protocol of CP immunotherapy transfusion for COVID-19 patients is depicted in **Figure 7**.

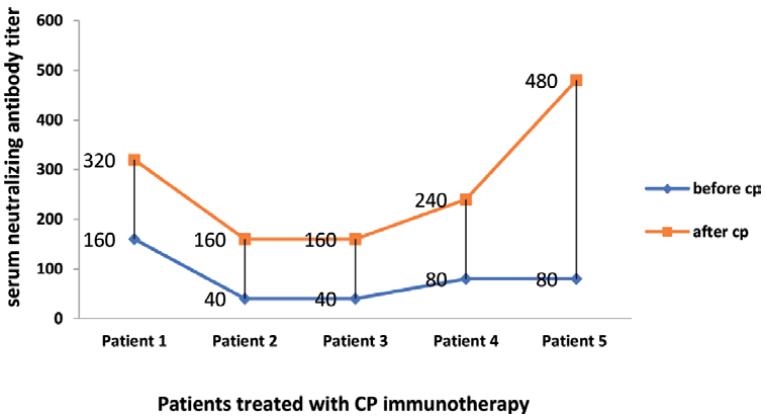

Figure 6.
Comparison of serum neutralizing antibody titer before and after CP immunotherapy. [CP, Convalescent plasma]. Source adapted from Shen et al. [30].

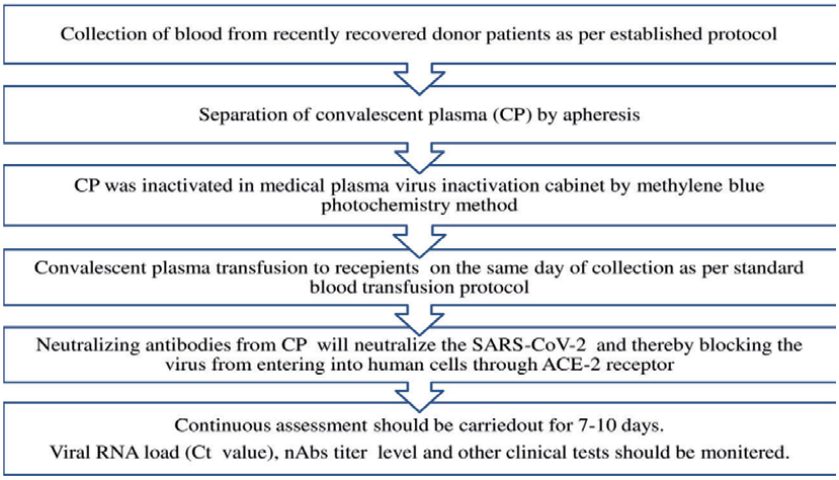

Figure 7.
Schematic representation of CP immunotherapy for SARS-CoV-2 patients highlighting the major steps.

4.2 Immunomodulatory effects

In a mouse model study, SARS-CoV pathogenesis is directly regulated by complement and its absence showed significantly reduced respiratory disease, decreased neutrophilia in their lungs, reduced systemic inflammation and viral load remains unchanged in a complement-deficient mice. Since SARS-CoV pathogenesis was mainly immune-driven, inhibiting the complement signaling pathway after SARS-CoV infection is also an effective immune therapeutic strategy [38]. By scavenging complement fragments of C3a and C5a, intravenous immunoglobulin prevents immune damage and restricts the development of immune complexes [39]. Similarly, passive antibody transfer may limit the cellular damage induced by the activation of complement cascade in an excessive inflammatory area.

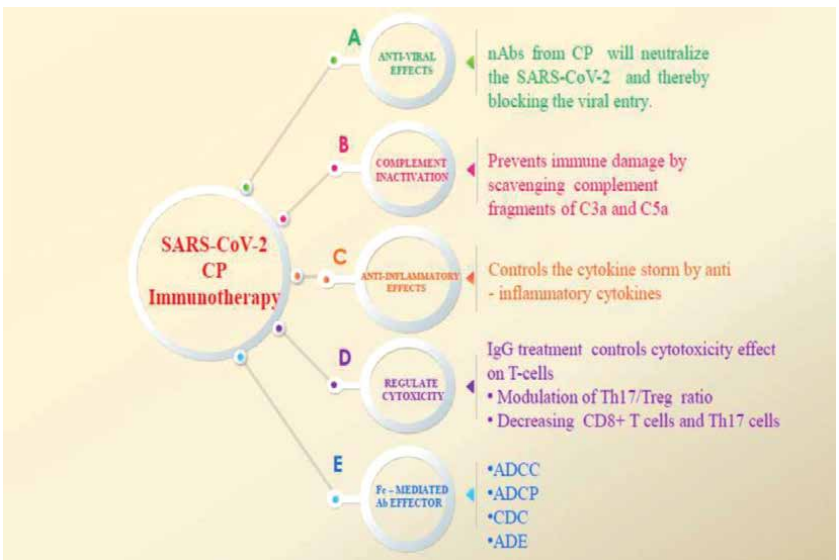

Figure 8.
A brief overview of possible mechanisms of action of CP immunotherapy. [nAb, neutralizing antibody titer; ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cell mediated phagocytosis; CDC, complement-dependent cytotoxicity; ADE, antibody-dependent enhancement.

IgG therapy controls the cytotoxic effect of T-cells by modulating the balance of Th17/Treg and decreasing CD8+ T cells and Th17 cells [40]. The possible mechanisms of action of CP immunotherapy are summarized in **Figure 8**.

Feline coronaviruses, HIV and dengue viruses use an antibody-dependent enhancement (ADE) phenomenon to take advantage of pre-existing poorly nAbs to effectively infect host target cells in order to combat anti-viral humoral immune response [41].

In vitro assays with human promonocyte cell lines HL-CZ demonstrated that ADE was primarily mediated by highly diluted antibodies against spike proteins, significantly increasing the rate of apoptosis of SARS-CoV infected cells [42].

5. Adverse effects

Previous studies did not find any adverse effects associated with CP immunotherapy during historical influenza A (H1N1), SARS-CoV, or MERS-CoV epidemics [18, 26, 43]. In case of Ebola, it was associated with mild adverse effects such as fever, nausea, skin erythema, and no other significant adverse events were found [44]. A self-limited facial erythema occurred in 2 out of 10 SARS-CoV-2 infected patients with no major adverse events found during the transfusion study [28]. The therapy was well tolerated in most of the patients while some reported only mild adverse effects. Several studies of SARS-CoV-2 have shown that CP immunotherapy is safe and not associated with any significant adverse effects.

6. Limitations

The risk of Hepatitis B virus, Hepatitis C virus and HIV disease transmission through the donated plasma should be thoroughly investigated before CP transfusion. The nucleic acid test for this viruses is strictly mandatory to ensure the safety of SARS-CoV-2 infected CP recipients [45].

Vaccine development should consider ADE phenomenon in COVID-19 patients as ADE may promote intensity of infection and administration of CP in those coronaviruses endemic areas should be carried out with caution since ADE appears to be harmful to actively infected patients [46].

All the recovered patients received not only the CP transfusion but also other standard care like antiviral treatment. As a consequence, these antiviral agents may also lead to the subsequent recovery of patients, or may synergize with the therapeutic effect of CP, which cannot be ruled out.

Taken together, these studies suggested that convalescent plasma from recently recovered patients with high neutralizing antibody titers against SARS-CoV-2 would be more effective for CP immunotherapy. A warranted random clinical trial in larger groups is required for dose optimization and to overcome possible adverse side effects of CP immunotherapy.

Furthermore, the kinetics of nAbs titers against SARS-CoV-2 need to be critically evaluated because of neutralizing antibodies represented short term humoral immune response.

7. Conclusion

The clinical trials of Convalescent plasma therapy conducted/initiated against SARS-CoV-2 pandemic across the world are getting increased day-by-day due to the

significant outcomes of infected patients with a high positive recovery rate when compared to all other modes of treatment against SARS-CoV-2 pandemic crisis. In SARS-CoV-2, many reports have shown that administration of CP immunotherapy is safe with significant potential efficacy and it was not associated with any major adverse events. Even in the autoimmune conditions also CP is considered to be safe. Thus, CP immunotherapy is considered as classical and historically noteworthy candidate to be further re-evaluated as a most suitable therapeutic option against SARS-CoV-2 pandemic.

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Conflict of interest

"The authors declare no conflict of interest."

Appendices and nomenclature

WHO	World Health Organization
PHEIC	Public Health Emergency of International Concern
CP	Convalescent Plasma
nAbs	Neutralizing Antibodies
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cell mediated phagocytosis
CDC	Complement-dependent cytotoxicity
ADE	Antibody-dependent enhancement

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Chapter 2

Recombinant Human Interferon-Gamma: Prospects for the Treatment of Chronic Epstein-Barr Viral Infection

Irina A. Rakityanskaya and Tatiana S. Ryabova

Abstract

Infection of Epstein-Barr virus (EBV) is about 90% among people over the age of 40. The EBV causes a chronic infection that is characterized by chronic or recurrent symptoms and persists for a long time. Recombinant interferon-gamma (IFN-γ) has high clinical and antiviral efficacy in the treatment of herpesvirus infections. 110 patients with chronic EBV infection were examined. The patients were divided into three groups for different treatment regimens: Group 1—IFN-γ therapy (15 injections of Ingaron i/m, 500,000 IU every other day); Group 2—valaciclovir (Valtrex 500 mg × 2 times/day, orally for 2 months); Group 3—valganciclovir (Valcyte 450 mg × 2 times/day, orally for 2 months) and IFN-γ (10–20 injections of Ingaron i/m, 500,000 IU every other day). The best results were obtained in group 3–73.07% negative PCR. In this group, the combination of valganciclovir + IFN-γ was different. We showed that the efficacy of therapy in patients with chronic EBV is determined by the duration of INF-γ administration. We also determined spontaneous and induced production of IFN-α and -γ cytokines in serum and in lymphocyte culture. We demonstrated that in patients with an initially low level of induced IFN-γ, the production of this cytokine significantly increased in three months after the end of therapy.

Keywords: Epstein-Barr virus, immunity, recombinant human interferon-γ, treatment, herpesvirus

1. Introduction

The development of immunodeficiency leads to the spread of persistent and/or chronic herpesvirus infection, in which the pathogen is not eliminated from the host's body for months, years, or even throughout life. Each herpesvirus in the host organism has its own target tissue, where the virus persists with the ability to enter and exit the tissue using a developed strategic mechanism, which consists of the minimum expression of viral genes in a small number of infected cells or their elimination at the protein level. This allows the virus to evade the immune response and remain in very small quantities (1 infected cell per 5 ml of blood) with minimal impact on the patient's body, remaining in it for the rest of its life. In this case, the immune response is unable to eliminate the infectious pathogen from the body.

1.1 Epstein-Barr virus

Epstein-Barr virus (EBV) or infectious mononucleosis virus, together with other herpesvirus infections, is a prototype of persistent viral infections characterized by latency. In the mid-1980s, the problem of chronic EBV infection or "chronic mononucleosis" attracted the attention of researchers. It has been shown that EBV causes the development of chronic or recurrent infectious mononucleosis-like symptoms in immunocompetent individuals, which persist for a long time and are characterized by constant fatigue, headaches, myalgias, lymphadenopathy, subfebrile fever (37.1–37.3°C), hepatosplenomegaly. Additionally, gastrointestinal diseases may develop hematological, neurological, and skin lesions [1].

EBV infection is accompanied by high production of IgG antibodies to viral capsid (VCA) and early antigens (EA) when compared with the control group, as well as very low production or absence of antibodies to Epstein-Barr virus nuclear antigen (EBNA) [2]. That is why it was suggested that the cause of the syndrome is a chronic EBV infection [3]. However, some patients may not have abnormally high antibody titers associated with EBV [4]. According to data published by Kanegane et al., the severity of the disease directly correlates with the level of EBV DNA copies number in serum or plasma [5]. In later studies, it was shown that an increased level of EBV DNA in the blood is a more specific criterion for chronic EBV than the levels of antibodies to EBV [6]. In 1983, Hellman et al. first proposed an abbreviation for this syndrome "Chronic Active EBV infection" (CAEBV) [7]. As a result of the analysis of the literature, including work on chronic EBV as well as work on herpesvirus infections, immunodeficiencies, and three types were identified based on clinical, pathological, and virological data characteristic of this syndrome [8, 9]:

1. Chronic fatigue syndrome of unknown etiology (CFS), which is characterized by profound, debilitating fatigue and a combination of symptoms leading to a significant decline in occupational, personal, social, and educational status;

2. CAEBV;

3. Severe CAEBV (SCAEBV).

EBV is known to spread through contact with saliva and penetrate the epithelium that lines the nasopharynx. Waldeyer's ring, which includes the adenoids, tonsils, and the lymphoid system surrounding the nasopharyngeal region, forms a continuous structure called lymphoepithelium [10]. The virus infects epithelial cells, replicates, then is released, followed by infection of resting naive B cells in nearby areas by activating latent proteins (six EBV nuclear antigens (EBNAs: EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP), two latent membrane proteins (LMPs: LMP1, LMP2) and viral anti-apoptotic protein BHRF1) encoded by the growth program. As a result, the cell becomes a proliferating lymphoblast (lymphoblastic burst). This program leads to the expansion of EBV-infected B cells in the lymphoid tissue of the oropharynx and the appearance of infected B cells in the blood. In some infected B cells, EBV inhibits its growth transformation program, allowing the cells to enter the memory B cell pool, while the virus persists as a truly latent infection devoid of viral gene expression. Therefore, the target of the incoming virus is a resting naive B cell, which becomes infected as it passes through the epithelium. Naive B cells are continuously recirculated throughout the body, extravasating from the peripheral circulation to secondary lymphoid tissue through specialized structures called high endothelial vesicles (HEVs) located in the lymphoepithelium. Naive B cells migrate to the mantle zone of the follicles below

the epithelium and remain there for several days, and then return to the circulation. Infection of new naive B cells occurs in the intraepithelial layer, and not in the mantle zone [11], that is, when an infected B cell enters the follicle, it is already a blast and cannot migrate to the mantle zone. EBV infects cells through the interaction of viral glycoproteins gp350/220 with CD21 and gp42/gH/gL with class II HLA in a B cell. Thus, memory B cells are a place of long-term viral persistence, where the virus can remain throughout the patient's life, because immunological memory is formed, and the virus ceases to be pathogenic for the host since the genes that induce cell proliferation and contribute to the development of neoplastic disease are disabled. However, EBV can infect T and NK (natural killer) cells of the tonsils [12] and peripheral blood [13]. Expression of CD21 on T cells [14] and NK cells can be induced by trogocytosis and the formation of an immunological synapse that occurs when EBV contacts B cells, which leads to a possible EBV infection [15].

It has been shown that the level of infected B cells in the population ranges from 5 to 3000 in every 107 memory B cells, both in peripheral blood (on average 110/107) and in Waldeyer's ring (average value 175/107), then there is a virus evenly distributed throughout the ring. Thus, the level of infected cells is similar between peripheral blood and Waldeyer's ring, but 20 times lower than in other lymphoid tissue (spleen and mesenteric lymph node) [16]. The total body load in humans amounts to 104–107 (on average 0.5/106) infected memory B-cells, representing a small, stable, and most critically "safe" pool of infected cells, which guarantees long-term persistence. Only about 1% of these cells are found in the peripheral blood. One latently infected memory B cell in the amygdala can differentiate into a plasma cell and secrete a virus that infects epithelial cells. The virus constantly seeps into the oral cavity, where it mixes with saliva for about 2 minutes before swallowing. Thus, the oral cavity is a reservoir for EBV flow and not a static reservoir. About 250 cells begin to replicate in the Waldeyer ring at any given time. The oral cavity and peripheral blood are important anatomical sites for the localization and persistence of EBV infection. These two compartments are connected by oropharyngeal lymphoid tissue such as the lingual, palatine, and pharyngeal tonsils. EBV-infected B cells can re-enter the tonsils, where memory lymphocytes express characteristic sets of adhesion receptors, through which they are able to return to target organs, where they first encountered antigens. Thus, EBV-infected cells can release viral particles through lytic replication, reinfect cells in lymphoepithelial tissue, and subsequently release viruses into the oral cavity [17].

The release of viruses into plasma from different anatomical sites indicates that different viral strains can persist in different tissue compartments. Therefore, EBV can be detected in the tissues of various anatomical structures of the human body.

Despite the fact that EBV is an oncogenic virus, the vast majority of EBV-infected people do not suffer from any long-term consequences. This is due to the antiviral immune response that develops during primary infection with EBV, and further supports subsequent lifelong control to ensure the mutual coexistence of the virus and its host. Early control of EBV infection is associated with the expansion of innate immune cells (primarily NK cells) and CD8+ and CD4+ T cells, specific for a wide range of EBV proteins expressed during the lytic and latent stages of viral infection. Patients with persistent EBV infection develop a specific CD8+ T-cell response to antigens of the lytic and latent cycles, the former being more frequent. An individual lytic epitope-specific response can account for up to 2% of the total population of CD8+ T cells. The response to immediate-early antigens dominates the response to early antigens, and the response to late expressed antigens rarely develops [18]. CD8+ T cells play a major role in the formation of responses to proteins EBNA3A, 3B, and 3C. A less specific immune response develops against EBNA1, EBNA2, and LMP2. Individuals expressing HLA-B*3801 have been shown

to have strong responses to the EBNA2 epitope, and carriers of HLA-A*0203 have strong responses to the epitope from EBNA-LP. In persistently infected individuals the EBV-specific T cell pool contains resting antigen-expressed T cells that are not active and do not proliferate. Lymphoid markers CCR7 and CD62L, specific for the latent antigen, are expressed on T cells. The phenotype, functional profile, and clonotypic composition of TCRs specific for CD8+ T cells remain stable for many years [19]. The EBV-specific CD4+ T cell response in healthy carriers is much weaker and may be 10 times less pronounced than the CD8+ T cell response to the same antigen. EBV-specific CD4+ memory T cells share the same phenotype regardless of whether they are specific for latent or lytic antigens. CD4+ T cells do not express perforin and granzyme, and upon ex vivo stimulation, the cytokine polyfunctionality of cells increases, and TNF-alpha production predominates [20]. NK-T cells are a conserved population of congenital T cells expressing the semi-invariant Va24-Ja18/Vβ11 T cell receptor. Only one study evaluated the frequency of NK T cells in the blood during EBV infection, and it was shown that the number of NK T cells was increased in the first month of infection. A change in cellular phenotype and function was noted with an increase in the content of CD56 (bright) with a high ability to destroy EBV-infected cells. NK-T cells play an important role in the control of primary EBV infection by eliminating infected B cells and increasing the antigen-specific response of T cells through the release of immunomodulatory cytokines [21]. It has been shown that patients with primary immunodeficiency are predisposed to the development of EBV-associated disease. The presence of NK T cells reduces the EBV transformation of B cells in vitro. With EBV infection of blood lymphocytes, the previous depletion of NK T cells leads to both an increase in the number of B cells infected with EBV and an increase in the total viral load in culture. It has been suggested that NK T cells play a role in the early immune recognition of newly EBV-infected B cells [22].

1.2 Interferon-γ

Interferons (IFNs) are important biological regulatory proteins called cytokines and mediators of cellular homeostatic reactions that are produced in response to viral infection inhibiting the replication of a wide range of DNA and RNA viruses, thereby creating negative feedback. Inhibition of the viral replication cycle is carried out with the help of the synthesis of viral polypeptides [23]. When IFNs are administered in vivo, the level of viremia decreases, that is, interferons can be used as antiviral drugs, and the antiviral effect is mediated both by the immune system itself and by intracellular antiviral mechanisms. All types of IFNs inhibit more than one step in the viral life cycle: viral entry and decay, viral mRNA transcription, viral protein synthesis, viral genome replication, and progeny assembly and isolation of virions. According to the amino acid sequence, IFNs are divided into three types: I, II, and III.

IFN-γ is the only representative of type II IFN. It is structurally unrelated to type I IFNs, binds to another receptor and is encoded by a separate chromosomal locus. Type II IFN or immune IFN—IFN-γ is a highly pleiotropic cytokine, secreted not in response to viral infection, but indirectly by mitogen-activated T cells and NK cells, which are the primary producers of IFN-γ during the innate and adaptive phases of the immune response to viral infection. Other cells such as B cells, NK T cells, and professional antigen-presenting cells (APCs) have now been shown to secrete IFN-γ. The production of IFN-γ by monocytes/macrophages and dendritic cells acting locally is important in cell activation [24].

IFN-γ plays an important role in the activation of macrophages for the production of tumor necrosis factor-α (TNF-α), increases macrophage phagocytosis and microbicidal activity, the formation of active nitrogen and oxygen intermediates,

including superoxide radicals - nitric oxide and hydrogen peroxide, which are powerful cytotoxic effectors, stimulates the Th1-T cell response and has a strong inflammatory activity. IFN-γ is the main product of Th1 cells and further shifts the immune response towards the Th1 phenotype. IFN-γ achieves this by stimulating characteristic Th1-effector mechanisms: innate cell-mediated immunity (through the activation of NK cell effector functions), specific cytotoxic immunity (through the interaction of T cells with APC), and macrophage activation. IFN-γ increases the content of lymphocytes and leads to their long-term persistence in the tissue, induces activation of the complement cascade and acute phase response, plays a role in the switch of IgG class production, and has a direct antiviral effect [24]. Normally, in the early stages of the host's immune response, IFN-γ production by NK cells, CD4+ T (Th1) cells, and CD8+ T cells is aimed at improving antigen recognition in APCs such as macrophages and dendritic cells. IFN-γ is one of the key cytokines that differentiate naive CD4 cells into Th1 effector T cells, which produce the main mediators of cellular immunity against viral and intracellular bacterial infections [25]. Together, IFN-γ and IL-12 generate a very strong Th1 response. Th1 cell-mediated immunity and Th2 cell-mediated humoral immunity are modulated by IFN-γ, which affects the differentiation of naive T cells into Th1 or Th2 cells.

When activated, almost all CD8+ T cells, NK cells, and Th1 lymphocytes produce IFN-γ, which stimulates cytokine activity and increases the expansion of low avid NK cells. Of all the interferons/cytokines of the Th1 response, IFN-γ is most strongly correlated with the Th1 response [26]. The effects induced by IFN-γ lead to increased immune surveillance. In addition, IFN-γ blocks the production of IL-4, an inducer of Th2 cell differentiation and proliferation. The synergistic effects of IL-21, IL-18, and IL-15 increase IFN-γ production. The most potent regulator of IFN-γ production is IL-15 compared to IL-21 in human NK and T cells. The cytokines IL-15 and IL-18 are produced by macrophages, while IL-21 is mainly produced by activated T cells. IFN-γ increases the expression of the HLA (major histocompatibility complex) class I and II antigen by increasing the expression of subunits, increasing the expression and activity of proteasomes, resulting in increased sensitivity of the host to an infectious pathogen and an increased ability to identify and respond to this pathogen [26]. Thus, IFN-γ has many important immunostimulatory and immunomodulatory effects.

With the development of inflammation, a high level of IFN-γ leads to the activation of both canonical and non-canonical pathways. In the canonical signaling pathway, IFN-γ dimerizes and binds to two IFN-γ receptors, which are composed of two different ligand-binding chains: high-affinity IFNGR-1 (α) with high expression and two signal-transforming low-affinity IFNGR-2 (β),—with related signaling mechanisms. The IFNGR1 and IFNGR2 chains belong to the class II cytokine receptor family. The IFNGR2 chain limits sensitivity to IFN-γ and the IFNGR1 chain is usually in excess. But the expression level of IFNGR2 can be tightly regulated depending on the state of cell differentiation or activation. Receptors are expressed on the surface of almost all cell types. The expression level is determined by the cell type and its activation status. Initially, IFN-γ binds to IFNGR1, and the formed IFN-γ*IFNGR1 complex facilitates its binding to IFNGR2, then downstream signaling pathway events are initiated [27]. IFN-γ gene transcription is induced through several mechanisms. The most studied response to IFN-γ, mediated by STAT-1-containing transcription factor GAF (gamma-activated factor), which is activated by tyrosine kinases Jak1 and Jak2 and binds to the gamma-activating sequence GAS (Gamma Activating Sequence), which is present in the promoter regions of many genes. As a result of gene activation, the formation of cellular immune response to a viral infection begins [28]. The JAK/STAT pathway is the main signaling pathway initiated by IFN-γ stimulation. Further, IFN-γ, together with one of its receptor

subunits IFNGR1 and pSTAT1, is translocated into the cytoplasmic domain in combination with endocytosis and induces gene expression by binding to GAS elements in the promoter region of inducible IFN genes [29]. When viruses inhibit the functions of STAT1 molecules, IFN-γ can independently induce a noncanonical signaling pathway [30]. That is, IFN-γ is capable of inducing gene expression in STAT1−/− bone marrow macrophages, suggesting that IFN-γ acts independently of STAT-1 or in an alternative non-canonical manner. Typically, activation of noncanonical pathways occurs later, after STAT1 activation. However, there is evidence that noncanonical pathways can be activated in the absence or presence of STAT1 in a dependent manner [31]. The IFN-γ and IFN-α/β signaling pathways intersect at several levels, partially overlap, which allows cross-interaction of certain functions within the cell. This crossover mechanism is relevant because in vivo cells are not stimulated in isolation by a single cytokine, but rather a cytokine cocktail that induces gene expression through the integration of multiple signaling pathways.

When infected with a virus, IFN-γ can induce apoptosis by regulating Fas ligands to remove virus-infected cells, enhancing the expression of type I IFNs, pro-inflammatory cytokines, and chemokines by endothelial, epithelial cells and fibroblasts to attract macrophages, neutrophils, and T cells to the sites of infection [32]. IFN-γ can also initiate the expression of dsRNA-specific adenosine deaminase (ADAR), which inhibits viral replication by editing or disrupting the translation of viral proteins [33].

Virus infection of a cell begins with the attachment of the virus to the surface of the host cell through a receptor and/or through cell membrane molecules such as glycans. Viruses can release their genomes directly into the cell after fusion of its membrane with the plasma membrane, while other viruses enter cells through cellular endocytosis, which allows the virus to release the core virion containing the viral genome directly into the cytoplasm [34]. The isolated genome, either naked or associated with viral proteins, moves to certain regions of the cytoplasm or nucleus for its replication [35]. IFN-γ can inhibit the entry of the virus from the endosome into the cytoplasm.

Virus replication is the primary goal of the virus life cycle [36]. Suppression of any stage of the life cycle can lead to suppression of viral genome replication during viral infection. IFN-γ is a potent antiviral cytokine that interferes with various stages of the viral life cycle in stimulated cells using the following mechanisms [35]:

1. Inhibition of the viral penetration, both at the extracellular and intracellular stages, by controlling the expression and/or distribution of the receptors necessary for the penetration of the virus;

2. Inhibition of the viral replication by disrupting the replication niche of the virus;

3. Disruption of gene expression, interfering with translation;

4. Violation of stability by interfering with nucleocapsid assembly;

5. Violation of virus shedding by breaking the disulfide bond of the required participant in cellular interaction;

6. Modified reactivation by suppressing the main regulator of viral transcription;

7. IFN-γ can also inhibit the penetration of the virus at the stage of transfer of the invading virus from the endosome into the cytoplasm [35].

1.3 The use of interferon-γ in the treatment of herpesvirus infections

In recent years, numerous works have been published in the world on the treatment of herpesvirus infections with recombinant IFN-γ, showing high clinical and antiviral efficacy [27, 37–39]. IFN-γ demonstrated a 7–10 times more potent antiviral effect than IFN-α or -β. When IFN-γ is added at the 3–4th days after infecting, there is a decrease in EBV-induced B cell proliferation and immunoglobulin secretion, while the addition of IFN-α and -β has an effect only within 24 h. Lotz et al. Found that EBV-infected cells can be regulated by all IFNs at an early stage. Subsequently, there comes an intermediate period when only IFN-γ is able to directly influence EBV-induced B-cell responses. In the third phase, B-lymphocytes become insensitive to the direct action of all IFNs and are exposed only to cytotoxic cells [40]. In 2002 the introduction of recombinant IFN-γ, as well as IFN-β, showed high efficiency of inhibition of replication of the herpes simplex virus type 1 (HSV-1) [41]. That is, the high level of inhibition achieved by the administration of exogenous IFN-γ was the result of a synergistic interaction with endogenous IFN-α/β, which is produced locally in response to HSV-1 infection. Other researchers revealed that IFN-β and IFN-γ interact synergistically, blocking viral DNA synthesis and nucleocapsid formation in HSV-1 infected cells, without affecting the viability of the host cells. Thus, the authors concluded that IFN-mediated suppression of HSV-1 replication plays the role of the main mechanism by which the host immune system limits the spread of infection in vivo [42]. In a double-blind, placebo-controlled study, it was shown that the introduction of recombinant IFN-γ three times a week subcutaneously reduces the incidence of severe infections in patients with various genetic types of chronic granulomatous disease [43].

In the Russian Federation, the only IFN-γ preparation has been registered under the trade name Ingaron, developed by SPP PHARMACLON Ltd. via the microbiological synthesis in a recombinant E. coli strain and purified by column chromatography. The drug consists of 144 amino acid residues, devoid of the first three of them (Cys-Tyr-Cys), replaced by Met.

The purpose of this study is to evaluate the efficacy of IFN-γ therapy for the content of the number of EBV DNA copies in saliva samples by the Real-time PCR method, for the dynamics of INF-α and INF-γ production (spontaneous, serum, and induced levels) and the clinical picture in patients suffering from chronic Epstein-Barr virus infection (CEBVI) one and three months after the end of therapy.

2. Material and methods

2.1 Schemes of therapy

All patients were divided into three groups for different therapy regimens.

- The first group consisted of 51 patients (from 22 to 49 years old) who received IFN-γ therapy (500,000 IU every other day, intramuscular injections (i/m)). The total course was 15 injections;

- The second group consisted of 42 patients (from 22 to 48 years old) who received prolonged therapy with a drug from the group of acyclic natural nucleosides - valaciclovir (Valtrex 500 mg × 2 times/day, orally) for two months;

- The third group consisted of 46 patients (from 19 to 52 years old) who received prolonged therapy with a synthetic nucleoside analog of guanosine—valganciclovir

(Valcyte 450 mg × 2 times a day, orally) for 2 months in combination with IFN-γ (10–20 intramuscular injections of Ingaron 500,000 IU every other day). Previously, all patients in this group, as prescribed by a doctor or independently (often repeatedly), received therapy with drugs from the group of acyclic natural nucleosides, including valaciclovir for short courses (7–10 days). There was no pronounced clinical and laboratory positive effect from the previous therapy, for this reason, these patients have prescribed valganciclovir in combination with IFN-γ.

To assess the efficacy, a comparative analysis of the amount of EBV DNA in saliva samples was carried out one month after the end of the treatment course. Clinical complaints were compared for the patients of Group 1 in one and three months after the treatment course.

Patient groups and therapy are presented in **Table 1**.

Patient group	Therapy in the main groups	Subgroup of patients	Therapy in subgroups
1st group (n = 51)	Ingaron 500,000 IU every other day, i/m. Course of 15 injections	—	—
2nd group (n = 42)	Valtrex 500 mg × 2 times/ day, 2 months	—	—
3d group (n = 46)	Valcyte 450 mg × 2 times/ day, 2 months + Ingaron	3A group (n = 22)	Valcyte 450 mg 2 times/day (2 months) + Ingaron 500,000 IU every other day, i/m. Course of 10 injections
		3B group (n = 24)	Valcyte 450 mg 2 times/day (2 months) + Ingaron 500,000 IU every other day, i/m. Course of 20 injections

Table 1.
Characteristics of therapy in patient groups.

The study procedures were in accordance with the Good Clinical Practice (GCP) guidelines and ethical principles of the Declaration of Helsinki. The study was approved by the Ethics Committee of Fresenius Medical Care (Dialysis Center St. Petersburg, Russia). Written informed consent was obtained from all participants before the study was initiated.

2.2 Survey methods

Clinical research methods included the collection of anamnesis, data on previous immuno- or antiviral therapy, and the presence of concomitant diseases. The clinical condition of patients was assessed according to the generally accepted method, including objective data and registration of patient complaints at the time of examination. The severity of patient complaints was recorded using a subjective assessment scale on a 3-point scale (0—no symptoms, 1—mild symptoms, 2—moderate severity of symptoms, 3—significant severity of symptoms).

Diagnosis of CEBVI was based on clinical data and positive results of EBV DNA studies in saliva samples conducted by polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection. The test systems "AmpliSens EBV/CMV/HHV6-screen-FL" (FBSI "Central Research Institute of Epidemiology", Russia) were used. The unit of measurement used to estimate the viral load during DNA extraction from saliva is the number of copies of EBV DNA per ml of sample. According to the instructions, this indicator is calculated using the formula: Number

of DNA copies = CDNA × 100, where CDNA is the number of copies of the viral DNA in the sample. The analytical sensitivity of the test system is 400 copies/ml.

We studied the dynamics of IFN-α and -γ production before the initiation of IFN-γ therapy and 1 and 3 months after the end of the course. Determined the level of IFN-α and IFN-γ in the blood serum, as well as the spontaneous and induced production of these cytokines in the culture of blood lymphocytes. Newcastle disease virus (NDV) (obtained at the L.A. Tarasevich State Institute of Culture, St. Petersburg) with an infectious titer of 8 lg EID/0.2 ml in a volume of 8 μl/hole and Phytohemagglutinin (PHA-P) (PanEko, Russia) at the dose of 10 μg/ml were used as inducers of IFN-α and -γ production respectively. The quantitative content of cytokines was determined in the serum and supernatant of a 24-h whole blood culture by solid-phase ELISA using the test systems "alpha-Interferon-IFA-BEST" and "gamma-Interferon-IFA-BEST" (JSC "VectorBest", Russia). Reference values for spontaneous, serum, and induced production of IFN-α and IFN-γ are provided by the manufacturer of the test systems.

2.3 Statistical analysis

Statistical analysis of the obtained results was carried out using the statistical software package IBM SPSS Statistics, version 26 (Armonk, NY: IBM Corp.). Group results are presented as mean ± standard error of the mean (SEM). Statistical processing of the results was carried out using parametric (Pearson's method) and nonparametric (Kendall's tau (τ)) criteria. To check the condition of independence of observations, linear regression analysis (with the calculation of the coefficient of determination (R Square) and the Durban-Watson criterion) and analysis of variance (ANOVA Analisis of Variance) with the calculation of the Fisher criterion (F) were carried out to test the significance of the model. The standardized coefficient ß was calculated with 95% Confidence Interval (95% CI). The critical level of significance of the difference in indicators was taken equal to 0.05.

3. Results

The examination was carried out in 139 patients with CEBVI: 86 women and 53 men with average age of 35.27 ± 1.28 years. The duration of CEBVI from the appearance of the first complaints to laboratory confirmation and the diagnosis was 2.23 ± 0.21 years. 98 (7.720%) and 27 (24.54%) patients suffered in childhood from chronic tonsillitis with no response to antibiotic therapy and infectious mononucleosis respectively. All patients underwent differential diagnosis of CEBVI with other viral infections (HIV, viral hepatitis, cytomegalovirus infection, toxoplasmosis), helminthic invasions, autoimmune diseases associated with EBV infection.

CEBVI is characterized by a long course and frequent relapses with clinical and laboratory signs of viral activity, described in detail in the literature [44–46]. Patients worried about prolonged subfebrile condition (37.1–37.3°C), weakness, unmotivated fatigue, excessive sweating (especially at night), a constant feeling of discomfort and/or pain in the throat, lymphadenitis, swelling of the nasal mucosa with abundant drainage mucus, stomatitis. Some patients have a cough, skin rashes, arthralgia, pain in the muscles of the trunk and extremities are possible. There may be manifestations of conjunctivitis, otitis media. Neurological disorders develop headaches, memory and sleep disorders, decreased concentration, irritability, tearfulness, a tendency to depression. Perhaps an increase in internal organs (according to ultrasound, hepato- or splenomegaly) and feeling of heaviness in the right hypochondrium. Also, patients complain of frequent colds, the addition of other herpesviral infections. In the history of such patients, long-term stressful situations,

psychoemotional and physical overloads often take place, against the background of which the patient's condition worsens.

3.1 Comparative analysis of the efficacy of antiviral therapy

In all patients (N = 139) CEBVI was confirmed by PCR reaction in saliva samples. **Table 2** shows the results of a comparative analysis of the dynamics of the number of EBV DNA copies in groups of patients who received different therapy regimens.

Patient group	Number of copies/ml before therapy	Number of copies/ml after therapy
1st group IFN-γ (N = 51)	294630.59 ± 72210.69	154786.97 ± 18671.15 (N = 36) in 15 patients—0.00 copies (29.41%)
2nd group valaciclovir (2 months) (N = 42)	278857.24 ± 44608.15	47108.18 ± 25928.62 (N = 30) in 12 patients—0.00 copies (28.57%)
3d group valganciclovir (2 months) + IFN-γ (N = 46)	425250.00 ± 62697.09	35934.50 ± 33764.56 (N = 13) in 33 patients—0.00 copies (71.74%)

Table 2.
Dynamics of the number of EBV DNA copies one month after the end of antiviral therapy in patients with CEBVI.

From the presented data in the 1st group after Ingaron therapy, only 15 (29.41%) patients had negative PCR results in saliva samples. In the 2nd group of patients taking valaciclovir negative PCR results were obtained in 12 (28.57%) patients. In group 3, one month after taking the combination therapy valganciclovir + Ingaron, 33 (71.74%) patients obtained negative PCR results in saliva samples.

The patients of the 3rd group, according to the combination therapy were distributed as follows:

- 3A subgroup—22 patients received Valcyte 900 mg/day (2 months) + Ingaron 10 injections of 500,000 IU every other day;
- 3B subgroup—24 patients received Valcyte 900 mg/day (2 months) + Ingaron 20 injections of 500,000 IU every other day.

Table 3 shows the results obtained in these subgroups.

Therapy scheme (Group 3)	Number of DNA copies/ml before therapy	Number of DNA copies/ml after therapy
Subgroup 3A: Valcyte 900 mg/day (2 months) + Ingaron 500,000 IU × 10 (N = 22)	334086.00 ± 95214.02	In 12 patients—0.00 copies; (54.54%) in 12 patients—6285.57 ± 2823.61
Subgroup 3B: Valcyte 900 mg/day (2 months) + Ingaron 500,000 IU × 20 (N = 24)	381745.32 ± 161946.09	In 21 patients—0.00 copies; (87.50%) in 3 patients—123469.51 ± 46615.32

Table 3.
Dynamics of the number of EBV DNA copies one month after the end of combined antiviral therapy in the 3rd group of patients.

From the data presented in **Table 2** one can see that in the total 3rd group of patients, there is a reliably positive dynamics of the number of DNA copies the decrease in a month after combination therapy. In subgroup 3A, negative PCR results were obtained in 54.54% of patients. The best result was observed in the patients of subgroup 3B (in 87.50% of patients) who received 20 injections of IFN-γ 500,000 IU every other day, in combination with valganciclovir. That is, the positive result of this therapy regimen is due not so much to the combination of medicines, but to the amount and duration of IFN-γ administration.

3.2 Dynamics of INF-α and INF-γ production

After the comparative analysis of the efficacy of different regimens of CEBVI therapy, we analyzed the dynamics of INF-α and INF-γ production (spontaneous, serum, and induced) in the culture of lymphocytes in the first group of patients (N = 51) before the start of therapy with IFN-γ, after one and three months after the end of therapy. We also assessed the dynamics of clinical complaints in these patients after IFN-γ therapy. **Tables 4** and **5** present the data obtained.

Research indicator	IFN-α level (pg/ml)before therapy	IFN-α level (pg/ml) after 1 month of therapy	IFN-α level (pg/ml) after 3 months of therapy	p
	1	2	3	
Spontaneous IFN-α	3.76 ± 0.58	5.80 ± 4.02	3.85 ± 19.24	P1,2 = 0.345 P2,3 = 0.435 P1,3 = 0.359
Serum IFN-α	5.09 ± 1.47	4.21 ± 0.70	5.57 ± 1.20	P1,2 = 0.289 P2,3 = 0.202 P1,3 = 0.380
Induced IFN-α	296.78 ± 127.43	578.154 ± 129.46	294.78 ± 60.67	P1,2 = 0.284 P2,3 = 0.360 P1,3 = 0.145

Table 4.
Dynamics of IFN-α production before the start, one and three months after therapy in the 1st group of CEBVI patients (N = 51).

Research indicator	IFN-γ level (pg/ml) before therapy	IFN-γ level (pg/ml) after 1 month of therapy	IFN-γ level (pg/ml) after 3 months of therapy	p
	1	2	3	
Spontaneous IFN-γ	2.07 ± 0.26	2.57 ± 0.75	2.00 ± 0.57	P1,2 = 0.34 P1,3 = 0.36 P2,3 = 0.57
Serum IFN-γ	1.85 ± 0.14	5.57 ± 1.20	2.10 ± 0.68	P1,2 = 0.024 P1,3 = 0.21 P2,3 = 0.38
Induced IFN-γ	1862.72 ± 624.52	2487.96 ± 437.73	4308.12 ± 3053.77	P1,2 = 0.38 P1,3 = 0.38 P2,3 = 0.27

Table 5.
Dynamics of IFN-γ production before the start, one and three months after therapy in the 1st group of CEBVI patients (N = 51).

One month after the end of therapy with IFN-γ, a tendency to an increase in the spontaneous production of IFN-α was revealed (statistically insignificant), but after three months the values returned to the initial values. Serum IFN-α production did not change after one and three months, remaining within the normal range. There was a tendency to an increase in the induced production of IFN-α one month after the end of therapy, followed by a normalization of the level after three months. Thus, IFN-γ had no significant effect on IFN-α production in the general group of patients after one and three months of therapy.

From the data presented in **Table 4**, it follows that in the group of patients a month after the end of therapy with IFN-γ, the serum ($p = 0.024$) production of IFN-γ increased, and after three months the serum level practically returned to the initial value ($p = 0.57$). The level of spontaneous production one and three months after the end of therapy did not change significantly. Induced production of IFN-γ also tended to increase one and three months after the end of therapy without significant dynamics.

When analyzing the initial data of the level of induced IFN-γ, it was found that these values sharply differed in patients, i.e. from the lower to the upper limit values of the reference (281-4335 pg/ml).

In this regard, the group of patients ($N = 51$) was divided into 2 groups in accordance with the induced production of IFN-γ before the start of therapy:

- 1st subgroup ($N = 30$)—the level of induced IFN-γ closer to the upper limit of the reference values (2706.00 ± 1058.94 pg/ml);
- 2nd subgroup ($N = 21$)—the level of induced IFN-γ closer to the lower limit of the reference values (287.20 ± 64.65 pg/ml).

Figure 1 shows the data on the dynamics of the induced IFN-γ production in these groups of patients.

The results of the study showed that after the course of therapy with IFN-γ in the 1st subgroup, the content of induced IFN-γ had a tendency to a gradual decrease, while in the 2nd subgroup there was a significant increase in the level of induced IFN-γ three months after therapy ($p = 0.027$). At the same time, the values of IFN-γ levels in both groups remained within the reference values. **Figure 2** shows the results of the dynamics of the spontaneous level of IFN-γ before and after IFN-γ therapy in both subgroups.

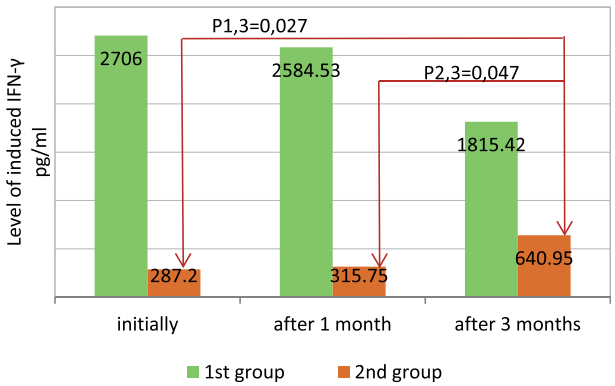

Figure 1.
Dynamics of the level of induced IFN-γ before the start, one and three months after IFN-γ therapy in patients with CEBVI in the subgroups 1 and 2.

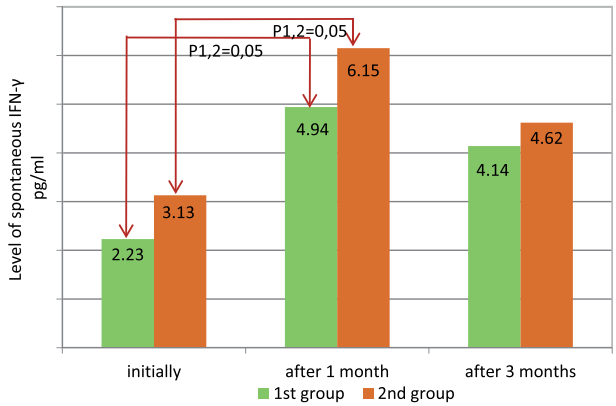

Figure 2.
Dynamics of the level of spontaneous IFN-γ before the start, one and three months after IFN-γ therapy in patients with CEBVI in subgroups 1 and 2.

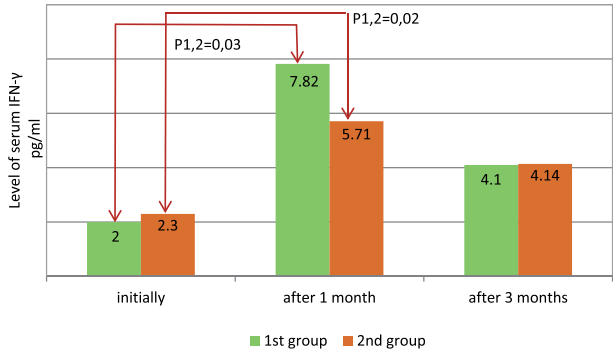

Figure 3.
Dynamics of the level of serum IFN-γ before the start, one and three months after IFN-γ therapy in patients with CEBVI in subgroups 1 and 2.

The presented data show that the values of the spontaneous level of IFN-γ in both groups significantly increased after one month. After three months, there was a trend towards a decrease in the spontaneous level of IFN-γ in both groups, while remaining above the baseline values. However, these values in both groups did not differ from the reference values (0–6 pg/ml). **Figure 3** shows the dynamics of serum IFN-γ levels after therapy in both groups of patients.

The results of the research showed that in both subgroups the increase in serum IFN-γ production was significant one month after the end of therapy ($p = 0.03$ and $p = 0.02$, respectively). In three months after the treatment course, there was a tendency to a slight decrease in serum IFN-γ, while the data obtained did not differ from the baseline (before the start of therapy levels) and from the reference values provided by the manufacturer of the test system (0–10 pg/ml).

3.3 Dynamics of clinical complaints

The next stage of the work was the analysis of the frequency of the main clinical complaints in patients of both subgroups before the start, one and three months after IFN-γ therapy (**Table 6**).

From the data in **Table 6**, one can see that in one and three months after the therapy with IFN-γ in patients of the 1st group there was a significant decrease in

Frequency of complaints (%)	Before therapy (n = 30)	after 1 month of therapy	after 3 months of therapy	p
	1	2	3	
Subfebrile temperature	83.33	30.76	30.76	P1,2 = 0.004 P1,3 = 0.004 P2,3 = 0.000
Lymphadenitis	53.33	43.33	26.66	P1,3 = 0.047 P2,3 = 0.05
Sore throat	93.33	43.33	36.66	P1,2 = 0.001 P1,3 = 0.001
Weakness	76.66	66.66	53.33	P1,3 = 0.001
Chills	70.00	13.33	20.00	P1,2 = 0.001 P1,3 = 0.001
Sweating	93.33	53.33	46.66	P1,2 = 0.001 P1,3 = 0.001
Runoff of mucus	33.33	13.33	16.66	P1,2 = 0.05
Decreased concentration of attention and memory	56.66	40.00	36.66	P1,3 = 0.050

Table 6.
Frequency of main clinical complaints (%) in patients before the start, one and three months after IFN-γ therapy in patients of the 1st group (1st subgroup—with the level of induced IFN-γ closer to the upper limit of the reference values).

subfebrile temperature, sore throat, chills, sweating, and decreased concentration. The rest of the complaints tended to decrease or remain unchanged. The dynamics of clinical complaints in the patients of the 2nd subgroup is presented in **Table 7**.

Frequency of complaints (%)	Before therapy (n = 21)	After 1 month of therapy	After 3 months of therapy	p
Lymphadenitis	66.66	14.28	19.04	P1,2 = 0.002 P1,3 = 0.05
Sore throat	33.33	23.80	19.04	P1,3 = 0.002 P1,3 = 0.002
Chills	47.67	28.57	23.80	P1,2 = 0.001 P1,3 = 0.001
Sweating	61.90	52.38	47.67	P1,2 = 0.029 P1,3 = 0.001
Runoff of mucus	21.05	10.52	10.52	P1,2 = 0.029 P1,3 = 0.029
Stomatitis	15.78	10.52	9.52	P1,2 = 0.004 P1,3 = 0.001
Joint pain	15.78	10.52	9.52	P1,2 = 0.004 P1,3 = 0.001
Decreased concentration of attention and memory	33.33	23.80	26.31	P1,2 = 0.002
Sleep disturbance	15.78	14.28	10.52	P1,3 = 0.004 P2,3 = 0.046

Table 7.
Frequency of clinical complaints (%) in patients before the start, one and three months after IFN-γ therapy in patients of the 1st group (2nd subgroup—with the level of induced IFN-γ closer to the lower limit of the reference values).

In the 2nd subgroup of patients, one and three months after IFN-γ therapy, a significant positive dynamics of the main clinical complaints were observed, in particular, a decrease in lymphadenitis, sore throat, chills, sweating, mucus drainage along the back of the throat, stomatitis, joint pain, decreased concentration attention, sleep disorders. That is, patients with an initially reduced level of induced IFN-γ before starting IFN-γ therapy have a more pronounced response to the therapy.

When analyzing the clinical picture, we revealed that in the group of patients with a higher level of induced IFN-γ production at the time of initiation of therapy complaints were more intensive and occurred with higher frequency (**Figures 4** and **5**).

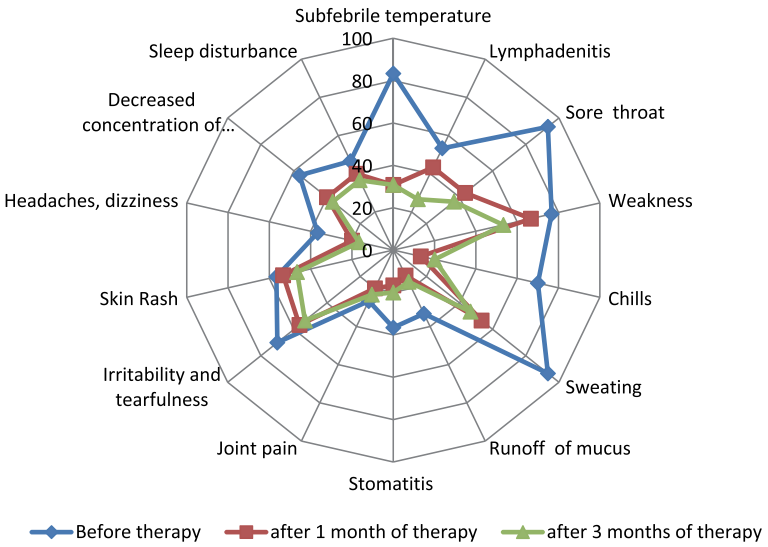

Figure 4.
Frequency of main clinical complaints (%) in patients before the start, one and three months after IFN-γ therapy in patients with CEBVI in the 1st subgroup.

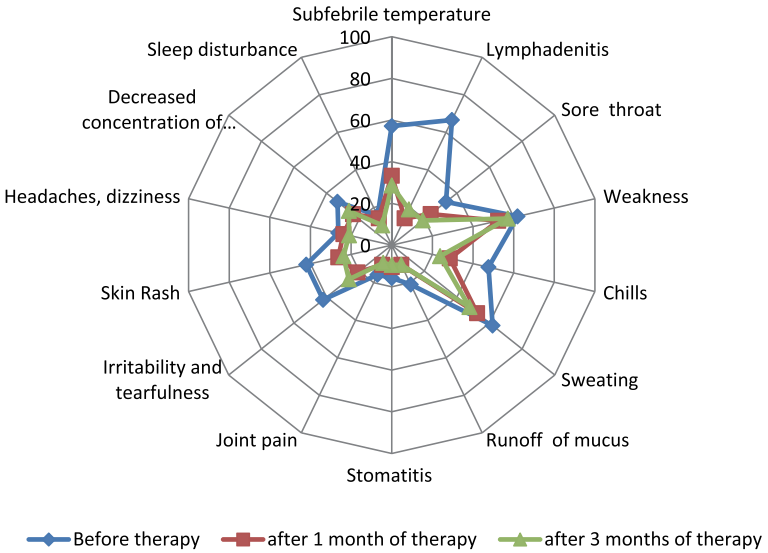

Figure 5.
Frequency of clinical complaints (%) in patients before the start, one and three months after IFN-γ therapy in patients with CVEBI in the 2nd subgroup.

3.4 Relationship between the number of EBV DNA copies and clinical complaints

Further, a correlation analysis was carried out to reveal the relationship between the numbers of EBV DNA copies in saliva samples before the start of IFN-γ therapy with the severity of clinical complaints in patients. The results are shown in **Table 8**.

Complaints	Correlation coefficient
Weakness	$\tau = 0.473$; $p = 0.026$ $r = 0.553$; $p = 0.026$
Sore throat	$\tau = 0.629$; $p = 0.002$ $r = 0.741$; $p = 0.001$
Joint pain	$\tau = -0.413$; $p = 0.052$ $r = -0.521$; $p = 0.039$

Table 8.
Influence of the number of EBV DNA copies on the severity of clinical complaints in patients with CEBVI (N = 51).

Thus, the number of copies of EBV DNA in saliva samples affects the development of weakness, sore throat, and arthralgia in patients with CEBVI.

3.5 Prognostic significance of the number of copies of EBV DNA

In order to clarify the prognostic significance of the number of EBV DNA in saliva samples, a linear regression analysis was carried out with the calculation of the coefficients of determination R2 (R Square) and using the Durban-Watson test and analysis of variance (ANOVA "Analysis of Variance") using criterion F and the calculation of the standardized coefficient beta (ß) with a 95% confidence interval. The results of the F criterion and the ß coefficient, indicating the significance of the obtained regression models, are presented below.

- The number of EBV DNA copies in saliva samples before the initiation of IFN-γ therapy affects the level of induced IFN-α production one month after the end of therapy ($F = 12.166$; $p = 0.002$; $\beta = 0.615$; CI: 75.999; 264.975; $p = 0.002$);

- The number of copies of EBV DNA in saliva samples before the initiation of IFN-γ therapy affects the level of induced IFN-γ production one month after the end of therapy ($F = 3.852$ $p = 0.061$; $\beta = -0.365$; CI:-0.011; −0.001; $p = 0.061$).

3.6 Influence of the induced IFN-γ level on clinical complaints

The next stage of the work was the correlation analysis of the influence of the initial level of induced IFN-γ on the clinical picture of the disease in patients of the 1st and 2nd subgroups (Group 1). It was shown that in the 1st subgroup, a high level of induced IFN-γ inversely influences the development of sweating in patients ($r = -0.506$; $p = 0.023$; $\tau = -0.419$; $p = 0.021$). In subgroup 2, the initially low level of induced IFN-γ inversely influences the development of weakness ($r = -0.405$; $p = 0.045$; $\tau = -0.419$; $p = 0.037$). It was not possible to identify other significant correlations in the subgroups.

4. Discussion

Currently, there is no single approach to the treatment of CEBVI, despite the fact that there are a number of specific antiviral drugs. In particular, acyclic nucleosides are widely used, such as acyclovir, valaciclovir (Valtrex), famciclovir (Famvir), and synthetic nucleoside analogs of guanosine: ganciclovir (cymevene), valganciclovir (Valcyte). In most cases, antiviral therapy is ineffective, which has been confirmed in numerous studies. In 2016, the results of efficacy analysis of infectious mononucleosis treatment were published according to the WHO World Register of Clinical Trials, both completed and ongoing. It was shown that the effectiveness of antiviral drugs (acyclovir, valaciclovir) in acute infectious mononucleosis is doubtful. Acyclovir and valaciclovir reduce EBV replication by inhibiting viral DNA polymerase and decreasing the oral secretion of EBV in patients with infectious mononucleosis. Balfour HH, et al. showed that taking the drug in a dose of 1 g every 8 h for 14 days leads to a decrease in clinical complaints in infectious mononucleosis [46]. In the case of viral shedding, shedding was observed to suppress the shedding against the background of antiviral therapy, but this effect ceased after the end of antiviral therapy [47]. The authors did not obtain a statistically significant difference between the groups of patients receiving antiviral drugs and the control groups. Most of the studies processed were unclear or at high risk of bias. Experimental studies in vitro have shown that EBV thymidine kinase has a variable affinity for antiherpetic antiviral drugs, that is, acyclovir and dihydroxypropylmethylguanine are relatively weak substrates for EBV thymidine kinase [48]. In our study, in the group of patients receiving therapy with Valtrex (valaciclovir) at a dose of 1 g per day for 2 months, suppression of EBV DNA replication in saliva samples was obtained in 28.57% of patients. This is confirmed by literature. The effectiveness of the use of valganciclovir in suppressing EBV replication and reducing the severity of clinical complaints in patients has been shown [49]. Taking valganciclovir leads to a decrease in the amount of EBV DNA from an average of 4.3 $\log_{10}$ copies/ml to 1.2 $\log_{10}$ copies/ml by 0.77 logs (95% CI, .62–.91 logs; $P < .001$) [50]. Valaciclovir and famciclovir suppress EBV DNA replication by 18% and 30%, respectively [51], while valganciclovir reduces EBV DNA secretion by 46% [52]. That is, valganciclovir can be used in the treatment of CEBVI.

The research by Kure S. et al., devoted to the study of the inhibitory effect of pure recombinant human (rh) IFN-α and IFN-γ on EBV infection in B-cell lines, BJAB lines, and in normal mature B-lymphocytes, showed that the pretreatment of cells within 24 h with rhIFN-α and rhIFN-γ suppressed the production of EBV specific nuclear antigen (EBNA-1) in BJAB cells 24 h after EBV infection. Both rhIFN types also effectively inhibited EBV infection in normal mature B lymphocytes, as evidenced by a decrease in [3H] thymidine incorporation 6 days after EBV infection and the total number of proliferating cells 21 days after infection. The authors showed that rhIFN-α exhibited a more pronounced inhibitory effect than rhIFN-γ. None of the rhIFNs showed a pronounced inhibitory effect on EBNA expression in covert EBV-infected Raji and Daudi cell lines. These results indicate that rhIFNs act predominantly at the early stage of EBV infection [53]. In our work, it was shown that in group 1 (N = 51) one month after rhIFN-γ therapy, 15 (29.41%) patients had negative PCR results in saliva samples, and 36 (70.59%) patients had copies of EBV DNA decreased. That is, rhIFN-γ can completely inhibit viral replication in 29.41% of patients. However, in this group of patients, a pronounced reliable dynamics of clinical complaints were obtained after the end of therapy. In 2002, it was shown that treatment of Vero cells with IFN-β or IFN-γ inhibits HSV-1 replication by less than 20-fold, while co-treatment with IFN-β and IFN-γ inhibits

HSV-1 replication by ~1000 times [41]. The authors suggested that the high level of inhibition achieved by the administration of exogenous IFN-γ is the result of a synergistic interaction with endogenous IFN-α/β, which are locally produced in response to HSV-1 infection. Our results confirm these in vitro data. If we compare the results we obtained in the groups of patients who received rhIFN-γ and monotherapy with valganciclovir in terms of the dynamics of the number of DNA copies in saliva samples, then no difference was obtained between these groups, that is, the effectiveness of monotherapy with rhIFN-γ or valaciclovir has similar efficacy (29.41% and 28.57% respectively). Our results are consistent with previously published data, in particular, the Russian literature describes the results of the study of rhIFN-γ (Ingaron) and presents evidence of the high efficiency of its use in the treatment of herpesvirus infections [54]. The authors showed that the drug has a direct antiviral effect, and the clinical effect is manifested through the activation of cellular immunity, which controls the viral antigen. In group 3, a month after taking the combination therapy valganciclovir+rhIFN-γ, a negative PCR result was obtained in 19 (71.74%) patients. The effectiveness of the therapy did not depend on the combination of drugs but on the duration of the course of rhIFN-γ administration. The best result from therapy was in patients who received 20 injections of rhIFN-γ in combination with valganciclovir. It was in this group that the number of copies of EBV DNA in saliva samples was negative in 87.50% of patients. Thus, a positive result on the number of EBV DNA copies during this treatment regimen is due not so much to the total combination course, but to the amount and duration of rhIFN-γ administration.

In 2003, an open, randomized, controlled, multicenter clinical study was conducted to study the anti-fibrotic effect of rhIFN-γ in 153 patients with chronic viral hepatitis B. RhIFN-γ was introduced i/m daily at a dose of 1 MU for three months and 1 MU every other day for the following six months. As a result, it was shown that rhIFN-γ has a pronounced anti-fibrotic effect in patients with chronic hepatitis B [55]. The effectiveness of treatment was 66% in the group of patients versus 16.2% in the control group. Later in 2011, the results of the study of rhIFN-γ monotherapy in 25 HBsAg-positive patients with stage 2-4 fibrosis who received long-term rhIFN-γ therapy were published [56]. The authors also showed that long-term therapy for nine months leads to pronounced positive dynamics of inflammation and fibrosis of the liver tissue. Our results with long-term administration of rhIFN-γ confirm these data.

With herpes viral infection, the secretion of cytokines is altered, modulating a strong and effective antiviral immune response against infected host cells. After primary infection, herpes viruses persist in the host organism for a long time [57]. One of the factors contributing to the persistence of herpes viruses is their ability to adopt two different modes of the life cycle: latent and lytic. After primary infection, herpes viruses pass into a latent, transcriptional-translational suppressed state, which can often be interrupted by lytic episodes. During the latency phase, transcripts were identified, in particular, such as microRNAs (miRs), which play a role in the mechanism of evasion of the virus from the host's immune response, including impaired interferon signaling [58].

It has been shown that the early EBV protein BZLF1 can block IFN-γ production by inhibiting the downstream IFN-γ signaling pathway. Essentially, BZLF1 stops the transcription of all expressed HLA class II molecules and, therefore, the activation of T-helper cells required for the induction of an immune response, inhibits IFN-γ-induced tyrosine STAT1 phosphorylation and nuclear translocation of BZLF1, reduces the expression of the IFN-γ receptor, stimulating the mechanism, with the help of which EBV can avoid the antiviral immune response during primary infection [59]. In addition, the EBV lytic transactivator Zta suppresses the production of IFN-β,

the EBV protein LMP1 inhibits TNF-α and induces the production and secretion of IL-10, and the miR-BHRF1-2-5p EBV blocks the proinflammatory signaling of IL-1 [60]. Cytokine signaling is a very early response to viral infection and explains the presence of corresponding inhibitory viral factors in the tegument. Thus, the dysregulation of the production of proinflammatory cytokines is based on the fact that virions already contain molecules that directly target the proper cytokine signaling. After infection of host cells and transcription of viral DNA leading to translation of viral miRs into viral peptides, other mechanisms of proper immune surveillance are targeted, including, in particular, presentation of HLA class I antigen, as well as decreased expression of NKG2D ligands [61].

INF-γ plays not only an important role in modulating T-cell immunity but also, having a direct antiviral activity is used as an effective therapeutic agent in the treatment of viral infection [62]. Okano et al. conducted a study of the efficacy of therapy with recombinant IFN-γ in a patient with infectious mononucleosis and X-linked lymphoproliferative syndrome (XLP). EBV-determined nuclear antigen and EBV DNA have been found in various tissues of the patient. After therapy with recombinant IFN-γ, there was positive dynamics in the reduction of virus-infected cells and a linear increase in the content of IFN-γ in the blood serum. NK cell activity remained within normal limits throughout the course of therapy. The authors suggested that cytotoxic cells can produce endogenous IFN-γ [63]. A. Linde et al. also revealed an increase in serum IFN-γ levels 24 h ($p = 0.05$) and 48 h ($p = 0.01$) after EBV infection, subsequently, the level of IFN-γ returned to baseline values [64]. In another study, in patients with acute infectious mononucleosis, an increase in the level of serum IFN-γ was shown only during the first week from the moment of infection, later the level of IFN-γ returned to normal [65]. Interesting data were obtained when studying the dynamics of IFN-γ level production in patients with tuberculosis, who showed a decrease in the average IFN-γ level over time ($p = 0.001$), but this decrease occurred during the first 8 weeks from the start of specific therapy ($p = 0.019$). When comparing baseline susceptible ($N = 55$) and drug-resistant patients ($N = 18$), there was no difference in the change in IFN-γ levels over time. Since the production of IFN-γ and secretion from T cells increase in response to an increase in antigenic load and then stabilize over 24 weeks, a decrease in the concentration of IFN-γ may indicate a positive response to the therapy and play the role of monitoring the response to therapy [66].

Our data indicate the absence of a significant increase in the production of the induced, serum, and spontaneous level of INF-γ three months after the end of therapy with INF-γ in the general group of patients, which is fully consistent with the previously published results of other authors. But when analyzed separately in each group of patients, it was shown that in the group with an initially low level, the administration of INF-γ led to a significant increase in the level of induced INF-γ three months after the end of therapy ($p = 0.027$). This is probably due to the initial low level of induced INF-γ and a more pronounced response to INF-γ therapy, which manifested itself in a significant positive dynamics of the main clinical complaints. Thus, we demonstrated that the dynamics of the production of the initially low level of induced INF-γ can be a marker of the positive effect of the therapy with INF-γ.

The absence of positive dynamics of the increase in the production of induced INF-γ in the general group of patients one and three months after the end of therapy with INF-γ indicates the absence of the effect of the drug on the level of production of endogenous INF-γ, which was previously demonstrated in studies by other authors. At the same time, INF-γ has a pronounced antiviral effect, which was shown earlier, and does not cause the increase of INF-γ production to the levels that would exceed the reference values.

When analyzing the clinical picture, we revealed that in the group of patients with a higher level of induced IFN-γ production at the time of initiation of therapy, complaints were more pronounced and more frequent. This is probably due to a more intensive inflammatory response in this group of patients. This conclusion is supported by previously published data that these inflammatory reactions are enhanced by the presence of IFN-γ, which dramatically increases the production of inflammatory mediators by macrophages [67].

5. Conclusions

1. RhINF-γ has a pronounced antiviral effect, which is expressed in a significant decrease in the number of EBV DNA copies in patients with CEBVI.

2. The introduction of rhINF-γ leads to positive dynamics of the clinical picture of the disease. The most pronounced positive dynamics were found in patients with an initially low level of induced INF-γ.

3. The positive dynamics of the production of the initially low level of induced INF-γ can be a marker of the effectiveness of the therapy with rhINF-γ in patients with CEBVI.

4. The efficacy of therapy in patients with CEBVI is determined by the duration of the introduction of rhINF-γ: 500,000 IU every other day at least 20 injections shows the best result.

5. In the group of CEBVI patients with an initial level of induced IFN-γ at the lower limit of reference values, therapy with rhINF-γ leads to a significant increase in the level of induced INF-γ three months after the end of therapy.

6. Therapy with rhINF-γ one and three months after the end of treatment of patients does not cause changes in the production of INF-α to levels that would exceed the reference values in patients with CEBVI.

Conflict of interest

The authors declare that they have no potential conflicts of interest.

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Chapter 3

A Narrative Review of the Measles Outbreak in North America and Globally

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Abstract

In the early twenty-first century, measles was completely eradicated in the United States of America (USA) and almost eliminated in Canada. This was greatly due to most of the population being vaccinated against the virus. In 2018 and 2019, the USA and Canada experienced a rapidly developing measles virus outbreak due to growing debates about vaccine efficacy and side effects. Therefore, some people refused to vaccinate their children against measles, as well as many other life-threatening preventable diseases. This led to a major measles outbreak and health concern in the USA, Canada, and globally. Some countries including the Democratic Republic of the Congo (DRC) reported a significant number of cases and casualties resulting from measles, mainly due to the lack of funding for vaccines, as well as inadequate vaccination coverage in certain socio-demographic areas. People traveling from these countries can easily transmit the disease, though there has been a steep decline in cases since the travel ban due to coronavirus disease-2019 (COVID-19). The number of unvaccinated children currently in the USA and Canada has quadrupled since 2001. Over the past couple of years, most of the measles cases have been diagnosed in those who either did not receive the measles vaccine or complete the recommended doses of the vaccine. This paper reviews the measles outbreak, in recent years, among unvaccinated individuals in the USA, Canada, and globally.

Keywords: measles, child, adult, vaccination, disease outbreaks, United States of America, Canada, global, the Democratic Republic of the Congo

1. Introduction

The measles virus also referred to as rubeola from the *Paramyxoviridae* family, is considered a rapidly transmissible, vaccine-preventable disease affecting global populations. It is an enveloped non-segmented, negative-strand RNA virus that encodes six known structural proteins: N, P, M, F, H, and L; as well as two non-structural proteins V and C [1]. Virus-host transmembrane fusion requires hemagglutinin and fusion glycoproteins, while only the hemagglutinin glycoprotein is

required for host-cell attachment [1, 2]. In humans, the virus has a receptor binding preference for the CD46 protein [3]. It is possible to trace viral ancestral lines; the wild type, for example, is organized into eight clades, and twenty-two confirmed genotypes [2].

The clinical presentation may provide a relative timeline of primary exposure, as well as the progression of symptoms. The incubation period averages about eleven days until presenting with high fever, cough, coryza, and conjunctivitis. Within two to three days, koplik spots typically appear, and from three to five days a rash may appear on the hairline. Rash grouping could be identified as it spreads downwards to the trunk and limbs, along with increasing high fever. The measles virus does not only have the designation as a highly contagious human disease, but it is also often linked to the global infant, childhood, and adult morbidity and mortality, with the age group five to twenty years, having fewer complications than the elderly. Common complications include diarrhea, otitis media, pneumonia, encephalitis, seizures, and death. It is spread by air droplets and is believed to infect the respiratory tract, initially infecting alveolar macrophages and dendritic cells, spreading deep into the lungs, lymphatics, and then the rest of the body [4, 5].

With over 100,000 deaths annually and growing, the measles virus remains one of the major causes of vaccine-preventable infant death worldwide [6, 7]. With the global emphasis on the COVID-19 pandemic, the number of administered vaccinations decreased, creating the potential for a surge in measles outbreaks [8]. The objective of this paper is to provide a much-needed review and study of measles outbreaks in unvaccinated and partially vaccinated children and adults globally.

2. Methodology

An electronic literature search was performed using PubMed, Google Scholar, EBSCOhost, Mendeley, and MedLine Plus. The search was limited to peer-reviewed articles published between January 1, 2010, and September 14, 2020. An article was selected if it included keywords such as measles, partially vaccinated, unvaccinated, vaccine against measles-mumps-rubella (MMR), and disease outbreaks within North America and globally. Articles were reviewed and included based on the applicability to the topic.

3. Review of measles cases in the recent outbreaks

3.1 Measles outbreaks in the USA

The numbers of cases as illustrated in **Figure 1** are laboratory-confirmed cases as reported by the Centers for Disease Control and Prevention (CDC). The data were for cases from January 2010 to May 7, 2020; whereas the data for 2019 showed cases from December 30, 2018. The CDC noted there were 12 confirmed cases in the USA, as of August 19, 2020. The highest number of measles cases reported in the USA since 1992 was in 2019 with 1,282 confirmed cases overall from 31 states [9]. The World Health Organization (WHO) has also reported 12 cases of measles in 2020 and 1,282 in 2019 within the USA [10]. Of the 1,282 individual cases, 128 were hospitalized and 61 cases had complications such as encephalitis and pneumonia. All the identified cases were caused by the wild-type D8 and B3 of measles.

Furthermore, most cases (73 percent), were associated with the outbreak in New York State [9]. Specifically, cases reported in New York were seen among Orthodox Jewish communities in Brooklyn, Rockland, and Orange counties [11].

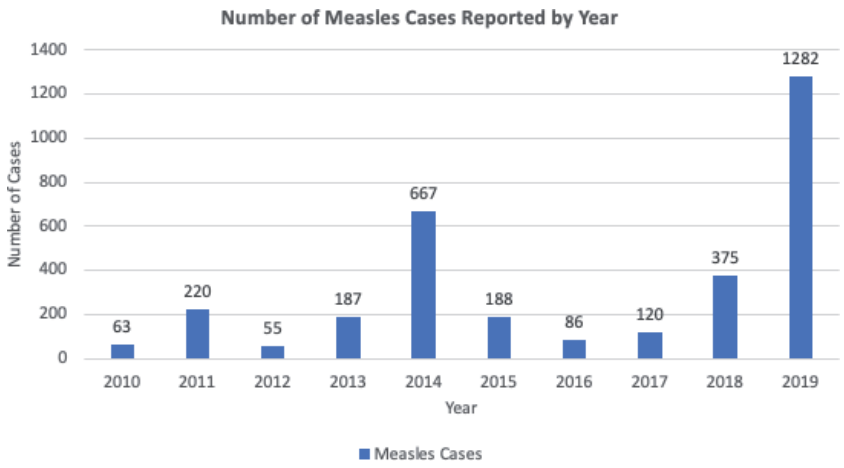

Figure 1.
The number of measles cases reported by year within the USA. Note: Data recreated and reported by the CDC, as of may 7, 2020 [9].

The neighboring state of New Jersey to date has no confirmed case of measles in 2020 but had 19 confirmed cases in 2019 [12]. Twelve of those cases were linked to the 2019 measles outbreak in Ocean County in individuals 5 to 51 years of age who were unvaccinated or had unknown vaccination status [13]. Moreover, on the West Coast during 2019, Washington State had two outbreaks of measles with a total of 86 cases. The first outbreak had 72 confirmed cases that contracted the disease between January and May 2019; whereas, the second outbreak in the state had 14 confirmed cases from May 9, 2019, through August 28, 2019, when the outbreak was declared over. This was the highest number of recorded cases seen in Washington State since 1990 [14].

Several factors contributed to the spread of measles in the USA. The most significant factor is measles infection in unvaccinated individuals, which then spread and caused outbreaks in communities throughout the USA among unvaccinated people [9]. Measles is still common globally and re-emerged in the USA when travelers who were infected with the virus brought the disease into the USA [15]. For example, in 2019, California had 73 confirmed cases, 41 of those cases were associated with six outbreaks. An outbreak is defined when there are three or more cases; hence, five of the six outbreaks were linked to patients exposed to international travelers who had measles [16].

3.2 Measles outbreaks in Canada

The number of confirmed measles cases in Canada in 2019 was 113 according to the Public Health Agency of Canada. **Figure 2** shows the measles rash onset per week from December 30, 2018, to December 28, 2019. The weeks are on the x-axis with a total of 52 weeks [17]. In contrast, there has been only one case of measles reported in Canada from December 29, 2019, until August 22, 2020 [18].

The 73 cases of measles in Canada were also genotyped and submitted to the National Microbiology Laboratory per the WHO guidelines in 2019. **Figure 3** shows the distribution of measles genotypes detected in 2019; genotype B3 (n = 20) and D8 (n = 53). Both genotypes are circulating globally [17]. The only case of measles infection in 2020 is of the genotype D8 [18].

The most significant factor for the spread of measles in Canada is when an unvaccinated visitor from an endemic area, or an unvaccinated Canadian returning

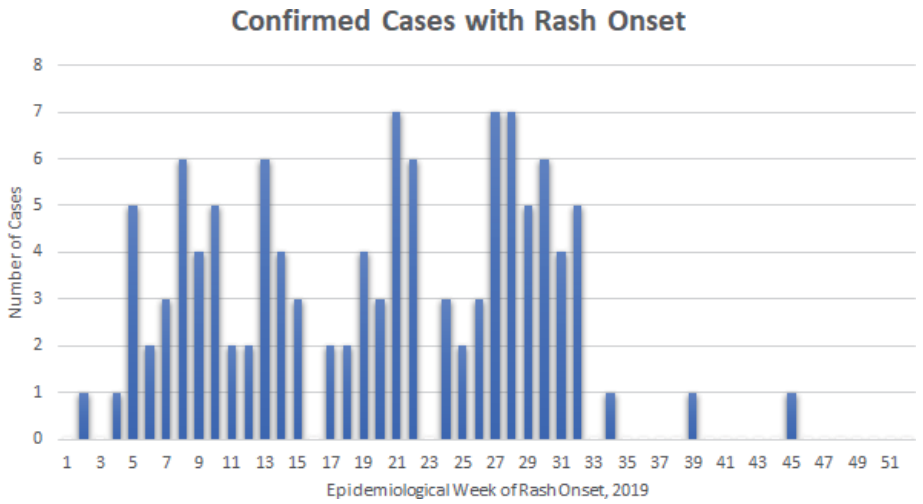

Figure 2.
The number of rash onset measles cases reported by weeks in 2019 within Canada. Note: Data recreated and reported by the Government of Canada, as of January 10, 2020 [17].

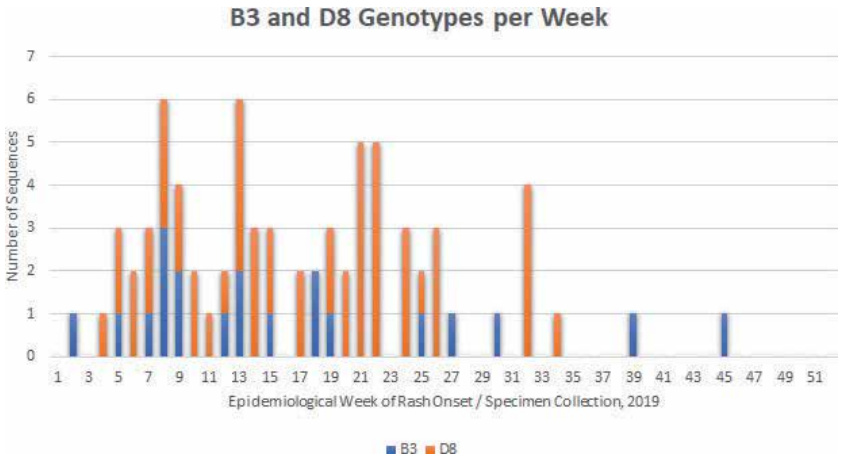

Figure 3.
The distribution of measles genotypes detected by weeks in 2019 based on rash onset in Canada. Note: Data recreated and reported by the Government of Canada, as of September 3, 2020 [17].

from such an area, imports the globally circulating measles genotype (B3, D8) into the country [19]. The stark contrast in the number of reported cases in 2020 speaks to this phenomenon. Due to an increased restriction on international travels to and from Canada, there has been a simultaneous decrease in reported cases of measles [20]. This trend is also seen in the USA where only 12 confirmed cases of measles were reported as of August 19, 2020, compared to 1,282 cases in 2019 [9].

Figure 4 shows a distribution of cases in the past in several areas according to the WHO reporting system for measles virus infection [10]. The highest confirmed years for Canada were seen in 2014, 2015, and 2019.

3.3 Measles outbreaks in the top affected countries

Although preventable, measles is a very contagious virus that is seen globally. As of September 9, 2020, the top ten countries with the highest cases of measles are as follows: DRC has 7,736 cases; Brazil follows with 6,241 cases; Nigeria is third with

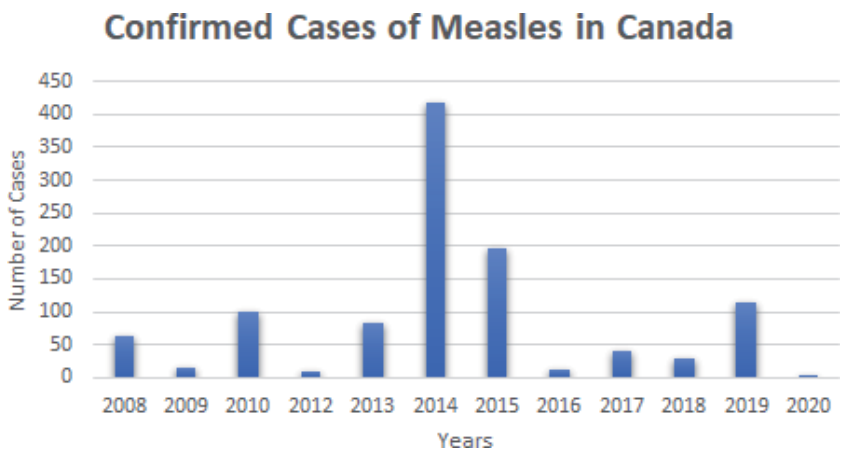

Figure 4.
Numbers of reported cases by year in Canada. Note: Data recreated and reported by WHO, as of September 9, 2020 [10].

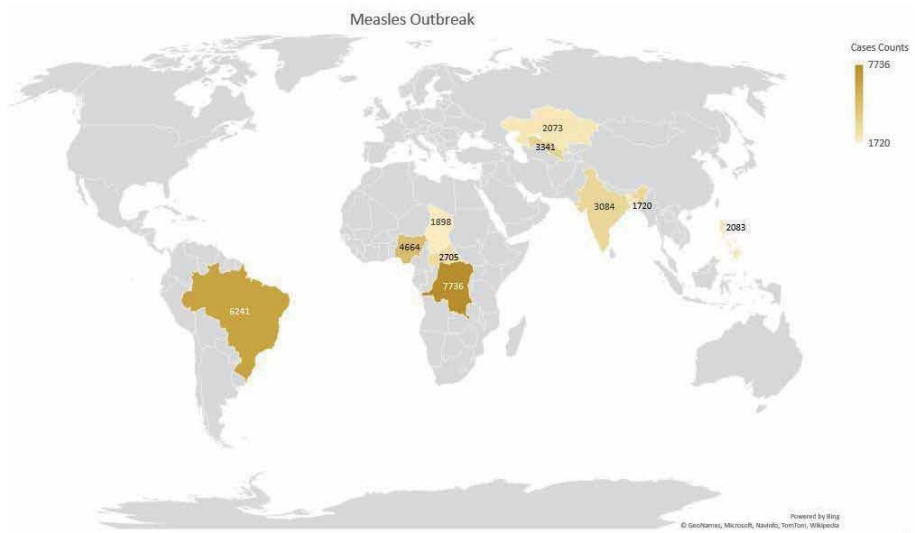

Figure 5.
Map depicting the case counts of measles in the top ten countries globally, as of September 9, 2020. Note: Data recreated and reported by the CDC [21].

4,664 cases; Uzbekistan (3,341); India (3,084); Central Africa Republic (2,705); Philippines (2,083); Kazakhstan (2,073); Chad (1,898); and lastly Bangladesh with 1,720 cases, as shown in **Figures 5** and **6** [21].

The DRC is currently the country with the highest measles cases and casualties. Prevalence is undeterred by the actions of the government and international aid agencies. This is mainly due to the lack of funding for vaccination, as well as poor vaccination coverage in certain socio-demographic areas due to weak public health system and insecurity [22]. In addition, outbreak of other epidemic prone diseases is occurring concurrently which diverted the resources needed to fight the measles outbreak [22].

In 2016, the Americas announced the eradication of measles, with imported virus resulting in isolated cases. However, at the beginning of 2018 measles was re-introduced to the Americas following a case from Venezuela that secondarily impacted Brazil [23]. Since then, Brazil has seen an increase in cases.

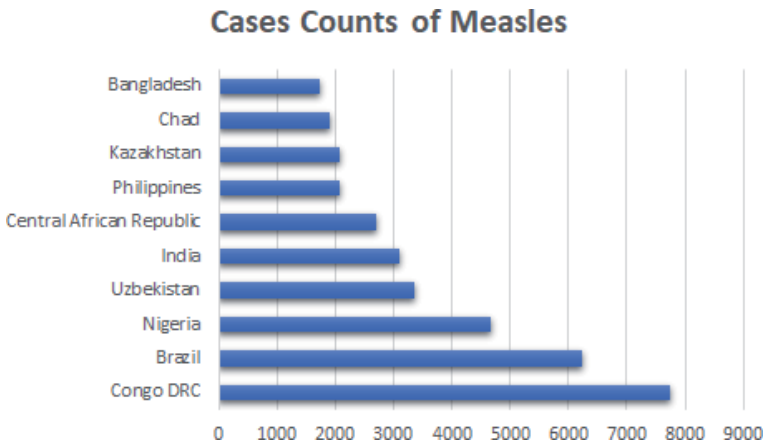

Figure 6.
Case counts of measles in the top ten affected countries globally, as of September 9, 2020. Note: Data recreated and reported by the CDC [21].

Nigeria first experienced a rise in cases beginning in 2019, which has not stopped to date. This has occurred despite the activation of the National Measles Emergency Operations Centre and the deployment of multi-disciplinary Rapid Response Teams [24]. Efforts to contain the spread of the outbreak has resulted to multiple reactive vaccination campaigns, deployment of multi-disciplinary Rapid Response Teams, intensification of surveillance activities and laboratory diagnostics as well as review of treatment protocols [24].

4. Discussion

In the year 2000, for more than twelve consecutive months, the USA did not have a single transmission of the measles virus [25]. This public health accomplishment was celebrated until recently, with measles making a comeback globally even in places where the virus had been eradicated. In 2019, the highest number of cases since 1992 was reported in the USA with 1,282 confirmed cases [9]. Outbreaks of measles can be triggered by travel into unvaccinated and/or under-vaccinated populations by infected persons. In certain parts of the USA, many areas have vaccination rates below 90 percent, and these areas experience an increase in measles transmission [26]. These outbreaks are seen mostly in densely populated areas and states, such as in New York or California. In general, the probability of contracting measles is 90 percent in those who are exposed and unvaccinated [25]. This cascade can continue to proliferate in tight-knit communities resulting in an outbreak.

In Canada, the method of measles transmission is concordant with the USA. In 2019, there were 113 confirmed measles cases in Canada. Although the number is significantly lower than in the USA, the transmission mechanism was identical; unvaccinated visitors from countries that have high measles infection rates, or unvaccinated Canadian citizens visiting those countries help to spread the disease [19]. It was also noted that outbreaks were linked to economic migrants who were from endemic areas for measles coming into Canada [15].

On a global scale, a high number of measles cases have been seen in countries where vaccination is not readily available and medical care is scarce. Most cases are seen in the DRC where over 7,000 cases have been recorded [21]. Other third-world countries with high proportions of socio-economic disparities are also in the same

situation. Without high rates of vaccination and availability of medical care, the transmission of measles along with the associated mortality will increase.

In 2019, there were more measles cases reported globally than in any year since 2006, as indicated by WHO. More than 180 countries were affected and over 500,000 confirmed cases of measles were reported. More specifically, the top three countries in terms of the number of confirmed measles cases are the DRC, Brazil, and Nigeria [21].

Once a person is exposed to measles, he/she will develop symptoms of fever, cough, coryza, and conjunctivitis. The last to appear is the rash, which can present 14 days after inoculation with the virus [9]. Once infected, there is no specific treatment for the disease and symptomatic care is the mainstay of treatment. Vitamin A has been shown to decrease the severity of measles. In a study conducted to assess the effectiveness of vitamin A, it was found that at least two doses of 200,000 IU vitamin A in children greater than 1-year old, decreased the mortality by 62 percent [27]. Since there is no treatment and infection with the disease can result in life-threatening complications, post-exposure prophylaxis is imperative when someone has an unknown immunity status. The CDC recommends that those who cannot provide evidence of immunity, either by vaccination paperwork or titers, should be offered the MMR vaccine within 72 hours of exposure or given immunoglobulin (IG) within six days of exposure. However, both should not be given together [9].

Ultimately, vaccination against the measles virus is the greatest defense against the disease. From 2000 to 2017, an estimated 21 million deaths were prevented with the measles vaccination [28]. The measles vaccine is often combined with mumps and rubella vaccines and given as a combined trivalent vaccine [29]. Millions of doses of the MMR vaccine have been given and have proven to be safe and effective with minimal adverse effects, such as mild fever or rash [30]. The vaccine should be given to children in two doses to attain maximum efficacy. The first dose of the MMR vaccine given at 12 months will result in the production of antibodies in 96 percent of the recipients, with the rest responding to the second dose, and the efficacy rate of the vaccine in preventing measles is greater than 99 percent [29].

In 2010, the World Health Assembly set three milestones for Measles Prevention that were to be achieved by 2015. The milestones were to increase coverage of measles vaccines to greater than 90 percent in children 1-year old on a national level, decrease the annual incidence of measles to less than five cases per million of the population, and to reduce the overall global mortality rate by 95 percent [28]. Even though great strides have been made, and mortality has decreased, these 2015 global milestones have not been reached [28]. Although vaccination is a clear method to prevent the spread of measles, many barriers limit mass vaccination globally.

Currently, in 2020, the rampant spread of COVID-19 has resulted in many countries placing travel restrictions and essentially halting the global travel industry. This has led to a substantial decrease in cases of measles globally. In the USA, 12 cases have been reported and one in Canada this year. In a study designed to trace the global transmission dynamics of the measles virus, it was concluded that the disease can spread not just within a given region, but also between regions that have huge distance in between them, given the massive number of travelers between those regions [31]. However, continuing restrictions as a method of control for measles is neither feasible nor reasonable.

Furthermore, specific populations and their ideology against vaccination become another barrier to the eradication of diseases such as measles. The anti-vaccination movements are fueled by claims, such as those in the 1990s suggesting that the MMR vaccine was linked to autism [26]. Even though many studies have disproved these claims, there is still strong opposition to vaccination. Also, many groups cite religious obligations as their reason for refusal of vaccination. These

groups specifically state that the rubella component of the trivalent MMR vaccine was originally derived from the cells of aborted fetuses [32]. Ultimately, these challenges need to be overcome. Public health organizations and populations against vaccination, irrespective of their reason, need to dialog for the benefit of society as a whole. By doing so, further strides can be achieved in the global fight against measles, resulting in the preservation of life and preventing complications associated with measles.

5. Conclusion

As shown in this review, the incidence of increased risk of the measles infection correlates with an increase in the proportion of unvaccinated individuals, especially in children 18 years of age and younger, those with certain religious beliefs opposing vaccinations, and those who reside in underprivileged nations. It is imperative to educate the general population on the importance of vaccination and to offer immunization to the underprivileged nations at very low or no cost, to aid in the direction of eradication of this virus. The general public's distrust of vaccines needs to be met with medically backed information that allows citizens of every nation to make better-informed decisions. In Canada and the USA, most measles cases were associated with unvaccinated individuals. In most daycares and schools, in the USA and Canada, vaccination against measles is generally required for children. It can be concluded from statistical data, that the measles vaccine has shown compelling evidence towards decreasing the prevalence of this disease. Individuals who are at higher risk such as university students, healthcare workers, and individuals who travel regularly should be vaccinated to achieve meaningful success. Ultimately, vaccines reduce disease burden by directly protecting the vaccinee and by indirectly protecting the non-immune population, and through identifying these positive impacts, the giant stride towards eradicating measles and other vaccine-preventable diseases can be replicated on a global scale.

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Chapter 4

Severe Acute Respiratory Syndromes and Coronaviruses (SARS-CoV, MERS-CoV, and SARS-CoV-2)

Bradley Fevrier

Abstract

The current SARS-CoV-2 (coronavirus) outbreak has reached pandemic proportions with a large global imprint. In December 2019, COVID-19 was first reported in Wuhan, Hubei Province, China and has continued largely unabated. The SARS-CoV-2 (coronavirus) is much talked about currently; however, it is worth noting that there are several different coronaviruses known to man, with most of them being responsible for causing illness in animals. Seven (7) types of coronaviruses are identified as causing illnesses in humans. Of the seven human coronavirus infections, four involve mild upper respiratory tract complaints that produce slight symptoms of the common cold. Conversely, the other three human coronavirus infections present more severe consequences as recently demonstrated by the SARS-CoV-2. These deadly outbreaks of pneumonia can have consequences that are far-reaching and are global in nature. SARS-CoV was the first new viral pandemic of the 21st century. It had its beginnings in southern China during November 2002 having started mysteriously; It was contained in 2004 after having spread to five continents and thirty-three countries, infecting approximately 8000 people. MERS-CoV the virus that the causes Middle East respiratory syndrome (MERS) was first identified in 2012 in Saudi Arabia and Jordan and has since registered roughly 2,220 confirmed cases and 790 deaths.

Keywords: Coronavirus, SARS-CoV, CoVid-19, SARS-CoV-2, MERS-CoV, Pandemic

1. Introduction

Coronavirus (CoV) is one of the leading pathogens primarily targeting that the human respiratory system [1]. Earlier outbreaks of coronaviruses include the severe acute respiratory syndrome (SARS)-CoV and the Middle East respiratory syndrome (MERS)-CoV which have been formerly considered as a serious threat to public health [2]. The first iteration of coronavirus was identified in the mid-1960s and categorized into four separate subfamilies: α−/β−/γ−/δ-Coronavirus. Alpha and beta-coronaviruses predominantly causes infection in mammals, whereas gamma and delta-coronaviruses primarily infect birds [3]. A new contagious coronavirus is presently holding much of the worldwide population hostage. This virus,

SARS-CoV-2, which causes the COVID-19 disease, emerged in Hubei, China and has spread to most countries with ongoing devastating effects [4].

Since the era of the pneumonic plague, emergent respiratory infections have enthralled the scientific and Public communities' and more recently have manifested in popular films depicting airborne viral outbreaks [5]. This has led to deliberations pertaining to the possibility for respiratory spread of these infections [6]. Although numerous emerging agents can exhibit respiratory involvement, this chapter will focus on emerging pathogens that involve the respiratory system and focus on 3 agents that exhibit a range of characteristics of emerging diseases: SARS-CoV, MERS, and SARS-CoV-2.

2. SARS-CoV

2.1 Etiology, epidemiology, and clinical presentation

Severe acute respiratory virus (SARS) is a deadly pulmonary infection caused by the SARS coronavirus (SARS-CoV), first reported in Guangdong Province, China, in November 2002 [7]. The emergence of SARS-CoV signaled the first time the public, as well as numerous scientists, observed this cluster of viruses, and its potential to cause severe infections and death in humans [8]. By July 3, 2003, SARS global infections were 8439 cases of which 812 were fatal [9]. This prompted a full-bodied international response estimated at roughly 40 billion dollars which aided in containing the outbreak [10]. By the close 2004 there were no new reported cases [9, 10]. Genetic classification indicates that the introduction into the human population took place from civets or other mammals found in live-animal markets of China [9]. Furthermore, it is prevailingly considered that SARS-CoV originated in a colony of horseshoe bats in southern China, with civets acting as the intermediate amplifying and transmitting host to humans [11].

3. SARS-CoV infection in humans

SARS-CoV is an airborne virus transmissible between humans through small respiratory droplets, in a similar manner to influenza [6]. SARS-CoV can also be spread indirectly via surfaces that have been touched by someone who is infected with the virus, and by close interactions with infected individuals acting as so-called "super spreaders" [6]. The incubation period of SARS-CoV is generally 2–7 days, but infected persons may present symptoms as long as 10 days after infection [6]. Several epidemiological studies conducted during the outbreak identified numerous deaths occurring disproportionately among the elderly, and individuals who were immunosuppressed. At the onset of SARS-CoV illness, patients present with flu-like symptoms typically non-specific, with mild respiratory symptoms identified as most common in some cases, while other symptoms included rash, malaise, fever, and myalgia [12, 13]. Approximately 70% of the SARS-CoV patients experience shortness of breath and lingering or persistent fever, while clinical improvements were observed in 30% patients after the first week [14]. Intensive care treatments such mechanical ventilation was required by about 20 to 30% of SARS-CoV patients [14, 15]. Individuals 12 years of age and younger displayed limited severe disease manifestations [6, 13, 16]. Prognostic studies indicate greater risk of severe outcomes associated with increased age, high pulse, and lactate dehydrogenase (LDH) levels [7, 17, 18].

4. Pathological changes and clinical diagnosis in SARS-COV infection

Histopathologic data existing on SARS-CoV patients have been mostly determined from autopsy cases. Pathological lesions in certain organs of SARS-CoV victims, such as the lungs and intestines, have been extensively studied [1, 4]. The primary pathological change in SARS-CoV patients occurs in the lungs [4, 6]. Gross examination of the lungs revealed edematous, heavy lungs weighing up to 2100 g with several areas of extensive consolidation (**Figure 1**) [1, 4].

Histopathologic data for SARS-CoV of infected lungs characteristically displayed diffuse alveolar damage [DAD] [19, 20]. Through the initial period of the disease (7 to 10 days), SARS lungs exhibited the following characteristics of acute exudative DAD: 1) Widespread edema, 2) desquamation of alveolar epithelial cells, 3) formation of hyaline membrane, 4) collapse of alveoli, and 5) fibrous tissue in alveolar spaces (**Figure 2**) [6, 12, 19, 21, 22].

In SARS cases of lengthier disease duration, fibrous organization features of DAD were visible after approximately 10–14 days. These features included interstitial and airspace fibrosis and pneumocytic hyperplasia [12, 23, 24]. The more extensive the disease period, the more widespread the fibrous organization of the lung tissue [14, 25, 26]. Dense septal and alveolar fibrosis were exhibited in SARS cases with duration of more than 2 to 3 weeks [12, 19, 23, 24]. The overall histological data presentation of SARS lung infection is non-specific and dependent on symptom onset; Acute DAD is most frequently associated with early phase disease (<10 days) [6, 27]. Furthermore, there is limited documentation on the pathologic demonstration of SARS-CoV in living patients, since the bulk of patient tissue samples were taken from autopsy [1, 4, 6].

The predominant changes involving SARS-CoV cases have been visceral and involve severe pulmonary changes [1, 19]. Accurate and easily implementable diagnostics formed an essential part of SARS-CoV disease control, due to the non-specific nature of the infection and its rapid spread. Following the initial disease outbreak, many laboratories rapidly developed SARS-CoV reverse transcription polymerase chain reaction test (RT-PCR) analyzes, to detect viral RNA. These tests have numerous advantages over traditional RT-PCR tests [28].

Real-time RT-PCR assays use amplification primers and internal probes as a result, can be designed to be extremely precise for SARS-CoV RNA [6]. Real-time

Figure 1.
SARS gross morphology of the lung [19]. Images ©John Wiley and Sons Ltd. as cited.

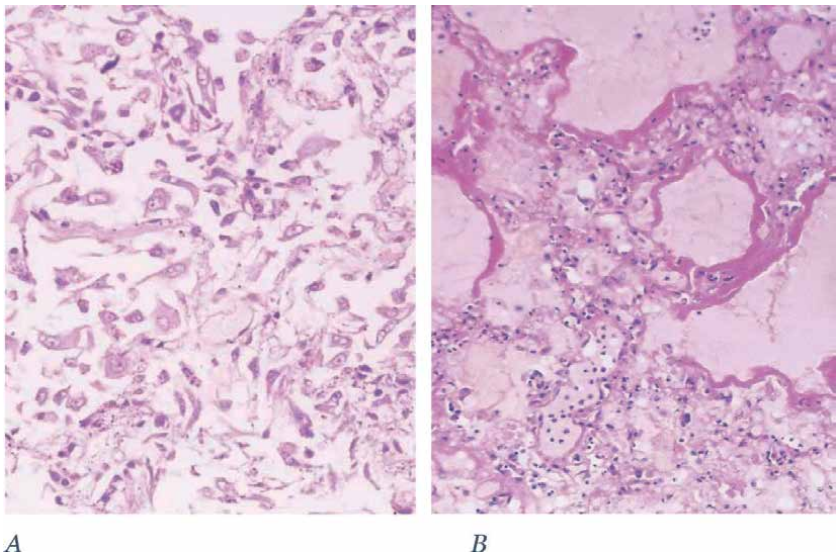

Figure 2.
(A) Alveoli filled with desquamated epithelial cells. (B) Formation of hyaline membrane (H&E, original magnification ×200).

RT-PCR analyzes can be extremely sensitive, with steady detection limits of between 1 and 10 SARS-CoV RNA copies per reaction [6, 29]. They can be completed quicker than traditional RT-PCR analyzes with reduced risk of contamination in the laboratory. Real-time RT-PCR assays often times give a very accurate estimate of the viral load present in a sample [29].

5. MERS-CoV

5.1 Etiology, epidemiology and clinical presentation

The Middle East respiratory syndrome coronavirus (MERS-CoV or MERS) was first identified in September 2012 in a fatal case of severe respiratory failure in a Saudi Arabian patient [30, 31]. Previous cases were retrospectively acknowledged from an outbreak of severe respiratory illness in Jordan in 2012 [32]. In contrast to the rapid spread and subsequent latency of SARS-CoV, MERS-CoV has moved continuously through the Arabian Peninsula and generated sporadic outbreaks in countries where infected persons have traveled [6]. As of January 2020, there have been 2519 laboratory-confirmed cases of MERS, and 866 associated deaths (case-fatality rate: 34.3%) reported globally [32].

A significant number of cases has been identified in Saudi Arabia and to a lesser extent the United Arab Emirates (UAE), Qatar and Jordan [33]. While the close relationship to numerous bat coronaviruses suggests a bat-related origin, overwhelming molecular and serological evidence points to the involvement of dromedary camels in the transmission of MERS-CoV to a human host [34]. While transmission from ancestral bats to camels cannot be excluded, and camels may have introduced the virus into human populations, the majority of reported MERS-CoV cases have ensued from human-to-human nosocomial transmission [6, 33].

A hospital outbreak was reported in Saudi Arabia with a cluster of six cases; Three of the cases were healthcare workers, two were patients (one of whom died) and one was a visitor. Another instance involved an ill patient admitted to a Korean

hospital which led to an outbreak of 186 infections including 36 fatal cases [35]. Person-to-person transmission has also been identified within households, where the highest risk of transmission involves patient respiratory secretions and individuals being within close proximity with each other. Individuals exhibiting signs and symptoms or other epidemiological characteristics suggestive of MERS should be promptly quarantined and tested for viral infection [32, 35].

6. MERS-CoV infection in humans

The clinical manifestation for MERS-CoV infection varies from asymptomatic/mild to severe disease. Generally, individuals with chronic comorbid conditions (diabetes, heart disease) and elderly patients are at increased risk for development of respiratory failure [35]. Although infection is commonly associated with respiratory disease, in some rare cases viral RNA has been discovered in blood, stool and urine signifying a systemic infection [33, 35].

Notwithstanding the increased mortality related to symptomatic cases, research studies have shown that roughly 25% of patients infected with MES-CoV are asymptomatic [36]. A seroepidemiological analysis of over 10,000 infected samples from Saudi Arabia revealed positive antibodies in approximately 0.15% of patients. Individuals with some level of camel-exposure had an increased likelihood of positive serology [33, 35]. Clinical symptoms are non-specific, and patients have reported an expansive range of diverse indicators including chest pain, fever, cough, myalgia, sore throat, shortness of breath, vomiting and diarrhea [32]. In more severe cases, mechanical ventilation is required for patients who are presented with acute hypoxic respiratory failure [15, 31]. Fatal outcome of MERS-CoV infection have been associated with underlying comorbidities such as hypertension, diabetes mellitus type II, obesity, and cardiac disease [37]. MERS-CoV infection has an incubation period that ranges from 2 to 14 days [30]. Signs and symptoms usually appear well before the patient reaches a detectable viremia i.e. the virus is present in the patients' bloodstream [30, 37]. Neurological sequelae, and gastrointestinal distress have also been documented in addition to these respiratory symptoms [37].

7. Pathological changes and clinical diagnosis in MERS-COV infection

The understanding of the pathological findings related to MERS-CoV infection have relied on a paucity of autopsy cases. Notwithstanding the limited number of autopsy cases, several studies have assessed the pathological features of MERS-CoV infection in human tissue. The pathogenesis of MERS-CoV infection in human tissue ex vivo revealed exudative diffuse alveolar damage (DAD) with hyaline membranes, interstitial pneumonia (which was primarily lymphocytic), pulmonary edema, multinucleate syncytial cells, and type II pneumocyte hyperplasia [38]. Researchers also observed bronchial submucosal gland necrosis in diseased lung tissue, where these bronchial lesions make up the pathologic origin for respiratory failure and radiologic anomalies of MERS-CoV infection [38]. Some of the cells in the lungs targeted by the MERS-CoV infection include: pneumocytes, multinucleated epithelial cells, and bronchial submucosal gland cells [39]. Microstructurally, viral particles were discovered in the pulmonary macrophages, pneumocytes, renal proximal tubular epithelial cells and macrophages infiltrating the skeletal muscles [38, 40]. Consistent with the microstructural results in the kidney, renal biopsies revealed acute tubulointerstitial nephritis and acute tubular sclerosis with proteinaceous cast formation [40].

Researchers discovered comparable replication of kinetics and cellular tropism in a study comparing the replication of camel-isolated MERS-CoV strains to human-isolated MERS-CoV strains. Non-ciliated bronchial epithelium and alveolar epithelial cells including type II pneumocytes were infected by all strains. It is important to note that no infection of the pulmonary macrophages was present [39]. Infection of several cell types including vascular endothelial cells, renal tubular cells, and podocytes was established in studies examining kidney explants [41]. Exploratory infection of small intestine tissue samples with MERS-CoV confirmed that infection was restricted to the surface enterocytes and formation of syncytial cells [6]. It has been observed that infected patients shed virus in their urine and stool, which is consistent with these findings.

RT-PCR has functioned as the main clinical laboratory diagnostic test throughout transmission events. Critical to the success of these tests is an understanding of the viral kinetics and tissue tropism discovered in MERS-CoV cases. Numerous studies have acknowledged that lower respiratory tract samples contain the highest viral loads, while upper respiratory swabs, whole blood or serum, feces, and urine may also contain significant viral load [35]. Samples from the upper respiratory tract, urine and blood may offer further diagnostic usefulness by delivering a convenient sample type, notwithstanding 10 to 100 times lower virus levels. Measurable viremia at the point of diagnosis has been linked with an increase in patient death due to the necessity for mechanical ventilation, despite blood only being positive in approximately one-half to one-third of cases [42]. The reduced viremia rate in MERS-CoV samples in comparison to SARS-CoV is significantly different, where RT-PCR on blood can be beneficial for preliminary diagnosis and is normally the primary positive site identified. Analyses of upper and lower respiratory samples as well as blood samples for MERS-CoV patients, has shown that it may benefit in maximizing the sensitivity while also stratifying risk [34]. Two RT-PCR testing approaches were approved for emergency use authorization by the FDA during the MERS-CoV outbreak: both targeted a region upstream of the envelope gene (principal target of the humoral immune response). Of these two tests, one additionally targets a specific region of the ORF1a gene, while the other targets two regions inside the nucleocapsid gene [6].

MERS-CoV serology tests share comparable kinetics to that of SARS-CoV infections. About 2–3 weeks following the onset of symptoms, a significant number of patients develop measurable levels of IgM and IgG antibodies. However, in many cases the detection of IgG has superior diagnostic value when compared to IgM [34]. Some researchers posit that if serologic testing is used to detect current infection, "a neutralization assay and 4-fold increase in titer after 14 days should be used to confirm a specific immune response" [6, 42]. Disease severity may affect antibody responses as numerous studies have established; PCR-positive patients exhibiting only mild disease symptoms often do not generate measurable quantities of antibodies, especially when monitored during the post-acute phase of disease [34].

8. SARS-CoV-2

8.1 Etiology, epidemiology, and clinical presentation

The coronavirus (SARS-CoV-2) (also known as the novel coronavirus) outbreak has reached pandemic proportions with a large global footprint [43, 44]. In late December 2019, SARS-CoV-2 was first reported in Wuhan, Hubei Province, China among clusters of patients with pneumonia of unknown etiology [43, 44]. In early

January 2020, the National Health Commission of People's Republic of China released information regarding the causative agent of an enigmatic pneumonia identified as a novel coronavirus (SARS-CoV-2). The novel coronavirus (SARS-CoV-2) was verified by several independent laboratories located in China [45, 46]. The World Health Organization (WHO) provisionally named the causative virus as 2019 novel coronavirus [2019-nCoV/SARS-CoV-2] [46]. Coronaviruses are known to cause respiratory, hepatic, and neurologic diseases and are generally spread among humans and animals [3]. The SARS-Cov-2 virus is illustrated by a spherical shape, and a characteristic "crown" appearance, and they belong to the family of coronaviruses of positive-stranded RNA viruses [47].

Genetically, SARS-CoV-2 has a closer resemblance to SARS-CoV than the Middle East respiratory syndrome coronavirus [MERS-CoV] [48]. Nevertheless, the span of the incubation period, clinical severity, and transmissibility of SARS-CoV-2 differs from SARS-CoV [49]. Public health and government efforts aimed at curbing the spread by implementing social practices through social distancing, mask wearing, isolating/quarantining and non-pharmacological and preventive treatments for psychophysical wellbeing, has been relatively successful in part, but SARS-CoV-2 has continued to increase globally [50, 51]. By the end of January 2021, SARS-CoV-2 accounted for more than two million deaths and more than 100 million confirmed cases of the disease [52]. Radiologically, SARS-CoV-2 has distinctive imaging features that constitute a visual identity. Besides, SARS-CoV-2 negatively impacts other organs in addition to the lungs. As a result of these developments, SARS-CoV-2 has grown exponentially with nearly 2000 articles being published per week [50].

9. SARS-CoV-2 infection in humans

SARS-CoV-2 infections are variable in nature, with some infections being asymptomatic with others causing minor to moderate illness with respiratory and flu-like symptoms, including sore throat, fever, chills, and cough [53]. Injury, inflammation and ensuing respiratory distress in SARS-CoV-2 patients occurs as a result of the SARS-CoV-2 spike protein binding to human angiotensin I-converting enzyme 2 (hACE2) predominantly targeting the virus to type II pneumocytes inside the lung [54, 55]. A substantial number (approximately 20%) of patients also develop severe infection and multi-organ failure which necessitates intensive care with mechanical ventilation or extracorporeal membrane oxygenation [50, 53]. In some cases, SARS-Cov-2 infection can be deadly, with a case fatality rate of ~5%. The incubation period of SARS-CoV-2 is generally 5–7 days, but the symptoms of infection may present itself well after that period [56]. The phase from the onset of symptoms to fatality usually varies from 7 to 40 days with a median of 14 days [57]. This phase is dependent on the patients' age, and the status of their immune system.

Similarities in the symptoms between SARS-CoV-2 and earlier beta-coronavirus such as fever, dry cough, and dyspnea are distinctive [50]. However, there are distinctive features presented by SARS-CoV-2 which involves affecting of the lower airway as evident by upper respiratory tract indicators like sneezing, rhinorrhoea, and sore throat [58]. Additionally, chest radiograph results taken upon admission, show an infiltrate in the upper lobe of the infected lungs, associated with increased difficulty breathing (dyspnea) resulting in low levels of oxygen in the blood (hypoxemia) [58]. Notably, while most SARS-CoV-2 patients exhibit gastrointestinal symptoms like diarrhea, very few MERS-CoV or SARS-CoV patients show similar gastrointestinal concerns. Thus, testing fecal and urine samples to exclude a potential alternative route of transmission among patients and healthcare workers [57].

10. Pathological changes and clinical diagnosis in SARS-COV-2 infection

Nasal droplets and saliva from infected patients function as the leading route of SARS-CoV-2 virus communicability [59]. According to Heydarloo et al., the virus accesses the alveolar-type 2 cells (AT2 cells) by attaching its viral spike (S1 and S2) proteins to the angiotensin-converting enzyme 2 (ACE2) receptor [60]. Researchers found that previous iterations of coronaviruses specifically SARS-CoV, replicated more aggressively in alveolar-type 2 cells than in alveolar type 1 cells in the lung [38]. This is significant since it has been reported that there is an 80% genetic similarity between the SARS-CoV and SARS-CoV-2 viruses [61]. SARS-CoV-2 has an extraordinary potential for binding with AT2 cells in the lungs as shown via molecular pathways [62].

The SARS-CoV-2 pandemic continues to affect much of the world and understanding its clinical diagnosis is important. Data on diagnostic testing for SARS-CoV-2 is still in its infancy, as such, understanding these tests and interpreting their results is imperative. The most frequently administered and dependable test for SARS-CoV-2 diagnosis thus far, has been the RT-PCR test completed using nasopharyngeal swabs. In some cases, alternative upper respiratory tract samples, comprising throat swabs and/or saliva have been used. Individual companies focus on a variety of RNA genes, with a significant number of tests affecting 1 or more of the envelope, RNA-dependent RNA polymerase (RdRp), and ORF1 genes [63].

In most SARS-CoV-2 patients with symptomatic infection, viral RNA in the nasopharyngeal swab becomes detectable as early as day 1 of symptoms and peaks within the first week of symptom onset. The cycle threshold (Ct) that is used to measure viral RNA, can be defined as "the number of replication cycles required to produce a fluorescent signal, with lower cycle threshold values representing higher viral RNA loads" [63]. A PCR positive is typically clinically reported as a Ct value of less than 40. By week three of infection, there is usually a decline in this positivity and subsequently becomes unnoticeable. In severely ill hospitalized SARS-CoV-2 patients, the cycle threshold values are lower than the cycle threshold values recorded in less severe cases. It is important to note, a "positive" PCR result reveals only the recognition of viral RNA and does not automatically suggest presence of viable virus [62]. It has been reported in a minority of positive test cases that viral RNA was detected by RT-PCR past week six. There have also been instances of a positive result being reported after consecutive negative PCR tests completed two days apart. Currently, it is unclear whether this is a testing error, reinfection, or recurrence.

SARSCoV-2 infection can also be identified indirectly by assessing the patients' immune response to infection. In patients who exhibit mild to moderate symptoms, serological diagnosis becomes extremely important past the first two weeks of illness onset. Serological diagnosis is an essential means of understanding the scope of SARSCoV-2 infection in the community and may assist in identifying individuals who are immune/protected from infection.

11. Conclusion

Wide-ranging efforts to decrease transmission of SARSCoV-2 infection are crucial to controlling the present epidemic. Lessons learned from the SARS-CoV and MERS-CoV outbreaks offer, valuable experiences and insights into how to fight the SARSCoV-2. Specific consideration aimed at decreasing spread must be applied in vulnerable populations specifically health care workers, and the elderly. Additionally, research into the pathogenesis of human coronavirus infection is crucial for finding suitable therapeutic objectives. Presently, no specific antiviral drug is available for SARS-CoV, MERS, and SARSCoV-2.

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Chapter 5

Chikungunya Neurological Manifestations: A Systematic Literature Review

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Abstract

Although the most common Chikungunya (do not capitalize the disease unless it is named after a proper noun such as Zika, Ebola or Carrion's Disease) manifestations are osteoarticular, those which bring the most morbidity and mortality are neurological, where thorough mapping through studies with a methodological outline have not yet been well structured. Therefore, the objective was to review the literature to identify neurological manifestations of CHIKV. We used the Virtual Health Library (VHL) and PubMed with the following descriptors: #1 "*Chikungunya*" [MeSH]; #2 "*neurological manifestations*" [MeSH] and their equivalents in the Portuguese language, selecting literature published between July 2007 to January 2018. From the 180 studies that were found, 30 were selected. Findings were divided into two subcategories: "Chikungunya: Typical Neurological Manifestations" and "Chikungunya: Severe Neurological Manifestations". The studies show that headaches were characterized as the most common symptom in adult patients affected by CHIKV, followed by meningeal involvement. Meningeal involvement is also a more serious clinical scenario associated with encephalitis, convulsions, polyneuropathies such as Guillain-Barré syndrome and death. CHIKV is a public health problem for many reasons including its chronic potential complications. Given the neurological symptoms, this disease is concerning in age extremes, for patients with comorbidities and for patients with more than one viral infection by arboviruses, in whom the most severe neurological manifestations are more common.

Keywords: Arboviruses, Chikungunya, Clinical Practice, Neurology, Systematic Review

1. Introduction

> *"In a true and perfect form, imperturbability is indissolubly associated with wide experience and an intimate knowledge of the varied aspects of disease"*
> *(Sir William Osler)*

Chikungunya virus is an *alphavírus* transmitted by arthropods, especially by mosquitos from the *Aedes* genus, being endemic in tropical areas in Africa and Asia. The virus is capable of causing large outbreaks in regions where the population has not previously immunized [1]. (Given the fact that there is not a currently available vaccine, is "immunized" the right term to use. I understand that immunity can occur through previous infection, but "immunized" often implies intetional vaccineation.) In Brazil, the first autochthonous cases of the disease were identified in Oiapoque, state of Amapá (North), and Feira de Santana, state of Bahia (Northeast), in September 2014 [2]. According to data from the Brazilian Ministry of Health, in 2017 up until epidemiological week 25, 131,749 probable cases of CHIKV were registered in the country with an incidence rate of 63.9 cases/100,000 inhabitants; of these, 66,576 (50.5%) were confirmed in the state of Ceará (893.0 cases/100,000inhabitants) [3]. During the year of 2018, Brazil registered 87,867 probable cases of Chikungunya in Brazil, with the Northeast region presenting 11,287 (12.9%) of these and the state of Ceará having an incidence of 17.6/100,000 inhabitants [4]. Chikungunya can evolve in three phases: acute with persistent symptoms up to 14 days, subacute with the sustainment of symptoms for up to three months and chronic, a phase in which symptoms last for more than three months [5]. Chikungunya commonly presents with symmetrical arthralgia and/or polyarthritis, disabling of hands, wrists, ankles, knees and feet and the associated symptoms of asthenia, myalgia, headache, nausea/vomiting, diarrhea, photophobia, retro-orbital pain, conjunctivitis, pruritic maculopapular rash, facial/peripheral edema and lymphadenopathy [6].

Between 20% and 50% of patients infected with CHIKV develop chronic arthralgia. Predicting who will develop chronic diseases is difficult because there are no markers for these [7]. The frequency of serious cases is 0.3%, being associated with a higher age (>65 years old) and the presence of comorbidities [8]. The most concerning manifestations are neurological (encephalitis, meningoencephalitis, myelitis, Guillain Barré syndrome), and bullous cutaneous and myocarditis [9]. Thus, although Chikungunya is frequently described as a self-limiting disease, rare, more severe disease forms have been observed with CHIKV, some beingassociated withdeaths [1].

Therefore, the purpose of this study was to perform a systematic review to determine the typical and serious neurological manifestations caused by Chikungunya. Our hypothesis is that, despite advances and epidemiological studies, as well as screening, neurological manifestations are more disabling and serious, and therefore require more attention from the scientific community.

2. Material and methods

A systematic review of the literature using the Virtual Health Library (VHL), which hosts recognized databases, and PubMed was performed. Initially, the following descriptors were used: #1 "*Chikungunya*" [MeSH]; #2 "*neurological manifestations*" [MeSH], as well as their equivalents in the Portuguese language.

The period reported in the literature ranged from July 2007 to January 2018 due to the scarcity of articles on the subject. Compilation of the data was performed in January and November of 2018. Manuscript selection occurred primarily through the analysis of titles and abstracts. Article analysis followed the eligibility criteria:

Authors (year)	Sample (N)	Main findings
Khatri [10]	One (64-year-old male)	Encephalomyelitis with quadriparesis and urinary retention
Hossain et al. [11]	1,326	Headache 165 in confirmed cases and 860 in probable cases
Hamilton and Cruickshank [12]	One (77-year-old woman)	T2 Weighted Imaging Fluid Attenuated Inversion Recovery (T2WI/FLAIR) changes in the right medial temporal lobe one year left for the acute infection
Huits et al. [13]	269	Headache (124) and the joints involved 45 are spine
Mahto et al. (2018)	Two	One patient had Guillain-Barré syndrome (GBS) with bilateral lower motor facial nerve palsy, and meningoencephalitis with epidermal necrosis
Mehta et al. [14]	Five	Paraesthesia and triparesis, hyperreflexic; urinary retention; confusion, dysarthria, headache, neck stiffness; spastic paraparesis; extensor plantars, palmomental reflex. Confusion, 1 x seizure, drowsiness, dysarthria. GSB. Hemiparesis. Confusion, impaired speech and swallow; flaccid hyporeflexic quadraparesis
Puccione-Soler et al. (2018)	One (69-year-old woman)	Slow thinking, inattention, and mild confusion
Cerny [15]	1196	encephalitis, optic neuropathy, neuroretinitis, and GBS
Méndez et al. [16]	830	Headache (633), photophobia (48), lethargia (1)
Sá et al. [17]	Four	Two patients had generalized tonic convulsive crises and a diminished level of consciousness. In third patient the ct revealed bilateral frontal hypoattenuation, whereas abnormal cellularity and elevated protein levels were detected in a csf analysis. The fourth patient had decreased level of consciousness and uncontrollable movements.
Acevedo et al. [18]	11	GBS. Symmetric motor polyneuropathy. Motor and sensory axonal neuropathy. Meningitis and Encephalitis. Meningoencephalitis
Langsjoen et al. [19]	194	Headache (7)
Torres et al. [20]	1069 (newborns)	Hyperalgesia/allodynia (97), Meningoencephalitis (12).
Macpherson et al. [21]	493	Headache (265)
Pinheiro et al. [22]	Three articles	One case of Encephalitis, one case of Meningitis and one case of GBS
Feldstein et al. [23]	1,929	Headache (316)
Kageguka et al. (2016)	381	Headache (92)
Mohite and Agius-Fernandez [24]	One (69-year-old woman)	Acute unilateral optic neuropathy as a delayed complication of Chikungunya virus (CHIKV) infection
Peper et al. (2016)	One (39-year-old female)	Headache and photophobia
Marimoutou et al. (2015)	646	Headache 39 in confirmed cases
Anderson et al. [25]	One (36-year-old)	Headache
Malik et al. [26]	10,715	Headache (5464)
Ramanchandran et al. (2014)	403	Headache (261)
Thiberville et al. [27]	76	Headache (38)

Authors (year)	Sample (N)	Main findings
Taraphdar et al. [28]	550	Headache (53)
Dupuis-Maguiraga [29]	876 (adults, children, and newborns)	Headache (613), Meningoencephalitis (140), SGB (8), Convulsion (192), encephalitis (52)
Mohan et al. [30]	2602	Headache (319), Consciousness altered (4), Photophobia (36)
Sissoko et al. [31]	1154 participants being 318 had confirmed chikungunya	Headache (258)
Singh et al. [32]	Ten cases of flaccid limb weakness following symptoms and signs suggestive of CHIKV	Four cases of flaccid limb weakness confirmed (4)
Ganesan et al. (2008)	AJNR Am J Neuroradiol	Three patients with CHIKV in CNS

Label: Guillain-Barré syndrome = GBS.

Table 1.
Main findings.

(1) At least a combination of the terms described in the search strategy were present in the title: (colon) (2) Articles were written in English, Portuguese or Spanish; (3) Articles addressed Chikungunya neurological manifestations; (4) Original articles with the full text available through the CAPES (Coordination of Personal Improvement of Higher Level) Periodicals Portal, a virtual library created by the Brazilian Ministry of Health where content is restricted to authorized users. Revision studies, letters to the editor, prefaces, brief communications corrections/recalls, comments, editorials, monographs, dissertations and theses were excluded. (It seems that corrections or recalls would be important if any addressed the manuscripts cited in the review.) Manuscripts that were repeated in more than one of the databases were counted only once. Some articles were excluded because they generally addressed arboviral disease symptomatology and/or showed *in vitro* or animal alterations. Since this is a systematic review, Resolution 510/16 of the Brazilian National Health Council (CNS) ensures the dispensation of submission to a Human Beings Research Ethics Committee.

To ensure trustworthiness of the findings, data collection was performed individually by two researchers with divergences being solved by a third senior researcher.

Each sample article was thoroughly read and the information was inserted in a spreadsheet (**Table 1**), including the author and publishing year; study sample and main study findings (PICOS). This review followed the Prism Protocol (http://www.prisma- statement.org/).

3. Results

According to the search strategy, from the 234 articles found, 30 were selected after the eligibility criteria were applied (**Figure 1**). These were then input in **Table 1**. The findings were then divided into two subcategories: "Chikungunya: Typical Neurological Manifestations" and "Chikungunya: Severe Neurological Manifestations".

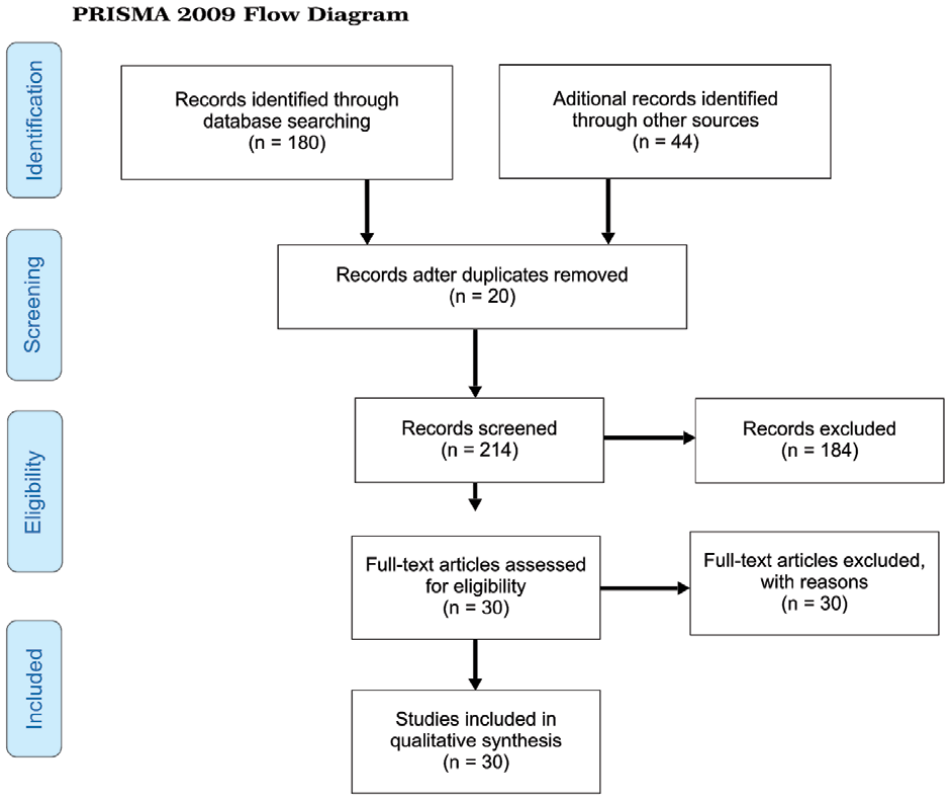

Figure 1.
PRISMA flow diagram (reproduced by Moher et al., 2009).

The studies [11, 14, 16, 19, 21, 23, 25–28, 30, 33–35] have shown that headache has been characterized as the most common symptom in adult patients with CHIKV, followed by meningeal involvement (meningitis or meningoencephalitis) [18, 20, 22], changes in consciousness level [17, 30, 36] and visual changes such as photophobia [16, 30, 33].

The most serious symptoms in patients exposed to CHIKV infection were central nervous system involvement: meningitis, encephalitis, meningoencephalitis [10, 14], seizures [12, 17]; and effects on the peripheral nervous system such as polyneuropathy - Guillain Barré syndrome [32] in addition to deaths [17, 18].

4. Discussion

4.1 Chikungunya: typical neurological manifestations

Our findings are in accordance with recent clinical studies published in Dehli [37]. Headache was the most common manifestation observed in the clinic, followed by meningoencephalitis which was consistent with the study by Huits et al. [13]. According to the authors, 73% of patients with CHIKV reported headache as a symptom. Sissoko et al. [31] reported a similar prevalence of headaches (81.4%) in a field evaluation during a Chikungunya outbreak in Mayotte.

Because Chikungunya is a febrile syndrome, it is common for a systemic cytokine elevation to displace the hypothalamic thermostat and thereby cause systemic signs of acute febrile infection. According to Chow et al., during an acute infection

arising from viremia, the production of alpha-interferon and IL-6, IL-1Ra, IL-12, IL-15, IP-10 and MCP-1 is observed. This Cytokines cause collapse accompanies a decrease in viral load, usually within 14 days [38].

On the other hand, studies in India and the Reunion Islands and Calcutta, the French colony in the Pacific Ocean, presented meningoencephalitis as the most common manifestation. Interestingly, other authors have also shown increased incidences of encephalitis in Asia [39, 40]. In Ecuador, 37.5% of patients with CHIKV viral RNA samples in their cerebrospinal fluid (CSF) had meningitis or encephalitis and 18.7% GBS [18]. A study carried out in the city of Rio de Janeiro, Southeastern Brazil, showed that of 212 cases of arboviruses (Zika and Chikungunya), 24.1% presented meningoencephalitis [18].

The involvement of the central nervous system seems to be associated with the patient's previous comorbidities [14, 15, 18, 41] and to the extremes of age [12, 29].

Studies with newborns have demonstrated hyperalgesia as the main symptom of neonates exposed to vertical transmission with an incidence ranging from 53–94% [20]. According to Castro, Lima and Nascimento [42], peripheral neuropathy with the predominance of a sensory component is the most common presentation in CHIKV, rarely being motor, and this would be explained in large part by the nervous compression process caused by CHIKV thus manifesting as pain and/or paresthesia.

4.2 Chikungunya: serious neurological manifestations

The most serious CHIKV cases are believed to be associated with tropism of the virus by to central nervous system (CNS) cells and, once again, with the extremes of age. According to recent data from experimental studies, CHIKV may attack the CNS by two mechanisms: direct injury and immune-mediated injury. Oligodendrocyte and astrocyte cultures displayed high susceptibility levels to CHIKV [22]. CHIKV has also previously shown tropism to the choroid plexus, cerebrospinal fluid, meninges and epididymal cells of fetuses, as well as extrauterine life. An increase in the incidence of encephalopathy in fetuses of mothers exposed to the virus during pregnancy has also been previously reported. In addition, its presence is believed to induce an immune mediated response by IL-6, G-CSF, GM-CSF, MCP1, TNF-α, CXCL9, CCL2 and CXCL10 resulting in immune response lesions [38]. The theory is that glial cells may have several pattern recognition receptors involved in viral particle detection, which would make them more sensitive to the virus's presence; in contrast, they may also have the potential production of antibodies which could cause great damage, providing an expressive synthesis of cytokines and chemokines in the presence of CHIKV [22]. An indication of this is the report of an acute unilateral optic neuropathy case as a late complication of CHIKV viral infection published in 2015 [24].

Another important factor which has been observed is the presence of co-infections by other arboviruses such as dengue and Zika [18]. In these cases, the aforementioned process is probably intensified, increasing therefore, the potential for CNS injury.

5. Final considerations

Chikungunya is a public health problem due to its capacity to develop chronic complications, causing morbidity and mortality given the severity of atypical manifestations. In the context of neurological manifestations, it appears that meningitis, meningoencephalitis and encephalitis are both common and severe complications, which indicate a worrying deleterious potential of this disease to the CNS,

especially in more vulnerable groups: extremes of age; patients with comorbidities; and patients with more than one viral infection by arboviruses.

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Conflicts of interests

The authors declare that there is no conflict of interests in this study.

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Authors' contributions

JGJ, ELC, LOC, LFCM, LRM, MOB, MMMP, MLRN and GVL were responsible for the study design, conducting the systematization and analysis of the data. All authors read and approved the final manuscript.

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Chapter 6

Herpesviridae and microRNAs

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Abstract

MicroRNAs (miRNAs), first discovered in the year 1993 in the nematode *C. elegans*, are small, approximately 22-nucleotide-long, non-coding RNAs that regulate gene expression. Cellular miRNAs have been implicated in the control of many biological processes, and their dysregulation is associated with different diseases. They can be significantly up/downregulated upon infection or disease, serving as excellent biomarkers and therapeutic targets. Several human DNA viruses, including many herpesviruses, have now been reported to encode viral miRNAs. There are a variety of possible interactions and mechanisms of viral microRNAs (vmiRNAs) which are yet to be remains obscure. Viral miRNAs can function as orthologs of cellular miRNAs and regulate their expression. Additionally, viruses have also developed vmiRNA mechanisms to avoid being targeted by the host miRNAs. Herpes Simplex Viruses (HSV-1 & HSV-2) cause genital and oral herpes, establishing lifelong latent infections in their hosts, and it is one of the most prevalent sexually transmitted infections (STIs) worldwide. vmiRNAs play essential roles in Herpesvirus biology. In this chapter, we will discuss the current knowledge about miRNAs and their role in different stages of Herpesvirus infection. It will also elaborate the biomarkers, therapeutic potential of these molecules, and the prospective areas of future research.

Keywords: Herpesviridae, HSV, miRNA, miRNA biogenesis, HHV, KSHV

1. Introduction

Eight out of the hundred reported herpesviruses, from the family herpesviridae (herpein meaning, "to creep"), cause lytic and latent infections in the humans. The human herpesviruses are classified into the alpha-, beta- and the gamma-herpesviruses, based on the range of hosts they infect. The alpha (α)-herpesviruses, involving the herpes Simplex Virus –1 (HSV-1), HSV-2 and the varicella zoster virus (VZV), are known to infect a broad range of hosts while having a short replication cycle in these hosts. The beta (β)-herpesviruses include the members, the human cytomegalovirus (HCMV) and the reseolo-human herpesvirus –6 and – 7, and infect a restricted range of hosts as compared to the α-herpesviruses while having a longer replication cycle within these hosts. The third group known as the gamma (γ)- herpesviruses, containing the Epstein–Barr virus (EBV) and the Kaposi's sarcoma associated herpesvirus (KSHV), have the most restricted host range amongst the three sub-categories of human herpesviruses [1]. As reported for the year 2016, 13.2% of the global population aged 15 to 49 years were harboring HSV-2 within themselves, whereas about 66.6% amongst the 0 to 49 years aged individuals had HSV-1 infection [2]. Persons infected with HSV-2 are at 3 times to risk of infection with HIV compared to persons who are not infected with HSV-2 [3]. This may or

may not be due to a biological process as implied, but behavioral and the specific populations are vulnerable to infections with both the viruses. Both HSV-1 and 2 have an envelope of lipid-bilayer encasing them and have a double-stranded DNA (~152 kb) as their genetic material. The 12 glycoproteins in the outer layer participate in the entry of the HSV into the cell. The viral genes are expressed in an orderly fashion with the immediate early (IE) genes expressed first, which encode the proteins for the regulation of the viral replication. This is followed by the expression of the early (E) genes, which encode for the enzymes involved in the replication process. Finally, the expression of the late (L) genes takes place, which encode for the structural proteins of HSV [4]. The completion of the replicative cycle results in the generation of assembled virions which are transported via the endoplasmic reticulum/Golgi cargo transport system to the cell membrane, where the virions are released by acquiring a part of the host's cell membrane. HSV infection leads to pain and suffering, which although not always, may be lethal to the host. HSV-1 mainly causes the stromal keratitis in the eye whereas HSV-2 is responsible for genital lesions [4]. No vaccines against HSV are available for public use, although some are undergoing clinical trials. Therefore, drugs like Acyclovir, Valacyclovir and Famciclovir are the only therapeutic solutions available, which are associated with side-effects and limitations in bioavailability [5]. Thus, more suitable therapeutic agents, in terms of optimal bioavailability and diminished adverse effects, is the need of the hour.

MicroRNAs (miRNAs) on the other hand, are gaining a growing attention from the scientific community as the self-molecules which are the key regulators in infection and disease. These 20–24 nucleotides are non-protein coding RNAs which act post-transcriptionally to regulate the expression of the genes [6]. Since its discovery, miRNAs have found their significance in the diagnostics and therapeutics of diseases such as cancers, diabetes and infections of bacteria and viruses [7–13]. Thus, in this chapter, we have made an attempt to review the facts known about miRNAs are discuss their role in herpesvirus infection with our main focus on HSV infections.

2. Biogenesis of miRNAs

2.1 Canonical pathway for miRNA biosynthesis

The biological synthesis of miRNAs may be either from intragenic or intergenic sequences. Most of the intragenetically synthesized miRNAs are from introns whereas some are from exons of the protein coding genes. miRNAs are also synthesized from intergenic sequences which are independent miRNA genes and have their own, specific promoters. There are canonical as well as non-canonical pathways for miRNA genesis [14]. The canonical pathway for miRNA biogenesis marks the transcription of the primary miRNA (pri-miRNA) from the miRNA genes by RNA Polymerase II (RNA Pol II) in the nucleus. After the transcription the pri-miRNA, which can be as long as 1000 nucleotides in length, they are processed by a microprocessor complex consisting of an RNA-binding protein DGCR8 and an RNase III Drosha. Pri-miRNA methylation by methyltransferase-like 3 (METTL3) marks it for recognition by DGCR8 of the microprocessor complex [15]. DGCR8 recognizes the intersection of the flanking single-stranded (ss) RNA and the stem loop in the pri-miRNA hairpin-structure after which, the Drosha is recruited and involves in a cleavage process [16]. This processing forms the precursor miRNAs or pre-miRNAs (~80 nucleotides in length) having 2 nucleotide 3′ overhangs, which are transported from the nucleus to the cytosol by Expotin-5 (XPO-5)/ Ran-GTP

complex. In the cytosol, the Ran GTPase- activating protein brings about the hydrolysis of GTP, changing the Ran conformation, thereby, releasing the pre-miRNA bound to the XPO5 [17]. After the release, the RNAse III endonuclease Dicer removes ~22 base pairs (bp) of the pre-miRNA terminal loop to form the mature miRNA duplex (**Figure 1**). This processing step of the miRNA allows them to be eligible for loading onto the Argonaute (Ago) complex of proteins which are the essential components of the RNA-induced Silencing Complex (RISC), therefore, the miRNAs mediate their action (**Figure 1**). The decision for the specific loading of a strand of miRNA duplex on the RISC complex is made on the basis of the thermodynamic stability of the two strands and is accompanied by ATP hydrolysis [18]. The strand with a lower 5′ stability or a 5' Uracil is named as the *guide strand* (~22 nucleotides in length) and is loaded onto the Ago protein, while the strand not loaded onto Ago, named as the *passenger strand*, is cleaved by the slicer activity of the Ago and degraded by the ribonucleases [14, 19].

2.2 Non-canonical pathways for miRNA biosynthesis

While most of the miRNAs are generated via the canonical pathway, there are many which are synthesized by the non-canonical pathways. Although there may be many such non-canonical miRNA synthesis pathways, they use up different combinations of proteins that are participants of the canonical pathway. Primarily, there are two types of the non-canonical pathways, the Drosha/DGCR8 independent route and the Dicer independent route. *Mitrons* are an example of miRNAs synthesized by the Drosha/DGCR8 independent pathway, where the pre-miRNAs

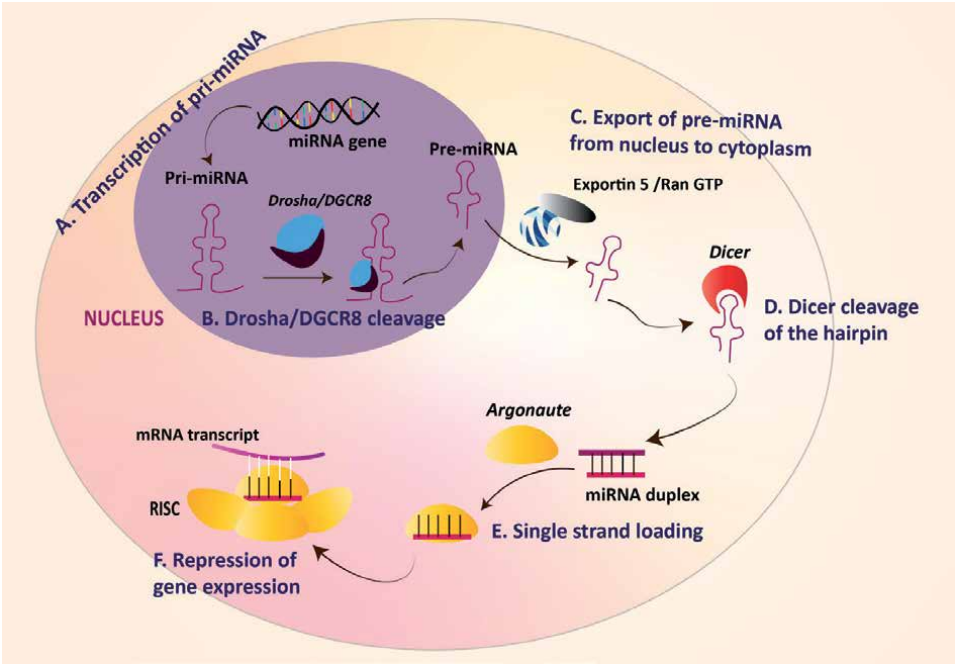

Figure 1.
Biogenesis of miRNAs: A. transcription of pri-miRNA from the miRNA gene by RNA pol II followed by B. Drosha/DGCR8 cleavage of the pri-miRNA to pre-miRNA. C. the pre-miRNA is then exported from the nucleus to the cytoplasm with the help of the Exportin 5 /ran GTP complex where D. dicer cleavage of the hairpin structure takes place. E. One of the strands of the miRNA duplex is loaded onto the RISC, via the ago protein which causes the F. repression of the gene expression by base complementarity-induced mRNA degradation or translation repression.

generated are similar to the Dicer substrates [20]. Here, Exponin-1 needs to transport the miRNA transcripts to the cytosol without Drosha processing it and allows a biased 3p strand loading onto Ago-2. On the other hand, the Dicer-independent pathway involves Drosha processing of the short hairpin RNA (shRNA) transcripts, but is too short to be cleaved by Dicer [21]. Hence, the entire pre-miRNA is loaded onto the Ago-2, which slices the 3p strand. Finally, the trimming from 3′-5′ of the 5p strand concludes the maturation of the miRNA [14, 22].

3. Role of miRNAs in HSV replication

miRNAs play a huge role in the crosstalk between the virus and the host. There are certain viral and cellular miRNAs that regulate the host responses to a viral infection as well as the progression of infection. Both the viral and the cellular miRNAs are capable of regulating the host and the viral mRNAs. Some of the miRNAs that have been identified to be involved during the HSV-1 infection have a direct or indirect impact on the viral replication. These cellular miRNAs may directly target the HSV genome or are manipulated by the HSV through the viral proteome/transcriptome [23]. HSV-1 infected HeLa cells have shown a downregulation of the miR-649 cellular miRNA that targets a ubiquitously expressed cytoplasmic protease, MALT-1, which activates the NF-κB signaling (**Figure 2**). Since NF-κB signaling inhibits HSV-1 replication, the downregulation of miR-649 elevates the expression of MALT-1, increasing the restriction on HSV-1 replication [24]. Similarly, another cellular miRNA which is involved in the suppression of HSV-1 replication is miR-101 (**Figure 2**). The HSV- viral immediate early protein ICP4 directly binds to the promoter of miR-101 to increase the expression of miR-101 in the infected cells thereby decreasing the expression levels of its target GRSP1, which is a scaffolding

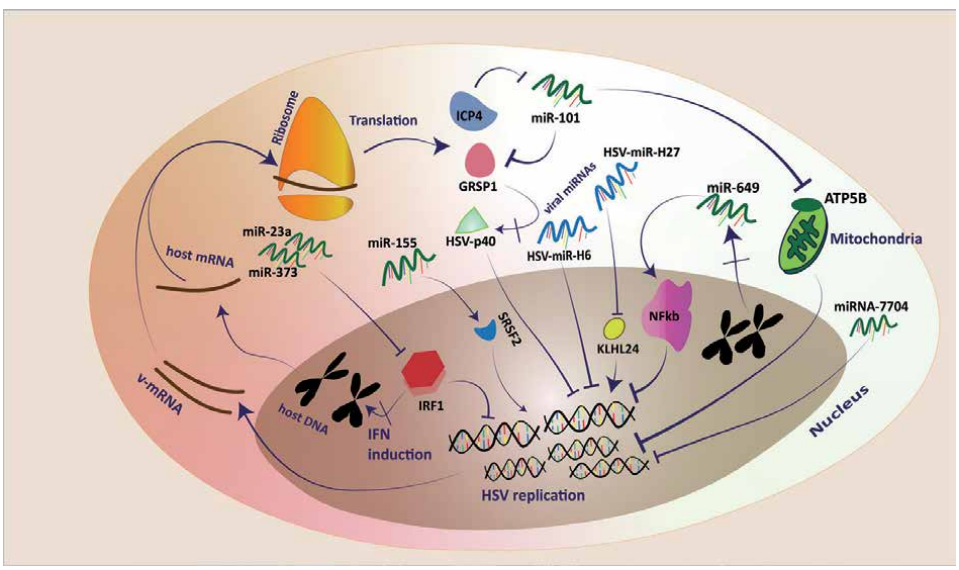

Figure 2.
miRNAs in HSV replication: The figure describes the mode of action of the various cellular miRNAs (green) and the viral miRNAs (blue) that regulate the HSV replication. While the cellular miRNAs, miR-649, –101, –23a, –373 and the viral miRNA, miR-H6 block the HSV replication, the cellular miRNA, miR-155 and the viral miRNA, H-27 promote the HSV viral replication. Both the viral and the cellular miRNAs regulate the host as well as the viral gene expression to modify the replication process either as a host response tying to fight the infection or to establish the infection within the host. Pointed arrowheads indicate progression of an event while blunt arrowheads indicate blockade of an event.

protein that may be involved in the polarization of epithelial cells. GRSP1 directly bind to the HSV-1 p40 and increases the replication of HSV-1. With enhanced suppression of GRSP1, the replication of HSV-1 is hindered. Although it seems surprising that HSV auto-downregulates its replication, it may be a necessary step for the virus to prevent host cell death due to lysis, therefore, maintaining a permissive milieu for virus harboring and replication within the cells [25]. Also, miR-101 can target ATP5B, a subunit of the ATP synthase, to restrict the HSV-1 replication [26]. These studies depict that a single miRNA such as miR-101 could regulate multiple targets to restrict the viral replication. In a study conducted by Shabani et al., a macrophage miR-7704, was capable of reducing the HSV-1 replication in HeLa cells [27]. Another cellular miRNA, miR-23a which targets the Interferon regulatory factor-1 (IRF-1), involved in innate antiviral immunity, is upregulated, so as to escape the host immune responses [28]. Also, a direct suppressor of IRF-1, miR-373, enhances HSV-1 replication by supressing the interferon stimulatory gene responses (**Figure 2**) [29]. Increased expressions of miR-155, a miRNA which could epigenetically increase the transcription of the serine/arginine-rich splicing factor 2 (SRSF2), enhances the HSV-1 replication, due to SRSF2- mediated transcriptional activation of the HSV-1 genes (**Figure 2**) [30]. Some HSV-1 viral miRNAs have also been studied that regulate the virus's replication. HSV-miR-H6, a viral miRNA which is generated profusely during HSV-1 infections, reduces HSV-1 replication by downregulating the viral protein ICP4 (**Figure 2**) [31]. Also, H-27 targets KLHL24 which is a transcriptional repressor (**Figure 2**). Therefore, increase in H-27 expression does not allow KLHL24 to suppress the HSV-1 immediate early and early gene expression, thus, promoting HSV-1 replication [23, 32].

4. miRNAs in acute HSV infection and latency

Encephalitis due to acute HSV-1 or − 2 infection could arise as a direct effect of the HSV invasion of the host or due to the heightened host responses to the virus which causes host damage in the process of virus destruction. Although the incidence of Herpes Simplex Encephalitis (HSE) cases is low, 20% of those affected face neurological after-effects [23]. The inflammation associated with encephalitis often involve the miRNAs, which is also the situation in HSE. miRNAs also contribute to the susceptibility of the cells to HSV infection. This may be the reason for the discovery of a number of cellular miRNAs in HSE. miRNAs miR-155, miR-146a and miR-15b were found to be upregulated in the mouse brain post-HSE [33] and have been associated with neuroinflammation (**Figure 3**) [34]. HSV-1 encoded miR-H28 and miR-H29 were identified to be expressed late during the infection and are exported out of the infected cells in exosomes, thus indicating a role of these miRNAs in viral spread [35]. Also, miR-200 family and miR-182 are the miRNAs identified to be involved in HSE as they target Syndecan-2 (sdc2) which contributes to the biosynthesis of the heparan sulphate required for the attachment and entry of HSV into the cells. Therefore, downregulation of sdc2 by miRNAs is a strategy maneuvered by the host to resist the spread of the virus [33]. This also depicts that mutations in the miRNA sequence or the mRNA target are key players in varied susceptibility to the HSV infection of the central nervous system.

The opposite of excessive HSV pathogenesis is HSV latency, a phase of HSV infection characterized by minimum genome replication. HSV uses neurons as the hideaway for escaping the spotlight for the immune responses while sheltering itself within the host. Since the discovery of HSV- miRNAs in 2006, many miRNAs have been identified to participate in the different stages of viral latency [36] miR-LAT (Latency associated transcript), a miRNA generated from the exon 1 of

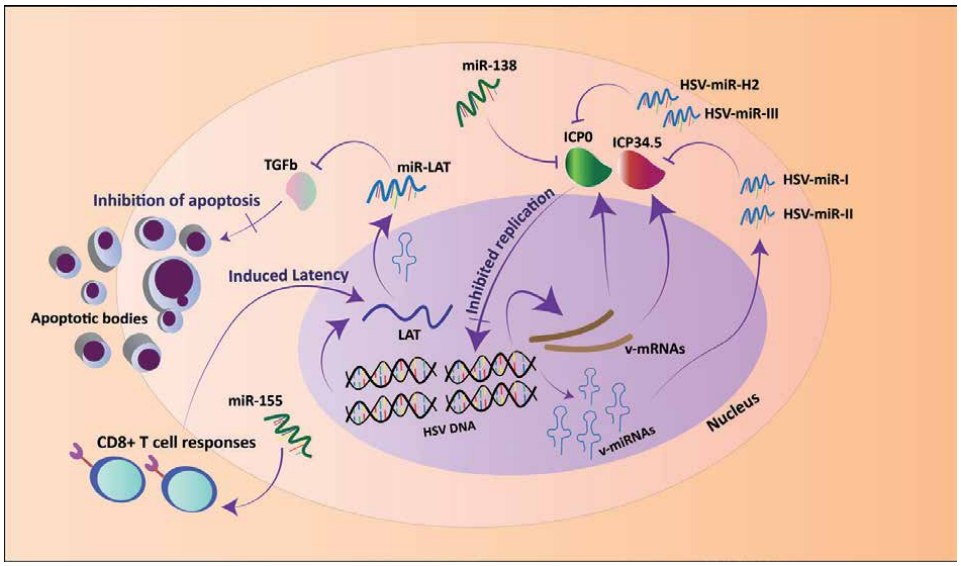

Figure 3.
miRNAs in HSV latency: Majorly, the LAT-associated v-miRNAs participate in the induction, establishment and maintenance of latency of the HSV in the cells. HSV-miR-I and miR-II suppress the ICP34.5 expression to restrict HSV replication and promotion of the HSV latency. HSV-miR-H2 and miR-III inhibit the ICP0 expression to suppress the HSV replication. HSV-miR-LAT inhibits apoptosis to allow the infected cell and the virus to survive and establish a latent infection. There are two cellular miRNAs that contribute to the latency. miR-138 promotes latency by preventing ICP0-triggered viral replication whereas miR-155 contribute to the maintenance of latency via the induction of the CD8+ responses.

the HSV1-LAT gene, is responsible for the TGF-β mediated anti-apoptotic effects on the cells during latency. HSV-miR-H2 has been found to suppress the immediate early protein ICP0, therefore, inhibiting the replication to promote HSV latency (**Figure 3**) [37]. On the other hand, HSV2-miR-H6, a miRNA associated with HSV2-LAT, contributes to the reactivation of the virus from latency [38]. Mutation studies on both these miRNAs confirm their effects. miR-I, is another HSV2-LAT associated miRNA which reduces the expression of ICP34.5, a neurovirulence factor, to establish latency in the dorsal root ganglia [39]. A similar study by the same group identified miR-II and miR-III miRNAs from the HSV2-LAT, with miR-II targeting ICP34.5 and miR-III targeting ICP0, to maintain latency (**Figure 3**) [40]. Furthermore, the cellular miRNAs also engage in the events of HSV latency. miR-155 is involved in the maintenance of latency via the elicitation of CD8 responses [41]. Similarly, enhanced expression of miR-138 in the neurons, to target ICP0 for suppression (**Figure 3**), is crucial in maintaining the HSV-1 latency and survival of the hosts [42]. The crosstalk between the viral/cellular miRNAs, amongst themselves, and with the host transcriptome decides for the maintenance of latency which may be for the lifetime, or trigger the reactivation of the virus.

5. Role of miRNAs in the immunological events of HSV infection

Ocular infection by HSV-1 may lead to chronic inflammation resulting in lesions of stromal keratitis (SK). The immunological events underlying the development of SK involve organized T-helper 1 (Th1) and T-helper-17 (Th17) cells, which produce IFN-γ and IL-17, respectively [43, 44]. Also, the involvement of the regulatory T cells (Treg) guides the exacerbation or abatement of the keratitis [45, 46] miRNAs also regulate the development, activation, function and recruitment of the Th and

the Treg cells [47]. A pro-inflammatory miRNA, miR-155 is known to be upregulated during SK and leads to the enhancement of the Th1 and Th17 responses and helps promoting the keratitis. Antagomir nanoparticles containing the anti-miR-155 sequences have been shown to downregulate miR-155 expression and suppress SK [48]. Also, miR-132 was found to be upregulated during SK, which helps in the advancement of angiogenesis via upregulation of the vascular endothelial growth factor (VEGF) by directly targeting a negative regulator of VEGF, a Ras-GTP inhibitor [49]. The Toll-like receptor (TLR) pathway is an integral innate immune response pathway that is altered in almost all infection scenarios. Similarly, the genes associated with the TLR pathway are modulated post-HSV-2 infection by the miRNAs, miR-124, miR-150, miR-342-5p, miR-1245b-5p, miR-1245b-3p, and miR-592 [50]. Speaking of the innate immune responses, type I Interferon (IFN) signal transduction plays a crucial role in curbing viral replication. Thus, for the virus to establish a progressive infection, it must manipulate the host machinery to overcome the IFN combat. So far, in case of HSV-1, miR-221 is manipulated by HSV-1 to suppress the IFN-β production and effector functions [51]. The major innate immune response involved immediately after the HSV infection is the series of events resulting in acute inflammation, which is essential for virus clearance. This also implies that the infiltrates need to be resolved and the cell debris need to be cleared off so as to maintain the immunological homeostasis of the host, preventing a chronic inflammation that could be lethal [52]. Certain pro-resolving mediators (SPMs) function to resolve the acute inflammatory state and may engage a few miRNAs to do so. The binding of Resolvin (RvD1), a SPM, to its receptor upregulates the miRNAs miR-21, miR-146b, miR-219, and downregulates miR-208a [53, 54]. The target mRNA of miR-219 is the transcript of the gene encoding the 5-lipoxygenase enzyme that leads to the decreased production of leukotriene B4 and increased production of the SPMs. miR-146b suppresses the expressions of IL-8 and RANTES, which are the leucocyte-recruiting chemokines at the inflamed regions [54]. miR-146a is also pro-inflammatory in nature and have been shown to trigger the arachidonic acid-mediated, overproduction of IL-1β, to induce an Alzheimer's-like neuropathological condition in the brain. Since it can directly suppress the complement factor H, it is considered as one of the mechanisms of complement evasion by HSV-1 [55]. Another miRNA upregulated as a result of RvD1 binding is miR-21 which contributes to the establishment of an anti-inflammatory milieu by increasing the production of IL-10 when the resolution of inflammation is vital [56, 57]. Contradictorily, miR-208a, which decreases the IL-10 production and enhances the NFκB activation to prolong the inflammatory events, was itself constrained [54]. It has also been noted that miRNAs can function in a cell/tissue-specific manner. miR-4661 is one such miRNA that promotes acute inflammation in the neutrophils, whereas mediates the cessation of the inflammatory responses in the monocytes and the macrophages via the augmented production of SPMs. It also polarizes the macrophages towards the resolution of inflammation [58]. HSV-1 miR-H8 reduces natural killer (NK) cell- dependent killing of the virus-infected cells by the suppression of the glycosylphosphatidylinositol gene expression [59]. HSV-1 viral miR-H28 induces the production of IFN-γ also to restrict the spread of HSV-1 between cells while not affecting the viral replication, so that optimal transmission between individuals take place [60].

6. Other herpesviridae viral miRNAs

The herpes simplex virus 1 and 2 discussed mainly in this chapter are also termed as human herpesvirus (HHV)-1 and 2. The other members of the

herpesviridae family of viruses include the Varicella zoster virus (VZV) or HHV-3, the Epstein Barr virus (EBV) or HHV-4, the human cytomegalovirus (HCMV) or HHV-5, the Roseoloviruses HHV-6 and HHV-7, and the Kaposi's sarcoma-associated herpesvirus (KSHV) or HHV-8 [61]. A common characteristic of the herpesviruses is that all the members are capable of establishing latent infections in their hosts and reactivate when the immune system has been compromised. Herpesvirus reactivation brings about changes in the host signaling pathways with the help of alterations in the miRNA expression [62]. VZV is a neurotropic virus that causes chickenpox in humans. Although there are not many reports on VZV v-miRNAs, nearly 20 miRNAs have been predicted from the VZV genome and some of these miRNAs have been shown to be involved in viral replication [63, 64]. EBV, the first human oncovirus reported to encode viral miRNAs and cause various cancers such as nasopharyngeal carcinoma and Burkitt's lymphoma, encodes 44 miRNAs. The EBV miRNAs target the viral as well as the host mRNAs to regulate carcinogenesis and cellular transformation of the EBV-associated cancers [65]. HCMV, which allows extensive replication in the endothelial as well as the epithelial cells and has a large dsDNA genome of 230 bp, encodes 22 miRNAs [66, 67]. KSHV, a gamma herpesvirus and the causative agent of Kaposi's sarcoma, encodes 25 miRNAs. The family also contains HHV-6 and HHV-7, which are the lesser explored members. A single miRNA, miR-U86, encoded by HHV-6A has been reported to target the expression of the U86 gene which is an IE gene of HHV-6A [68]. Therefore, apart from HSV-1 and HSV-2, the other members of the herpesviridae family also encode a number of miRNAs to regulate their infection cycles in the host. Owing to similarities amongst them, investigations pertaining to one will provide insights regarding the other. Here, we discuss some of the miRNA effector functions that are employed during other herpesvirus infections.

6.1 The human cytomegalovirus (HCMV) miRNAs

The HCMV have co-evolved with their hosts and encoding miRNAs that are capable regulators of cell cycle progression, viral gene expression, apoptosis and host immune response evasion (**Figure 4**). In order to maintain the latent infection, the virus attempts to maintain the hematopoietic progenitor cells (HPCs) in the dormant state. EGR-1 (Early growth response gene-1) is critical in maintenance of the HPC quiescence. miR-US22 is a HCMV miRNA that is expressed during the early infection stages or reactivation to restrict the proliferation of the HPCs. It does so by targeting the host transcription factor, EGR-1, so that the HCMV can overtake the host replication machinery for its own replication. Since viral replication is close to negligible during latency, the HCMV miR-US22 is not expressed during this state [69]. Another miRNA mechanism to limit cell proliferation during latency is the suppression of the RhoA, a regulator of the actin dynamics. miR-US25–1 targets the RhoA GTPase to disable mitosis in the cells [66, 67]. The lytic and latent infections are characterized by the abundant expression of the miRNAs, miR-US25–1, miR-UL112-3p, and miR-UL22A. Although most of the HCMV miRNAs are detected during the first 4 hours of infection, miR-UL22A, miR-UL112-3p and miR-UL148D continue to be detected post-IE infection. miR-UL112-3p targets the UL123 viral mRNA that codes for IE72 which is actively involved in viral replication and cell lysis [70] miR-UL148D targets the IE response 5 transcript to indirectly regulate the expression of the viral IE genes through the signaling transduction events induced by Cyclin-dependent kinase 1 (CDK-1) in order to promote latent HCMV infections [71]. The FOXO transcription factors are the facilitators of both mitochondria-dependent and -independent pathways for the induction of apoptosis in cells [72] miR-US5–1 and miR-UL112-3p of HCMV have known to

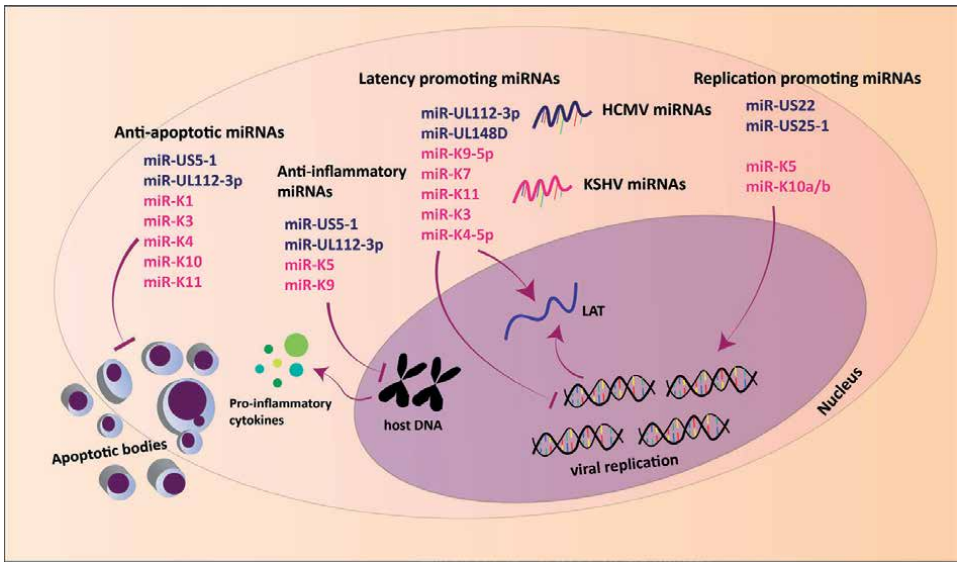

Figure 4.
miRNAs in other herpesvirus infections: The figure summarizes the involvement of the various miRNAs in the different events of other herpesvirus infection. Here, we have taken the example of two herpesviruses, HCMV (navy blue) and KSHV (magenta) to show the extent of v-miRNA involvement during the viral pathogenesis. Mostly, the viral miRNAs involved in replication, latency, cell survival and immune modulation have been mentioned in the figure and are the ones widely explored.

target a member of the FOXO family, FOXO3a [73]. The mechanism involves a downregulation of FOXO3a expression such that its pro-apoptotic functions are limited and binding to the promoter of Bcl-2-like protein 11 (Bim) is restricted. In profusely HCMV replicating cells, the IKK expression was directly constrained by the miR-US5–1 and miR-UL112-3p miRNAs of HCMV, such that production of the pro-inflammatory cytokines, IL-6 and RANTES via the NF-κB pathway was considerably suppressed [74] miR-UL148D directly targets the RANTES transcript to suppress its pro-inflammatory functions [75]. Also, miRUL112-3p directly targets MICB, an MHC I-related chain B, to indirectly repress the NK-cell killing activity so as to protect the infected cells from destruction during the lytic and preferably also during the latent HCMV infections [66, 67, 76].

6.2 Kaposi's sarcoma (KS)- associated herpesvirus (KSHV) miRNAs

KSHV miRNAs regulate the viral mRNAs directly as well as indirectly via the regulation of the cellular mRNAs (**Figure 4**). Certain viral miRNAs participate to establish latency in the KSHV-infected cells. miR-K7 and miR-K9–5p target the KSHV RTA protein, which acts as a switch between the lytic and latency cycles [77, 78]. Also, miR-K11 and miR-K3 function to maintain latency by indirectly suppressing the RTA transcript [79–81]. Another latency-inducing miRNA is the miR-K4-5p which inhibits the retinoblastoma-like protein-2 (RBl2) expression due to which the DNMT1 (methylation enzyme) expression increases. Methylation of the RTA promoter by DNMT1 suppresses RTA expression, inducing latency in the cells [79, 81]. Angiogenesis is an important event in the KSHV infection as it allows the spread of the latency-induced malignancies. GRK2 which regulates the AKT/CXCR2 pathway to establish a reciprocity between viral replication and induction of angiogenesis, is targeted by miR-K3 to decrease viral replication and induce angiogenesis, thus establishing latency [82]. Contradictorily, BCLAF1, Bcl 2-associated factor-1, is the common target for miR-K5, miR-K9 and miR-K10a/b and has been known to

trigger the lytic infection cycle [83] miR-K5 and K9 also suppress the TLR-mediated production of the inflammatory cytokines IL-6, IL-8 and IL-1α by directing interfering with the intermediates, MyD88 and IRAK-1, respectively [84, 85]. The KSHV miRNAs also attempt to inhibit apoptosis and promote cell survival, which is crucial for both malignancy and latency. Therefore, miR-K1, -K3 and -K4 inhibit apoptosis by directly targeting the Caspase-3 protease [86] miR-K10, the viral orthologue of hsa-miR-142-3p, targets TGF-β to promote cell survival and cellular transformation [87, 88] miR-K10a is also the negative regulator of the TWEAK receptor, which is the receptor for the TNF-like weak inducer of apoptosis, thereby, inhibiting apoptosis as well as downregulating the production of IL-8 and MCP-1 to contribute to the KS progression [89] miR-K1 also promotes cell cycle progression by inhibiting p21 [90]. Another target for miR-K1, IκBα, which usually retains NF-κB in the cytoplasm by blocking the nuclear localization signals, is constrained to promote latency and survival of the transformed cells [91]. Activation of NF-κB also inhibits the Warburg effect leading to the declined expressions of GLUT-1 and GLUT-3, suggesting the significance of the metabolic regulation for the proliferation of the KS cells [92]. Just as miR-155 have been considered as the regulatory epicenter of various cancers and viral infections, the KSHV miRNA, miR-K11 mimics some of the functions of this cellular miRNA. miR-K11 blocks the C/EBPβ resulting in the enhanced, IL-6-mediated proliferation of the B cells [93]. It also targets JARID2 leading to increased B cell transformation [94]. Moreover, miR-K11 also inhibits the IFN type I signaling by directly targeting IKKε mRNA [95, 96]. Thus, the generation of cellular miRNA orthologues by the herpesviruses is clearly evident of its co-evolution with its host, such that it diverts the cellular machinery towards its own viral processes while hiding itself efficiently from the host responses.

7. miRNAs as potential biomarkers in herpesvirus infections

With as many miRNAs that are expressed during each of the herpesvirus infections, the recognition of certain miRNAs are biomarkers for these infections is promising. There are a number of reasons that back the concept of miRNA biomarkers in human infections. The distinct pathophysiological events occurring during the infection is reflected in the miRNA expression patterns. For example, where miR-649 is considerably downregulated in HSV-1 infected cells as compared to the uninfected cells to mark the overwhelming participation of NF-κB is fighting the infection, there are certain miRNAs exclusively generated by the HSV (miR-H6, -H27 etc.). miRNAs are also the biomarkers of the HSV infection status within the host as certain miRNAs are specific to the lytic phase (miR-H1) whereas some are specifically expressed during the latency phase (miR-H2–6). miRNAs are easily available for detection in the body fluids such as blood, serum and human dental pulps [97, 98] and so are the v-miRNAs of the *herpesviridae* family members [99]. Although there are a few ongoing clinical studies on miRNAs biomarkers in cancer, the reports on EBV-related cancer miRNAs also being detected in the blood and urine, will boost the EBV research in the same direction [100]. Furthermore, the molecular techniques used for the miRNA detection, such as, the real-time PCR array, microarray profiling and the next-generation sequencing techniques are popular and well established in case of herpesvirus infections as well [101–104]. One of the greatest advantages of using miRNAs as biomarkers is their sustained expressions *in vivo* as compared to the mRNAs. This is because of the presence of exosomes which enclose the miRNAs. Moreover, the miRNAs released extracellularly are bound to the Ago proteins. These mechanisms protect the miRNAs against degradation by the nucleases [35, 105, 106]. Whether v-miRNAs or cellular

miRNAs are better biomarker candidates might be debatable. However, an important consideration in this context is that v-miRNAs are virus-specific whereas host miRNAs are not exclusive for a virus infection. For example, miR-155, which is crucial in HSV-1 latency is also involved in pro-inflammatory functions in other diseases such as cancer, asthma, arthritis, Cystic fibrosis and also recently reported in other viral infections, such as that of the Sars-CoV-2 [41, 107, 108]. Therefore, a panel of host and viral miRNA combinations may serve as an appropriate biomarker in case of each of the herpesvirus infection.

8. Therapeutic considerations of miRNAs in herpesvirus infection

miRNAs can also be referred to as the endogenous post-transcriptional gene regulators and have a special advantage over drugs. miRNAs are self-molecules and hence are safer than any of the synthetic or natural compounds used as therapeutics against the infections. Another feature of miRNAs that make them interesting candidates for therapeutic considerations is their ability to regulate more that one gene expression, and inversely, the expression of a single gene can be modulated by more than one miRNA. Being the regulators of gene expression, modification of the miRNAs is an approach for changing the course of an infection or a disease. There are two ways by which the miRNA expression may be modified. One of modifications include reintroduction of the miRNA expressions by the use of specific miRNA mimics, while the second is to block the infection-induced/modified miRNA expression by the use of the specific-miRNA inhibitors [23]. The use of miRNA mimics and inhibitors have been trending in the field of research on infectious diseases since the successful progression of miRavisen through the clinical trials to be established as therapeutics against the Hepatitis C virus [109] miRavisen is a miRNA inhibitor of miR-122, a miRNA which increases the HCV viral replication [110, 111]. Similarly, inhibitors of miR-373 or HSV-miR-H27 can be administered as therapeutics to decrease the HSV viral load in the hosts. There have also been studies where mimics of miRNAs have proved to be useful in restricting viral replication. Recently, a study identifying the significance of miR-29b mimics have been reported to decrease the Rotavirus infection considerably [112]. Likewise, mimics of miR-7704 and miR-101 could be encouraged in the HSV therapeutic research. Furthermore, the reported miRNA research could be compiled to develop therapeutic formulations involving combinations of mimics and inhibitors to synergistically suppress the HSV infection in the host. However, miRNAs, like any other therapeutics, face the challenges of target-specific delivery and off-target effects. These challenges are also being addressed by the researchers with the synthesis of appropriate miRNA-loaded nanoparticles which ensures on-site targeted delivery and optimal bioavailability while maximally reducing the off-target effects [6]. All-in-all, the potentiality of miRNAs as therapeutic agents against viral infections is being explored explicitly, also implying that their clinical applications in herpesvirus infections is inevitable.

9. Conclusion

Elucidation of miRNAs have emerged as a promising field of research due to the capability of the miRNAs to be easily manipulated to alleviate an infection. The fact that a single miRNA can target more than one gene expression (viral, cellular, or both) to suppress the viral infection is equally fascinating and efficient. The significance of miRNAs as biomarkers and therapeutic agents in infection makes

them acceptable to the researchers. All the more, the commercially available mimics and inhibitors of the miRNAs makes the research pertaining to them affordable. However, miRNAs face the challenges of specific, on-site delivery, long-term miRNA stability and off-target consequences, and yet, have been FDA-approved under the small molecule therapeutics category. The challenges of targeted delivery and optimum bioavailability is met with the miRNA-loaded nanoparticles, which diminish the off-target effects as well. Thus, with miRNAs finding their utility into a variety of applications, expansion of the miRNA investigations, both in basic and applied research, is evident.

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Chapter 7

Immunological and Virological Failure among Individuals on Highly-Active Antiretroviral Therapy

Hadush Negash, Brhane Berhe and Miglas Welay

Abstract

Initiation of antiretroviral treatment decreased HIV related mortality and morbidity. Virological failure (a condition defined when the plasma viral load of HIV infected individuals greater than 1000 RNA copies/ml based on two consecutive viral load measurements with adherence support) have an increased risk of clinical progression to acquired immune deficiency syndrome (AIDS) and death. Nowadays, combination of highly active antiretroviral therapy is recommended to decrease the likelihood of drug resistance. However, there is emergence of drug resistance and treatment failure during treatment. Hence, managing and detecting antiretroviral treatment response is important to monitor the effectiveness of medication and possible drug switching for treatment regimens. Additionally, mechanisms of drug resistance and factors associated with immunological and virological treatment failure should be addressed.

Keywords: immunological response, virologic response, adverse drug reactions, HAART, drug resistance, mechanism

1. Introduction

Globally, in 2017, there were about 20.9 million HIV patients receiving highly active antiretroviral therapy (HAART) [1]. The treatment is targeted at inhibiting viral attachment and replication leading to the recovery of immune function [2, 3]. Since the introduction of HAART in 1996, there is a decline in mortality and morbidity associated with HIV [4, 5]. Due to HAART, the impact of HIV infection decreased [6] due to viral suppression, restoring and preserving immune function, improving quality of life, and reducing HIV related morbidity and mortality along the course of treatment [7–9]. There are a number of problems associated with HAART, including emergence of drug resistance, difficulties of maintaining long-term adherence, and drug-related toxicities [5] which lead to virological failure, immunological failure and clinical progression [10].

Virological failure increases the risk of clinical progression of HIV to AIDS [11]. World health organization (WHO) recommended combination therapy of HAART [12, 13]. The combination therapy improved the natural history of HIV infection from a life-ending event to a manageable chronic condition [14]. Adverse drug reactions (ADRs) associated with HAART resulted some considerable health consequences like co-morbidity with opportunistic infections [15–17].

Treatment failure to first-line regimens resulting from ADRs creates the need for very expensive and difficult-to-implement second-line regimens. Second-line HAART regimens are not easily affordable and largely donor dependent in resource-limited settings [18]. Effective viral suppression is targeted by UNAIDS to meet "90-90-90" in 90% of persons on HAART by 2020 [19]. Use of an effective and safer HAART regimen helps to promote maximal viral suppression and improved immunological and virological response [20, 21].

2. Occurrence and determinants of immunological and virological failure

2.1 Prevalence of immunological failure/immune reconstitution among HIV infected individuals on HAART

Immune reconstitution is the immunological response of immune cells (mostly CD4+ T cells) due to initiation of HAART. The response is either success or failure. Previously conducted studies reported different rates of immunological failure among HIV infected individuals (**Table 1**).

The variation among the above studies might be explained due to the differences in patient adherence, differences to define immunological failure whereby some studies defined as the reduction in number of CD4 + T cell count to baseline or below, or persistently low CD4+ T cell count (below 100 cells/μL) [31] and others defined immunological failure as reduction in number of CD4+ T cell count to baseline or below severe immune suppression (CD4+ T cell count <200 cells/μL) [24, 25, 28, 30, 32, 33]. Other studies defined immunological failure as 50% decline of CD4+ T cells from treatment peak value [28, 34] or at least 30% decline from treatment peak value [28, 35].

Moreover, the prevalence of immunological failure can be expressed with person-years among the HIV patients on treatment. As per recent studies, the rate was 2.966 [22], 2.57 [36], 8.7 [37] and 8 [38] person-years. The variation among these reports might be due to the differences in treatment adherence and the presence of opportunistic infections (status of co-morbidity). Hence, the coverage of good adherence toward the HAART was one of the determining factors for immunological success. The likelihood of having good adherence of HAART increases the treatment success rate considering the results of previous studies (**Table 2**).

Number	Prevalence of immunological failure of HAART	Reference
1.	24.7%	[22]
2.	6.5%	[23]
3.	9.8%	[24]
4.	11.5%	[25]
5.	15.1%	[26]
6.	18.4%	[27]
7.	64.4%	[28]
8.	33.5%	[29]
9.	35%	[30]

Table 1.
Occurrence of immunological failure of HIV infected individuals on HAART.

Number	Percentage of adherence from previous studies	Reference
1.	72.5%	[22]
2.	81.8%	[25]
3.	82.7%	[26]
4.	82.4%	[39]
5.	78.5%	[32]
6.	92%	[33]
7.	85.8%	[25]
8.	97.7%	[34]
9.	100%	[24]

Table 2.
Coverage of good treatment adherence of clients from different studies.

Treatment adherence differs among studies due to differences in psychosocial support of relatives or the society to strictly adhere to the treatment guidelines, stigma, and lack of commitment to take medications so that HIV patients might drop themselves from on course ART treatment, perceived feeling of unwellness from medication, and scaring of treatment side effects [31, 40, 41].

Previously, baseline CD4+ T cell counts were playing an important role for an HIV patient to initiate HAART. The median baseline CD4+ T cell counts for an HIV infected individual to start HAART is explained below (**Table 3**). However, nowadays, the target is to test and treat regardless of their CD4+ T cell counts.

2.2 Associated factors of immunological failure among HIV infected individuals on HAART

Poor adherence to HAART increased to experience failure in CD4+ T cell recovery [22, 26, 28, 33, 43]. This allows increased viral replication which in turn increases infection of new CD4+ T cells and ultimately depletion of immune cells [41]. Additionally, lower baseline CD4+ T cell count is one of the determining factors for increased immunological failure because immune recovery mostly depends on the number of baseline CD4+ T cell counts [22, 29, 38]. The timing of HAART initiation is important to optimize the CD4+ T cell immune response to medication [44].

Number	Median baseline CD4+ T cell counts	Reference
1.	196 cells/μL	[22]
2.	191 cells/μL	[24]
3.	162 cells/μL	[23]
4.	156 cell/μL	[24]
5.	152 cells/μL	[28]
6.	115 cells/μL	[32]
7.	177 cells/μL	[34]
8.	238 cell/μL	[42]

Table 3.
Median baseline CD4+ T cell counts of HIV infected individuals to initiate HAART as per previously published studies.

Number	Percentage of virological failure	Reference
1.	12.47%	[54]
2.	14.7%	[55]
3.	15%	[56]
4.	5.3%	[57]
5.	7.5%	[58]
6.	23.2%	[59]
7.	24%	[60]
8.	47%	[61]
9.	30.6%	[62]
10.	57.1%	[63]
11.	32%	[64]
12.	20.6%	[65]
13.	16.7%	[66]
14.	51.6%	[67]
15.	20.9%	[68]
16.	69.6%	[69]

Table 4.
Prevalence of virological failure of HAART among individuals infected with HIV.

These reports may highlight that patients with low CD4+ T cell count have poor long term CD4+ T cell immune response [22].

TB/HIV co-infection worsen the rate of immunological failure [22, 25, 37, 45–48]. TB infection impairs cellular immune responses through MTB-induced apoptosis of CD4+ T cells which subsequently lead to depletion of CD4+ T cells [49]. Incidence of TB during the course of HAART decreased the likelihood of treatment adherence due to its high pill burden and side-effects [50]. Although the risk of acquiring TB remains elevated, much less is known about the effect of HAART on recurrent TB [51]. All in all, recurrences of TB are lower among HIV infected individuals with higher CD4+ T cell counts [52].

2.3 Occurrence of virological failure among HIV infected individuals on HAART

Virological failure among individuals on HAART is defined as plasma viral load of the HIV infected individual is greater than 1000 RNA copies/ml based on two consecutive viral load measurements with adherence support [53]. The occurrence of virological failure of HAART among HIV infected individuals is reported below (**Table 4**).

The variation might be due to the differences in cutoff values of viral RNA copies per ml of plasma to consider virological failure [60, 63, 70], the duration of follow-up, differences of co-morbidity, variations on the treatment adherence of HAART, clinical/immunological failure [61, 71], perinatally infected [67], lower mean age [65] and shorter duration on HAART [66].

2.4 Associated factors of virological failure among HIV infected individuals on HAART

Magnitude of virological failure of HAART is not the same for all HIV infected individuals. There are factors that affect the likelihood of virological failure like

malnourishment and overweight [22, 54, 72, 73]. Abnormal body mass index (BMI) is significantly correlated with decreased CD4+ T cell counts that increases viral load by progressing into the advanced stage of the disease [74, 75].

Moreover, the occurrence of virological failure is higher among TB co-infected individuals [54, 76, 77]. Concurrent HAART and TB treatment increases the rate of virological failure due to impaired treatment adherence and pharmacokinetics drug reactions. Hence, clients on HAART with active TB should be prioritized for viral load monitoring and follow-up. Moreover, to prevent incident TB during ART INH prophylaxis is recommended. Increased viral copies compromise immunity and negatively affect treatment by contributing to the double burden side effects of TB and HIV [78].

Failure for an immune-reconstitution increases the rate of virological failure [54, 79–81]. This leads to an increase in viral replication [22]. Hence, it is a surrogate marker for virological failure. During HAART, detecting and monitoring immunological response should also be emphasized to prevent drug resistance [22, 82].

3. Immune reconstitution during chronic HAART

Despite the successful suppression of viral replication, some clients on HAART might fail to recover immune cells (immune reconstitution). It is estimated that half of HIV infected individuals on HAART might fail to reconstitute their CD4+ T cell counts to levels above 500 cells/μL. Additionally, up to 16% might fail to achieve a CD4+ T cell counts greater than 200 cells/μL, even with long term therapy of HAART [83]. There are factors determining rates of immune reconstitution including older age [84], lower baseline CD4+ T cell counts prior to HAART [85], and co-infection with hepatitis C virus (HCV) and TB [86, 87].

3.1 HAART is the most effective strategy for successful immune reconstitution

Initiation of HAART in early stages of HIV infection is associated with not detectable viral load suggesting effective inhibition of viral replication [88, 89]. During treatment initiation (during the first 4 months of HIV infection), there is lower recovery of immune cells due to a narrow restorative time. It is explained that starting HAART initiates both the rate and extent of CD4+ T cells reconstitution [90].

While treating HIV infected individuals, combining cytokine, IL-7 therapy, prevents apoptosis of T cells and is required for naive T-cell survival. This increases CD4+ T cell counts among clients on treatment [91]. The T cells remain capable of responding to antigenic stimuli and produce cytokines after polyclonal and specific antigenic stimulation. Then repopulation appears to extend cells from thymus to effector sites of the immune system [92, 93].

4. HIV drug resistance

Detecting HIV drug resistance is one of the major limiting factors in the successful treatment of HIV infection [94]. This includes genotypic and phenotypic assays. The genotypic detection is performed by matching results with lists of frequently updated HIV mutations that are known to confer drug resistance which are relatively inexpensive. However, it can only identify documented HIV mutations and may not detect new mutations that arise in a particular HIV variant. Although it is not easy to predict the actual degree of drug resistance, phenotypic testing yields

drug susceptibility of a particular HIV variant. It provides information on the sensitivity of a particular HIV variant in comparison to a control isolate with full drug sensitivity. Moreover, there is inconsistency in interpreting the detected decrease in viral drug sensitivity into actual decreases in clinical sensitivity. Hence, it needs to have large-scale clinical trials for a correlation might be made between changes in phenotypic sensitivity and actual drug resistance [95].

Investigators have assessed the effectiveness of HIV drug resistance testing in improving clinical response to pharmacotherapy, like the GART [96], the VIRADAPT [97] and the ARGENTA [98]. They reported that individuals whose drug selection was based on genotypic resistance testing had significantly lower viral loads than patients who did not receive resistance testing prior to starting therapy. This reflects reduced morbidity and mortality [97].

Several factors related to the life cycle and replication of HIV are key contributors toward the rapid and widespread emergence of resistance. Initially, the reverse transcriptase enzyme is highly prone to errors during the process of reverse transcription. Hence it makes errors in each HIV genome per round of replication process which enhances its mutation for every 2000 nucleotide bases [99]. In addition to these base substitutions, insertions can occur. The especial scenario of HIV drug resistance mechanism is also due to high rate of replication. When untreated, plasma HIV RNA levels range from 103 to 105 copies/ml or sometimes greater than 106 copies/ml in acute infection [100].

The HIV virus continues to infect new cells at a very high rate to maintain the infection to a stationary state. Additionally, increased rate of errors in the reverse transcription yields new variants with drug resistance. Some HIV variants manifest drug resistance due to intrinsic factors. Hence, antiretroviral resistance can still occur even during successful therapy of HIV infection [101].

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Section 2

Vaccines and Other Public Health Measures

Chapter 8

Trained Immunity-Based Vaccines: A Ready-to-Act Strategy to Tackle Viral Outbreaks

Laura Conejero, Paula Saz-Leal and José Luis Subiza

Abstract

Viral outbreaks have become significant threats to global human public health. New emerging viruses, pathogen mutations, and even the progressive loss of efficacy in some existing vaccines are behind this problem, which is amplified by the rapid virus spread given the ease of current mobility. Taking into account that these outbreaks arise in the absence of conventional effective vaccines, alternative approaches based on trained (innate) immunity are being considered. This immunity is dependent on a functional reprogramming of innate immune cells, leading to an enhanced nonspecific response towards different pathogens, including viruses. Trained immunity-based vaccines (TIbVs), defined as vaccine formulations containing trained immunity inducers, could be used during viral outbreaks to confer non-specific protection but also to enhance adaptive specific immune responses. In this chapter, we aim to illustrate how TIbVs could tackle the above-mentioned situations derived from viral outbreaks, reviewing the potential of available TIbVs in such urgent situations with a special mention to COVID-19.

Keywords: trained immunity-based vaccines, innate immune training, pandemic, viral outbreak, BCG, MV130

1. Introduction

Pandemics and epidemics of infectious origin are large-scale outbreaks that can greatly increase morbidity and mortality globally or over a wide geographic area, respectively [1]. Pandemics have occurred throughout history and appear to be increasing in frequency in the last centuries. Noteworthy examples include the Black Death at the end of the Middle Ages, Spanish flu in 1918, the 2014 West Africa Ebola epidemic or the current COVID-19 pandemic. The direct impact of pandemics on health can be dramatic. These large outbreaks can disproportionally affect younger or active workers, but vulnerable populations such as the elderly are at a particular high-risk. Pandemics can cause acute, short-term as well as longer-term damage to economic growth due to public health efforts, health system expenditures, and aid to affected sectors. Evidence suggests that epidemics and pandemics can have significant social and political consequences too, by debilitating institutions, amplifying political tensions, stigmatizing minority populations, or encouraging sharp differences between social classes [2].

Outbreaks by respiratory ribonucleic acid (RNA) viruses such as influenza or coronaviruses entail the principal threat due to their ease of spreading among humans, their potential severity and recurrence. However, other RNA viruses such as flaviviruses (Zika) or filoviruses (Ebola) must be taken into consideration due to a great overall burden of morbidity and mortality [3]. Antiviral drugs can help mitigate a viral outbreak by reducing the disease in infected patients or their infectiousness. While these drugs can be very successful against some viruses (*e.g.* hepatitis C virus [HCV]) [4], they are not universally effective as exemplified in the current SARS-CoV-2 pandemic [5]. Nowadays, having effective vaccines may be the only tool to reduce susceptibility to infection and thus, prevent the rate of virus spread [2].

Vaccination has dramatically decreased the burden of infectious diseases. Vaccines have saved hundreds of millions of lives over the years [6]. It has been estimated that approximately 103 million cases of childhood diseases were prevented in the United States through vaccination between 1924 and 2010 [7]. The eradication of smallpox in 1980 through vaccination is considered one of the crown accomplishments of medicine. Despite these achievements, effective vaccines have been developed against just over 30 pathogens among bacteria and viruses. There are many pathogens, including viruses such as human immunodeficiency virus (HIV) or respiratory syncytial virus (RSV), for which all efforts for vaccine development have failed so far. In addition, current available vaccines for worldwide important viral diseases like influenza are suboptimal, especially in the elderly, resulting in vulnerability among billions of at-risk populations [6]. On the other hand, having a new effective and safe vaccine in time to control highly contagious emerging viruses that cause epidemic or pandemic threats is an almost impossible task considering the timeframes for vaccine development. This includes preclinical and clinical research, its approval by the regulatory authorities, as well as its production and distribution [3].

Altogether, it has been postulated that one possibility of filling the gap between the appearance of a viral outbreak by an emerging pathogen and the availability of a specific vaccine is to take advantage of the heterologous protection of some existing vaccines, in order to increase the non-specific resistance of the host through trained immunity [8, 9].

2. Specific anti-infectious vaccines

Conventional (specific) anti-infectious vaccines are biological preparations containing live-attenuated or dead microorganisms, their antigens or nucleic acids encoding for them, designed for specific pathogens. The purpose of vaccination is to induce a long lasting adaptive immune response against key antigens able to confer host resistance for future encounters with the corresponding pathogen. Either the production of antibodies, generation of T helper/effector cells, or both, may play a critical role in such a resistance, which greatly depends on the type of pathogen, the route of entrance and the host-pathogen relationship (*e.g.,* extracellular and/or intracellular) [10]. Successful vaccines are highly effective not only in inducing long-lasting immunity against disease-causing pathogens, but also in providing herd immunity to the community that substantially restricts the spread of infection [6].

Most of the vaccines available today have been developed empirically and used successfully long before their mechanism of action on the immune system was understood. Early protection is associated to induction of antigen-specific antibodies, being their quality (avidity, specificity, or neutralizing capacity) key factors for

their efficacy. Long-term protection relies on the persistence of vaccine antibodies and availability of immune memory cells capable of rapid and effective reactivation with subsequent microbial exposure. On the other hand, T cells have a critical role in the induction of high affinity antibodies and immune memory. Furthermore, T cells have a direct role in protection conferred by some vaccines, including the tuberculosis Bacille Calmette-Guérin (BCG) vaccine [11].

Vaccines using whole pathogens have been classically classified as either live attenuated or inactivated (killed). Subunit vaccines contain just selected antigens (*e.g.,* proteins, polysaccharides). Recently, due to a growing availability of bioinformatics and sequencing tools, there has been an increase interest on so-called "rational" vaccine design approaches for subunit vaccines, such as the reverse vaccinology [12]. In this regard, modern vaccines include recombinant proteins or nucleic acids [13]. Rather than administering the antigen itself, DNA and mRNA vaccines targeting dendritic cells (DCs) encode the antigen of interest that will be produced by the vaccinated host, representing a new era in vaccinology [14]. In fact, the first RNA vaccine licensed for humans in Western countries has been recently developed for SARS-CoV-2.

As commented before, a vaccine response is linked to the induction of T and B cell specific responses to the antigens contained in the vaccine. This requires lymphocyte activation, proliferation and differentiation on specialized lymphoid tissues (*e.g* lymph nodes), where antigen presenting cells, like DCs for T cells or follicular dendritic cells (FDCs) for B cells, are present. Mature DCs are recruited into the T cell areas of lymph nodes from the periphery, *e.g.,* at the site of injection of the vaccine. DCs express pattern recognition receptors (PRR) that recognize evolutionary conserved pathogen-associated molecular patterns (PAMPs) that are not contained in self-antigens and are identified as "danger signals" [15]. When immature DCs are exposed to the vaccine-derived antigens at the site of vaccination, they uptake them and become activated [16]. This activation will lead to their maturation with the expression of homing receptors at their surface, triggering DC migration to the draining lymph node through afferent lymphatic vessels, where the activation of T and B lymphocytes will occur. Mature DCs process the up-taken antigens and present them to naïve T cells associated to molecules of the major histocompatibility complex (MHC) within the T cell areas of lymph nodes. On the other hand, unprocessed native antigens, either free or complexed with antibodies or complement, access the B cell areas of lymph nodes (lymphoid follicles) where they are captured by FDCs and displayed from their cell surface to the B cells. Antigen-specific B cells will rapidly proliferate forming a germinal center and differentiate into plasma cells producing low-affinity immunoglobulin (Ig) M antibodies. The B cells will then receive additional signals from activated T cells, undergoing isotype antibody switch from IgM to IgG or IgA and affinity maturation of the antibodies produced.

For a vaccine to be immunogenic enough, DC activation, that can be achieved by adjuvants, is essential. Live attenuated and inactivated whole-cell vaccines are considered "self-adjuvanted" as they naturally present sufficient PAMPs to activate innate immune cells, including DCs; thus, promoting a robust antigen-specific immune response. In contrast, subunit vaccines generally require different types of adjuvants to enhance and/or drive the immune response in the desired direction [15, 17].

2.1 Difficulties for novel specific vaccines in a viral outbreak situation

Viral outbreaks appear when there is a sufficient number of susceptible individuals within a nearby population. Although susceptibility is a balance between host factors (high/low resistance) and pathogens (high/low virulence), in many cases it reflects a

lack of prior contact with a given pathogen. In general, this is related to the emergence of new viruses or the lack of effective vaccines against known viruses. As pointed above, the development of effective vaccines is not an easy task against certain viruses. We are still lacking vaccines for some of the most lethal viral infections, including HIV and MERS-CoV, among others. These pathogens are difficult to tackle, as we do not fully understand their mechanisms to evade the immune system or how to elicit protective immunity against them [13]. However, encouraging progress is being made against these pathogens and there are currently several "pipeline vaccines" in development, such as RSV, universal influenza vaccine, and SARS-CoV-2 [18–20]. Apart of SARS-CoV-2 for obvious reasons in the current pandemic, there is an urgency to have a universal influenza vaccine that provides a broad and durable protection from influenza virus infection. Yet, the high level of antigenic diversity and variability, and antigenic drift in the surface antigens, enable these viruses to escape antibody-mediating neutralization [21]. On the other hand, there is a number of vaccines currently licensed, including the influenza A virus vaccine, that provide incomplete protection, especially in high-risk groups [22]. Mumps outbreaks observed in Ireland, United Kingdom and United States in vaccinated subjects with Measles Mumps Rubella (MMR) vaccine is another example [23]. Different factors have been postulated to contribute to mumps outbreak, including waning immunity and primary and secondary vaccine failure. Yet, their actual contribution is not fully understood [23].

Vaccine efficacy must consider different target populations as well. Adaptive immune response to vaccines may be limited in newborn and the elderly. Early in life, immune responses are dampened compared to adults [24, 25]. Neonates have underdeveloped germinal centers in lymph nodes and the spleen, and low expression of B cell receptors which in turn results in low levels of primary IgG responses to infections and vaccines [26]. As we age, our immune system undergoes age-related changes that lead to progressive deterioration of the innate and the adaptive immune responses, this is termed immunosenescence. The most common features of immunosenescence are short-lived memory responses, impaired response to new antigens, increased predisposition to autoimmune diseases and low-grade systemic inflammation (*inflammaging*) [27, 28]. Immunosenesence results in increased susceptibility to infections and deficient response to vaccination causing high hospitalization and mortality rates. For example, influenza vaccine efficiency has been reported to be 17–53% in the elderly, compared with the 70–90% efficacy in young adults [29]; and vaccination with Varicella zoster virus (VZV), also an important pathogen in elderly people, only partially prevents reactivation of herpes zoster [27].

If the difficulties listed above are outlined for existing or developing vaccines, quickly obtaining an effective vaccine to urgently control a new virus outbreak is almost an impossible task in the short-term as pointed above. This is well exemplified by the SARS-CoV-2 vaccine race pushed by the devastating COVID-19, with more than 100 vaccine candidates in the running. It is considered that no less than 1 year will last the time until the first licensed vaccine can provide protection in the best scenario [30]. This, in spite of greatly shortening the usual clinical development time and regulatory obstacles for a new vaccine and, therefore, without knowing its true performance and/or safety in the medium term compared to other authorized vaccines [31].

3. Trained immunity and infections

It has become evident from epidemiological, clinical and experimental data that some conventional whole-cell vaccines, like BCG and others, also provide resistance to infectious diseases not related with the specific pathogen targeted by the vaccine [32–34]. Much of these non-specific "heterologous" effects appear to depend on

the activation of innate immune cells by the PAMPs contained naturally in these vaccines [10], although other mechanisms such as cross-reactive epitopes between different pathogens could also account for this protection in some cases [35].

Immunological memory, understood as the ability to "remember" past encounters with pathogens, has been classically attributed to the adaptive branch of the immune system exclusively, by virtue of the antigen-driven clonal expansion of T and B lymphocytes and exemplified by the mechanism of conventional specific vaccines pointed above. However, the notion that innate immunity was unable to induce immunological memory has been challenged in recent years, particularly from studies in organisms that lack adaptive immunity, such as plants or invertebrates, as well as early studies in mice lacking the adaptive immune system [8, 36]. Altogether, the term 'trained immunity' was coined to define an innate immune memory that lead the innate immune system to an enhanced response to secondary challenges [37]. Importantly, trained immunity seems to be underlying the heterologous effects of an increasing number of vaccines [38–40].

3.1 The concept

What is trained immunity? - Trained immunity is defined as the memory of the innate immune system, where an encounter with a first stimulus (*e.g.* a microbial insult) results in a subsequent long-term adaptation and enhanced non-specific response by innate immune cells against a secondary challenge (the same or unrelated), thus providing non-specific, broad-spectrum, long-term protection in case of infection [8, 9, 37, 41].

Which cells can be trained? - Trained immunity properties have been defined for distinct cell subsets of the innate immune system [9, 42], including natural killer (NK) cells and innate lymphoid cells [43]. Of note, training of myeloid cells [42], particularly monocytes and macrophages [44, 45], and more recently DCs [46, 47] and hematopoietic stem cells [48], have been extensively studied. Finally, the acquisition of this immunological memory has also been demonstrated to a lesser extent for non-immune cells [49].

How to get trained? - A wide variety of stimuli can train innate immune cells, particularly when considering monocytes and macrophages [9, 50]. Among infectious agents, live microorganisms such as the tuberculosis vaccine BCG [51], *Candida spp* [52] or viruses [53, 54]; bacterial components, such as flagellin, lipopolysaccharide, muramyl dipeptide [55], fungal components as β-glucan [52] or even helminth products [56]. In general, microbial ligands engaging some PRR, like C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain-like receptors (NLRs) are well established training inducers, whereas those engaging toll-like receptors (TLRs) may have opposite effects depending on the TLR type and concentration [55, 57]. Intriguingly, not only infectious agents but also endogenous inducers and metabolites such as oxidized low-density lipoprotein or mevalonate can induce trained immunity [50].

What hallmarks define trained immunity? - In contrast to adaptive immune responses, epigenetic reprogramming of transcriptional pathways — rather than gene recombination — mediates trained immunity. This training phenomenon comprises three key hallmarks that occur at the intracellular level: increased cytokine production upon rechallenge, changes in the metabolism and epigenetic reprogramming [9, 58, 59], which eventually support increased protection upon infection.

Among those cytokines whose production is augmented after re-exposure in trained cells, proinflammatory molecules such as tumor necrosis factor α (TNF-α), interleukin (IL)-6, IL-1β and interferon γ (IFN-γ) are fairly constant [45, 52, 55, 60, 61]. Modulation of IL-10 varies between studies [45, 52, 56, 62, 63]. A noted

shift from oxidative phosphorylation to aerobic glycolysis (Warburg effect) is the main change in cellular metabolism during the induction trained immunity [64]. Moreover, glutaminolysis, cholesterol synthesis and the tricarboxylic acid cycle are non-redundant pathways required for trained immunity to take place [64, 65]. Epigenetic reprogramming, mainly mediated by histone modifications, is one of the bases for the long-lasting effect of trained immunity [8, 66–68]. Immune pathway activation and changes in metabolism serve as basis for epigenetic rewiring [65]. As a result, epigenetic modifications have been found at the level of important promoters for the training process, which makes chromatin more accessible and conditions gene expression patterns of trained cells upon stimulation with a secondary challenge [69].

As a result of the whole process, enhanced, broad-spectrum, non-specific protection mediated by innate immune cells is found upon infection. This cross-protection has been observed for a wide range of human pathogens including fungi [51, 52], parasites [70, 71] and different bacterial infections [72–75]. Importantly, induction of trained immunity has been proved to be effective against viral infections including yellow fever [76], influenza A virus [77] and others [78, 79]. In this line, the induction of this phenomenon has been also proposed as a tool for reducing susceptibility to emergent SARS-CoV-2 infection, as will be described at the end of the chapter [78, 80].

How long does trained immunity last? – Trained immunity phenotypes have been observed for months and up to one year after the training insult. This was initially controversial, as trained immunity properties had been attributed to short-lived myeloid cells such as monocytes or DCs [38]. In this regard, several studies have shown that modulation of bone marrow progenitors is also an integral component of trained immunity, supporting the long-lasting effect of this phenomenon [9, 81]. In this way, trained immunity inducers [82–85] would be able to reprogram and induce expansion of hematopoietic progenitors with a particular bias to the myeloid lineage. Thus, bone marrow-derived mature cells would be also trained [86], showing improved clearance of infection [83].

Complementary to progenitor reprogramming, peripheral trained immunity induction would take place in tissue-resident cells [9]. This is especially relevant at the mucosal level, where cells encounter most of the infectious training inducers. Alveolar macrophage (AM) memory was demonstrated following viral infection [87, 88]. Training of these long-living cells led to increase antimicrobial properties, independently of systemic immunity [87, 89]. This local training of AM was further reproduced following respiratory mucosal administration of tuberculosis vaccine, being crucial for *Mycobacterium tuberculosis* clearance [90]. On the other hand, training of NK cells lead to long-lived, self-renewing, stable expanded cells with memory-like properties, both in an antigen-dependent or independent manner [91–93]. Finally, it was also reported that self-renewing long-living skin epithelial stem cells exhibited local trained immunity, providing faster wound healing in primed mice than in naïve mice [94, 95].

3.2 Trained immunity on ongoing immune responses

3.2.1 Effect on adaptive immunity

Non-specific effects of vaccines have been extensively studied and reported over the last decades. Although trained innate cells could partially account for these effects, involvement of adaptive immunity has also been suggested [96]. An adaptive immune mechanism of non-specific effects could be heterologous immunity; vaccine antigens can give rise to T cell cross-reactivity against other antigens that may confer some protection against unrelated pathogens [96, 97].

However, innate immune cells constitute the bridge between the intrusion of microbial threats and the activation of adaptive immunity. As said before, following sensing of pathogens by PRRs, activated innate immune cells secrete different factors and act as antigen-presenting cells (APCs) to initiate activation of adaptive immunity [98]. Thus, it would not be unexpected that trained innate immune cells, within their acquired enhanced properties, would be able to induce stronger adaptive immune responses [39]. In this regard, BCG vaccine, a well-known trained immunity inducer, has shown to enhance the antibody titer and alter heterologous T cell responses against a wide range of vaccines and unrelated infections [99–101]. In different experimental models, BCG-mediated protection against viral and *Plasmodium* infections was abrogated in the absence of T cells. In these models, BCG vaccination has been mainly associated with modulation of $CD4^+$ T helper (Th) 1 responses. Similar observations have been found in different clinical studies [99]. Of note, BCG vaccinated human volunteers displayed a long-lasting heterologous Th1 and Th17 response upon stimulation with unrelated pathogens and TLR-ligands [38]. To some extent, similar observations have been found in other vaccines such as diphtheria-tetanus-pertussis (DTP) or measles vaccine [99].

As said before, trained immunity properties have been recently described also for DCs. As being the most professional APCs, they emerge as crucial bridge for potentiating adaptive immune responses. In this sense, DCs with high immunostimulatory properties that enhance adaptive immune responses via IL-1β release had been described [102]. More recently, programmed memory DCs have shown to increase Th1/Th17 immunity and confer protection during cryptococcosis [46]. Finally, different polybacterial preparations of whole-cell inactivated bacteria, have shown to prime DCs and induce enhanced Th1, Th17 and IL-10 T cell responses against related and unrelated stimuli [103, 104]. This capability of modulating heterologous T cell responses by APCs have been also described to suppress pathogenic T cell immunity in experimental models of autoimmune encephalomyelitis [56].

3.2.2 Effector functions on trained innate immune cells

As noted above, a hallmark of trained innate immune cells is the enhancement of some effector functions leading to increased non-specific resistance against a variety of pathogens. In this regard, β-glucan-trained monocytes show enhanced candidacidal activity and efficiently inhibit the *C. albicans* outgrowth [52]. Production of reactive oxygen species (ROS) has shown to be also affected by the induction of training. Thus, BCG-trained monocytes [45], β-glucan-trained macrophages [105] or β-glucan-trained neutrophils [106] produced increased amount of ROS following different challenges. Finally, increased phagocytosis and production of microbicidal molecules have been observed in β-glucan-trained macrophages [70, 105]. Mechanisms underlying this enhanced effector function could be an intrinsic cell reprogramming as consequence of the training, as well as be supported increased expression of different PRRs and surface molecules [45, 60, 87]. Altogether, these enhanced effector responses could improve pathogen clearance by increasing host resistance.

On the other hand, a substantial part of the adaptive immune response is directed at recruiting other effector cells from the innate immune system to eventually resolve an infection. Both T helper and B responding cells release cytokines, antibodies, and other mediators that activate monocytes, macrophages, NK cells or neutrophils to clear extracellular and intracellular pathogens [107]. Multiple studies have demonstrated the importance of IFN-γ-mediated priming in the activation of macrophages [108, 109], produced by $CD4^+$ Th1 and $CD8^+$ T cells [107]. In this sense, it has been previously demonstrated that adaptive T cells render innate macrophage memory via IFN-γ-dependent priming [87, 89]. Furthermore, a

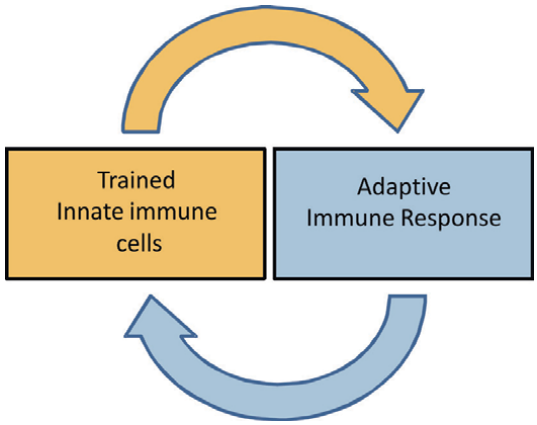

Figure 1.
Effect of trained immunity on ongoing immune responses. Induction of trained immunity allows trained cells to enhance adaptive immune responses and vice versa, final effector functions of trained cells can be further potentiated by enhanced adaptive responses.

deep crosstalk between Th17 and neutrophils have been widely demonstrated, via production of IL-17 and other related cytokines [110].

Taken into account the potential role of trained innate cells in both the induction of adaptive and effector responses, a notable amplification loop in the global immune response could be considered (**Figure 1**).

4. Trained immunity-based vaccines

Based on trained immunity pillars, a next generation of anti-infectious vaccines has been postulated, coined as 'Trained Immunity-based Vaccines' (TIbVs). TIbVs would be conceived to confer a broad protection far beyond the antigens they contain. By proper targeting of innate immune cells to promote trained immunity, a TIbV may confer non-specific resistance to unrelated pathogens while trained immunity memory is still present, in addition to the specific response given by intrinsic antigens [39].

A *bona fide* TIbV would consist of two main components: the trained immunity inducer(s) and the specific antigen(s). The antigen(s) mission is to generate an adaptive (specific) immune response as any conventional vaccine. The trained immunity inducers aim to promote the training of innate immune cells. This innate immune training would confer non-specific resistance against unrelated pathogens for a window of time (months) plus an enhanced adaptive immune response to the antigens present in the vaccine itself or from other sources (*e.g.,* coming from eventual infections or bystander pathogens) [39].

Two additional concepts arise under the TIbV umbrella: i) trained immunity-based immunostimulants (TIbIs) and ii) trained-immunity-based adjuvants (TIbAs). The former (TIbIs) would induce the training of innate immune cells, so they would be ready-to-act against upcoming infections conferring broad non-specific protection while trained immunity is present, still enhancing adaptive immune responses following any eventual natural infection. The latter (TIbAs) would enhance adaptive responses against specific antigens incorporated either to the trained inducers as in *bona fide* TIbVs, or in a separated but combined vaccine [39] (**Figure 2**).

Following the above features, the TIbV concept can be applied to existing anti-infectious vaccines composed of microorganisms that show heterologous protection ascribed to trained immunity.

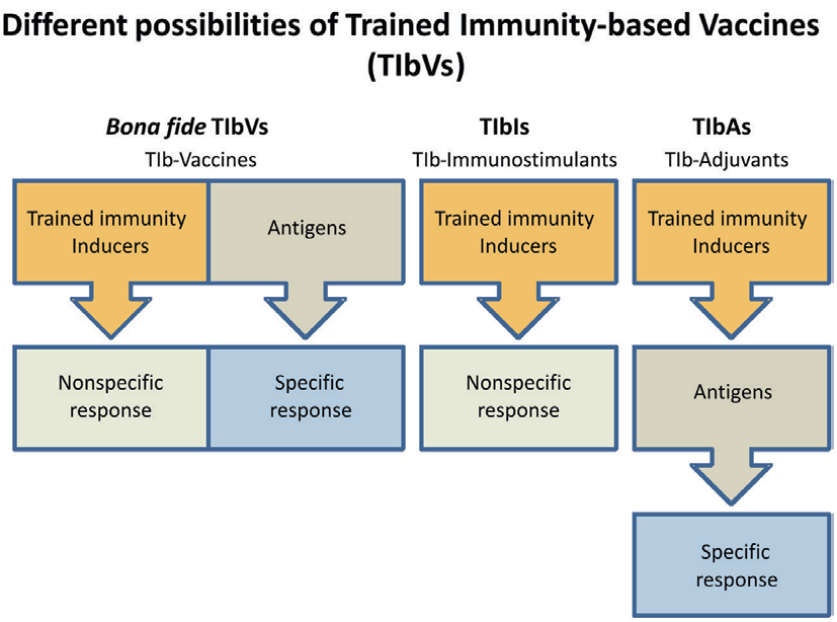

Figure 2.
Different possibilities of trained immunity-based vaccines (TIbVs). Under the umbrella of trained immunity-based vaccines (TIbVs) different possibilities exist depending on their design and purpose. Bona fide TIbVs are those containing both trained immunity inducers and antigens in the same vaccine as occurs in conventional vaccines with trained immunity inducing properties. These vaccines show heterologous protection in addition to the specific response to the target antigen. TIbIs are intended just to confer non-specific protection by means of trained immunity induction beyond the intrinsic antigens they may contain. TIbAs are intended to enhance the specific response of other vaccines that are administered later, once trained immunity has been induced, or specific antigens combined in the same vaccine as any other adjuvant.

4.1 Available vaccines with heterologous protection against viral infections

During the last decades, robust epidemiological data has demonstrated the role of certain vaccines leading to protection against heterologous infection with a high impact on overall mortality in children [111–113]. This protection could not only be explained by protection achieved by the target disease. Studies on MMR vaccination in high-income settings have also evidenced a reduction in non-target infections, particularly in respiratory infections [114]. A limitation for most of these epidemiological studies is that they do not identify the agent (viral, bacterium or parasite) responsible for the infection. These heterologous effects of certain vaccines conferring non-specific protection for a quite long time are believed to be largely due to non-specific stimulation of the innate immune system. It is not yet clear whether this is a direct reflection of trained immunity induction (*i.e.,* acting as TIbVs) in every case. The fact that most of these vaccines use live-attenuated microorganisms, *i.e.,* self-replicating agents, may suggest that a continuous stimulation of innate immune cells is necessary to obtain protection and/or to achieve a proper trained immunity for this purpose.

4.2 Live attenuated vaccines

4.2.1 BCG

The BCG-Denmark strain was tested in randomized-controlled trials (RCT) in infants who normally did not receive the BCG vaccine at birth. These studies carried out in Guinea-Bissau demonstrated that vaccination at birth was associated

with lower neonatal mortality, especially due to neonatal sepsis, respiratory infections, and fever [111, 115]. In these lines, a meta-analysis commissioned by the WHO concluded that BCG administration during the first month of life reduces all-cause mortality by 30% [116]. In these studies authors did not discriminate the etiology of infection (bacterial vs. virus); therefore, a reduction in viral infections may explain, to some extent, this result. However, in two studied carried out in India in neonates with BCG-Russian strain no such effect was observed [117]; suggesting that different immunological effect of diverse BCG strains may account for these discrepancies. A study carried out in infants to assess the impact of BCG vaccination on the incidence of RSV infection suggested a possible protective role for BCG vaccination against acute lower respiratory tract infection [118]. Other clinical studies have provided evidence suggesting a protective role for BCG on secondary viral infections [79]. In this regard, the impact of BCG vaccination on viral infection in human healthy volunteers has been assessed using the live attenuated yellow fever vaccine (YFV) as a model of viral human infection [76]. BCG vaccination induced epigenetic reprogramming in human monocytes, and these modifications correlated with IL-1β upregulation and the reduction of viremia, all these features being the hallmarks of trained immunity [76].

Similar protective effect of BCG was observed in several studies in elderly people regarding respiratory tract infections. BCG vaccination in subjects of 60–75 years old once a month for three consecutive months resulted in reduction of acute upper respiratory tract infection, concomitant to significant increase in IFN-γ and IL-10 compared with those receiving placebo [119]. A recent randomized trial of BCG vaccination was carried out in elderly patients (age ≥ 65 years) returning home from hospital admission, these subjects are at high risk to develop infections. The BCG vaccination increased the time to first infection (primary outcome) and decreased the incidence of a new infection [120]. Besides, results demonstrated that BCG vaccination resulted in lower number of infections of all causes, especially respiratory tract infections of probable viral origin, although no discrimination was made between respiratory tract infections caused by bacteria or viruses.

BCG has also been shown to enhance the response to vaccines directed against viral infections [79]. A clinical study in healthy volunteers demonstrated that BCG administration prior to influenza vaccination increases antibody titers against the 2009 pandemic influenza A (H1N1) vaccine strain, concomitantly with an enhanced IFN-γ production to influenza antigens compared with the control group [121].

4.2.2 Influenza vaccine

The cold-adapted, live attenuated influenza vaccine (CAIV) has been shown to provide non-specific cross-protection against RSV in an experimental model of infection [122].

In a randomized pilot study conducted in healthy volunteers receiving a trivalent influenza vaccine, cytokine responses against unrelated pathogens were observed [121]. During the 2003–2004 influenza A (H3N2) outbreak, an open-labeled, nonrandomized vaccine trial was carried out in children 5 to 18 years old. Subjects received either trivalent live attenuated or inactivated influenza vaccine. Live attenuated influenza vaccine but not trivalent inactivated vaccine was effective in children administered during influenza outbreak, despite the dominant circulating influenza virus was antigenically different from the vaccine strain [123].

4.2.3 Measles vaccine

Measles vaccine (MV) is among the live vaccines that have been shown to have beneficial effects reducing all-cause mortality [124]. Randomized clinical trials and observational studies from low-income countries have concluded that measles vaccination is associated with decreased overall mortality and morbidity [100]. However, a systematic review carried out by Higgins and colleagues has pointed out that most of these studies were considered at high risk of bias [116]. Nevertheless, MV seems to induce a transient suppressive effect on both the lymphoproliferative and innate response evaluated in peripheral blood mononuclear cells (PBMCs) from children, with slight increase in innate immune response, measured by non-specific cytokine production [100]. It has been reported that following measles vaccination, the *ex vivo* production of both innate (IL-6 and TNF-α) and adaptive (IFN-γ and IL-2) cytokines decreases for 2 weeks, but levels of IL-2, IL-6 and IFN-γ are increased at day 30 post vaccination compared with baseline [125]. Differences in males and females have been reported, where girls seem to receive stronger beneficial effects. In this regard, a study of MV-specific innate responses following MMR vaccination found higher TNFα, IL-6 and IFN-α secretion, cytokines associated to trained immunity, in adolescent girls than boys [126].

4.2.4 Oral polio vaccine

There are currently only three countries where polio remains endemic. Thus, polio-free, high income countries are introducing the use of the inactivated polio vaccine (IPV). However, there are still many countries that use the live-attenuated oral polio vaccine (OPV). Despite current WHO policy to replace OPV by IPV, there is epidemiological evidence that supports that replacing OPV by IPV might have an impact on overall mortality [96], since OPV has shown strong non-specific beneficial effects even in settings where the incidence of the targeted infection is low. In this regard, campaigns to eliminate polio in West Africa have been associated with lower child mortality rates [127].

4.3 Inactivated vaccines

As pointed above, most of the vaccines described so far showing non-specific heterologous effects contain live-attenuated microorganisms. Nevertheless, fully inactivated bacterial vaccines have also been described conferring protection against viral infections, and some of them for a fairly long period of time. Interestingly, these vaccines are mucosal preparations that are administered daily for long periods of time (weeks/months) rather than single, or seldom, doses used in live attenuated vaccines. Thus, it seems that the much longer administration of these inactivated mucosal vaccines resembles the effect achieved by live vaccines on heterologous protection associated to trained immunity (**Figure 3**).

4.3.1 Polybacterial whole-cell vaccines

These vaccines are used for the prevention of recurrent infections in susceptible subjects, mainly associated to the respiratory and urogenital tracts [128–134]. Since they target infections occurring in these tracts, their administration is generally through mucosal tissues to obtain a better mucosal response [135, 136].

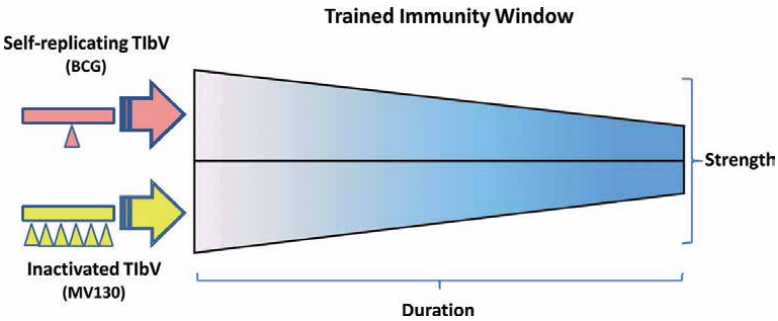

Figure 3.
Trained immunity window by self-replicating and inactivated TIbVs. Trained immunity-based vaccines (TIbVs) containing live-attenuated self-replicating microorganisms (e.g. BCG) may require fewer administrations to induce an adequate trained immunity window of sufficient intensity, quality and/or duration than vaccines with dead microorganisms. Fully-inactivated TIbVs can be enhanced to induce trained immunity with a multiple dose schedule (e.g. MV130).

MV130 is a sublingual vaccine used to prevent recurrent respiratory tract infections [128, 129] containing inactivated whole-cell bacteria that are common pathogens in the airways. Its ability immunomodulating DCs has been addressed experimentally *in vitro* and *in vivo*. MV130 triggers the release of cytokines ascribed to trained immunity in different setting, including TNF-α, IL-1β and IL-6 [103, 137, 138]. Sublingual immunization of mice with MV130 induces a systemic Th1/Th17 and IL-10 enhanced responses against unrelated antigens [103]. Similar enhancement was shown in patients treated with MV130 where an increased T cell response to flu antigens were described [128]. MV130 was successfully used in infants with recurrent wheezing, a condition triggered in most cases by viral infections. It is noteworthy that the protective effect was also shown 6 months after discontinuation of treatment, which points to a long-lasting effect that fits with the memory ascribed to trained immunity (Nieto et al., under review). In this regard, MV130 has been shown to induce trained immunity and to confer protection against experimental virus infections (Brandi et al., under review). Recent studies have assessed the clinical benefit of MV130 as a TIbV in the context of recurrent respiratory infections in vulnerable populations such as patients with different primary and secondary immunodeficiencies showing a reduced rate of respiratory infections [130, 139] (Ochoa-Grullón et al., in press).

4.3.2 Polybacterial lysates

Although not considered vaccines but immunostimulants, these bacterial preparations are, like MV130, used for the prevention of recurrent respiratory infections. OM-85, one of the best studied, is composed of chemically treated bacterial lysates for oral administration, acting through the gastro-intestinal mucosa. OM-85 has been shown to be effective in experimental viral infections [140] and in children with recurrent wheezing [141], a condition triggered by viruses as noted above. OM-85 stimulates the release of proinflammatory cytokines such as IL-1β, TNF-α and IL-6 by macrophages [142], typical of trained immunity induction, as well as Th1 cytokines including IFN-γ [143]. It is not known, however, the role of trained immunity in their mechanism of protection. A recent study conducted in infants, the observed protection against respiratory infections under OM-85 treatment stopped when treatment was discontinued [144], which may point against the memory ascribed to trained immunity.

4.4 The potential clinical applications of TIbVs in the context of viral outbreaks

The non-specific mechanism of TIbVs against widely differing pathogens associated to the induction of trained immunity can be exploited clinically. This makes TIbVs as a ready-to-act tool to tackle disease outbreaks from different angles where conventional specific vaccines have proven their limitations:

Newly emerging disease outbreaks, with no conventional vaccines available. Even in the presence of therapeutic options, vaccines are the best tool to prevent infections. However, even with worldwide efforts, getting a vaccine to the public takes time. In addition, side effects, dosing issues, and manufacturing problems can all cause delays [3]. Herein, using available TIbVs could mitigate the devastating consequences of emergent outbreaks by means of non-specific protection, until a suitable specific vaccine is available.

Newly emerging disease outbreaks, first coming vaccines with partial efficacy. Even if a vaccine gets available to the market, conventional strategies might raise some issues. The unpredictable identity of largely unknown emerging pathogens, the lack of appropriate experimental animal models, and the time for faster developing may give raise to an upcoming vaccine with no full efficacy [3]. On the other hand, limitations of current vaccines, such as mumps, also include a low efficacy resulting from an unacceptable drop in the immune response over time, requiring re-immunization [145]. In these contexts, the administration of a TIbV prior to the specific vaccine may enhance the response to the latter (111).

Re-emerging disease outbreaks, pathogens with high mutation rates and loss of vaccine efficacy. Mutations are the building blocks of evolution in any organism. Viruses are among the fastest evolving entities, especially RNA viruses such as influenza. Implications in conventional vaccine design are numerous, as a high mutation rate makes it hard to design a vaccine that is universally effective across many years. As a result, this makes a vaccine effective for shorter and raises the need for yearly vaccination programs [22, 146]. Since the underlying mechanism of TIbVs extend well beyond their nominal antigens and have a broad-spectrum of protection, TIbVs could overcome the troublesome of highly specific vaccines that promote antigen variant switching [147].

Disease outbreaks in vulnerable populations. During infectious disease outbreaks, vulnerable populations are usually disproportionately affected due to an interplay of immunological, epidemiological, and medical factors, which leads to sub-optimal or even under-vaccination [148]. This is well exemplified in the elderly population, where successful vaccination against important infectious pathogens which cause high morbidity and mortality represents a growing public health priority. Age-related immunosenescence and 'inflammaging' have been postulated as underlying mechanisms responsible for decreased response and high mortality, including during COVID-19 pandemic or influenza season [80, 149]. Therefore, more potent vaccines are needed. In this regard, the induction of trained immunity by the use of TIbVs is proposed to overcome the immune dysfunction found in these individuals [28]. Thus, elderly has been proposed as one of the groups to benefit from the use of TIbVs, including severe COVID-19 disease, with the aim of potentiating the immunogenicity of their vaccination [80]. Moreover, some types of immunodeficiencies or immunosuppression may benefit from TIbVs. These formulations, by means of tackling both branches of immunity, especially the innate compartment, may be an achievable alternative to reinforce protection or optimize immunogenicity of vaccination in this population [130, 139].

Altogether, harnessing the TIbV concept has been suggested as a crucial step in future vaccine development and implementation, because a wide range of clinical applications may benefit to some extent from their use [150].

4.5 TIbVs in the time of COVID-19

Despite the tremendous financial and scientific effort invested to rapidly obtain a prophylactic vaccine against SARS-CoV-2, only the first one has been licensed in December 2020. Although this means less than a year since the declaration of the pandemic by the WHO, which is an unprecedented achievement, in the meantime, two pandemic waves of COVID-19 and more than 1.5 million deaths have been declared worldwide. Therefore, alternative strategies have been considered to fill the gap until a safe and effective vaccine is available. As noted earlier in this chapter, TIbVs can play an important role for this purpose by increasing host resistance to other pathogens, including viruses.

A bunch of recent studies have been published supporting the role of certain vaccines, including BCG, OPV and measles, as a possible successful strategy to reduce susceptibility and severity to SARS-CoV-2 through trained immunity induction [80, 151, 152]. Thus, clinical trials are currently being conducted to find out the contribution of trained immunity as a preventive tool in the context of COVID-19 pandemics [153]. In a prospective observational trial, 255 MMR vaccinated subjects were followed searching for COVID-19 cases, thirty-six presented COVID-19 but all with a remarkable mild course [154]. Recent studies have also suggested a potential benefit of influenza vaccine on the susceptibility and the outcome of certain infections including SARS-CoV-2. In this sense, a particular attention has been focused on a high-risk population, the elderly. In a study conducted in Italy, influenza vaccination in people aged 65 and over was associated with a reduced spread and a less severe clinical expression of COVID-19 [155].

Finally, in addition to the potential role of TIbV conferring resistance against SARS-CoV-2 infection, they can eventually be used to increase efficacy of specific anti-COVID-19 vaccines, when available, especially in vulnerable population. In this sense, implications of vaccination route and mucosal immunity have also been raised as a key aspect in the development of safe and effective prophylaxis interventions against SARS-CoV-2. Most formulations in development are parentally administered; only a few COVID-19 vaccine candidates are administered by mucosal routes. Still, studies indicate that even if mucosal immunization against coronavirus does not confer sterilizing immunity, the ability to induce anti-SARS-CoV-2 IgA responses in the airways may prevent virus spread to the lung and avoid respiratory distress [156]. In this regard, mucosal TIbVs could enhance the mucosal response of specific COVID-19 vaccines acting as TIbAs by combining them as pointed above in those especially vulnerable subjects.

5. Conclusions and future perspectives

Viral outbreaks can cause epidemics and pandemics if the route of transmission allows for the rapid virus spread. Given the ease of travel and the global exchange of potential transmitting agents, these situations will be increasingly frequent in the future. Preventing the spread of a virus outbreak caused by a highly contagious agent is not easy in the absence of effective therapies or preventive measures. Although the development of effective prophylactic vaccines specific for the threatening virus is the final goal when possible, this requires a minimum time of almost a year in the best possible scenario. Meanwhile, the consequences of the spread of a deadly virus can be devastating, as it is exemplified during the COVID-19 pandemic. This scenario may also take place in the case of re-emerging viruses tackled by partial efficacious vaccines. In such situations, harnessing the heterologous non-specific protection of some existing vaccines with a known safety track record

is an interesting possibility. This protection may be critical for vulnerable subjects and/or for highly exposed individuals, like healthcare workers.

Non-specific protection of some vaccines is thought to be mainly dependent on their effect on the innate immune system. Increasing evidence gathered over the past few years points that innate immune cells show memory-like features when properly activated. This memory termed "trained immunity" has been associated with the non-specific protection of vaccines. The concept of "trained immunity-based vaccine" (TIbV) has been drawn to exploit the potential of trained immunity in designing novel vaccines or to redefine bacterial-derived preparations conferring broad protection against widely differing pathogens. As trained immunity may have implications on the adaptive immune response and *vice-versa*, its potential to provide enhanced immune responses is quite broad whether considering natural infections or following vaccination.

Taken advantage of the current COVID-19 pandemic, a number of clinical trials have been launched with putative TIbVs in order to address protection in highly exposed subjects. The results are eagerly expected as these initiatives may be considered as a proof-of-concept supporting their use in future epidemics/pandemics to fill the gap until a specific vaccine is available. Nevertheless, as trained immunity can be achieved by different inducers, it is unlikely to obtain the same degree of protection, duration, etc. for all of them, which may also depend on the biological behavior and the route of transmission of the threatening pathogen. As in most instances rapidly spreading viruses are airborne and primarily infect the mucosa of the airway tract, induction of trained immunity at the local mucosal level can confer a more adequate protection. This may be an opportunity for mucosal TIbVs as compared to those given parenterally.

Trained immunity may justify heterologous protection of vaccines, help to explain their underlying mechanisms, open avenues for next generation of vaccines, or be proposed to tackle outbreaks by new pathogens as described here. However, this is an emerging field that requires more clinical data before being a reality in the clinical practice; not only to be used against infectious outbreaks, but to fight against recurrent infections in vulnerable subjects for whom no effective vaccines are yet available.

Conflict of interest

JLS is the founder and CEO of Inmunotek SL, Spain, a pharmaceutical company that manufactures bacterial vaccines. LC and PS-L are employees of Inmunotek.

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Chapter 9

The MONITOR Ecosystem: A Digital Health Intervention for the Early Detection, Control, Follow-Up, and Management of COVID-19 in Mexico

Miguel Betancourt-Cravioto, Jorge Falcón-Lezama, Fernando Rojas-Estrella, Rodrigo Saucedo-Martínez and Roberto Tapia-Conyer

Abstract

In fighting infectious disease outbreaks, a basic epidemiological principle is to detect cases quickly and to isolate each case, to interrupt transmission. This principle has been the cornerstone of the Carso Group (CG) COVID Protocol, a systematic blueprint for the reopening of operations of workplaces in the context of ongoing disease transmission in Mexico. The CG comprises over 50 companies with approximately 180,000 employees engaged in economic activities including telecommunications, retail, construction, banking, mining, and manufacturing, among others. To cope with the COVID-19 pandemic within the CG, the Carlos Slim Foundation designed, developed and implemented MONITOR, a digital health ecosystem comprising a mobile phone application, web portal, and analytics platform, to assess the infection risk of each employee, follow-up their health status, and detect early symptoms of COVID-19. MONITOR provides daily notifications for any suspected cases and activates a COVID-19 testing request and follow-up of results. This intervention helps rapidly identify and isolate suspected cases, as well as follow-up of work and family contacts, to prevent further outbreaks. Use of MONITOR has thus enabled containment of COVID-19 in workplaces and safe return to work. MONITOR is an example of the implementation of public health practices in workplaces and can serve as the basis for larger deployment in population-wide settings.

Keywords: COVID-19, SARS-CoV-2, digital health, outbreak, prevention, pandemic, surveillance

1. Introduction

1.1 The SARS-CoV-2 outbreak

A cluster of cases of pneumonia with unknown etiology was first reported in Wuhan, Hubei Province, China in December 2019. Subsequent analysis identified

a novel coronavirus, later named severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). The disease caused by the virus was eventually called coronavirus disease 2019 (COVID-19) [1, 2]. On January 30, 2020, the World Health Organization (WHO) declared the outbreak a Public Health Emergency of International Concern; on March 11, a pandemic was declared [3].

Coronaviruses are widely distributed globally [2]. Most human coronavirus infections are mild; however, two previous outbreaks led to many illnesses and deaths. The epidemics of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002–2003 and Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 resulted in many people developing severe pneumonia, with mortality rates of 10% and 36%, respectively [4].

According to the WHO, a patient with suspected COVID-19 infection is anyone meeting the clinical criteria with respect to signs and symptoms associated with the disease, including fever, dry cough, and fatigue. Other less common symptoms include myalgia, nasal congestion, headache, conjunctivitis, sore throat, diarrhea, and smell or taste disorders (anosmia and dysgeusia). Epidemiologic criteria include exposure to an area with a high risk of viral transmission or community transmission owing to work, residence, or travel [5].

COVID-19 infection exhibits different effects in different groups and individuals. Most infected people have mild- or moderate-intensity symptoms and approximately 80% recover without the need for hospitalization. One in five people develop severe illness and experience breathing difficulties, among other serious symptoms. Importantly, it is possible that transmission can occur from someone who either has mild symptoms, remains asymptomatic, or is in the incubation period [6].

According to the United States Centers for Disease Control and Prevention (CDC), the incubation period (time from infection to illness onset) is from 2 to 14 days after exposure, and symptoms develop in 97.5% of infected individuals within 12 days [7]. Adopting a 14-day period of quarantine has become the standard procedure for individuals who have been in contact with someone with a confirmed COVID-19 diagnosis or who have traveled to high-risk areas [8].

SARS-CoV-2 is transmitted through droplets produced when an infected person coughs, sneezes or talks, which are ingested or inhaled by an individual nearby (within a distance of approximately 2 meters). A person can also become infected by touching a contaminated surface or object that has been contaminated with the virus and subsequently touching the mouth, nose, or eyes [9]. The diagnosis of COVID-19 is confirmed via a positive nucleic acid test result using sputum, a throat swab, or lower respiratory tract secretion sample [10].

The Lancet COVID-19 Commission has offered a list of practical solutions to the challenges during the current pandemic. One set of solutions is focused on non-pharmaceutical interventions in which governments, non-governmental organizations (NGOs), and the private sector collaborate to mitigate negative consequences of the pandemic [11]. In line with this, the WHO has recommended the use of face masks, proper hand hygiene, physical distancing and mobility restrictions, among other measures [12–14]. In Mexico, the federal government announced the National Social Distancing Period Intervention, which included closure of non-essential public and private activities with the objective of reducing population mobility to limit community transmission, especially among those with a high risk of developing complications (age 60 years and older; people with hypertension, type 2 diabetes, obesity, respiratory diseases, or immunosuppression [15–17]; and pregnant women) [18].

2. About the Carso Group

The Carso Group (CG) is a Mexico-based conglomerate of companies spanning five sectors: commercial, infrastructure and construction, industrial, energy, and banking [19]. CG comprises more than 50 companies, with operations in over 5000 locations throughout Mexico and approximately 180,000 employees. CG companies are located in 1333 municipalities (54.2% of the total municipalities) within the 32 Mexican states, and 83% of these are located in 20 metropolitan areas: 45.6% in the Mexico City Metropolitan Area, 4.1% in Puebla-Tlaxcala, 4.1% in Guadalajara, 3.9% in Tijuana, and 3.8% in Monterrey.

The COVID-19 pandemic has affected all economic activities globally, forcing governments and entities to implement actions to contain its transmission [20–22]. Among the most important of these, most economic activity has shifted to telework, with the exception of activities that are deemed essential [12, 15, 23]. Importantly, some CG companies are engaged in essential economic activities in areas such as telecommunications, banking, mining, and energy. In addition, CG employees have varying levels of risk, either owing to older age or underlying non communicable diseases (NCDs). Some employees are more vulnerable because the nature of their work requires constant interaction with others, such as those working in retail stores, or because their household or workplace is located in a municipality with a high risk of contagion.

In response to the COVID-19 pandemic, the CG Board of Directors commissioned the Carlos Slim Foundation (CSF), a non-profit independent organization linked with the Group, to design and implement a comprehensive response protocol to contain the spread of COVID-19 in the workplace, with the goal of protecting the health of employees and their families, as well as suppliers and clients.

The CSF was founded in 1986. Its aim is to address social inequalities by improving quality of life for the most vulnerable populations by training human resources and promoting greater opportunities. The scope of work of the CSF spans education, employment, health, nutrition, social justice, culture, human development, natural disaster support, economic development, and environmental protection [24].

The Carso COVID Protocol is the result of the abovementioned efforts. The protocol is divided into two components, launched at different times during the COVID-19 pandemic in Mexico; Annex 1 provides greater detail regarding the policies and recommendations outlined in the Protocol:

- Prevention and containment of COVID-19, launched on March 13, 2020
- Safe return to work, launched on May 22, 2020.

The CSF has extensive expertise in the design, development, implementation, and scale-up of digital health (DH)-based solutions aimed at improving health care service delivery to strengthen primary care, with a strong focus on prevention [25]. Hence, the CSF convened a team of experts in different disciplines to design a COVID-19 digital health ecosystem to facilitate implementation of the Carso COVID Protocol.

3. Digital health in the response to viral outbreaks

One of the basic epidemiological principles in mitigating infectious disease outbreaks is to stop transmission through timely detection and isolation of cases [26]. Hence, leveraging DH contributes to an effective public health strategy to prevent and mitigate the spread of an infectious disease [27].

DH is the new paradigm in health care, defined as "the convergence of four disruptive technologies: 1) the use of digital platforms, connected through micro-services, 2) cloud-based services with robust infrastructure to support big data transactions, 3) use of inter-connected wearables, devices and tracers, and 4) connecting communities through mobile phone apps and social media. These technologies are enhanced by Artificial Intelligence." [28]. The potential of DH has been demonstrated in detecting and mitigating infectious disease outbreaks and epidemics in countries worldwide [29]. Some examples of successful DH-based strategies include the 2014–2016 Ebola outbreak across West Africa [27] and the 2003 global outbreak of SARS-CoV [29]. In Mexico, there is evidence of the benefits of a mobile phone-based intervention to support surveillance during the H1N1 influenza pandemic in 2009 [30].

The current COVID-19 pandemic highlights the need for innovation in continuous surveillance, rapid diagnosis, and real-time contact-tracing of emerging infectious diseases. Health systems are structured around face-to-face medical visits, which involve direct interaction between patients and their health care providers [31]. This operating model can contribute to faster spread of infection among uninfected individuals, quickly overwhelming available critical care services [31, 32]. Additionally, health care providers are at high risk of exposure to COVID-19 infection, which leads to reduced availability of skilled clinicians over time. When health care resources are strained to a breaking point, patient care may be compromised, which is associated with negative outcomes and increased mortality rates [33].

Given the aforementioned scenario, there is a window of opportunity to implement, expand, and integrate DH technologies across the health care system [34]. The use of digital platforms has become the primary means of communication whereby people, governments, organizations, and health institutions can communicate, work, interact, share and exchange knowledge and information, and generate data [35].

DH has become increasingly instrumental in the response of governments and organizations to COVID-19 [36]. DH solutions include, but are not limited to, telemedicine/telehealth, electronic medical records, public health surveillance leveraging on big data and AI algorithms, wireless health sensors, georreference-based tracing technologies, mobile health applications (apps), and health analytics platforms for public health and clinical decision-making [33]. In the case of

Type of solution	Type of tool	Country and developer
A. COVID-19 general information		
Coronavirus symptoms	Government websites, apps, chatbots, forums, SMS text messaging, call centers	WHO: Chatbot [38], website [39] PAHO: Website [40] Uruguay: Call center [41] Paraguay: Ministry of Health free call center [42] Mexico: COVID-19MX App [43], chatbot [44], websites [44–46] Trinidad and Tobago: Website [47] Argentina: Chatbot [48] Jamaica: Website [49] USA: CDC website [8] Spain: Ministry of Health website [50] and AsistenciaCOVID19 App [51] Canada: COVID-19 Virtual Assistant and website [52] Colombia: Government website, telephone lines, and virtual assistant [53] Bolivia: Government COVID-19 call center and BoliviaSegura App [54]

Type of solution	Type of tool	Country and developer
Real-time information regarding the status of the epidemic in each country	Dashboards, websites, apps	WHO: Interactive dashboard [55] PAHO: Big data tool [56] Google: COVID-19 Situation Dashboard [57] Humanitarian response: Interactive map [58] HealthMap: Interactive map [59] Johns Hopkins: Interactive dashboard [60, 61] Spain: Ministry of Health website [62] Brazil: Ministry of Health Interactive map [63] Mexico: COVID-19 Situation Dashboard UNAM [64] and COVID-19 Traffic Light Monitoring System SSA [65] Jamaica: Interactive dashboard [66] Colombia: Interactive map [67]
General questions regarding COVID-19	Government websites	PAHO: COVID-19 website [68] WHO: COVID-19 website [39] Mexico: COVID-19 web page [44] Spain: Ministry of Health website [62] USA: CDC website [69] and Department of Health and Human Services [70] Canada: COVID-19 website [71] Colombia: Government website [53] Bolivia: Government COVID-19 website [54]
Self-diagnosis support to seek medical care	Apps, chatbots, websites, call centers, self-assessment tools	WHO: Chatbot [38] Google: COVID-19 self-assessment [72] Johns Hopkins: Coronavirus self-checker tool [73] Peru: COVID-19 coronavirus evaluation CDC: Coronavirus self-checker tool [74] Jamaica: Call centers [75] MAYO Clinic: Coronavirus self-assessment tool [76] Canada: COVID-19 self-assessment tool [77] Colombia: COVID-19 self-diagnosis tool [78] and virtual assistant [53] USA: Veterans Crisis Line Veterans Health Administration - COVID-19 Response Plan [79]
General guidance from health care professionals	Apps, call centers	Argentina: National call center [80] Uruguay: Chatbot and call center [81] Mexico: National Call Center [82], UNAM call center [83]
B. Teleconsultation and patient monitoring		
Patient monitoring and follow-up, contact tracing	Apps, call centers, teleconsultations	Brazil: Monitora COVID-19 App [84] Uruguay: Coronavirus UY App [85] France: StopCovid App [86] China: Beijing Cares App [87] Australia: COVIDSafe App [88] South Korea: Self-quarantine Safety Protection App [89] India: AarogyaSetu App [90] USA: Veterans Health Administration - COVID-19 Response Plan [79] Indonesia: PeduliLindungi App [86] Germany: Corona-Datenspende [91] and Corona-Warn-App [86] Hong-Kong: StayHomeSafe App [92] Colombia: CoronApp [93]

Type of solution	Type of tool	Country and developer
Clinical support from health professionals	Apps, call centers, teleconsultations	Guatemala: Online doctor app [94] Peru: Telephone line [95] USA: Veterans Health Administration - COVID-19 Response Plan [79] USA: Department of Health and Human Services [70]
Follow-up of suspected cases in quarantine	Apps, call centers, teleconsultations	Brazil: Monitora COVID-19 App [84] Colombia: CoronApp [93] Costa Rica: EDUS COVID-19 app [96] Poland: Kwarantanna domowa App [86]
Remote clinical management	Call centers, teleconsultations	Mexico: phone line, WhatsApp for emotional, nutritional, and medical attention for TecSalud workers [97]
C. Education and training		
Evidence-based public health information regarding COVID-19	Web-based information, specialized interactive websites for researchers	WHO: Virtual Health Library COVID-19 [98] Cochrane Library on COVID-19 [99] PubMed: LitCovid literature hub [100] OAS: Organization of American States COVID-19 Repository [101] Elsevier: National Library of Medicine Elsevier Information Center [102]
Evidence-based materials for ongoing training	Virtual campuses, webinars, interactive platforms	WHO: Virtual Campus [103] PAHO: Virtual Campus [104] Mexico: Mexican Government website COVID-19 courses [105] Coursera: Virtual Campus [106] CDC: Virtual Campus [107]

Table 1.
Digital solutions developed to prevent and manage COVID-19 around the world.

COVID-19, DH can offer real-time access to comprehensive individualized reliable data, to enable personalized monitoring and provide AI-based assistance. DH can also be implemented to access reliable data and information, participate in social media, use risk-based algorithms to support self-diagnosis, seek health professionals to receive clinical support, and maintain work activities, among other applications [35, 37]. **Table 1** shows some DH solutions that have been developed and are being used by institutions, governments, and NGOs around the world to prevent, manage, and mitigate COVID-19.

4. MONITOR digital health ecosystem

The first case of COVID-19 in Mexico was reported on February 27, 2020 [108, 109]. Fully aware of the benefits of DH, the CSF had begun to work on the design of a robust response plan in January 2020. On March 13, 2020, the Carso COVID Protocol was launched, including a comprehensive set of actions and recommendations to prevent and contain COVID-19 in the workplace. As part of implementation of the Protocol, the CSF launched the MONITOR digital health ecosystem (MDHE), aimed at monitoring the wellbeing of CG employees and their families.[1]

[1] Annex 1 provides a detailed description of the recommendations and actions in the Carso COVID-19 protocol.

MONITOR was initially designed based on WHO and CDC guidelines, and its recommendations are in compliance with current regulations of Mexico's Ministry of Health. Furthermore, MONITOR has been continuously updated with the latest available scientific evidence.

The MDHE comprises three interconnected platforms operating concurrently: a mobile phone application and a web portal for employees and their families to register and report symptoms on a daily basis, the Integrated Measurement for Early Detection (MIDO)-COVID Platform to assess NCDs and COVID-19 serological status among employees at worksites, and the Health Intelligence Platform with robust analytics to support decision making. Importantly, the MDHE is compliant with Mexico's regulatory standards in terms of confidentiality, security and privacy.

4.1 Prevention and containment of COVID-19 in the workplace

MONITOR enables the implementation of a COVID-19 prevention and containment strategy according to the following stepwise process:

1. An employee registers in MONITOR using either the mobile phone app or a secure web portal. During registration, the employee is asked to provide information on any existing NCDs.

2. On a daily basis, individuals report whether they have any symptoms and if so, describe those symptoms. Using a point-based risk algorithm, MONITOR automatically assesses and classifies each person's risk and provides immediate recommendations, as follows:

    a. No risk (no symptoms): the employee is encouraged to continue notifying on a daily basis.

    b. Mild risk: the employee is encouraged to increase the number of notifications to twice a day and to continue monitoring.

    c. Moderate risk: the employee is encouraged to increase notifications to twice a day and if necessary, to call a dedicated call medical center operating 24/7/365.

    d. Severe risk: the employee is encouraged to increase notifications to twice a day and to call a medical call center. In addition, an alert is triggered to the human resources (HR) department of the employee's company, to signal a potential complication.

3. At the medical call center, a general physician provides remote counseling, and assesses whether the employee requires a reverse transcription polymerase chain reaction (RT-PCR) test to confirm a diagnosis of COVID-19 infection, or whether they need to be referred for immediate medical assessment.

4. If a person is referred for an RT-PCR test, the general physician schedules an appointment at any of the CG-dedicated lab facilities in Mexico City and 27 cities throughout the country. Once the employee has been tested, they are instructed to remain in isolation until their test results are obtained, within 24–48 hours.

5. Upon confirmation of COVID-19 infection:

    a. The employee receives a pulse oximeter for self-monitoring during the 14-day isolation period. The employee is required to notify symptoms via MONITOR.

b. The CSF epidemiology team, in coordination with company HR departments, conducts an outbreak investigation for each employee with confirmed COVID-19 infection, including tracing of all work and family contacts. A contact is defined as someone who has remained in close proximity (less than 2-meter distance) with the employee for at least 15 minutes while not wearing personal protective equipment; this definition is in line with WHO recommendations [9, 10].

c. All identified contacts are sent for confirmatory laboratory testing and are closely monitored throughout the period in which they are ill.

6. All collected data are available through the Health Intelligence Platform for real-time analytics, follow-up, and clinical support, for both HR departments and the CSF epidemiology team.

4.2 Safe return to work with MIDO-COVID

With ongoing transmission of SARS-CoV-2 during the phased reopening of non-essential activities in Mexico, a series of measures were implemented in CG's workplaces to ensure the safe return of employees. These measures are described in Annex 1.

There is ample scientific evidence demonstrating that the presence of comorbidities increase the risk of severity and complications of COVID-19, particularly cardiovascular disease, diabetes, hypertension, chronic lung or renal disease, and obesity [17]. In light of this evidence and building on previous experience [110–112], the CSF designed and developed the MIDO-COVID Digital Platform, aimed at assessing NCDs and COVID-19 serological status among employees resuming operations in the workplace.

Like MONITOR, MIDO-COVID is implemented following a stepwise process, performed by a MIDO expert[2]:

1. Registration of employees in MIDO-COVID, retrieving their data from the MDHE.

2. Measurement of weight/waist circumference and height, blood pressure, and capillary blood glucose, either fasting or random.

3. Performance of rapid antibody tests.

4. Recording of measurements and serologic test results.

5. Analytical algorithm with integrated risk profiling:

   a. NCDs profile with interpretation and recommendations.

   b. Serology test results with interpretation and recommendations.

The MIDO-COVID Digital Platform automatically delivers certified serologic test results. Importantly, this assessment confirms the presence of NCDs and

[2] In each workplace, employees are chosen to be trained as MIDO-COVID experts. These employees complete an online course in which they learn to measure weight, height, blood pressure, and capillary blood glucose; and how to record these measurements in the digital platform, perform serological tests, and provide brief counseling when informing employees of their test results.

Registrants in MONITOR	**N = 254,043**
Employees	184,117
Family relatives	69,926
1. Prevention and containment of COVID-19 in workplaces	
Medical call center calls	257,803
Laboratory tests performed	29,693
Positive cases (positivity rate)	5124 (17.2%)
Outbreak studies in workplaces	1840
2. Safe return to work with MIDO-COVID	
Total assessments	46,740
Main results of serology tests	
IgM – / IgG – (not exposed)	39,505 (84.5%)
IgM + / IgG – (early-stage infection)	322 (0.7%)
IgM + / IgG + (acute infection)	2013 (4.3%)
IgM – / IgG + (past infection)	4812 (10.3%)

Table 2.
Main results of the MONITOR digital health ecosystem (13 march to 31 October, 2020).

validates the self-reports provided by employees using the mobile phone app. Finally, in the case of active COVID-19 infection, the employee is quarantined and the contact tracing protocol is begun, as described above.

In sum, joint coordination of the HR department at each company with the epidemiology team at the CSF has enabled effective deployment of the Carso COVID Protocol through the use of MONITOR. **Table 2** shows the main results of the MDHE as of October 31, 2020.

5. Permanent strategies following the current public health emergency

Given that the COVID-19 pandemic has changed the way that companies function, the CG intends to retain certain strategies to protect employees' health. The COVID-19 pandemic has provided an excellent opportunity to improve the workplace environment in terms of health and safety at every worksite. The CG is fully committed to providing every employee with all the preventive tools, measures, and strategies needed to maintain a physically and mentally healthy community. In this sense, the CSF encourages joining MONITOR together with MIDO, with the recognition that poor control of NCDs increases the risk for COVID-19 complications [113]. MIDO screening can facilitate early detection of type 2 diabetes, hypertension, and dyslipidemia, focusing specifically on pre-disease stages and early treatment. MIDO offers a systematic risk assessment of screened individuals, identifying those with a healthy, at-risk (pre-disease), or disease status [112].

Inter-connecting MONITOR and MIDO-COVID in a digital ecosystem allows HR personnel to identify COVID-19 positive employees or those with high-risk of complications. Daily information can be used to better monitor, diagnose, track and control employees' infection risk and overall health. Moreover, data are stored in a secure cloud, where it can be retrieved to generate predictive models for each

company and type of workplace, as a strategy to better understand and control risk factors in each sector.

Flexible schedules, in which employees alternate teleworking and working at a CG location when necessary, are very important. If an employee feels unwell, they must notify their direct manager and should not present to their work location. In worksite dining rooms, menus should be based on nutritional recommendations following the EAT-LANCET Commission on healthy diets and sustainable food production. Every meal is to be prepared according to the planetary health plate, characterized by at least 50% vegetables (fruits and vegetables) and the remainder comprising whole grains, plant-source protein, animal-source protein, dairy foods, and unsaturated plant oils [114].

Workplaces are implementing programs to promote wellbeing and healthy lifestyles among employees and their household members. In this way, the CG seeks to empower its employees through health promotion initiatives conducted by trained multidisciplinary health professionals in topics including nutrition, NCDs prevention, physical activity, vaccination, mental health awareness, and wellbeing.

As part of its response to COVID-19 as well as other novel pathogens, the CG plans to create emergency response teams and permanent communication via DH among HR departments across all CG businesses. Frequent intervention assessments will be carried out to gauge adherence to protocols and determine where improvements are needed.

6. Conclusions

This chapter describes the CG COVID-19 mitigation strategy within a corporate group in Mexico. Priorities for the CG during this outbreak have been to protect employees' health and wellbeing by implementing protocols and strategies based on scientific evidence. Consequently, occupational safety and health have taken on greater relevance in all kinds of workplaces.

First, our experience shows that Digital Health can be used to quickly identify people with any infection risk, during early stages. CG employees are empowered through advice and counseling using IT tools such as a mobile phone app or website. The MONITOR strategy has proven to be an effective intervention. The use of DH has been instrumental in outbreak control and maintaining workplace activities. Second, we have learned that the use of a Digital Health ecosystem is effective in detecting and controlling COVID-19 outbreaks in work settings.

This experience can be useful for other organizations in the process of implementing and operating digital health based strategies to cope with outbreaks of viral disease.

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Conflict of interest

All authors declare no conflicts of interest.

Notes/thanks/other declarations

None

Abbreviations and acronyms

AI	artificial intelligence
CDC	Centers for Disease Control and Prevention
CG	Carso Group
CSF	Carlos Slim Foundation
DH	digital health
HR	human resources
MDHE	MONITOR digital health ecosystem
MERS-CoV	Middle East respiratory syndrome coronavirus
MIDO	Integrated Measurement for Early Detection
NCDs	non-communicable diseases
NGO	non-governmental organization
PAHO	Pan American Health Organization
RT-PCR	reverse transcription polymerase chain reaction
SARS-CoV	severe acute respiratory syndrome coronavirus
SARS-CoV-2	severe acute respiratory syndrome coronavirus-2
SSA	Ministry of Health
WHO	World Health Organization

Appendix 1. The Carso COVID Protocol

To support the Carso Group (CG) COVID-19 response plan, the Carlos Slim Foundation prepared and designed the Carso COVID Protocol, called the "Recommendations and guidelines for the prevention and management of COVID-19 in an organization". The Protocol has two components, launched at two time points during the course of the pandemic:

- Prevention and containment of COVID-19, launched on March 13, 2020 (**Supplementary Table 1**)
- Safe return to work, launched on May 22, 2020 (**Supplementary Table 2**).

This Protocol is based on scientific evidence regarding infectious diseases and the most important available evidence regarding COVID-19 to date. Most recommendations are based on WHO and CDC guidelines and are continuously updated as new evidence becomes available.

The Protocol was designed with consideration for the needs of the different CG workplaces. Nonetheless, each company within the CG has adapted the recommendations to the operational needs of each workplace. For example, most employees in workplace settings that were not considered essential switched to teleworking whereas employees performing activities classified as essential implemented different schemes, such as staggered working schedules. It is noteworthy that the Protocol was implemented at all CG worksites.

Section	Description	Main actions/recommendations
Continuity of Operations Group	In each company, this refers to a team comprising employees with decision-making capacity. This group designs, coordinates, and establishes the organization's continuous operations policies and guidelines.	• Oversee adequate implementation of the COVID-19 protocol. • Communicate general preventive measures. • Ensure the implementation of proper cleaning and disinfection policies. • Define and implement work-at-home policies and provide guidance to staff regarding their implementation to maintain operations. • Monitor employees' health status and implement quarantine and isolation policies according to guidelines. • Monitor the evolution of employees with COVID-19 infection in isolation. • Update COVID-19 prevention and care policies according to the latest evidence and government regulations.
Communication strategies	Identifying the point of contact in each workplace and department to communicate new policies and guidelines.	• Communication of Protocol policies through available channels: billboards, intranet, email, social media, mobile phone text messaging, etc. • Implementation of a "hotline" available to employees 24/7, to address any questions or request support.
General containment measures	This refers to general containment measures for employees.	Measures are classified according to risk: • General (employees, suppliers & visitors). • Asymptomatic employees. • Employees with any symptoms of respiratory infection. • Employees diagnosed with COVID-19 (suspected or confirmed) and/or in contact with a confirmed COVID-19 case.
General preventive measures	This describes hygiene and physical distancing measures that must be implemented in each workplace. The supplies needed to implement these policies are also listed. Recommendations for working from home are given.	• Placement of posters and communication materials. • Availability of face masks at workplaces, as well as bins for their disposal. • Equipment for cleaning staff. • Use of 0.1% chlorine solution or 70% ethanol for disinfection of surfaces. • Handwashing and use of alcohol-based hand sanitizer. • Suspension of air conditioner use and proper ventilation measures. • Physical distancing. • Staggered work schedules in confined or crowded workspaces. • Working from home. • Recommendations for vulnerable populations • Timely notification of symptoms. • Establishment of a COVID-19 team in each workplace.

Section	Description	Main actions/recommendations
Employee dining rooms	Describes sanitary measures that dining room staff must implement during the preparation of meals and cleaning of the dining room.	• Measures at the dining room entrance and restriction of access for employees who do not comply with policies. • Use of personal protective equipment among dining room staff. • Safe use of trays, glasses, and cups. • Elimination of self-service areas, such as salad bars or shared condiments. • Isolation of dining room staff with COVID-19 infection or symptoms.
Cleaning company staff	Describes policies and guidelines that all cleaning staff must comply with.	• Policies for work attendance. • Use of uniforms and restrictions regarding jewelry and beards. • Use of personal protective equipment. • Use of 0.1% chlorine solution or 70% ethanol to disinfect surfaces. • Suspension of air conditioner use and proper ventilation measures. • Adequate disposal of garbage.
Travel, business meetings and events	Describes general policies for travel, meetings, and events. This section emphasizes the importance of transitioning to tele- or video conferences.	• Cancelation or restriction of national and international travel. • Isolation policies after returning from essential travel. • Suspension of conferences, events, or summits, regardless of their nature. • Restriction of visitors and suppliers in the workplace. • Promotion of tele- and video conferences. • Restrictions regarding face-to-face work meetings.
Customer service workplaces	This section describes the general policies for workplaces that provide customer service, such as retail and post-sales services and banking.	• Use of face masks and personal protective equipment. • Cleaning of workspaces, and use of 0.1% chlorine solution or 70% ethanol for disinfection. • Identification and management of suspected cases of COVID-19 infection.
Call centers		• Alternating work shifts to facilitate physical distancing. • Identification and management of suspected cases of COVID-19 infection. • Use of 0.1% chlorine solution or 70% ethanol to disinfect surfaces.

Table S1.
Supplementary Table 1. Prevention and containment of COVID-19 (summary).

Section	Main actions/recommendations
Preparation and adaptation of workspaces	• Reinforcement of basic preventive measures. • Adaptation of workspaces: ○ Screens and partitions between workstations. ○ Signs outlining requirements and recommendations posted in areas with high visibility. ○ Strategically located alcohol-based hand sanitizer dispensers that are constantly stocked. • Sanitization and disinfection of workspaces.
Measures to reduce physical interaction	• Staggered work shifts and working from home. • Implementation of flexible work schedules. • Restrictions in common areas, e.g., dining rooms, hallways, and reception desks. • Protocols for interaction with suppliers and customers. • Restriction of meetings and promotion of tele- and video conferences.
Measures to reduce risk of infection	• Organization of teams to supervise the correct application of basic preventive measures. • Point-of-entry screening in each workplace. • Identification and management of suspected COVID-19 cases during working hours.
Employee training and awareness campaigns	• Permanent social media campaigns to raise awareness. • Development and adaptation of content and materials: the CSF developed a web portal with curated information about COVID-19. As of October 31, the CSF has produced more than 150 informational materials including infographics, videos, and audio recordings to support CG companies in raising awareness about COVID-19. • Two online courses for COVID-19 teams and employees

Table S2.
Supplementary Table 2. Safe return to work (summary).

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Chapter 10

The One-Health Approach to Infectious Disease Outbreaks Control

Sima Ernest Rugarabamu

Abstract

Close contact between people, animals, plants, and their shared environment provides more disease transmission opportunities. Host characteristics, environmental conditions, and habitat disruption can provide new opportunities for disease to occur. These changes may lead to the spread of existing and new diseases. Bacteria, viruses, fungi, protozoans, sporozoans, worms, and others cause infectious diseases. Some of these diseases may be prone to explosive outbreaks and may constitute deadly epidemic threats that could rapidly reach pandemic proportions. Drugs and vaccines can successfully control many infectious diseases; however, this is challenged by the lack of facilities and resources. In all parts of the world, infectious disease is an essential constraint to increased human, animal, and environmental interactions. Identifying hot-spot and interventions for prevention while considering the heterogeneity of target diseases to places, population time, or situation is essential. Therefore, successful infectious disease control measures must be based on understanding disease transmission pathways, strengthening surveillance systems, and intervention. Application of the One Health method is a responsive approach to infectious disease control. Much of the One-Health based approach to managing an infectious disease has been utilized with a promising effect on controlling current outbreaks. More deliberate efforts should encourage understanding of disease determinants to analyze infectious disease issues through a One-Health lens. Only through the extensive participation of all related field stakeholders can One-Health truly reach its potential to mitigate infectious disease outbreaks. This chapter reviews utilization of the One Health approach to infectious disease outbreak control.

Keywords: virus, one health, infectious disease, outbreak, control

1. Introduction

Infectious disease remains responsible for a large part of the world's premature death and disability burden [1]. Bacteria, viruses, and protozoa, among other agents, cause infectious diseases to humans [2]. Usually, infected cases are present in numbers at an expected level, but an outbreak may occur every once in a while [3]. A new strain of the disease agent can significantly impact either the local or global levels [4]. For example, global pandemics of smallpox, cholera, and influenza periodically threatened populations before developing improved living conditions, especially in high-income countries [5]. To date, safe, effective, and affordable

vaccines and the increasing availability of antibiotics have reduced the burden of many such diseases in high-income countries, though there is a lack of adequate control in many middle and low-income countries [5, 6].

Close contact between people, animals, plants, and their shared environment provides an increased risk of disease transmission [7]. Host characteristics, environmental conditions, and habitat disruption can provide new disease opportunities [6, 7]. These changes may lead to the spread of new or emerging diseases. For instance, viral diseases have become as common as emerging and re-emerging diseases [8]. They occur worldwide, in a variety of ecological settings [9]. Others are found only in limited ecologic and geographic foci. Over 60 percent of infectious diseases and 70 percent of humans' emerging infections are zoonotic, with two-thirds originating in wildlife or domestic animals [9, 10]. Notable examples of globally emerging and re-emerging infectious diseases include Ebola hemorrhagic fever, dengue, chikungunya, yellow fever, and other respiratory viral infections such as pandemic influenza H1N1 2009, SARS, Avian Influenza (H5N1) and (H7N9) [11, 12]. These diseases are prone to explosive outbreaks and constitute deadly epidemic threats that could rapidly reach pandemic proportions, affecting people's lives [13]. On the other hand, a global increase in antibiotic-resistant bacteria has resulted in pathogens resistant to most or essentially all of the available antimicrobials [14]. Problems facing scientists today include the emergence of diseases from the fast-changing human-animal ecosystems due to multiple factors [15]. Increasing human-environment interaction provides ingredients for outbreaks of infectious diseases [16].

In a world of increased frequency of interaction, animal food production for human consumption, increased use of transportation, and increased movement of people across national borders, these factors act as determinants for infectious diseases by directly or indirectly influencing the occurrence and distribution of infectious diseases [17]. Measures for successful disease control must be based on understanding disease transmission pathways, strengthening surveillance systems, and intervention [18, 19]. This is possible by the application of the One Health approach. Much of the One-Health based approach to managing outbreaks of infectious disease has been utilized with a promising effect to control current outbreaks. More deliberate efforts should encourage understanding of disease determinants to analyze infectious disease issues through a One-Health lens. Only through the extensive participation of all related field stakeholders can One-Health truly reach its potential to mitigate infectious disease outbreaks.

One Health is a collaborative, multisector, and transdisciplinary approach — working from local to global levels — to achieve optimal health outcomes by recognizing the interconnection between people, animals, plants, and their shared environment [20–23]. Therefore, One Health is an approach that recognizes that people's health is closely connected to animals' health and shared environment. One Health has become more important recently because many factors have changed interactions between the environment, people, animals, and plants [24].

Human populations continue to grow and expand. They increase close contact with wild and domestic animals, both livestock and pets [25]. Animals play an important role in lives, whether for food, fiber, livelihoods, travel, sport, education, or companionship [26].

Animals also share susceptibility to environmental hazards and some diseases, which can sometimes serve as early warning signs of potential human illness. For instance, birds often die of the West Nile virus before people in the same area get sick with West Nile virus infection [24–27].

The One Health issue includes a focus on zoonotic and vector-borne diseases, antimicrobial resistance, food safety, food security, environmental contamination, and other health threats shared by humans, the environment, and animals. Other

fields such as chronic disease, mental health, injury, occupational health, and non-communicable diseases also benefit from a One Health approach involving collaboration across disciplines and sectors [28, 29].

One Health is gaining recognition globally as an effective way to fight health issues at the human-animal-environment interface, including zoonotic diseases.

The complexity of health and environmental challenges needs to be evaluated in an integrated and holistic manner to provide a more comprehensive understanding of problems and potential solutions [30, 31]. Concerted efforts in the paradigm shift from the silo-based health systems to the One Health approach is important [29–32]. Decision-makers for disease prevention and control should utilize the One Health approach to prepare for and prevent illness, hospitalization, death, and the economic burden experienced during disease epidemics. In any public health emergency, an early warning system to combat epidemics is usually immediately implemented. Response networks, e.g., the Global Outbreak Alert and Response Network (G.O.A.R.N.), collaborate with institutions and networks to pool their human and technical resources to fight outbreaks [33]. The critical decision to initiate disease response is often reactive and urgently needed in a rapidly changing environment with little or incomplete information available and biased [33, 34]. Traditional surveillance systems provide regular data updates. However, these systems are inherently retrospective and delayed, limiting their utility for real-time epidemic response [35].

Additionally, a silo-based health system deals with present conditions or those immediately expected [30–36]. The One Health approach could help fill these holes by controlling the utility, scale, and timing of counteraction techniques [36]. For example, scientists recognized the link between human and animal health and its threats to Ebola epidemics' welfare and economies. This resulted from the importance of collaborative and cross-disciplinary approaches for responding to emerging and resurging diseases, particularly the inclusion of a wildlife component for global disease prevention and control.

During infectious disease outbreaks, the coordination and communication of prevention strategies – such as vaccination and treatment – support the deployment and management of crucial public health resources [37]. However, earlier trial vaccines that are protective in animals and safe to humans are not useful because most existing beneficial trials are not standardized or validated. Patients' safety remains unknown because the resources are usually limited and not enough to conduct conclusive trials [38]. Testing the drugs in animals is challenged by the unavailability of facilities to conduct research. Specifically, there are not enough biosafety laboratories. A significant issue surrounding this and other potential disease preventive measures is ensuring the availability and affordability of any useful drugs and vaccines [39]. The One Health approach could bring together scientists, public health officials, and researchers from academia, industry, and government in an open project and develop tools to address specific disease prevention challenges [40]. The tool could be a program to predict disease trends while addressing specific needs by engaging decision-makers and researchers in real-world scenarios. For instance, a collaborative effort that focuses on geographic risk can provide greater insight into which geographic areas emergent pathogens may be circulating in but are undetected [41]. These predictive models allow for more strategic focusing of resources for monitoring the emergence and spread of threats [42]. Continuous surveillance of wildlife and domestic livestock in these limited areas for early detection of pathogens may yield faster and more economical results than spreading resources worldwide to detect pathogens [39–42]. Wildlife is a reservoir of an extraordinarily deep and diverse pool of novel microbial agents [43]. Even considering such overwhelming diversity, the actual numbers of microbial agents reported to infect humans and cause disease are probably many

viral infections that remain undetected [44]. Continuous surveillance focusing on these few microbial agents for early detection of pathogens may yield faster and more economical results than spreading resources to all possible microbial agents to detect pathogens [43–45]. As a result, chance-based interceptions permit the utilization of information about the heterogeneity of hazard to target outbreak location to those spots, populaces, times, or circumstances where the danger of sickness is generally considerable and the probability of discovering high [46].

While the specific disease sources remain unknown, many pathogens are thought to be harbored in wild animals or the environment, with initial transmission to humans via contact with infected animal species or fomites, and later spread through human-to-human transmission [47]. The world-ecology provides habitats for diverse fauna [48]. Changes of the natural ecosystem because of social-cultural and environmental procedures have increased closeness between the human populace, domesticated animals, and wildlife, promoting increased contacts with the disease-causing microbe [49]. The One Health approach is expected to provide a good understanding of the drivers of spillover events from hot spot dwelling fauna to interfacing humans, which will enable disease prevention and control at the source while forecasting accuracy, visualization and communication, collaboration and partner engagement, state and local health department perspectives, pilot projects, and other issues at hand.

Using infectious disease outbreaks response as an example, we propose in this chapter utilization of the One Health concept approach, such as identifying spillover sources in both human and animal populations, designing comprehensive surveillance systems, and implementing an intervention approach to combat infectious disease outbreak.

2. Understanding disease transmission pathways

Complex interactions of humans with the biotic and abiotic components of the environment facilitate spillover events [3, 50]. Documenting how diseases occur is the key to understanding disease transmission pathways and different meanings attached to infectious diseases in different communities [34, 51]. This includes identifying potential sources and reservoirs of viruses in environmental, human, and animal systems.

Data collection is crucial to attaining preliminary information for the identification of sources of transmission. Numerous agencies publish data regarding clinical cases of human and animal disease, both spatially and temporally. These data can be collected and analyzed through integrated human-animal disease surveillance to assess infectious disease occurrence [51]. Additionally, spatial and temporal patterns, the likelihood of infectious disease in certain areas or periods, land use [52], human/livestock/wildlife population density [53], and other data may be collected and analyzed. Potential pathways can be prioritized to determine the most relevant regions [54]. A system of weight factors to perform prioritization using statistical models could be developed, such as the degree of human-animal interactions [55]. Regulation of environment and livestock waste could impact the importance of the potential pathways associated with human-animal ecosystem interactions.

3. Strengthening surveillance systems

Surveillance systems of the critical environmental reservoirs and pathways will allow for the early detection of outbreaks. It is essential to quickly identify

critical times and critical locations for the onset of outbreaks by monitoring disease indicators such as the pathogen presence or burden in a particular community with associated risk factors. This can be achieved by the environment, livestock, wildlife, and human sampling [56]. Regular monitoring of critical reservoirs will identify peaks in presence or indicators related to early signals of disease outbreaks [57].

Traditional human and livestock disease detection and management systems are based on diagnostic analyses of clinical samples [58–60]. However, these systems fail to detect early warnings of public health threats at a broad population level and fail to predict outbreaks promptly [61]. An alternative to this could be using human-wildlife contaminated ecosystems such as community-based urine, fecal, and other samples to identify public, wildlife, or livestock health [62]. This kind of monitoring, together with unique sampling, allows early detection and prediction of outbreaks by understanding pathogens, including shedding rates, risks, and magnitudes, critical in disease surveillance [63]. Recently, raw sewage has been used to monitor the presence and abundance of COVID-19 in communities. An epidemiological tool developed and refined by environmental scientists over the last 20 years (Wastewater-Based Epidemiology — WBE) holds the potential to contain and mitigate Covid-19 outbreaks while also minimizing domino effects such as unnecessarily long stay-at-home policies that stress humans and economies alike. WBE measures chemical signatures in sewage, such as fragment biomarkers from the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), only by applying the type of clinical diagnostic testing to the collective signature of entire communities [64]. As such, it could rapidly establish Covid-19 infections across an entire community. The One Health surveillance model aims to identify risks before clinical cases are reported [65]. Mapping pandemic potential can facilitate data collection representative of at-risk regions followed by risk mitigations [66]. Microbial source tracking can be more complicated, especially in limited-resource areas, which necessitates determining the environment to sample. Specific population, shedding rate and natural degradation and comparison, and correlation with clinical data are vital tools for getting reliable information for strengthening surveillance systems [66–68].

Intervention approaches in the One Health approach involve utilizing feasible innovation technology for human and animal ecosystems management, medical and veterinary interventions to oversee diseases, and education of local communities and governments to change human behavior, practices, and policy based on relationships between the environments or human and animal health [69].

The first intervention for vaccine-preventable infectious diseases is wildlife animal vaccination and treatment strategies [70]. Disease preventive measures act as a barrier between human-animal disease transmissions. For example, encouraging the disinfection of clean water could remove pathogens from the community. Interventions to prevent pathogens shedding are among the possible management that requires multiple strategies for accomplishment [71, 72]. For instance, rabies prevention by oral vaccination of wildlife with live vaccines has proven a powerful tool to eliminate or control rabies in multiple countries in Europe and North America. In 2012–2013 U.S. Department of Agriculture's Animal and Plant Health Inspection Service through Wildlife Services program, conducted the field trial involving the distribution of new oral rabies live recombinant human adenovirus type 5 vector, expressing rabies virus glycoprotein (AdRG1.3) (Onrab) vaccine bait in five states [73]. Baits laden with oral rabies vaccines are essential for managing wildlife rabies to monitor human contacts and potential vaccine virus exposure. Continued surveillance like these is needed because of the potential for vaccine virus infection [74].

This approach and several others can be considered examples of the complementary policies for the permanent implementation of interventions. There is a need to regulate animal pathogen shedding in waste products, especially in rural areas and forest ecosystems, and other previously reported critical transmission pathways [75–77].

The modification of human behavior is also imperative to minimize the transmittance of infectious diseases and pathways in which interventions cannot be performed for cost, capability, or convenience [78–81]. One primary health behavior-changing method is educating medical, veterinarians, and environmental professionals in the One-Health approach [82]. It is also crucial to educate the public where people are vulnerable to disease transmission [83]. Especially in impoverished, high-risk areas, robust measures should be taken to educate the public on the critical pathways of transmission of viral disease [84–88].

4. Conclusions

This chapter advocate utilizing the One Health model as part of the solution to the ultimate control of infectious disease outbreaks. Disease transmission includes complex frameworks that incorporate associations between humans, animals, and the environment. These systems have spatial and temporal variations that require a deep understanding of the interaction and the processes within. The most significant advance in understanding disease transmission is identifying reservoirs and primary transmission pathways.

Traditional infectious disease control measures such as case management, vaccination, active surveillance, case identification and isolation, and strategic community engagement have helped contain outbreaks. However, many people still die, and more epidemics are anticipated in previously affected and new geographical areas; new control approaches, including One Health, are essential. Research on the role of wildlife in disease causation should be undertaken to improve the situation. Wildlife surveillance data on the biodiversity of animal interface found in the hot spot regions and the pathogen's activity in animals and humans should also be included in strategic interventions. Overall, infectious disease control's success requires a balance between medicine, veterinary science, bioscience, epidemiology, health systems, socioanthropology, and political science, to facilitate early detection and response to unusual events.

Moreover, documenting how diseases occur is the key to understanding disease transmission pathways and different meanings attached to infectious diseases in various communities. A multipronged approach with data and tracking systems' support is an equally important component in attaining national and global health security. The One-Health-based approach to managing an infectious disease has been utilized with a promising effect to control few current outbreaks; there has still more principally that needs to be grasped by the veterinary network. Increasingly purposeful endeavors ought to urge other professionals to examine infectious disease issues through a One-Health focal point. Only through the broad cooperation of all related field partners can One-Health arrive at its capability to control infectious diseases.

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Chapter 11

Human Cultural Dimensions and Behavior during COVID-19 Can Lead to Policy Resistance and Economic Losses: A Perspective from Game Theory Analysis

Tamer Oraby, Michael G. Tyshenko and Samit Bhattacharyya

Abstract

The recent COVID-19 pandemic has caused significant societal impacts. Besides loss of life there were large additional costs incurred by every country including the treatment of patients and costs to implement response plans. The pandemic resulted in major economic disruptions and stalled growth worldwide due to travel bans, lockdowns, social distancing, and non-essential business closures. Public health officials in almost every country implemented and encouraged Nonpharmaceutical Interventions (NPIs) such as contact tracing, social distancing, masks, and isolation. Human behavioral decision-making concerning social isolation was a major hindrance to the success in curbing the pandemic worldwide. In many developing countries individuals' choices were motivated by the competing risk of losing jobs, and daily income. In this chapter we focus on human behavior concerning social isolation in the context of decision-making during the pandemic. We developed a conceptual framework and deterministic model that integrated evolutionary game theory within our disease transmission model. We illustrate scenarios numerically simulating the model. This study highlights the idea that human behavior is an important component in successful disease control strategies. Economic resilience, especially in low-income countries, can improve public understanding and uptake of NPIs.

Keywords: COVID-19, nonpharmaceutical interventions, cultural dimensions, human behavior, policy resistance, communication, mathematical model, game theory

1. Introduction

A month before the Chinese Spring Festival, the Chinese government reported multiple cases of pneumonia of unknown etiology in Wuhan, Hubei Province, China in December 2019. On January 20, 2020, there were 282 confirmed cases in and around Wuhan, of which 51 were severely ill, 12 were in a critical condition and six deaths as reported to the World Health Organization (WHO) [1]. Three days later public health officials in China implemented strict control measures in Wuhan with a complete lockdown of the population that lasted 76-days. Wuhan is the largest city in Hubei province with a population of over 14 million people [2].

A week later on January 30 2020, WHO declared this outbreak a public health emergency of international concern (PHEIC). The outbreak was caused by a novel coronavirus, SARS-CoV-2, and the disease was named COVID-19 [3, 4]. Since then, almost all countries started implementing several Nonpharmaceutical Interventions (NPIs) such as contact tracing, social distancing, mask wearing, self- isolation, school closures, business closures and countrywide lockdowns at different levels of strictness to stop the spread of the disease.

At the beginning of a pandemic several NPIs can be implemented by public health officials as a way to slow disease transmission until an effective vaccine or antiviral treatment becomes available. Implemented public health measures place restrictions on individuals and understanding how individuals respond and whether they are likely to comply or break new rules is extremely important. Measures can theoretically greatly influence and reduce the spread of the infection. However, human choice and self-interest chosen over altruism, among many other factors, can hamper NPI effectiveness and disease control efforts.

For example, lockdowns and self-isolation (self-quarantine) can be highly effective in reducing transmission but can result in population-wide socioeconomic and psychosocial impacts [5]. Adverse effects from extended isolation have been reported in a number of groups including children and adolescents [6, 7], immigrant workers [8, 9] and adults [10, 11]. Children experienced changes to their eating habits, sleep disturbances, depression and symptoms of anxiety [12–14]. Adults reported increased mental health issues, anxiety, stigma, depression, alcohol related harm, and domestic violence [10, 11, 15].

There are a number of demographics, social and psychological factors underpinning engagement with quarantine, lockdown, and compliance with public health directives regarding personal protective behaviors. Factors include perception of susceptibility to the infection, severity of the infection, perception of the effectiveness of ongoing public health measures, and their ability to conduct the activity safely (self-efficacy) [16]. One of the main reasons identified in research literature for non-adherence to quarantine and self-isolation is the perception of lower risk for the disease or having fewer risk factors [17]. Psychological fatigue is also suggested as a possible reason for NPI non-compliance [18, 19].

While cultural and social factors might be challenged by fear [20], the economic difficulty faced by some groups and especially minorities in some places, plays a role in human choice. This might partly explain the disproportionate COVID-19 incidence and mortality faced by minorities in the US, Australia, Canada, and the UK [21–24]. Similarly, migrant workers in low-income countries are also an economically vulnerable population group [25]. Thus, cultural dimensions (see **Figure 1**) can greatly affect uptake and adherence to NPIs [26–30] as well as disease transmission and mortality [31].

Initial and ongoing compliance by individuals is promoted by the existing level of infrastructure, resources, stockpiles, inter-pandemic planning, communication efforts from authoritative sources and the country's capacity. People afraid of contracting a viral infection will adhere to the best hygienic procedures, use masks, practice social distancing and avoid crowded places. While such measures act to delay the spread of viral diseases, like COVID-19, it will not completely protect the population. Public health directives that seek to reduce population-level risk factors and disease transmission are closely aligned with the idea of each individual practicing the best hygienic procedures, collectively, to achieve high compliance.

Indeed, economic growth and capacity as measured by gross domestic product (GDP) provides a measure of the pre-existing infrastructure to maintain and enforce law and order, regulate economic activity, and provide public goods during a protracted pandemic wave [32]. Many countries in less-developed parts of the

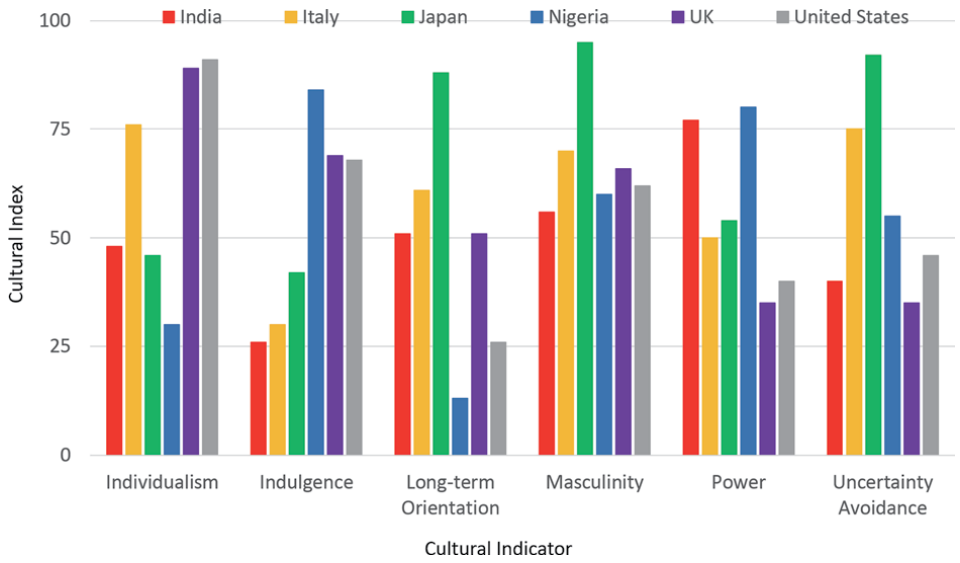

Figure 1.
A comparison of six cultural dimensions among six countries. For discussion see the text. (data source: https://www.hofstede-insights.com/).

world lack this capacity and are more vulnerable to system shocks like pandemics that disrupt economic growth and reduce GDP (**Figure 2**) [33].

Two decades ago, British psychologist James Reason introduced the Swiss Cheese Model to describe how failures in complex systems occur [34]. In his model he suggested that multiple defenses can be in place, whose function is to protect individuals from hazards, but these can possess inherent weaknesses. Multiple safeguards or barriers are like slices of Swiss cheese, having many transient holes. Having holes in any one "slice" does not normally cause a bad outcome. If the holes in many layers line up so they permit a trajectory of accident opportunity through the layers, then it allows for hazard exposure resulting in victims. The holes in the established defenses arise for two reasons: active failures and latent conditions. Nearly all adverse events involve a combination of these two sets of factors.

Google mobility data trends reported from mid-February to mid-December 2020 provide insight into the conditions and active failures during the COVID-19

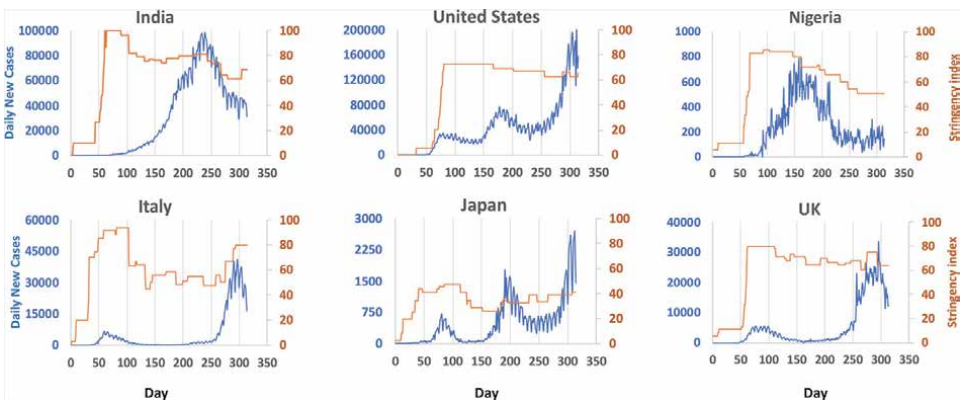

Figure 2.
Daily new cases and lockdown stringency index for the six countries. The day '0' starts with the date January 22, 2020. The percentage reduction in the growth rates of GDP in 2020 due to COVID are as follows: India −10.29%, US -5.91%, Nigeria −3.41%, Italy −10.6%, Japan −5.27%, UK -10.2%. Data source: Our world in data, https://ourworldindata.org/covid-mobility-trends, and https://www.statista.com.

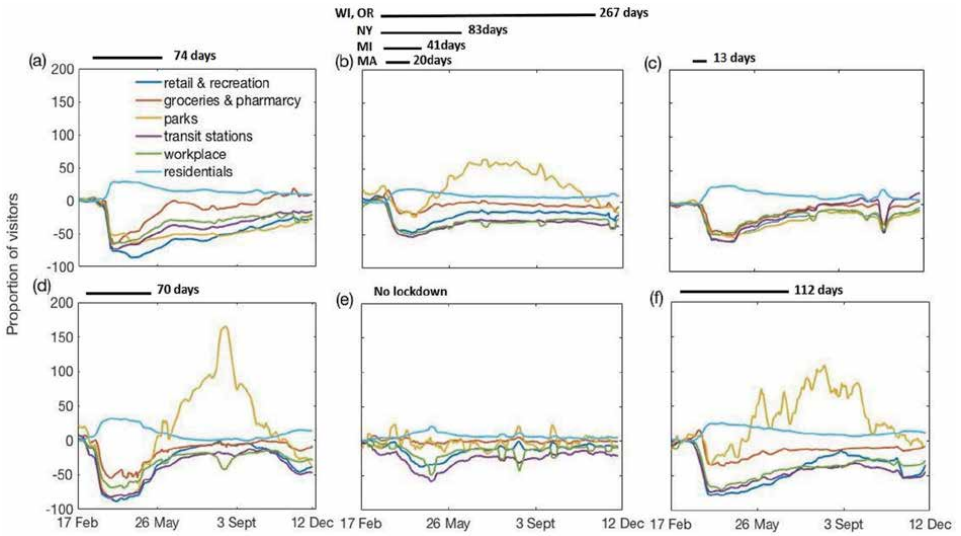

Figure 3.
Google mobility trends: Movement of people during COVID-19 lockdown period: (a) India, (b) United States (MA-Massachusetts, MI-Michigan, NY-New York, and WI, OR - Wisconsin, Oregon), (c) Nigeria, (d) Italy, (e) Japan, and (f) UK. For discussion, see the text. (data source: Our world in data, https://ourworldindata.org/covid-mobility-trends).

pandemic stemming from changes in human behaviors. In India (**Figure 3a**) there was good compliance at the beginning of the 74-day lockdown that began on March 25, 2020. However, as the lockdown progressed movement in all tracked mobility categories slowly increased until the end of lockdown. Retail and recreation showed an increase at the beginning of the lockdown as some people ignored social isolation to maintain their livelihoods.

Unlike India, the United States (**Figure 3b**) did not implement a nationwide lockdown, instead many states put in place lockdowns of various lengths ranging from 20–267 days (many states began lockdowns during the third week of March 2020). Compliance remained high for the first month and slowly mobility in all categories increased. Notably, mobility to parks and other open spaces increased significantly as shorter lockdowns in some states ended as spring weather arrived.

Nigeria (**Figure 3c**) imposed a 13-day lockdown on March 30 2020 with good compliance. Once the short lockdown ended mobility trended back upwards towards normal levels over the next two months.

Italy (**Figure 3d**) implemented a 70-day nationwide lockdown that began on March 9 2020 after large clusters of cases were reported in Northern regions of the country. Compliance was good with decreased mobility in all categories except visits to parks and outside spaces.

Japan (**Figure 3e**) was one of the few countries that did not use a lockdown strategy, mobility decreased to transit stations, retail businesses and workplaces as people followed government guidance and avoided hotspot areas and mass gatherings.

The UK (**Figure 3f**) used a 112-day nationwide lockdown that began on March 23, 2020 with good compliance during the first month then mobility increased in all categories. Changes in mobility were similar to what was observed in the United States and Italy. People in the UK spent increasing amounts of time outdoors and in parks during the lockdown [35].

The Swiss Cheese Model can be applied to pandemic defenses or safeguards showing that there are two levels protecting people: personal and interpersonal safeguards. When applying the Swiss Cheese Model to COVID-19 the pandemic barriers which can fail are the early NPIs such as social distancing, self-isolation and

lockdowns. For the model we group these NPIs collectively as "social isolation" barriers. In this chapter, we focus on human behavior of social isolation decision-making during the pandemic and its impact on socio-economic growth. Integrating evolutionary game theory, economic growth model and a deterministic disease transmission model, we develop a conceptual framework to analyze the situation using a Swiss Cheese Model approach. We illustrate the main scenario of social isolation versus no social isolation and its effects on growth by numerically simulating the model.

2. Model and methods

We use a deterministic model of ordinary differential equations (ODE):

$$\frac{dS}{dt} = -\beta(1-x)(A+I)\,S \tag{1}$$

$$\frac{dE}{dt} = \beta(1-x)(A+I)\,S - \alpha E \tag{2}$$

$$\frac{dA}{dt} = \alpha(1-p)E - \mu_A A \tag{3}$$

$$\frac{dI}{dt} = \alpha p E - \mu_I I - \mu_H I - \mu_D I \tag{4}$$

$$\frac{dH}{dt} = \mu_H\, I - \mu_R\, H - \mu_D\, H \tag{5}$$

$$\frac{dD}{dt} = \mu_D\, I + \mu_D\, H \tag{6}$$

$$\frac{dR}{dt} = \mu_A A + \mu_I\, I + \mu_R\, H \tag{7}$$

$$\frac{dx}{dt} = rx(1-x)(c_1 I + c_2 D - c_3(K_0 - k)/K_0)) - \xi\, x \tag{8}$$

$$\frac{dk}{dt} = \sigma\,((S+A+R)((1-x)+qx))^{\gamma} k^{1-\gamma} - \delta k - c_h H, 0 < q < 1. \tag{9}$$

with seven states/compartments: susceptible (S), exposed but not infectious (E), infected but asymptomatic (A), infected and symptomatic (I), isolated or hospitalized (H), dead (D), and recovered (R) (see **Figure 4**). The same letters (*S, E, A, I, H, R,* and *D*) are used notations for the variables that represent the proportion of individuals in each compartment. In this model, the effective transmission rate $\beta(1-x)$ is dependent on the proportion practicing social isolation x whose complement modulates the disease transmission rate β. See **Table 1** for definitions of parameters and their values.

We also use a population behavior dynamical Eq. (8) to model the dynamical changes of x in which people abiding to social isolation compare the risks of infection and fear of death to the relative economic loss. They can also break out of isolation after an average of $1/\xi$ days due to fatigue from social isolation. We postulate that the rate of fatigue ξ is dependent on the six cultural dimensions of Hofstede (see **Figure 1**); especially, individualism, long-term orientation, and indulgence. The constants c_1, c_2, and c_3 reflect also perceptions of risk of infection, fear of death and degree of damage due to the relative drop in GDP. Those factors are also related to cultural, social, and economical characteristics of the society. For instance, the perception of risk of infection might be related to uncertainty

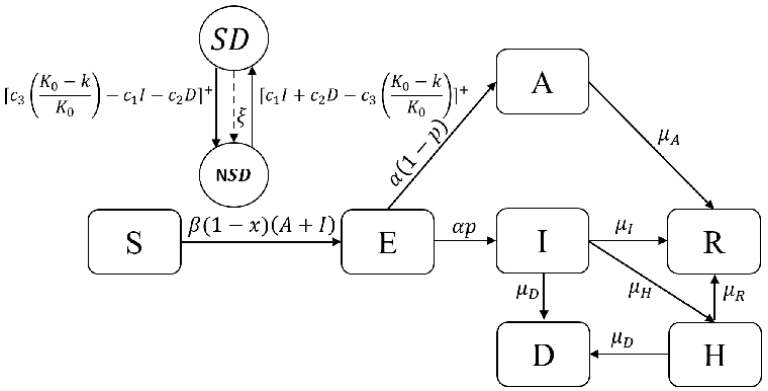

Figure 4.
Schematic illustration of the COVID-19 SEAIHRD model showing the force of infection $\beta(1-x)(A+I)$. Parameters α, μ_A, μ_I, μ_H, μ_R and μ_D are the rates of transition between the compartments. The fraction p is the probability of becoming symptomatic and infectious. The proportion of those who choose to maintain the social isolation is given by x. The pandemic fatigue rate is ξ.

Parameters	Definitions	Values	References
β	Disease transmission rate	0.2306	Calibrated
α	Rate of leaving exposed state	1/7	[36]
p	Probability of becoming symptomatic	0.75	[37]
μ_A	Recovery rate of asymptomatic	1/14	[38]
μ_I	Recovery rate of infectious	1/30	[38]
μ_R	Recovery rate of hospitalized	1/13	Calculated
μ_H	Rate of hospitalization	1/17	Calculated
μ_D	Death rate from disease	0.01	[39]
r	Imitation rate	20	Calibrated
ξ	Pandemic fatigue rate	0–0.5	Calibrated
c_1	Cost of infection	10–1,000	Calibrated
c_2	Fear of death	100–10,000	Calibrated
c_3	Sensitivity to relative economic loss	5–500	Calibrated
c_h	Cost of hospitalization	20,000	Calibrated
σ	Investment rate	0.02/365	Calibrated
γ	Elasticity	0.3	Calibrated
δ	Depreciation rate	0.01/365	Calibrated
K_0	Initial per-capita GDP	55,000	Calibrated
q	Fraction of labor working with social isolation	0.3	Calibrated

Table 1.
Parameters, their definitions, values and references.

avoidance, whereas the economic damage might be related to long-term orientation, masculinity, and socioeconomic status.

The population economic growth/decline is modeled using the Solow economic model of the per-capita GDP ($k = GDP/N$) in $1000 with Cobb–Douglas functional form of investment and production. We assume an initial per-capita GDP of K_0. The per-capita GDP suffers from lack of labor due to isolation except for a fraction q who are working from home. Also, it decreases due to the hospitalization burden that costs c_h per patient-day.

We use the method of Next-generation matrix [40] to find the basic reproduction number R_0 for the disease model without social isolation (in the beginning of the epidemic). The basic reproduction number is given by

$$R_0 = \beta\left[\frac{1-p}{\mu_A} + \frac{p}{\mu_I + \mu_H + \mu_D}\right]. \tag{10}$$

We use this formula for the basic reproduction number to calibrate some of the disease model's parameters at $R_0 = 2.5$ [41].

3. Results

3.1 Model simulation

We simulated the model using the Runge–Kutta method via the function ode45 in MATLAB. The time unit is day. We assume that the epidemic started with 100

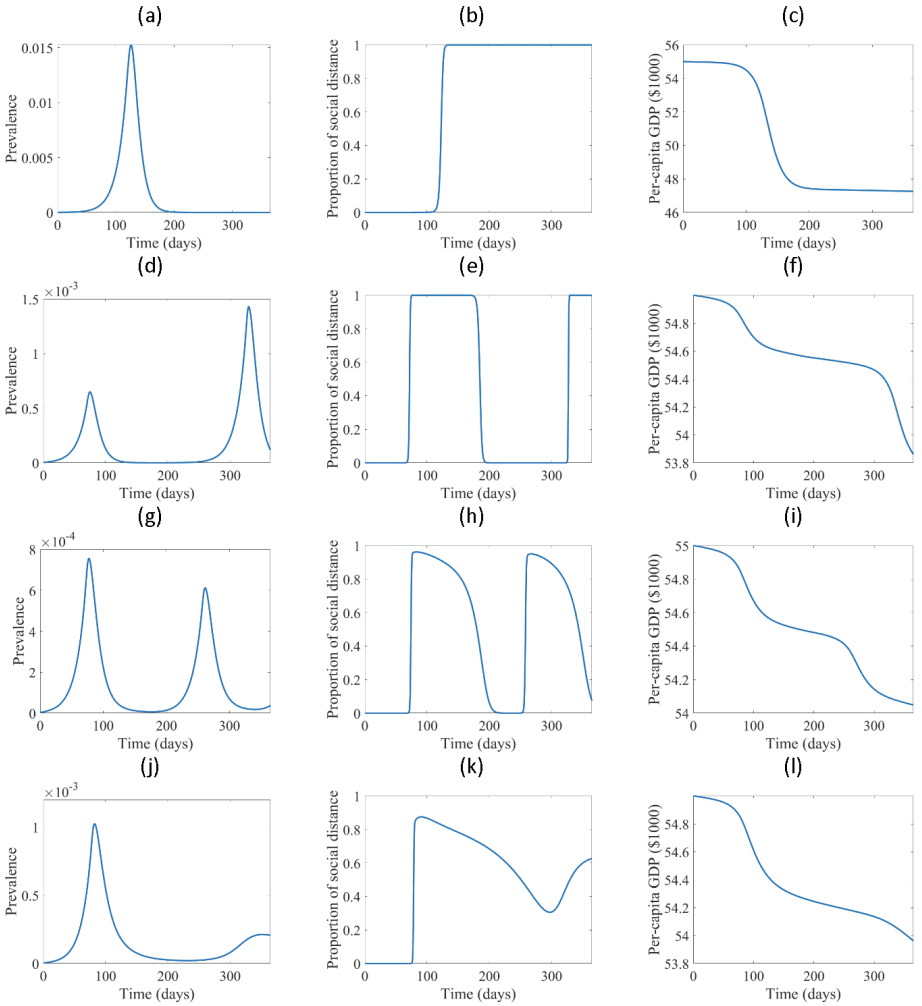

Figure 5.
Simulation of (from left to right) the disease prevalence, the proportion of those practicing social isolation, and per-capita GDP ($1000). The human behavior parameter values are $\xi = 0$, $c_1 = 10$, $c_2 = 100$, and $c_3 = 5$ for figure panels (a-b), $\xi = 0$, $c_1 = 100$, $c_2 = 1000$, and $c_3 = 50$ for figure panels (d-f), $\xi = 0.1$, $c_1 = 100$, $c_2 = 1000$, and $c_3 = 50$ for figure panels (g-i), and $\xi = 0.5$, $c_1 = 100$, $c_2 = 1000$, and $c_3 = 50$ for figure panels (j-l). Prevalence is shown as per 1.

exposed, 50 asymptomatic and 30 infected individuals in a population of size 11,000,000.

Simulations were performed with values given in **Table 1**. In particular, when there is no pandemic fatigue ($\xi = 0$), we found that people can adhere closely to social isolation (policy compliance), resulting in a curb in the disease prevalence, and inflecting and accepting a significant economic burden (at $c_1 = 10$, $c_2 = 100$, and $c_3 = 5$), see **Figure 5 (a)**, **(b)**, and **(c)**. We found also some fluctuations in prevalence occurring from human behavior (at $c_1 = 100$, $c_2 = 1000$, and $c_3 = 50$) (**Figure 5(d)**) and the choice between performing social distance (policy compliance) (**Figure 5(e)**) or ignoring public health directives to maintain economic benefits (i.e. no loss of personal income) (**Figure 5(f)**). The competing interests result in waves of the disease due to changing population level of social isolation versus economic loss from compliance.

In the presence of pandemic fatigue ($\xi = 0.1$), and at the same perceived costs ($c_1 = 100$, $c_2 = 1000$, and $c_3 = 50$), fluctuations continue to occur with a smaller magnitude second wave having a shorter inter-wavelength due to the reduced periods of compliance to social isolation (**Figure 5(g)–(i)**). That is, at a high brunt of economic loss and pandemic fatigue, people might be seen to abandon social isolation which results in a continuation in the spread of the disease, even when fear of death was also high. Increasing the pandemic fatigue rate ($\xi = 0.5$), results in faster decline in policy compliance and a relatively larger epidemic that does not seem to be abated nor fluctuating.

In all of the cases, the per-capita GDP dwindles fast during the waves of the epidemic and slows down as the waves subside, due to the availability of labor and the decreased hospitalizations.

4. Discussion

4.1 Public health guidance and human choice as influencers

Human choice is an important influencer on disease dynamics, and it is dependent on cultural, social and economic factors that might lead to lack of choice. Our model results (**Figure 5**) exhibit that risk of infection, fear of death and the effect of economic loss are important factors as they influence the behaviors of individuals in both lower and higher GDP countries. In lower income countries, an individual's daily wages depend on socioeconomic growth and GDP of the population. The majority of the population in low-income countries survive at or below the poverty line. The World Bank reports there are 33 countries with one-third of the population below the extreme poverty line ($1.90 international dollars/day income) and 69 countries with more than half their population living on less than $5.50 international dollars/day. The definitions of the poverty line vary considerably among nations, however, according to the World bank there are 23 countries with 50% or more of the population living below the nationally designated poverty line deemed appropriate - as defined by its own authorities [42]. The low-income countries include many African countries, Latin American countries (Guatemala, Honduras) or areas suffering military conflicts (Afghanistan, Yemen).

Thus, even small changes in income and GDP will be perceived as a larger income shock to individuals living near or below the poverty line. Individuals with very little capacity will ignore pandemic social distancing directives quicker than those with higher capacity, otherwise they will not have money for day-to-day food and basic necessities.

The perceived relative economical loss (c_3) displays sensitivity of the society to the change in the GDP. If a country is affluent (as reflected by its higher GDP) then c_3 must be of a small value. These countries are less sensitive to any drop, or relative drop, in their GDP. Countries with greater capacity are able to erect more stringent and additional Swiss Cheese Model safeguards. Low-income GDP countries are more sensitive to the changes in the economic cost, thus their c_3 value will be larger. This results in a fluctuation in human behaviors in relation to the economical cost which leads to waves of infections. During a pandemic, social isolation invoked by public health results in a decline in the economy and personal incomes but when the disease transmission (or the perception of disease transmission and risk) wanes individuals with lower capacity will relax their social distancing efforts and change behaviors, returning to work. It results in a resurgence in infectious disease case numbers, which in turn, often results in public health oversight increasing social isolation measures. This effect was observed during the "second wave" of COVID-19 as relaxed NPI measures resulted in a resurgence of detected positive cases in the EU, Africa, Asia, North America and South America [43–45].

4.2 Efficacy, media amplification, and fear as policy resistance influencers

Policy resistance is often cast as a conflict between the Nash equilibrium and the *social optimum* coverage [46]. This can be thought of as the tendency for interventions to be defeated by the system's response to the intervention itself. The role of fear and fatigue in compliance with policy can lead to resistance. Fear as a construct can be driven by media coverage.

Previous coronavirus outbreaks Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS) displayed an amplification of risk perception due to media coverage of the outbreaks [47, 48]. Studies affirm that individuals obtain their news about health and medicine from both mass and social media sources. Daily newspapers, TV channels are one of the biggest influencers of public perceptions of risk. The media plays an important role informing individuals about health risks, but it can also distort perceptions through social amplification of risk. The Social Amplification of Risk Framework (SARF) describes the process where some hazards and events become the focus of intense social and political concern and activity (amplification). This occurs even though experts and risk assessment can establish that the risk is of a relatively low probability, while other potentially more serious events receive comparatively little public attention (attenuation). Media coverage can magnify and change perceptions of risk. The alteration of risk by social amplification creates secondary effects such as stigmatization (of people, places, objects, technologies, and ideas), economic losses, and changes to regulatory oversight due to mass distortion of public risk perception [49, 50].

The efficacies of social distancing and media coverage causing amplification of risk perceptions during COVID-19 are crucial in developing policy acceptance or resistance. In many countries public health risk communication promoted a collectivist and altruistic approach while in other countries policy resistance arose to NPIs through social media. Evidence suggests that belief in conspiracy theories undermines engagement in pro-health behaviors and support for public health policies [51].

For example, in the USA expert messaging carried out by the US CDC regarding mask wearing to protect vulnerable individuals in society became co-opted by social media's distortion of risk (ineffectiveness of masks, lowered perception of SARS-CoV2 infection risk, and as an infringement of personal choice) [52]. Under our

model social media misinformation regarding the risk factors can alter the effective transmission rate through the proportion $1 - x$ of those individuals disregarding mask wearing and social distancing.

4.3 Pandemic fatigue and policy resistance as an influencer

Pandemic fatigue is recognized by the WHO to be natural and expected and is manifested through the decline in motivation of people to adhere to the recommended protective behaviors [53]. It is believed that fatigue emerges gradually [54] and is affected by a number of emotions, experiences and perceptions as well as the demographic, socio-economical, cultural, structural and legislative environment [55, 56]. During those periods, people will perceive personal, social and economic consequences of the social isolations [53]. Later, the perceived cost of infection and potential death will become smaller than the felt loss. For instance, college students reported physical exhaustion and decreased motivation among other feelings with more resilience expressed by senior students [57]. An increased adherence to preventive behavior and avoidance of risky behavior is positively associated with age [55]. A continued preventive behavior was found to be related to older ages; however, all ages grew weary of avoiding risky behaviors like meeting non-household members [55]. The needs of work and low socioeconomic status intensified the risky behaviors whereas lower education exacerbated both low adoption of preventive measures and high practice of risky behaviors [55]. Moreover, reports of regional COVID-19 cases and the fear of death increased the likelihood to implement both preventive measures and avoiding risky behaviors [55]. The disease-behavior-economic model presented in this chapter, including many of those aforementioned factors, showed that human behavior through pandemic fatigue can determine the fate of the epidemic as well as the economic growth.

One factor to overcome pandemic fatigue is resilience or the human ability to adapt to the new circumstances and to accept the existence of the disease risk while coping with it. The WHO recommended four strategies for governments to address pandemic fatigue: understanding people, engagement of people, acknowledgment of hardship, and allowing people to live with reduced risk [53].

4.4 Policy reinforcement of social distancing as an influencer

While most countries around the world implemented early, stringent social distancing policy including lockdowns once the virus began spreading domestically, the Japanese strategy for the COVID-19 outbreak used voluntary guidance for social distancing measures and persuasive messaging. Public health authorities implemented voluntary measures with contact tracing and diagnostic testing. Widely adopted voluntary compliance behaviors appears to have achieved results similar to other countries that used more stringent social interventions (e.g., lockdowns). The policy strategy comes as a trade-off with more healthcare demand and more deaths than if early stringent control was implemented [58]. The strategy's success depends on continued public good will and compliant behaviors. Hofstede cultural dimensions (see **Figure 1**) of high uncertainty avoidance, long-term orientation and masculinity in Japan resulted in high compliance with social isolation. Google mobility data confirms that even in the absence of lockdown the population avoided public transit (e.g., subways, busses, trains), retail stores, and workplaces (see **Figure 3**). The Japanese strategy requires ongoing public health risk communication efforts to maintain high levels of voluntary compliance.

Sweden used no lockdown approach with the public health goals of obtaining herd immunity to COVID-19 (where a threshold is reached where enough of the population would possess immunity to the virus), and secondly as a strategy to minimize economic shock impacts [59]. A similar no lockdown approach was also used in Japan.

In contrast to Japan's voluntary approach, on January 23 2020 China implemented an early mandatory, stringent lockdown strategy in Hubei province affecting 16 cities (including Wuhan) restricting movement of about 57 million people [60]. The unprecedented scale of this lockdown was controversial resulting in an exodus of people out of Wuhan just prior to the lockdown which could have spread the virus. The strategy placed a cordon sanitaire around the city of 11 million people which raised ethical concerns [61]. After 76 days on April 8 2020 Wuhan ended its lockdown [62]. While the Wuhan lockdown was considered a draconian and unprecedented strategy, experts estimated that lockdown in the city of Wuhan prevented between 0.5–3 million infections and 18,000–70,000 deaths at the expense of the economy and in terms of restrictions to personal freedoms [63]. Other countries followed and implemented similar Wuhan-style lockdowns including Italy (provinces of Lombardy and Veneto), Spain, Russia, India and the Philippines [64, 65]. In this way China acted as an "influencer" or role model for other countries that adopted the same type of lockdown, this is an example of reinforcement.

4.5 Economy and outcome inelasticity - social intervention failure as an influencer

Economic downfall due to social interventions including lockdown during COVID-19 have occurred especially in Low- and Middle-Income Countries (LMICs). Other countries like India and Kuwait showed that social interventions failed to effectively reduce local transmission occurring within large migrant laborer populations. The inelasticity occurred with migrant workers in another country (e.g., Indian migrant workers in Kuwait) or workers moving from one state to another state in their home country (e.g., India) [25, 66].

The vast majority of the migrant workers who traveled to Kuwait for work had very limited means. Non-Kuwaiti migrant workers make up more than 60% of the total population and are mostly employed in low-skilled sectors and domestic work. Migrant workers in Kuwait live in cramped dormitories with poor housing conditions having unmaintained and shared toilets, and poor or no ventilation. The lack of social distance and sanitation among occupants resulted in increased COVID-19 transmission among migrant workers [67].

In India, migrant workers usually live and work in megacities under crowded conditions that do not permit social distancing, putting them at an increased risk for disease transmission. Moreover, migrant workers in many LMICs have difficulty gaining access to health care services since they lack health insurance and lack of access to healthcare facilities as a result of administrative barriers [25]. During the COVID-19 pandemic migrant workers from LMICs face conditions that promote inelasticity (communal overcrowded housing, fear of job loss, unsanitary conditions, withheld income and lack of social distancing). Higher GDP countries also encounter this effect but to a much lesser degree with migrant workers (e.g., Canada's Temporary Foreign Worker Program that allows an employer to hire a foreign worker to help harvest crops and fruit) [68]. Many low-income individuals and migrant workers simply cannot adhere to social interventions that reduce transmission risk due to their situation. Their behavioral responses result in unintentional non-compliance and outcome inelasticity.

5. Conclusion

In controlling and managing infectious diseases through social isolation, distancing or vaccination, the role of individual choice is becoming an increasingly important driver that subsequently affects underlying disease burden among the population. In particular, human behavior and social interactions played a significant role affecting the magnitude of the COVID-19 pandemic. Major factors behind such behavioral interactions are losing jobs and forgoing daily income from social distancing, fatigue from social isolation, and/or conscious or unconscious exploitation of uncertainty due to lack of awareness and knowledge. Thus, the dynamics of controlling infection through social isolation is a potentially complex interplay between individual behaviors and disease dynamics, informed by the perceived cost of being socially isolated and infection risks [69]. This complex interplay can be seen as a strategic game and is conveniently modeled and analyzed using the mathematical framework provided by Game Theory [70, 71]. Such behavior-prevalence game theoretical models have already explored vaccine exemption behavior for endemic diseases [72] but there is less emphasis on behavioral interactions like social distancing, especially analysis from the perspective of cultural dimensions of populations and also their socioeconomic conditions. The current study opens up a forum for further research on how individual choice, especially at the population level, is of utmost concern for public health policymakers to curb a pandemic.

Our model scenario highlights the interplay between economic impact and human choice in social distancing measures. Individuals with limited resources must choose between complying with public health guidance (a collectivist approach where personal actions can help the population) at the expense of losing income that is necessary for basic sustenance (an individualist approach). Changes in public policy are essential to combat the long-standing problems associated with health and economic inequities since these are more pronounced during a health care crisis, such as the COVID-19 pandemic.

To address these inequities there needs to be changes in public policy during inter-pandemic phases to ensure planning in place that is activated at the beginning of an outbreak. Policies should act to provide increased resilience and capacity at the beginning of an outbreak to minimize economic losses. Both the public and private sectors can put planning in place to reduce the magnitude of the economic disruption from NPI compliance in the workforce, supply chains, and healthcare system to prevent unforeseen economic crises.

It was suggested that sharing or pooling of available resources and networking can occur at several different levels including: the individual, household, local community, city, state or province, regional and national scale as a strategy to increase resilience and avoid negative mental health and economic outcomes [73].

Pandemic crises such as COVID-19 have particular characteristics within a complex system requiring a number of different types of resilience be addressed including population health resilience (the population recovering from the disease), healthcare system resilience (the recovery of the healthcare system), economic resilience (recovery from the economic consequences) and psychological resilience (individual recovery from fear, anxiety, depression) [74].

In the context of the COVID-19 pandemic drawing on the different types of resilience can reduce psychosocial effects such as depression, anxiety, stress and non-compliance to public health NPIs during curfew, self-isolation and lockdowns. Indeed, previous studies have shown that resilience decreases the negative effects of stress both at the individual and regional levels [75, 76].

The city, regional and country-level attention and support for designated essential workers is important to ensure that they are adequately equipped and compensated for vital services performed to maintain public health standards [74, 75, 77].

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Chapter 12

Antiphospholipid Antibodies in Patients with COVID-19

Cristiana Isabel Sieiro Santos

Abstract

Patients infected with COVID-19 are at higher risk of thrombosis, suggesting an important role of COVID-19 induced coagulopathy. Abnormal coagulation parameters such as elevation in D-dimer are found in patients, with frequent thrombotic events ranging from peripheral ischemia, pulmonary thromboembolism to disseminated intravascular coagulation. Recently, the role of antiphospholipid antibodies (aPL) in the pathophysiology of COVID-19 have been questioned but it remains unclear whether they contribute to coagulopathy. We aim to evaluate the presence of aPL, including LAC, aCL (IgG, IgM), aβ2GPI (IgG, IgM) in a cohort of patients with SARS-CoV-2, study clinical associations and discuss the relevance. The relevance of aPLs in patients with COVID-19 is yet to be determined. Inflammation is closely associated to thrombosis and the presence of inflammatory mediators in COVID-19 infection can lead to thrombosis. Further studies are needed before to determine the role of aPL in COVID-19 patients and their relationship with thrombosis. The presence of aPL should be carefully interpreted as it is important to evaluate the persistence of aPL positivity in patients infected with COVID-19.

Keywords: antiphospholipid antibodies, covid-19, thrombotic, cardiolipin, lupus anticoagulant

1. Introduction

COVID-19 has swept through the world in the last 6 months with 28,584,158 confirmed cases, including 916,955 deaths, being reported to the World Health Organization (WHO), on September 13th 2020 [1].

COVID-19 is caused by SARS-CoV2-virus, a member of coronaviridae that includes MERS-CoV2 which is responsible for severe respiratory illness and causes acute respiratory distress syndrome [2]. Many patients infected with COVID-19 develop a hyperinflammatory response due to cytokine storm syndrome which associates with high mortality [3]. Recent studies demonstrated an association between COVID-19 severity and inflammatory biomarkers such as C-reactive protein, procalcitonin, IL-6 and ferritin. In addition, a high incidence of thrombotic events suggests an important role of COVID-19-induced coagulopathy, despite the use of prophylactic doses of low molecular weight heparin [4]. Advanced age and comorbidities are predictors of increased mortality in COVID-19, which may facilitate thrombosis in these individuals [5].

Several hemostasis laboratory parameters are altered in patients with COVID-19 which constitutes an argument in favor of coagulopathy [6–10]. Abnormal

coagulation parameters such as prolonged aPTT coagulation times, D-dimer elevation and fibrinogen degradation products correlate with COVID-19 severity and are risk factors for higher mortality [10–12].

There are increasing cases of thrombotic events ranging from venous thromboembolic disease, pulmonary thromboembolism to disseminated intravascular coagulation, as well as reports of arterial thrombosis including strokes and myocardial infarctions [13–15]. The activation of leukocytes, endothelium and plaquettes due to cytokine storm, as well as hypoxic vaso-occlusion and direct activation of cells by viral transduction are several mechanisms by which COVID-19 infection may lead to thrombosis [16].

Recently studies have been published about the role of antiphospholipid antibodies (aPL) in SARSCoV-2 patients, leading investigators to start measuring aPL in these patients because of the hypercoagulable state [17–21].

Antiphospholipid syndrome is an autoimmune condition that leads to autoantibodies creation. These autoantibodies react against phospholipids and phospholipid-binding proteins such as beta-2-glycoprotein I (β2GPI) and activate endothelial cells, platelets, and neutrophils, leading to thrombosis [22, 23]. The ability to promote thrombosis in arterial and venous circuits is a defining characteristic of antiphospholipid syndrome. The catastrophic variant of antiphospholipid syndrome is sometimes fatal and resembles to diffuse coagulopathy seen in patients with COVID-19 [24]. Classification criteria for APS includes lupus anticoagulant (LAC), anticardiolipin (aCL) and antibeta2-glycoprotein I antibodies (aβ2GPI) IgG or IgM, if persistently present [25].

The theory involving aPL in COVID-19 infected patients is intriguing however, most studies published so far only include one point of measurement, usually during the acute phase, without confirmation after at least three months, as defined in laboratory criteria of antiphospholipid syndrome [26].

Lupus anticoagulant (LA) is a well-known case of aPTT prolongation, which can be detected in a significant percentage of COVID-19 infected patients. Several biological causes such as high fibrinogen, factor VIII levels and biomarkers such as elevated C-reactive protein (CRP) and the presence of antiphospholipid antibodies may affect the aPTT [27].

We should also keep in mind that aPL can arise transiently in patients with critical illness and various infections [28]. The presence of these antibodies can lead to thrombotic events, making it difficult to differentiate from other types of thrombosis. Viral infections, such as VIH, hepatitis C and parvovirus B19 are triggers of transient aPL, due to a mechanism of molecular mimicry mechanism [29].

To investigate the role of aPL in COVID-19, it is important to evaluate all criteria of aPL, including LAC, aCL and aβ2GPI antibodies with their isotype and to obtain confirmation after at least 3 months, in concordance with the laboratory criteria of APS.

In this report, we illustrate the presence of aPL, including LAC, aCL (IgG, IgM) and aβ2GPI (IgG, IgM) in a cohort of patients with SARS-CoV-2 and discuss the relevance.

2. Materials and methods

We performed a single-centre, cross-sectional study between March 15th and September 15th 2020. The data from all patients who had tested positive for COVID-19 and had presented with thromboembolic complications were collected in a prospectively maintained database and compared to the data from patients without thromboembolic complications. Inclusion criteria: (a) aged >18 years old; (b) no

previous diagnosis of APS (c) positive for COVID-19 (d) patients that required hospitalization. Registry data included the following co-variables: (1) sociodemographic baseline characteristics, such as sex and age; (2) baseline comorbidities including hypertension, dyslipidaemia, hyperuricemia, diabetes mellitus, heart disease, lung disease; (3) need for anticoagulation prior to hospitalization (4) thromboembolic complications during admission (5) mean time of hospitalization (days) (6) laboratorial work (7) aPL positity and isotype determination during hospitalization and confirmed after a 3 month period.

2.1 COVID-19 detection

The diagnosis of SARS-CoV-2 infection was confirmed in all the patients by reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assay or serologic testing.

2.2 Analytical parameters

Screening blood tests included hemogram, D-dimer, C-reactive protein (CRP), procalcitonin, transaminases (serum alanine aminotransferase, serum aspartate aminotransferase), serum lactate dehydrogenase, ferritin, fibrinogen, coagulation times (PTT and aPTT).

2.3 Thrombosis diagnosis

Computerized tomography was performed to identify COVID-19-thromboembolic related complications. Duplex ultrasound was systematically performed to diagnose proximal and distal lower extremity deep vein thrombosis. Thoracic computer-tomography/angiography was performed if pulmonary embolism was suspected and brain computer-tomography in the case of stroke.

All patients received prophylactic or therapeutic dose low molecular weight heparin (LMWH) (enoxaparin) or unfractionated heparin (UFH), accordingly to thrombotic risk evaluated at admission.

2.4 Detection of aPL

Determination Serum aCL and aβ2GP1 (IgG, IgM) were determined by the chemiluminescence assay (CIA) (INOVA) and IgG/IgM aPS/PT were determined by ELISA (INOVA).

The detection of LA in human citrated plasma was performed by the HemosIL dRVVT Screen and HemosIL dRVVT confirm assays, as recommended by the International Society on Thrombosis and Hemostasis (ISTH).

aPL positivity was determined at admission and confirmed after 3 months.

2.5 Statistical analysis

Mann–Whitney U test, χ^2 test, or Fisher's exact test were utilized to compare differences between group A (with thromboembolic complications) and group B (without thromboembolic complications). A two-sided α of less than 0.05 was considered statistically significant. Statistical analyses were performed on SPSS 20.0 package (SPSS Inc.).

Patient dRVVT screen (low phospholipid concentration) and confirm (high phospholipid concentration) results were normalized. Cut-off value was 1.20 for both screen ratio and screen ratio/confirm ratio, demonstrating the

phospholipo-dependence; Anticardiolipin IgG/IgM and/or anti-β2-glycoprotein-I IgG antibodies was defined as elevated if the titer was >20 CU (99th percentile).

3. Results

Median age in the patient population was 63.2 (range 36-83) years, with a female predominance (66%). The median stay at the hospital was 9 (range 2-19) days. Sociodemographic baseline characteristics, medical history, and comorbidities, as well as thrombotic complications are shown in **Table 1**.

On admission, they received prophylactic (45%) or therapeutic (55%) dose low molecular heparin. None had received any anticoagulant drug nor had thromboembolic events prior to admission. Thrombotic events were reported in 18 patients and included 7 pulmonary embolisms, 1 aortic thrombosis, 1 brachial vein thrombosis, 1 stroke, 1 heart attack, and 7 deep vein thrombosis.

Patients with thrombosis were older (64.8 (IQR 36-83) vs. 60.4 (43-79), p 0.04), more likely to have diabetes (9(50%) vs. 2 (12.5%), p 0.03), dyslipidaemia (12 (66%) vs. 5 (36%), p 0.04) and higher levels of D-dimer (3797 (IQR 671-6407) vs. 480 (362-944), p 0.03), serum lactate dehydrogenase (451.3 (IQR 286.3-637.5) vs. 277.7 (205.8-317.5), p 0.02) and a higher mean hospitalization time (9 (6-11) vs. 5.7 (2.5-9.5), p 0.04).

Overall, 6 out of 18 patients with thrombotic complications were negative for all criteria aPL (LAC, aCL and aβ2GPI IgG and IgM), 10 patients had at least one aPL positive, 8 of which were LAC positive. 2 out of 8 positive LAC patients were positive but that this is not a false positive result since CRP was elevated up to 20-40 mg/L and routine aPTT was more prolonged then expected according to the CRP level and aCL IgG was positive. Two patients with negative for LAC were single positive for aCL IgG. 2 patients out of the 16 patients without thrombotic complications was positive for LAC, and aCL and 1 was positive for LAC alone.

Repeat positive aPL results three months after the first occasion could be performed in all patients except for 3, since two patients died from thrombotic complications associated with COVID-19 and the other patient could not be reached. aPL was repeated in 13 patients that were positive during the first period of testing. 6 out of 13 patients were LAC negative on the second occasion. Out of the 10 with positive aPL and thrombotic complications during the first period of testing, 1 was triple positive for LAC, aCL and aβ2GPI and another was positive for LAC and aCL. As for patients without thrombotic complications, one patient was positive for LAC and aCL and another was positive for LAC alone.

The group with thrombotic complications had a higher aTPP time than the group without thrombotic complications however, the difference between the two was not significative. Patients with positive LAC had a more prolonged aTPP time than those with negative LAC. 5 out of 8 patients with thrombotic complications that had positive LAC had a mean aTPP of 46.2 s. Out of the 5, 3 had positive LAC after 3 months.

When exploring the effect of hyperinflammation and thrombosis, we found that C-reactive protein (CRP) levels significantly associated with D-dimer levels (p 0.004) and aPL elevation (p 0.02) in the group with thrombotic events during the first testing period however, no association was found between CPR and aPL (p 0.5) and D-dimer elevation (0.8) during the second testing period.

We found no differences between both groups in aPL positivity, aTTP and TP times (s), fibrinogen, lymphocytes, C-reactive protein, serum creatine, serum ferritin and procalcitonin.

Demographics and past medical history	Patients with thrombotic complications (n = 18)	Patients without thrombotic complications (n = 16)	OR (Cl 95%)	P value
Male	7 (39%)	9 (64%)	0.35 (0.08-1.50)	0.16
Age (years)	64.8 (IQR 36-83)	60.4 (43-79)	—	0.04
Smokers (%)	10 (55%)	4 (25%)	3.75 (0.89-16.2)	0.08
Hypertension (%)	9 (50%)	7 (43%)	1.28 (0.33-4.97)	0.74
Diabetes (%)	9 (50%)	2 (12.5%)	7 (1.22-40.1)	0.03
Dyslipidaemia (%)	12 (66%)	5 (36%)	4.4 (1.05-18.6)	0.04
Hyperuricemia (%)	2 (11%)	1 (0.06%)	1.85 (0.15-22.9)	0.62
Cardiovascular disease (%)	3 (17%)	2 (12.5%)	1.2 (0.17-8.4)	0.85
Pulmonary disease (%)	2 (11%)	0 (0%)	5 (0.22-112.3)	0.31
Coagulation tests Prothrombin time (s)	13.5 (12-34)	12.6 (10.8-13.4)	—	0.47
Activated partial thromboplastin time (s)	36.3 (30.2-46.7)	33.5 (31.3-36.7)	—	0.86
Fibrinogen (g/L)	536 (393-947)	556 (569-962)	—	0.31
D-dimer (ng/mL)	3797 (671-6407)	480 (362-944)	—	0.03
aPL during hospitalization Positive dRVVT	8 (44%)	3 (19%)	3.47 (0.72-16.5)	0.12
Elevated cardiolipin (IgG/IgM)	5 (27%)	2 (13%)	2.70 (0.44-16.3)	0.28
Elevated anti-β2-glycoprotein-I IgG antibodies	5 (27%)	0 (0%)	13.40 (0.68-265.5)	0.08
aPL confirmed after 3 months Positive dRVVT	3 (20%)	2 (19%)	1.08 (0.13-8.8)	0.95
Elvated cardiolipin (IgG/IgM)	2 (13%)	1 (5%)	1.87 (0.15-22.8)	0.62
Elevated anti-β2-glycoprotein-I (IgM/IgG)	1 (7%)	0 (0%)	2.82 (0.10-74.5)	0.53
Other laboratory parameters Blood leucocytes (mm3)	111,063 (6550-12,000)	7264 (6500-8650)	—	0.28
Lymphocytes (mm3)	1415 (1050-1740)	1575 (740-1710)	—	0.81
Platelets (mm3)	258,266 (201000-317,000)	254.294 (202.000-313.000)	—	0.86
Serum creatinine (μmol/L)	1.04 (0.7-1.21)	0.87 (0.715-1.03)	—	0,87
Serum alanine aminotransferase (IU/L)	33 (17.3-58.8)	38 (22.5-53.5)	—	0.60
Serum aspartate aminotransferase (U/L)	27 (18.8-101.8)	32 (21-50)	—	0.39
Serum lactate dehydrogenase (IU/L)	451 (286.3-637.5)	277.7 (205.8-317.5)	—	0.02

Demographics and past medical history	Patients with thrombotic complications (n = 18)	Patients without thrombotic complications (n = 16)	OR (CI 95%)	P value
C reactive protein (mg/L)	100 (16.5-195.9)	30.6 (1.7-85.2)	—	0.39
Procalcitonin (μg/L)	0.18 (0.047-0.23)	0.067 (0.022-0.096)	—	0.18
Serum ferritin (ng/ml)	539.4 (140-792)	559.5 (70-897.5)	—	0.60
Mean hospitalization	9 (6-11) days	5.7 (2.5-9.5) days	—	0.04
Outcome Outcome (death/ discharged), N (%)	2/18 (11%)	1/16 (6.25%)	— —	0.70

Table 1.
Data are presented as median (25th-75th percentile) or numbers (%) as appropriate. Abbreviations: COVID-19: coronavirus disease 2019. aPL: antiphospholipid antibodies. Patient dRVVT screen (low phospholipid concentration) and confirm (high phospholipid concentration) results were normalized, i.e. expressed as ratios versus reference plasma results. Results are expressed as screen ratio/ confirm ratio. Cut-off value was 1.20 for both screen ratio and screen ratio/confirm ratio; Anticardiolipin IgG/IgM and/or anti-β2-glycoprotein-I IgG antibodies was defined as elevated if the titer was >20 CU (99th percentile), a cut-off provided by the manufacturer.

To control for possible confounding variables, sequential multivariate regression analyses were performed. In the multivariate analysis, the following risk factors were associated with thrombosis: age ($p = 0.02$) and D-dimer ($p = 0.03$). Diabetes and dyslipidaemia were significant predictors in univariate but not in multivariate analysis.

4. Discussion

The incidence of arterial and venous thrombosis associated with COVID-19 and laboratorial parameters has raised questions about a possible COVID-19 related coagulopathy. aPL has been considered as one of the mechanisms leading to a proinflammatory and hypercoagulable state. Hemostatic changes observed in COVID-19 patients have been previously associated with other coronavirus, which can activate the coagulation system and lead to thrombotic events [30].

Our study evaluates the incidence of aPL in a cohort of patients with COVID-19 and compares the incidence of aPL between two groups: the first group with thrombotic complications and the second group without thrombotic complications because of SARS-CoV2.

Several studies have suggested that aPL may be associated with thrombotic complications in COVID-19 patients and discussed the relevance of measuring aPL titers.

In most studies, the aPL confirmation after 12 weeks is often missing. Measuring LAC, aCL and aβ2GPI is useful for identifying patients at risk. Current criteria recommend increased levels of IgG and IgM aCL and aβ2GPI to confirm APS. The role of IgM aPL has been discussed based on a less strong association with thrombosis compared to IgG [31].

Zhang et al. has recently described the case of three patients with multiple cerebral infarctions that tested positive for aCL IgA and aβ2GPI IgG and IgA, without referring the titer or confirmation [28].

Harzallah et al. tested 56 patients for aPL and discovered that 45% had LAC positive, 10% aCL or aβ2GPI IgG or IgM positive. Titers of aCL or aβ2GPI were not reported and no association with thrombosis was mentioned [32].

In our study, patients with thrombosis 56% showed positivity for aPL, in the patients without thrombosis 19% tested positive for at least one aPL during admission. We should also take into consideration that most patients were treated with heparins to prevent thrombotic complications.

The majority of aPL measuring studies in the break of COVID-19 lack confirmed positivity of aPL after three months. Positive results of LAC, aCL or aβ2GPI need to be confirmed after 12 week-period to confirm persistent positivity. In our study, we had the opportunity to retest most patients at a second time point. In the group with thrombotic complications, out of 8 patients who had positive LAC on a first occasion, 3 had persistent LAC after 12 weeks and 5 turned into negative. Out of the 5 patients with positive aCL during admission, 2 had persistent aCL and out of the 5 patients with positive aβ2GPI, 1 had persistent aβ2GPI after 12 weeks, with one patient being triple positive for LAC, aCL and aβ2GPI.

Transient antibodies have been described in viral diseases or drugs; therefore, re-testing is crucial to avoid overdiagnosis of APS patients that were not persistently positive [25].

Several studies have demonstrated that viral and bacterial infections, due to molecular mimicry between viral and bacterial products and β2GPI- derived amino acid sequences can induce autoantibodies such as aPL. In most cases, these infection induced-aPL are transient and can associate with thrombosis. It has been mentioned that only with the appropriate genetic background can these antibodies become pathogenic and induce thrombosis [33].

The most common laboratory abnormalities identified in patients with COVID-19 include decreased albumin and lymphocyte count and elevated C-reactive protein (CRP), lactate dehydrogenase (LDH), erythrocyte sedimentation rate (ESR), aspartate transaminase (AST), alanine transaminase (ALT), and D-dimer [34, 35]. These abnormalities are associated with worse outcome. In our study, we have found that most patients with COVID-19 had hemostatic abnormalities, such as decreased lymphocyte count, elevated lactate dehydrogenase and elevated D-dimer and found significant differences between patients with thrombotic events and patients without thrombotic events.

Many of the laboratory abnormalities represent a balance between an acute phase reaction to the infection, including high CRP, high fibrinogen, high factor VIII, and high von Willebrand factor (VWF) and consumption of coagulation factors due to systemic or localized thrombosis and increase in D-dimer and fibrinogen [36, 37].

aPL analyses was performed during the acute phase in our study, which is mostly discouraged by current guidelines since, elevated levels of CRP may result in false positive LAC. In our cohort, we have found a significant association between CRP and aPL elevation and D-Dimer levels during the first testing period which could be interpreted as an argument in favor of these theory.

Comparing our research to previous studies that highlight the association of aPL and thrombosis, it remains unclear whether all these patients were prophylactically anticoagulated, as it was the case in our cohort.

Our study has some limitations: (1) In the analysis, for some patients, we only had one time-point (2) small sample size may influence statistical analysis (3) determination of aPL did not included aCL and aβ2GPI IgA.

5. Conclusion

The relevance of aPLs in patients with COVID-19 is yet to be determined. Inflammation is closely associated to thrombosis and the presence of inflammatory

mediators in COVID-19 infection can lead to thrombosis. Further studies are needed before to determine the role of aPL in COVID-19 patients and their relationship with thrombosis. The presence of aPL should be carefully interpreted as it is important to evaluate the persistence of aPL positivity in patients infected with COVID-19.

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Conflict of interest

The authors have declared no conflicts of interest.

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Chapter 13

The Power of Computational Intelligence Methods in the Containment of COVID-19 Pandemic from Detection to Recovery

Abdullahi Isa and Barka Piyinkir Ndahi

Abstract

The coronavirus disease (SARS-CoV-2) pandemic has caused unprecedented economic crises, and changes in our lifestyle to different things that we have not experienced before in this century, which cause by movement restriction order by the authority to halt the spread of the disease around the globe. Researchers around the globe applied computational intelligence methods in numerous fields which exhibits a successful story. The computational intelligence methods play an important role in dealing with coronavirus pandemics. This research will focus on the use of computational intelligence methods in understanding the infection, accelerating drugs and treatments research, detecting, diagnosis, and predicting the virus, surveillance, and contact tracing to prevent or slow the virus from the spread, monitoring the recovery of the infected individuals. This study points out promising CI techniques utilized as an adjunct along with the current methods used in containments of COVID-19. It is imagined that this study will give CI researchers and the wider community an outline of the current status of CI applications and motivate CI researchers in harnessing CI technique possibilities in the battle against COVID-19.

Keywords: COVID-19, computational intelligence, coronavirus, drug discovery, detection, diagnosis, prediction, contact tracing, treatment, recovery

1. Introduction

Emerging coronavirus disease (SARS-CoV-2) has posed a significant problem in global public health, and economic crises that we have not experienced before in this century. The disease appeared in December 2019 (COVID-19) which put a large number of individuals around the globe in quarantine, isolation, and lockdown in other to curtail the spreading of the disease [1, 2]. Guidelines have been issued by the Centers for Disease Control and Prevention (CDC) and the World Health Organization to curtail the spreading of the disease and protect the healthy population from contacting the SARS-CoV-2 virus from the infected individuals [3]. Throughout the globe, including the USA do not have the facilities

to accommodates such large individuals infected with the SARS-CoV-2 virus while managing quarantine. The authorities all over the globe had built several new facilities (Hospitals) to manage individuals infected with the disease [4]. In this context, it's important to use other alternative models as an adjunct along the current methods using by [3] to curtail how this SARS-CoV-2 virus spreading rapidly like wildfire. Several studies show, how computational intelligence techniques can be applied as an adjunct along with the current guidelines show by [3] to yield timely intervention in faster detection, diagnosis, prediction, contact tracing, drug discovery, treatment, and recovery of infected individuals.

CI is defined by [5] "as a branch of artificial intelligence (AI) which includes the study of versatile components to empower or encourage savvy practices in intricate and evolving situations". CI covers all pieces of AI and underlines the improvement and advancement of real-world applications. However, the virtuous circle of synergy between computational intelligence (CI), life science, and nature, show how CI techniques got their inspiration from natural phenomena that are utilized to solve different field of science complex problem, more specifically medicine [6]. The power of computational intelligence techniques has shown different success stories and always been fruitful since its inception with different novel ideas that are inspired by biology, and nature with powerful computational models. Two "fathers of computer science", Alan M. Turing and John von Neumann, in the year 1940s and 1950s, utilized natural phenomena of pattern formation and self-reproduction to formulate the basis of the computational model known as Cellular Automata [7, 8]. Perceptron was created based on working inner neurons in the brain [9], which is the fundamentals component of artificial neural networks that were advocated during the current "deep learning revolution" [10].

Computational Intelligence (CI) is the hypothesis, design, application, and advancement of "biologically and linguistically" spurred computational standards [11]. Generally, the three primary types of CI have been Neural Networks, Fuzzy Systems, and Evolutionary Computation. Nonetheless, in time numerous nature-propelled processing standards have developed. In this manner, CI is a developing field and at present notwithstanding the three fundamental constituents, it includes figuring ideal models like "ambient intelligence, artificial life, cultural learning, artificial endocrine networks, social reasoning, and artificial hormone networks" [11]. CI assumes a significant part in creating effective insightful frameworks, including games and cognitive developmental systems. Throughout the most recent couple of years, there has been a blast of research on Deep Learning, specifically deep convolutional neural networks. The best AI framework depends on CI.

The core designing objectives of computational intelligence are to show the methods for the design of intelligence and the central scientific task of computational intelligence is to perceive the philosophies that make intelligent behavior possible, regardless of whether in artificial or natural systems. The center methodologies of computational intelligence-like fuzzy systems, neural computing, and evolutionary computing- have as of late arose as promising devices for the application, development, improvement, and execution of intelligent agents/systems in medical services. Indeed, computational intelligence advancement play important role in bringing reforms to medical services practice.

However, computational intelligence discovered its way into the field of medical science since its inception, this is due to the colossal need for CI techniques in the medical arena. Several applications of computational intelligence techniques exist in the field of the medical arena, for example using neural networks which includes, but not limited to "Cancer prediction [12, 13], Clinical diagnosis of COVID-19 [14], Length of stay prediction [15–17], Speech recognition [18–20], Ophthalmology [21, 22], Radiology (MRI, adaptive medical image

visualization, ultrasound images) [23, 24], Neurology (aphasia, electroencephalogram—EEG, and EEG analysis) [25, 26], Image interpretation and analysis [27], Development of drugs [28–30]". However, an investigation by [14] utilized deep learning techniques to identify acoustic signatures of the presence and severity of COVID-19 using a standardized dataset of digital lung auscultations. The researchers estimate that automated translation of lung auscultation could better democratize the accuracy of this basic clinical test beyond the individual capacities of the doctors. Also, they intend to consolidate their algorithm into an autonomous computerized stethoscope (right now a work in progress), that could help decentralize great respiratory examination and observing, and maybe even engage patients to survey themselves, which would lessen nosocomial contaminations happening during a traditional clinical test. Even patients would be able to examine themself at home. This chapter discusses the utility of CI as an adjunct along with the current other methods used in the containment of COVID-19.

2. Computational intelligence

The computational intelligence arena is another discipline with ancient roots. Innovation always relies on discovery, and discovery depends on the advancement of technology. Certainly, same situation with Computational Intelligence. Theories produce by sciences that are investigated through experiment and the experiment rely on the direction of theories. Computational intelligence definition by [31] as the study of the design of "intelligent agents." Sound confusing, and so the researchers go on to define an agent as "something that acts in an environment." Agents act. Worms do that, and so do folks and thermostats. An intelligent agent acts in a way that is appropriate for the circumstances to achieve a goal. The intelligent agent learns and adapts [32]. CI is characterized by automatic adaptation and organizes accordingly concerning the implementation environment [33].

There is a lot of impressive opportunities provides by computational intelligence for advancing medical services such as diagnosis, treatment, prediction, etc. However, administrative management of the patient is not exempted by computational intelligence such as the personal information of patients. Screening of COVID-19 using polymerase chain reaction (PCR) can take a few hours/days which is problematic [6]. This shows the need for researchers to apply computational intelligence methods to improve the screening process and provide other alternative tools in screening, diagnosis of covid19, and treatment.

Also, applications areas of CI categorized by [33] are based on four main pillars of CI which include Neural networks which are applied to the category of the following problems such as ("clustering, classification, prediction, composition and control systems"). Secondly, Evolutionary computation is applied to ("route or path optimization, scheduling problem and medical diagnosis of diseases"). Furthermore, Fuzzy logic applied to ("vehicle monitoring, sensor data in home appliances and control systems"). Lastly, Expert systems are applied in ("financial applications, robot production, diagnostics, and various industry-based operations").

3. Application of computational intelligence in containing the COVID-19 pandemic

This section presents, how computational intelligence approaches were applied in many ways in other to enhance the containment of the COVID-19 pandemic.

Such areas where CI techniques are applied are detection and diagnosis, prediction, contact tracing, drug discovery, treatment, and recovery.

3.1 Computational intelligence methods for detection and diagnosis of (SARS-CoV-2): Use cases examples

One of the issues confronting all nations, including the USA during this coronavirus pandemic was inadequate testing tools for detecting and diagnosis COVID-19. There is a need for other alternative tools for diagnosis and detection of COVID-19 different from Real-Time Polymerase Chain Reaction (RT-PCR) [34]. Lack of diagnostic tools and efficient tests continue to cause a major problem in controlling the spread of the disease [2]. Research shows lack of RTPCR test units was enormous and it takes 4–6 Hours to acquire results. Thus, results to many infected patients cannot be distinguished from healthy individuals and keep on infecting the other healthy folks. Therefore, to halt the spread of the disease, there is a need for fast diagnosis and detection of COVID-19. Since the results of diagnosis of COVID-19 show symptoms associated with pneumonia symptoms which identity in the image and genetic test.

However, researchers all over the globe working tirelessly to control the spread of the disease using the medical image to explored computational intelligence approaches on digitized images. Several CI techniques play a significant role in the diagnoses of COVID-19 using Chest X-ray (CXR) and Computed Tomography (CT). Recently, several pieces of research have been conducted from the digitized image using neural network (CNN) to detect and diagnose COVID-19 [35, 36]. For instance, a study by [37] using the digitized image of computed tomography (*CT*) to detect COVID-19 based on Convolutional neural network (CNN) approaches. Also, in [38] classification of *CT* images into three classes: healthy, COVID-19 and bacterial pneumonia have been experimented with using a modified version of the ResNet-50 pre-trained network. in [39] Chest X-ray images (*CXR*) were utilized by a CNN to extract the high-level features based on various ImageNet pre-trained models. To detect COVID-19 those features extracted were pass as input into SVM as a machine learning classifier. Furthermore, in [40] based on transfer learning approaches, a proposed model on CNN algorithms called COVID-Net applied to classify the *CXR* images into four classes: COVID-19 viral infection, non-COVID, bacterial infection, and normal.

Moreover, a study by [20], aimed to diagnose COVID-19 using deep learning techniques and a transfer learning system. The system utilized a combination of convolutional neural network (CNN) architecture (one convolutional layer with 16 filters followed by batch normalization, rectified linear unit (ReLU), two fully-connected layers), and a modified AlexNet [21]. Their proposed model shows an accuracy result of 94.00%. In addition, an investigation by [22] to ascertain the uncertainty and interpretability of deep learning-based techniques for COVID-19 diagnoses in X-ray images, in other to provides the diagnostic confidence for a clinician, a Bayesian Convolutional Neural Networks (BCNN) was utilized to estimate the uncertainty on their proposed model. The results for detection accuracy of 92.86% on X-ray images were obtained by the proposed model.

3.2 The use of computational intelligence approaches for COVID-19 prediction

Prediction [41] refers to the output of an algorithm after it has been trained on a historical dataset and applied to new data when forecasting the likelihood of a particular outcome. The algorithm generates probabilistic values for an unknown variable for each record in the new data, allowing the model builder to identify

what that value will most likely be. This is heavily used in computational intelligence methods and we shall see how it has been implemented to mitigate the spread of COVID-19.

COVID-19 data was explored based on a proposed model [42] from Hikvision's temperature screening thermographic and hotspot non-contact infrared device using an acoustic device for collecting and analyzing COVID-19 data embedded with the pervasive computing devices. The principal component analysis (PCA) model was used to pre-process the collected data while Mode and Mean Missing data imputation (MMM-DI) method for removing outliers and filling missing data. To reduce noise and prevent false alarms, an Artificial Intelligence detector is also embedded with the sensor device. Using coding in MATLAB, the Susceptible, Infected, and Recovered (SIR) epidemic model is implemented to classify the cases as suspected, infected, and recovered generated for classification of the demographic data. With the previous history stored in the hidden layer, the data is then fed into a Recurrent Neural Network (RNN) with Long Short-Term Memory (LSTM) model to forecast the coronavirus disease cases. The proposed model helps in further treatment by exploring pervasive computing technologies in coronavirus disease prediction and detection. The issue of trust and privacy has to be handled to find a favorable rectification. Redundancy and noise challenges can be overcome by a new algorithm to assist in the area of data conversion.

For the diagnosis and prediction of coronavirus disease, prediction models such as autoregressive integrated moving average (ARIMA), LSTM, and prophet algorithm (PA) were utilized over the next 7 days to predict the number of coronavirus disease confirmations, recoveries, and death in a Computational Intelligence based technique that was proposed [43]. The algorithm with the best performance was PA. It gave a prediction of the number of coronavirus disease confirmations, recoveries, and deaths in Australia and gave accuracies of 99.94%, 90.29%, and 94.18%, consecutively.

In Jordan, the PA technique obtained the number of coronavirus disease confirmations, recoveries, and deaths with accuracy in prediction of 99.08%, 79.39%, and 86.82%, consecutively. More advanced prediction models are expected in future work. Using X-ray images of the chest, a diagnosis model which implemented VGG16 was proposed to find coronavirus disease. Being capable of obtaining an F-measure of 99% the technique allowed quick and reliable coronavirus disease detection, using a dataset that is augmented. The researchers believe that future studies will aid in diagnosing coronavirus disease using the VGG-XX versions in chest CT scans and compare their performances using larger datasets. The analysis of the spread of coronavirus disease and its related statistical data based on worldwide regional distributions was a further contribution of their study. Using their Artificial Intelligence-based analysis; two major conclusions were arrived at: (1) similar characteristics are observed in the most highly infected areas (2) in coastal environments, the spread of COVID-19 is tremendously higher than in other non-coastal environments. Henceforth, extra attention and care ought to be rendered to coastal cities. Effects of terrain, humidity, and temperature on the coronavirus disease and its spread in countries and cities would be good to be investigated in upcoming work.

3.3 Contact tracing based applications for COVID-19

The act of identifying all people that a coronavirus disease patient has come in contact with in the last fortnight is known as contact tracing. The infection is known to spread to people through coughing, sneezing, saliva, droplets, or discharge from the nose through contact transmission. Various applications, methodology, and

tools have been considered for use to curtail the spread of the virus and in this section, we shall discuss some computational intelligence approaches.

One of the major strategies for the containment of COVID-19 is contact tracing. In ordinary contact tracing, medical doctors interview the infected patients to trace and find others who may be contaminated through contact with the patient. The major problem of the above methods was the difficulty for the individual to recalled all his contact. In addition, other strategies may require experience clinicians and other resources. However, recent technology innovation enhanced contact tracing methods by reducing human intervention in the process, using a smart methodology known as digital proximity (DP) contact tracing. The DP method uses network technologies to recognize and find people who could be conceivably contaminated through contact.

With the boundless accessibility of computing networks and mobile applications - and their related technologies including cell phones, smartwatches, and others - the majority of the innovation-based contact tracing frameworks are based on mobile platforms [44, 45]. However, computational intelligence is right now used through the whole life cycle of COVID-19 starting from identification to mitigation [46]. A virtual computational intelligence Agent is an option in contrast to a medical doctor on account of traditional contact tracing. In digital contact tracing (DCT) frameworks, Bluetooth innovation is generally utilized as a vicinity identifier for COVID cases. Notwithstanding, the presentation of Bluetooth-based contact tracing applications might be influenced by changing sign power, which can be shown by various cell phones, versatile positions, body positions, and actual boundaries [47].

COVI was a Computational intelligence-based contact tracing application created in Canada that uses probabilistic risk levels to profile a person's contamination hazard level [48]. COVI utilizes the advantages of CI algorithms to improve and automate the mix of pseudonymized client information in surveying the danger levels. A deduced variant of an epidemiological model-based reproduced dataset is utilized to pre-train the CI models. Upon assortment of genuine data through an application, the test system boundaries are tuned to coordinate with genuine data. The effect of CI in the COVI application is seen by utilizing the CI predictor inside the test system to impact the conduct of the specialist in suggesting the danger levels. The contact tracing application can be utilized to foresee the lockdown territory dependent on places visited by a contaminated patient. In [45] the researchers proposed a K-Means clustering algorithm with DASV seeding to foresee the lockdown region. The proposed technique has been tried in Denver, USA, and effectively distinguished the territory to be locked down as clients strolling around there approach each other regularly.

3.4 The used of CI methods in case of COVID-19 treatment

Treatment in the context of COVID-19 is the approach that could be harnessed to help a patient get back on his feet. This could be by the use of medications or other methods. Computational intelligence techniques help with decision-making tools amongst other things to bring this to pass.

The introduced [49] platforms and conceptual structures in the research area of artificial intelligence-based methods suitable for fighting coronavirus disease were addressed. Extreme Learning Machine (ELM), Generative Adversarial Network (GAN), LSTM, and RNN are varying techniques that have been developed, incorporating coronavirus disease diagnostic systems. The major issues with coronavirus disease included geographical problems, radiology, recognizing, and high-risk people according to their studies. A mechanism was revealed that helped

in selecting the right models for predicting and estimating desired parameters using several nonclinical and clinical datasets. Considering these platforms help artificial intelligence specialists to analyze large datasets and assist physicians train machines, set algorithms, or optimize the data being analyzed for fighting COVID-19 with greater accuracy and speed. They are desirable because of their potential for creating a workspace while physicians could work side by side with artificial intelligence specialists as discussed. However, while artificial intelligence speeds up the methods to defeat coronavirus disease, real experiments ought to occur because a comprehensive knowledge of the advantages and limitations of computational intelligence-based methods for coronavirus disease is yet to be achieved, and new approaches need to be in place for challenges of this level of complexity. Building an arsenal of methods, platforms, approaches, and tools that converge to solve the sought goals and help in saving more lives is going to greatly assist in the combat against the coronavirus disease and its eventual annihilation.

Fingerprint and differentially expressed genes (DEGs), two types of drug data were clustered by a multimodal restricted Boltzmann machine (mm-RBM) according to a study [50]. Showing the chemical structures, the first type of data is binary data. From drug-induced perturbations in cell lines, the second one was extracted. First, the intrinsic correlations within each input modality were encoded using the modality-specific hidden variables in the proposed multimodal RBM model. By merging unknown variables, the intra-modality features were fused next and a typical representation of cross-platform features was formed. Data integration yields significant clusters based on the indications of the proposed approach. Henceforth, to discover medications that may prove useful in treating COVID-19, the clusters consisting of drugs used for curing coronavirus disease were chosen. Having antiviral properties, the introduced drugs are similar to sophisticated drugs that have been used to control coronavirus disease. Although the outcomes seem to yield a satisfactory explanation and are significant, further clinical research such as in vivo or in vitro tests needs to be carried out.

However, COVID-19 treatment is categorized into two drug discovery and vaccine development. As we all know, without drug discovery and vaccination there will no be any treatment of COVID-19 patients which indicated its high importance and urgent need. Computational intelligence methods have been utilized in search of new chemical combinations that can lead to effective medicine, provided integrated characteristics predictions, behavior prediction, reaction prediction, and ligand-protein interactions. Proteomics and genomics investigation have been suggested on the development of mDiverse drugs and vaccines for SARS-CoV-2. CI approaches in the development of new drugs and vaccines contributed immensely to the battle against COVID-19. Integrating CI methods in the pharmaceutical arena has proven both cost-effective and less time-consuming.

Many pharmaceutical companies embraced the use of computational intelligence techniques such as artificial neural networks, Support Vector Machines (SVM), deep learning, and many others to develop various drugs and vaccines [36]. A review of recently developed algorithms in [36] to design drug development pipelines consisting of drug discovery, drug testing, and drug re-purposing. Generative Adversarial Networks (GAN) were utilized to identify DNA sequences associated with specific functions, and proteins of interest produced with lower costs using Bayesian Optimization (BO) during drug discovery. To determine the best treatment, Bayesian-based Multi-Armed Bandit (MAB) algorithms which is a sequential decision-making algorithm are utilized in drug testing to test several drug candidates. Text mining methods and graph-based recommender systems were used in repurposing to identify correlations and predict drug-disease interactions.

Several pharmaceutical companies have employed ML-based algorithms such as artificial neural networks, Support Vector Machines (SVM), deep learning, and many others to develop various drugs and vaccines [51]. The authors in [51] provide a review of recently developed algorithms to design automated drug development pipelines consisting of drug discovery, drug testing, and drug re-purposing. In drug discovery, the deep learning algorithm Generative Adversarial Networks (GAN) is used to identify DNA sequences associated with specific functions, and Bayesian Optimization (BO) is used to produce proteins of interest with lower costs. In drug testing, sequential decision-making algorithms such as the Bayesian-based Multi-Armed Bandit (MAB) algorithms are used to test several drug candidates and determine the best treatments. In drug re-purposing, text mining methods and graph-based recommender systems are used to identify correlations and predict drug-disease interactions. The authors compiled a list of relevant data sets for drug development pipeline studies.

In an attempt to identify probable vaccine candidates and constructing an epitope-based vaccine against COVID-19 authors in [52] developed a computational intelligence system that incorporated reverse vaccinology, bioinformatics, immunoinformatic and deep learning techniques. Also, in a study by [53] to predict and evaluate potential vaccine candidates for COVID-19, the authors utilized Vaxign Reserve Vaccinology (VRV) tool and Vaxign-ML, a computational intelligence-based prediction and analysis framework. The results in their research showed the second-highest protective antigenicity as a non-structural protein (nsp3), in addition to the commonly used S protein.

3.5 COVID-19 recovery methods based on the use of CI techniques

COVID-19 recovery could be evaluated as the phase when we can say a patient has gotten back to his feet after being infected. Computational intelligence offers models that could assist reach this phase.

Data mining models were developed for forecasting coronavirus disease infected patients' recovery using the epidemiological dataset of coronavirus disease patients of South Korea in a study by [54]. Using a python programming language, support vector machine (SVM), logistic regression (LR), k-nearest neighbor (K-NN), decision tree (DT), naïve Bayes (NB), random forest (RF) algorithms were applied directly to the dataset. The most efficient was found to be the model developed by DT with the highest percentage of accuracy of 99.85%, followed by RF with 99.60% accuracy, then SVM with 98.85% accuracy, then K-NN with 98.06% accuracy, then NB with 97.52% accuracy and LR with 97.49% accuracy. The developed models would be very helpful in healthcare for the combat against COVID-19.

As people's way of life has changed amongst many other things due to the coronavirus disease pandemic, many losses have also been incurred and means of sustenance of a lot of people [55]. It greatly affected economic and commercial activities due to the suspension of both at certain intervals of time to control the spread of COVID-19.

Through technology management, accelerated COVID-19 recovery is emphasized as an approach to utilize with the advancements in healthcare and expansion in the access to electronic data. The area of healthcare can apply AI to address problems in the area, using substantial computation power, especially during an ongoing pandemic. Many of these machine learning systems ultimately present the most substantial transformative role in healthcare governance though many of them remain experimental [55]. Machine learning modeling has evaluated multiple scenarios to focus on the COVID-19 recovery index with the proposed research. To identify specific patterns and help the masses overcome the

impending outcome of coronavirus disease, the research presents a strong case where machine learning models can be used.

The generalization of developed machine learning models is possible as the study [55] feeds on near-time data and comprehensive academic underpinning. Developed and developing countries can use insights from this work as they apply to national and global levels for developing strategies. Machine learning should consider the limitations on algorithm development and understanding its appropriateness to apply like other revolutionary technologies.

Machine learning has the potential to play a key role in the advancement of healthcare and societal health enhancement as researchers are continuously attracted by predictive modeling techniques. The presented work [55] could offer counsel to make policy recommendations to help authorities develop well-informed health policies and accelerate the COVID-19 recovery.

3.6 Computational intelligence (CI) based quest for COVID-19 drug discovery

Recently, Computational intelligence approaches have revolutionized many fields in medical sciences and beyond. It has generally changed our everyday lives, from speech and face recognition [56]. Two of the most affected areas influenced by CI techniques are drug and vaccine discovery [57], in which CI methods have offered compound property prediction [58], activity prediction [59], response expectation [60], and ligand-protein cooperation. Graph Convolutional Neural Network (GCNN) has been the front runner on the prediction of drug discovery applications [61, 62]. Several studies show, drug property prediction can handle by (GCNN) and extract features through encoding the adjacency information within the features [63–65]. Protein interface assessment [66], reactivity forecast [67], and drug–target connections etc. [68, 69].

Noteworthy, CI methods have additionally improved in the field of vaccine design recently. VaxiJen was the first implementation of CI techniques in RV approaches and has shown promising outcomes for antigen forecast [70, 71]. Also, drug candidate created during the process of drug discovery needs to be safe for human utilization. This means confirmation that the drug is non-poisonous is required during the drug side effect observation. To achieve the above, it requires the creation of a database that can be utilized to facilitate modeling toxicology. Several investigations, based on CI techniques were implemented to identify the cardiotoxicity of a candidate drug, hydroxychloroquine, using ECG data from smartwatches [72].

In the case of COVID-19 drug discovery, several studies used CI approaches for both repurposed drug candidates and new chemical entities. The former aimed to exploit and predict interconnected biological pathways or the off-target biology of existing medicines that are proven safe and can thus be readily tested in new clinical trials. However, studies by Gordon et al. experimentally identifying 66 human proteins linked with 26 SARS-CoV-2 proteins, paved the way for the repurposing of candidate drugs [73]. Furthermore, for analyzing the virus-host interactome network-based model simulation has been the main computational approach used over wet-lab approaches [74]. Li et al. analyze the genome sequence of three main viral family members of the coronavirus and then relating them to the human disease-based pathways lead to the discovery of 30 drugs for repurposing [75]. Using an alternative approach by Zhou et al. offered a combination of network-based methodologies for repurposed drug combination [76]. Research has shown that the experimental evaluation of all drug and vaccine candidates was extremely challenging. However, researchers believed that leveraging computational intelligence approaches will speed up the discovery effort,

and capable of filtering generating therapy. Utilizing artificial neural networks and supervised learning methods has proven to be a vital game-changer when used for virtual filtering and *de novo* design. Large-scale training datasets and relevant bio targets are required in other to achieve desired performance using computational approaches.

3.7 CI methods used in COVID-19 19 surveillance

Surveillance is the art of monitoring people or things via various techniques like directly looking at them or using tools such as binoculars, sensing them via sensors, and generally keeping track of them often in relationship to time. Since the outbreak of COVID-19, it became imperative to monitor those that were infected especially after putting them in hospitals and isolation centers, and closely watch them as treatment was rendered to them and as an effective remedy was researched by the scientific and medical community. Those that were infected that came close to other individuals contributed to initiating the method referred to as contact tracing which is a form of monitoring to trace all those who are likely to be infected by the virus. When they are found via contact tracing, they are often put under isolation for several days so they do not infect others if they contact the virus. Surveillance was also carried out on the general public by ensuring they maintain social distancing, wear a mask, and use sanitizers by enforcers. In this section, we are primarily concerned about computational intelligence techniques that were used and can be used with surveillance to mitigate the spread of COVID-19.

To fight and overcome coronavirus disease like pandemics, a beyond 5G (B5G) enabled smart health care framework was proposed [77]. A cloud layer, an edge layer, and a stakeholder layer are all contained in the framework. Into the system was the integration of a mass surveillance system in terms of mask-wearing, social distancing, and body temperature detection. Analysis at the edge utilizing the latest generation of high-power edge computers was done on human vital signs and hospital test data. This diagnostic method for coronavirus disease could be extended to any infectious disease. Protecting sensitive personal data at the edge to protect anonymity, verifying non-coronavirus disease patients, and reducing overcrowding in health centers will all be helpful. Other protease sequence analyses and deep learning models will be tested in the framework in the upcoming work. A time-series analysis model and a prediction model could be embedded in the framework also in future work. For low latency and better security, pervasive edge computing could also be added.

To assist in reducing the coronavirus disease outbreak, an embedded surveillance system was presented [78] which detects the elderly ones who are more affected by COVID-19 in the recent pandemic. To determine the age of an individual an age estimation is used. To enhance the results of pre-trained deep networks, an enhancement age estimation method is used by utilizing face alignment. To refer to the presence of the elderly in an environment, a notification is sent to mobile or any other device systems using the Internet of Things. Using a public database, the proposed system was evaluated and the results obtained show that the system was satisfactory in its performance. Two types of comparison were additionally used to compare the accuracy of the proposed system. Pre-trained deep networks and face alignment were implemented in the first one for the enhancement of the deep learning model. The combination of face alignment and pre-trained deep networks proved age estimation performance from the obtained results. Implementation using two kinds of hardware and comparison between them was further done in the proposed system.

4. Conclusion

The current ongoing COVID-19 pandemic has become a global health emergency due to the continued growth of the high rate of infected patients globally. As of the time of writing this, there are approved therapeutic drugs for the curing of COVID-19 disease. However, the drugs were not sufficient globally, including in the USA. There is still a need for the early detection, diagnosis, and treatment of COVID-19 patients globally. The use of non-pharmaceutical methods such as quarantine, isolation of suspected patients is the most effective method for preventing the spread of COVID-19 before the approval of the therapeutic drugs for the curing of COVID-19 disease. Nonetheless, computational intelligence techniques are also served as an alternative tool for preventing the spread of the disease as well as monitoring the progression and severity of the disease in patients. The power of computational intelligence approaches has proven a game-changer in the fight against the spread of the COVID-19 pandemic. This paper presented how computational intelligence methods were utilized to fight against COVID-19 or containment of the COVID-19 pandemic. The paper demonstrates how computational intelligence methods were used in detection and diagnosis, prediction, contact tracing, treatment, recovery, drug discovery, and surveillance. We have seen how several studies leveraged computational intelligence methods, from a different perspective for containment of the COVID-19 pandemic. However, the clinical and non-clinical application of CI techniques in the containment of COVID-19 is promising, and additional extensive research is required. However, this study points out promising CI techniques utilized as an adjunct along with the current methods used in containments of COVID-19. It is imagined that this study will give CI researchers and the wider community an outline of the current status of CI applications and motivate CI researchers in harnessing CI technique possibilities in the battle against COVID-19.

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Chapter 14

Docking-Based Screening of Cell-Penetrating Peptides with Antiviral Features and Ebola Virus Proteins as a Drug Discovery Approach to Develop a Treatment for Ebola Virus Disease

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Abstract

Ebola drug discovery continues to be challenging as yet. Proteins of the virus should be targeted at the relevant biologically active site for drug or inhibitor binding to be effective. In this regard, by considering the important role of Ebola virus proteins in the viral mechanisms of this viral disease, the Ebola proteins are selected as our drug targets in this study. The discovery of novel therapeutic molecules or peptides will be highly expensive; therefore, we attempted to identify possible antigens of EBOV proteins by conducting docking-based screening of cell penetrating peptides (CPPs) that have antiviral potential features utilizing Hex software version 8.0.0. The E-value scores obtained in this research were very much higher than the previously reported docking studies. CPPs that possess suitable interaction with the targets would be specified as promising candidates for further in vitro and in vivo examination aimed at developing new drugs for Ebola infection treatment.

Keywords: bioinformatics, protein-peptide interactions, biological targets, drug development, HEX software, biological computation, drug design, CPP

1. Introduction

Ebola virus disease or EVD is a frequently fatal disease caused by a member of the *Filoviridae* family known as Ebola virus (EBOV) [1]. The pathogen was initially discovered in Africa in 1976 and then leaded to two serious outbreaks including the 2013–2016 outbreak of EVD in Western Africa that infected 28,652 people with

11,323 documented deaths; and 2018–2020 outbreak of EVD in the Democratic Republic of the Congo that affected 3481 people with 2299 documented deaths [2]. Ebola virus is transmitted to people from wild animals and spreads out in the mankind population by way of human-to-human transmission. The potential reservoirs of EBOV RNA are three species of African fruit bats [3]. The genome of this virus contains a negative-strand RNA that encodes six structural and one non-structural proteins, which can be employed as potential drug targets, including transmembrane glycoprotein (GP), nucleoprotein (NP), four viral protein (VP24, VP30, VP35, and VP40) and RNA polymerase (L) [4–6]. EBOV immediately suppresses the host's innate immune response and causes a severe febrile illness along with intense weakness, muscle pain, hypotension, coagulation disorders, sore throat, diarrhea, and vomiting [7–9].

Drug discovery and development for prevention of EBOV infections can be strikingly problematic due to the essential requirement of bio-safety level four (BSL-4) facilities that are needed for carrying out preclinical studies of Ebola virus [10, 11]. To date, there is only one approved vaccine for prevention of Ebola virus disease [12] and several recent FDA approved monoclonal antibody treatments for the patients [13–15]. The traditional drug discovery process remains time-consuming and faces rising costs, labors and challenges. Therefore, computational drug design assists to defeat these difficulties and is promising to meet the need for anti-Ebola medicines [16, 17]. As reported in many recent studies, docking based approaches have been effectively employed in drug development, for prediction of the potential ability of a given molecule to bind the other targets [18] and to provide productive results for accelerating identification and optimization of drug formulation [4].

Over the past decade, the pharmaceutical industry has come to appreciate the task of therapeutic peptides that can play in the improvement of medical needs and how this type of compounds can be either complement or preferable alternative to small molecules and other biological therapeutics [19]. Due to the particular features of protein-peptide interfaces (PPIs) the application of small molecules could be limited for target PPIs. Contact surfaces involved in PPIs are large in size and this resulted in small molecules not to be outstanding for modeling of new therapeutic drugs [20]. Adversely, peptide molecules are much more efficient to be developed for interaction with large and flat protein surfaces and appear to be better adaptive. In this regard natural or synthetic peptides that are capable of interfering with PPIs, termed interfering peptides (IPs), possess increasing application [21, 22]. Peptides have an extended history of avail in therapy and are recognized as being safe and well tolerated. Novel improvements in peptide administration, bio-delivery, safety and stability are also remarkable in the preference of peptidic drug design and formulation. IPs have the potential to modify various cellular processes and may affirm the idea that they would have a significant potential to become promptly valuable therapeutic instruments [22].

Cell-penetrating peptides or CPPs (also known as protein transduction domains) are short-length peptides, generally made up of 5–30 amino acids, which are able to pass drugs or CPP/cargo complexes across plasma membrane into the cells [23]. CPPs have tremendous potential for mimicking PPI and can be great options to be used as drugs. Several studies on CPPs are presently undergoing pre-clinical and clinical trials that will offer new treatment options in the near future [24]. For further information, examples of which studies are available on references [25–27].

In this in silico study we identified 25 cell penetrating peptides (CPPs) that have antiviral potentials. Using them, we deployed series of peptide-docking screening against Ebola virus proteins as a drug discovery approach to develop a potential treatment for Ebola virus disease.

2. Methods

2.1 Ligand CPPs collection

CPPsite2.0 is a simple-to-use updated database that presents miscellaneous information about CPPs and includes 1855 entries [28]. In this study, CPPsite2.0 database was used for selection and extraction of the cell-penetrating peptide sequences. By using AVPpred web server, which is the first antiviral peptides prediction algorithm [29], CPPs that had antiviral properties were chosen (**Table 1** lists the selected CPPs). Default machine learning technique was SVM. Threshold 50 was used for screening. Four parameters used in AVPpred server for prediction of the viral features including:

1. Motif search using MEME/MAST (Bailey and Elkan 1994; Bailey, Boden et al. 2009)

2. Amino acid composition

Peptide	Peptide Sequence	Length
MPGNLS	GALFLGFLGAAGSTMGAWSQPKSKRKV	27
Melittin	CIGAVLKVLTTGLPALISWIKRKRQQ	26
MPG-NLS	GALFLGWLGAAGSTMGAPKSKRKVGGC	27
hLFWT	KCFQWQRNMRKVRGPPVSCIKR	22
EGFP-MPG	GALFLGWLGAAGSTMGAPKKKRKV	24
Transportan10	AGYLLGKINLKALAALAKKIL	21
b-WT1-pTj	CGGKDCERRFSRSDQLKRHQRRHTGVKPFQ	30
TatLK15	RKKRRQRRRGGGKLLKLLLKLLLKLLK	27
MPGα	GALFLAFLAAALSLMGLWSQPKKKRKV	27
MPGβ	GALFLGFLGAAGSTMGAWSQPKKKRKV	27
pepM	KLFMALVAFLRFLTIPPTAGILKRWGTI	28
MPG-EGFP	GALFLGWLGAAGSTMGAPKKKRKV	24
A6	KLLKLLLKLWKKLLKLLKGGGRRRRRRR	28
Res1	KLIKGRTPIKFGKADCDRPPKHSQNGMGK	29
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	27
DermaseptinS4	ALWMTLLKKVLKAAAKAALNAVLVGANA	28
MousePrp(1–28)	MANLGYWLLALFVTMWTDVGLCKKRPKP	28
MG2d	GIGKFLHSAKKWGKAFVGQIMNC	23
P(beta)	GALFLGFLGAAGSTMGAWSQPKKKRKV	27
SN50	AAVALLPAVLLALLAPVQRKRQKLMP	26
Peptide1	MGLGLHLLVLAAALQGAWSQPKKKRKV	27
MCoTI-II	GGVCPKILKKCRRDSDCPGACICRGNGYCGSGSD	34
CL22	KKKKKKGGFLGFWRGENGRKTRSAYERMCILKGK	34
Camptide	RKLTTIFPLNWKYRKALSLG	20
MAP	KLALKLALKALKAALKLAGC	20

Table 1.
List of selected CPPs, their sequences and lengths.

3. Sequence alignment using BLAST

4. Physico-chemical parameters including secondary structure, charge, size, hydrophobicity and amphiphilic character as these yielded an appreciable accuracy using machine learning technique. The values of physico-chemical properties were retrieved from AA index database (Kawashima and Kanehisa 2000)

The three-dimensional model of the peptides was fabricated by Chimera 1.8.1 software.

ToxinPred tool was run with default parameters to calculate toxicity prediction of the determined peptides. All 25 peptides were non-toxic [30].

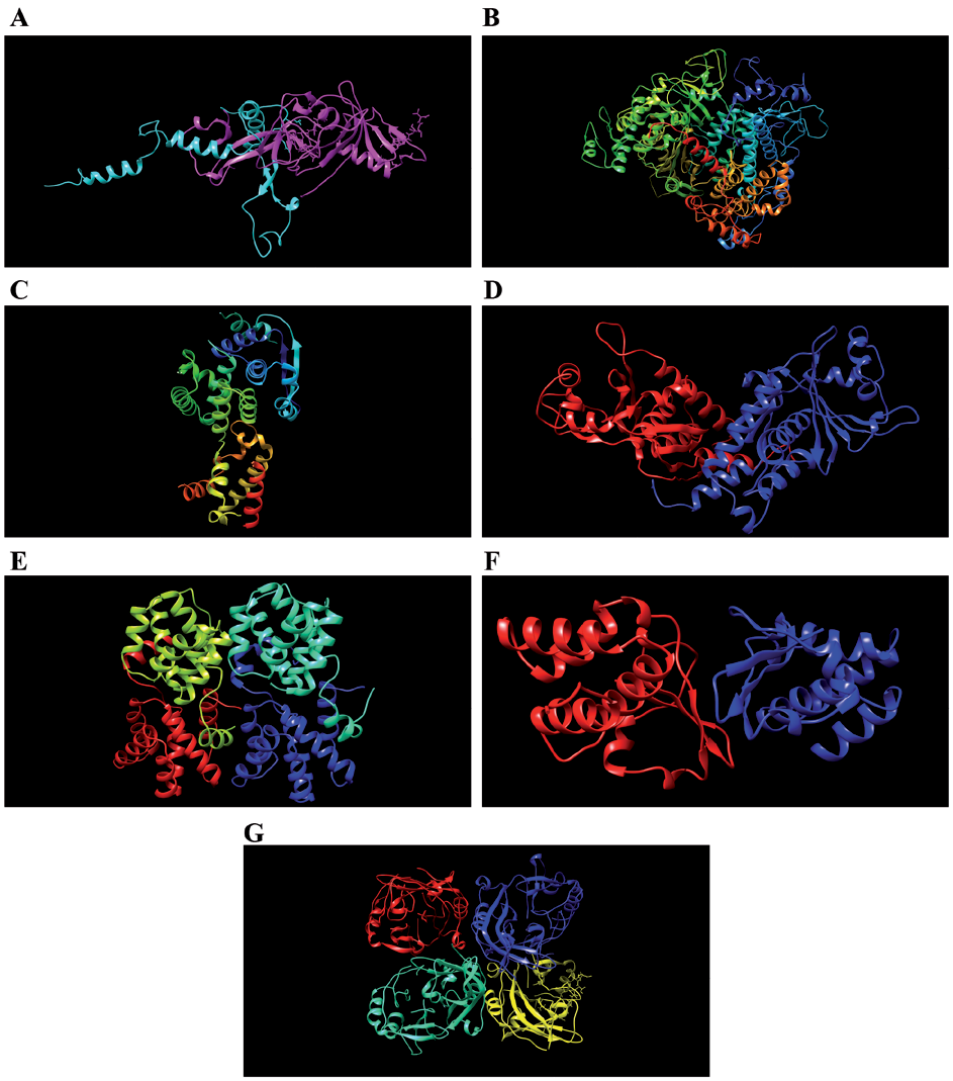

Figure 1.
Structures of EBVO proteins obtained from PDB; (A) GP; (B) L, (C) NP, (D) VP24, (E) VP30, (F) VP35, (G) VP40.

2.2 Target proteins collection

All the seven proteins of Ebola virus were chosen as targets. The structures of GP (PDBID: 5JQB), VP35 (PDBID: 3FKE), VP24 (PDBID: 4M0Q), VP30 (PDBID: 5DVW), VP40 (PDBID: 4LDB) and NP (PDBID: 4Z9P) proteins of EBOV were collected from Protein Data Bank which is an open access online database for the 3D structural data of biological macromolecules [31]. The retrieved structure of GP protein had one mutation at position no 42. The mutation was returned to the reference state through the utility of Spdbv software (Alanine nucleotide was converted to Threonine nucleotide). There was no structure of EBOV L protein in the PBD. The sequence of L protein was taken from UniPort and modeling was performed by Swiss-model [32–34]. All water molecules and hetero-molecules that attached to the protein structures were eliminated. The drawn structures of Ebola proteins are shown below in **Figure 1**.

2.3 Energy minimization

Energy minimization was carried out for the selected peptides by making use of Chimera software and for all of the target proteins by using Spdbv software. The aim of energy minimization is to discover a set of coordinates indicating the least energy conformation for the given structure.

2.4 Peptide – Protein docking of viral targets with ligands

Hex software is a tool for calculating and representing possible docking modes of pair of protein and peptide molecules [35]. Docking screening of the target proteins and antiviral CPPs was done by HEX software version 8.0.0 and finally, the free energy of binding between receptor-peptide was obtained that is shown in **Figure 2**. The parameters used in the docking process were:

1. Correlation type: shape only

2. FFT mode: 3D

3. Grid Dimension: 0.6

4. Receptor Range: 180

5. Ligand Range: 180

6. Twist Range: 360

7. Distance Range: 40

2.5 Results

The binding energies obtained from the docking method are publicized in **Figure 2**. The docking pose of the best peptide in protein cavity of each of the target proteins are well shown from the below docked **Figures 3–9**.

Peptide/Target	GP	L	NP	VP24	VP30	VP35	VP40
A6	-496.17	-683.74	-663.4	-594.04	-721.91	-563.78	-404.14
b-WT1-pTj	-358.98	-550.85	-573.37	-612.75	-626.5	-580.08	-420.93
Camptide	-378.94	-615.77	-485.78	-582.82	-620.38	-390.66	-494.4
CL22	-511.08	-577.85	-605.75	-650.13	-636.09	-560.84	-535.43
DermaseptinS4	-390.85	-531.73	-575.27	-614.92	-638.04	-625.71	-400.38
EGFP-MPG	-358.35	-562.85	-552.36	-529.89	-574.69	-502.78	-338.7
hLFWT	-354.97	-568.18	-552.13	-560.74	-540.04	-562.43	-333.88
MAP	-294.94	-543.51	-463.39	-613.29	-580.94	-357.94	-443.75
MCoTI-II	-370.08	-564.92	-545.8	-678.08	-588.13	-466.23	-491.89
Melittin	-378.63	-557.77	-572.52	-584.93	-617.02	-601.16	-334.3
MG2d	-358.28	-544.1	-497.29	-528.38	-587.99	-472.31	-387.07
MousePrp(1-28)	-396.81	-562.25	-592.58	-617.27	-611.68	-497.34	-407.69
MPG	-388.73	-605.78	-610.68	-568.28	-664.07	-554.02	-429.42
MPG-EGFP	-326.52	-554.77	-567.43	-538.48	-566.99	-566.18	-343.57
MPGNLS	-341.62	-602.47	-590.88	-562.08	-644.37	-570.6	-311.24
MPG-NLS	-327.33	-559.61	-556.75	-543.95	-593.77	-543.51	-409.1
MPGα	-317.08	-618.87	-557.53	-538.5	-668.5	-582.29	-332.87
MPGβ	-335.57	-581.02	-558.2	-533.64	-712.66	-574.42	-313.42
P(beta)	-390.33	-607.33	-541.65	-671.07	-667.55	-545.77	-491.08
pepM	-391.46	-601.41	-577.31	-593.6	-658.94	-644.36	-365.71
Peptide1	-381.54	-577.24	-608.68	-647.26	-618.47	-479.81	-481.43
Res1	-456.89	-557.41	-611.73	-635.45	-677.4	-531.16	-440.27
SN50	-386.69	-553.81	-548.98	-570.48	-609.27	-471.95	-443.61
TatLK15	-364.67	-569.16	-579.49	-614.37	-609.66	-580.46	-455.45
Transportan10	-305.62	-543.68	-520.11	-547.36	-592.08	-497.26	-290.36

E-value
Lower
Higher

Figure 2.
The binding energy scores obtained from Hex docking software.

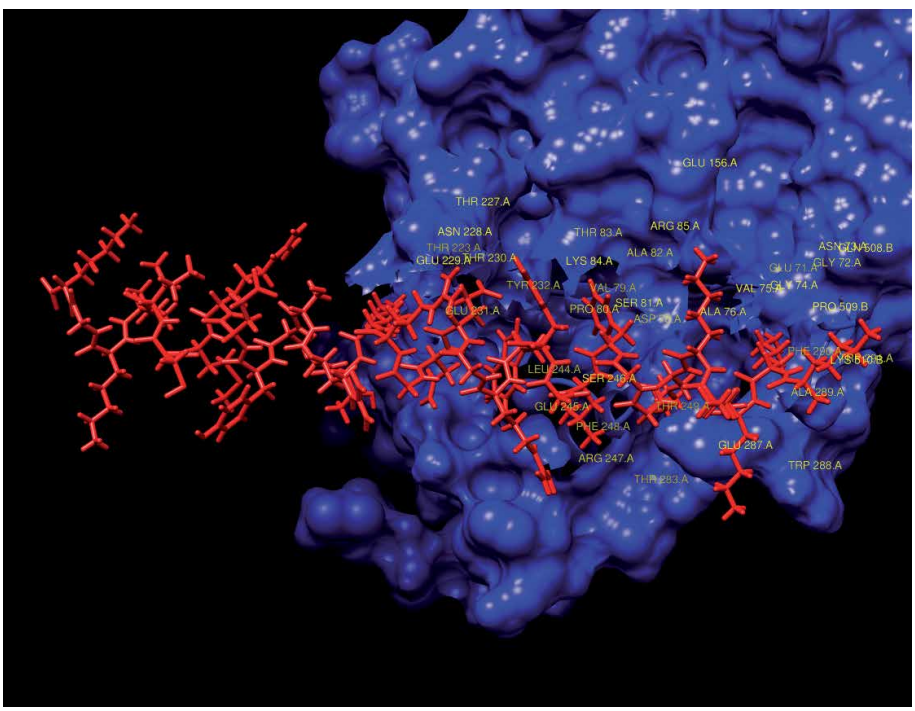

Figure 3.
Docking pose of GP with CL22 peptide; amino acid residues within 5 Å distance of binding site are labeled.

Figure 4.
Docking pose of L with A6 peptide; amino acid residues within 5 Å distance of binding site are labeled.

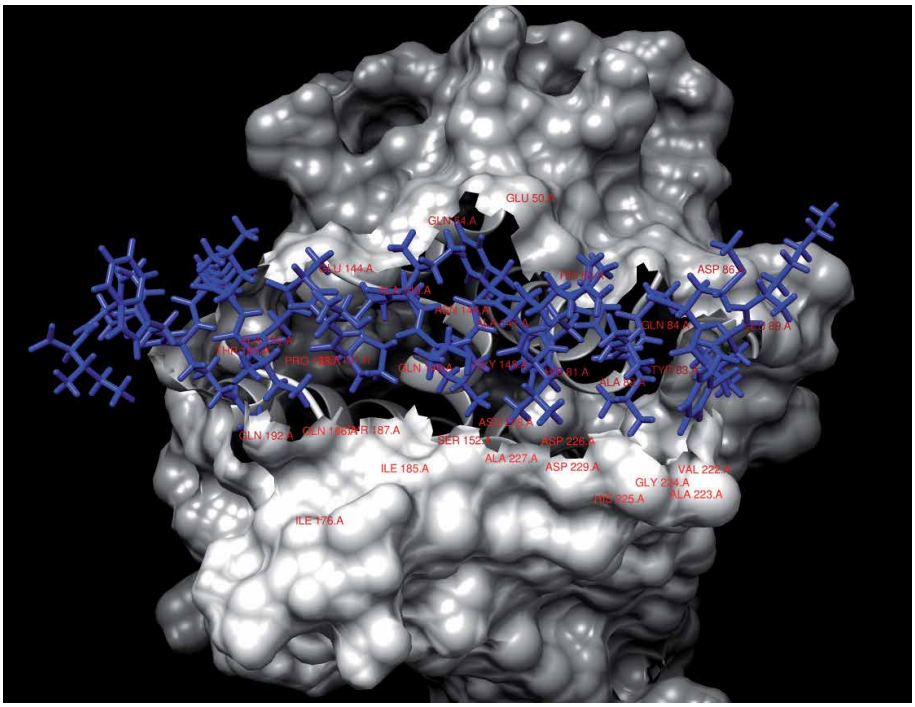

Figure 5.
Docking pose of NP with A6 peptide; amino acid residues within 5 Å distance of binding site are labeled.

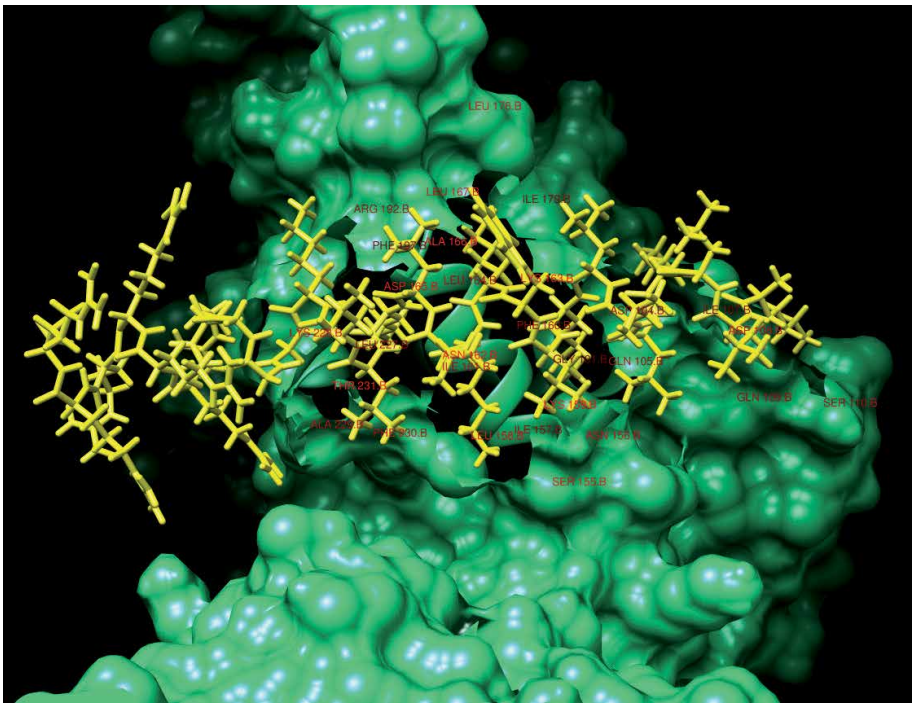

Figure 6.
Docking pose of VP24 with MCoTI-II peptide; amino acid residues within 5 Å distance of binding site are labeled.

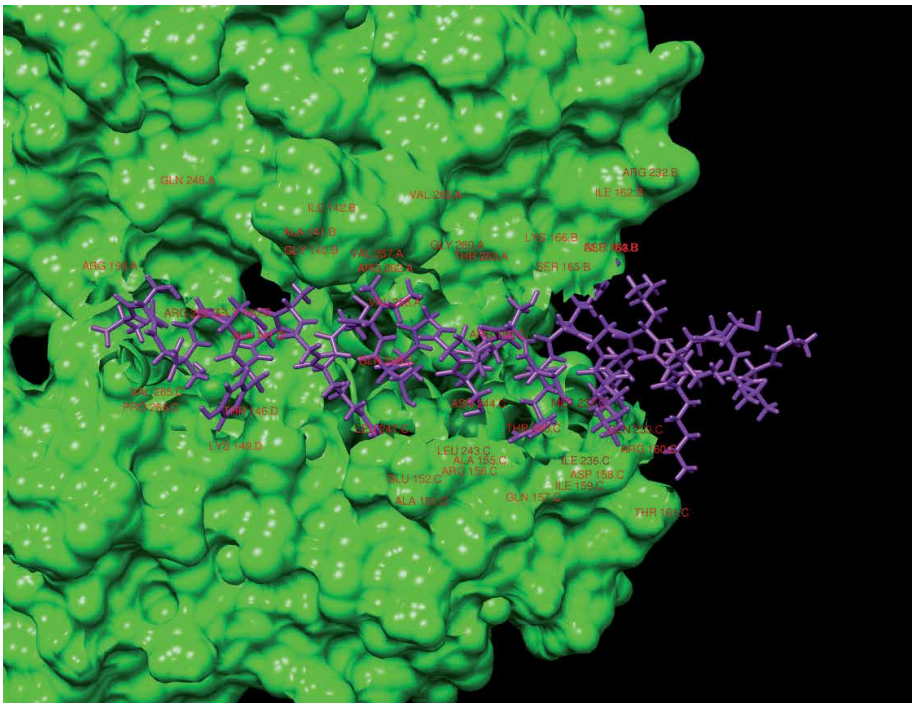

Figure 7.
Docking pose of VP30 with A6 peptide; amino acid residues within 5 Å distance of binding site are labeled.

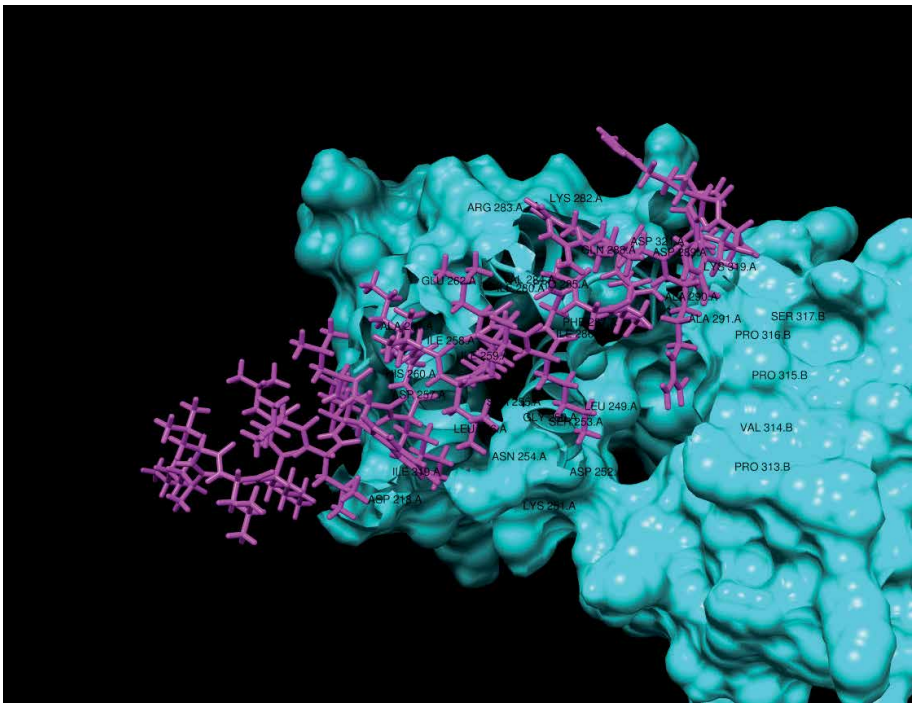

Figure 8.
Docking pose of VP35 with pepM peptide; amino acid residues within 5 Å distance of binding site are labeled.

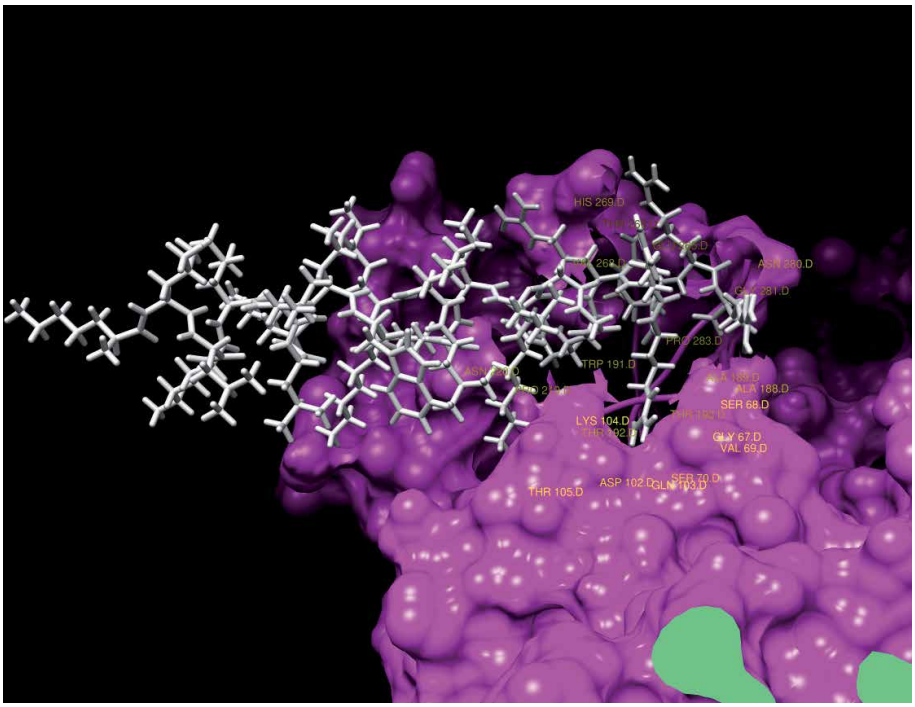

Figure 9.
Docking pose of VP40 with CL22 peptide; amino acid residues within 5 Å distance of binding site are labeled.

3. Conclusions

In our present study, protein – peptide docking technique were accomplished to assess the binding orientations of the seven viral targets from Ebola virus with the 25 selected cell penetrating peptide ligands. Each one of the targets was tightly bound to every 25 different types of CPPs with good score. The E-value scores were very much higher than the previously reported docking studies. Moreover, this research concluded that among all ligands, the CL22, A6 and Res1 peptides interacted efficiently with four of the Ebola proteins and would be considered as potent promising antiviral agents. It is anticipated that this study could pave a way for further in vitro and in vivo investigations to discover new approaches and candidates for Ebola drug design and formulation.

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Section 3

Non-Human Perspectives on Viral Outbreaks

Chapter 15

Survey for a Vector of Zika Virus and Two Other Mosquito Species in Four Ecoregions of Missouri: An *A Posteriori* Analysis

David M. Claborn, Sapana Subedi Chowi, Matthew Flint and Clement Acheampong

Abstract

In 2015, Zika emerged as a vector-borne disease in the Americas, causing a variety of health issues ranging from Guillain-Barre syndrome in adults to microcephaly in newborns. Following the documentation of mosquito-borne transmission of the disease in the southern United States, the Missouri Department of Health and Senior Services contracted with researchers at Missouri State University to complete a survey of possible mosquito vectors of the Zika in the state. The primary vector of the disease, *Aedes aegypti*, had been reported from Missouri in previous surveys from several decades ago, but a comprehensive survey of the state mosquitoes and never been completed. Researchers focused on mosquitoes that spend the immature stages in artificial containers because this is descriptive of the most important Zika vectors. The large survey over three years provided an opportunity for post hoc analysis of mosquito occurrence data across a variety of ecoregions inside the state, documenting changes in the vector populations as a result of invasive species. The survey also allowed an analysis of different trapping techniques for important species in the state. The results are reported in this chapter along with a discussion of the potential impact on human health of changes to the mosquito population.

Keywords: mosquito traps, *Aedes albopictus*, Missouri, ecoregion

1. Introduction

Following the 2015 emergence of the Zika virus in Brazil, the virus rapidly spread through much of the Americas. Although historically associated with a relatively mild, self-limiting disease, the modern pandemic was linked to the severe manifestations of Guillain-Barre syndrome in adults and microcephaly in babies born to infected mothers [1]. Primarily mosquito-borne, the virus is unusual in that it can also be transmitted between humans sexually. Most cases detected in the United States were associated with travel to infected areas and some perhaps by sexual transmission; however, several cases of mosquito-borne Zika virus were reported in Puerto Rico and other American territories. In 2016, probable mosquito borne transmission involving the mosquito *Aedes aegypti* was reported in Florida [2].

The introduction of the Zika virus into North America and reports of mosquito-borne transmission in 2016 prompted public health officials in the state of Missouri to investigate potential vectors of this virus in the state. Previous mosquito surveys in the state were old and tended to cover only small geographic areas. In addition, many changes to the mosquito fauna had occurred with the introduction of invasive species thus increasing potential for disease transmission, so an extensive survey of mosquitoes associated with artificial containers was initiated in the summer of 2016, then continued in the summers of 2017 and 2018. Due to concerns about the Zika virus and the potential for local transmission, state public health officials focused the survey on mosquitoes that inhabit artificial containers during the larval and pupal stages in areas, especially those near human habitation or businesses. This focus was justified by the fact that the primary vector of the Zika virus in the Western Hemisphere is *Aedes aegypti*, a mosquito that is well known for developing in artificial containers near human habitations and one that has historically been reported in Missouri [3]. In addition, there was some concern that the invasive species and vector of the Zika virus, *Ae. albopictus*, might be widely distributed in the state. This latter species was also associated with artificial containers during immature stages.

The survey emphasized automobile salvage yards, used tire dealerships and cemeteries because these environments have historically provided large numbers of container-inhabiting mosquitoes. A complete list of the species obtained in both adult and larval surveillance and species occurrence by county for the first two years of the survey is available in Claborn et al. [4]. Two important findings from that survey were the absence of *Ae. aegypti* and the ubiquitous presence of *Ae. albopictus*. The latter of these two findings confirmed a potential for vector-borne transmission of Zika virus in the state, though no such transmission has been confirmed at the time of writing for this chapter.

Due to the original purpose of the survey, the traps were not used in an experimental design specifically suitable for comparing effectiveness between trap types, such as the Greco-Latin Square design often used to compare trap efficacy [5]. We used an analysis of variance with a protected mean separation test to analyze all data for this study. The extensive survey provides an *a posteriori* opportunity to compare results of trapping potential vectors of Zika and other species using different trap types in Missouri. The comparison allows an analysis of how trap type may affect the results of a survey. In addition, the traps were used in a variety of Missouri ecoregions as described by Nigh and Schroeder [6]. There is no current data on the difference in mosquito fauna between ecoregions in Missouri.

The choice of trap and bait types has an obvious effect on the results of a mosquito survey. Numerous studies have demonstrated differing results of trap effectiveness. To date, most trapping studies in Missouri have relied largely on the use of the venerable Centers for Disease Control Miniature Light trap and its variations [3, 7]. Development of newer traps and baiting technologies provides the opportunity to obtain more complete knowledge of the species composition and abundance in the state as well as the effect of ecoregion habitat on the abundance of mosquito species.

2. Materials and method

2.1 Study areas

We chose the survey sites based on the theoretical range of the primary vector of Zika virus, *Ae. aegypti,* as described by the CDC [8]. The surveyed area including

most of Missouri south of the Missouri River as well as a few places north of the river on the western side of the state near and inside Kansas City, MO. This large region included four ecoregions: the Central Dissected Till Plains, the Osage Plains, the Ozark Highlands and the Mississippi River Alluvial Basin [6]. Only two surveyed counties were in the Central Dissected Till Plains, both near Kansas City. Most of the surveyed region lies within the Ozark Highlands, a region south of the Missouri River and covered with heavily forested hills. The western part of the surveyed region includes part of the Osage Plains region, a fertile prairie land with several streams and rivers. The southeastern portion of the surveyed region (the "Bootheel" of Missouri) includes parts of the Mississippi Alluvial Plain and is bordered by the Mississippi river. It supports large tracts of agriculture, including rice, soybean and cotton crops. The counties included in the survey (as well as the ecoregion for each) are depicted in **Figure 1**. Due to interest in potential vectors of human disease, the survey focused on locations near human habitations with many artificial containers, especially automobile salvage yards, used tire shops, and cemeteries. We also collected larvae from these sites and those data will be reported elsewhere. We resurveyed some sites as many as six times during three summers. Trapping occurred between June 3 and September 23 in 2016, between July 17 and October 29 in 2017, and between June 19 and August 19 in 2018.

We used three types of traps: the Fay-Prince Omnidirectional trap, the BG Sentinel trap and the Centers for Disease Control miniature light trap. All traps were baited with approximately five pounds of dry ice in a plastic cooler with a hole in the bottom to let the gas disperse, but the BG Sentinel trap also used a

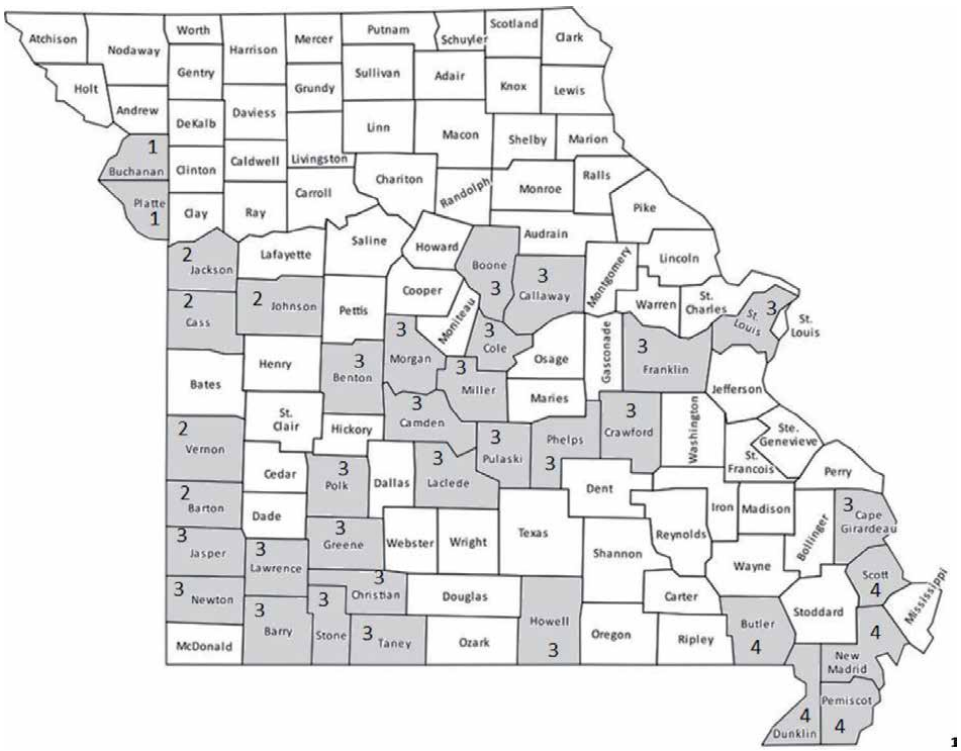

Figure 1.
Missouri counties where mosquito survey was performed are shaded gray (summers 2016–2018) (counties noted with a 1 are in the dissected till plains ecoregion. Those noted with a 2 are in the Osage Plains Ecoregion. Counties noted with a 3 are in the Ozarks Highlands ecoregion and those noted with a 4 are in the Mississippi River Alluvial Plain ecoregion).

commercial lure (BG Lure) as it was designed to do. We placed the traps with baits on the sites in early afternoon and retrieved them in late morning. All traps were at least 50 paces apart from other traps. Trap contents were placed in a large cooler with a small amount of ice, then transported to the laboratory in Springfield, Missouri, where all mosquitoes were killed by freezing. Laboratory workers then pinned the female specimens and identified them microscopically using dichotomous keys in Darsie and Ward [9] and Burkett-Cadena [10].

2.2 Analysis

Data points consisted of the number of mosquitoes from each of the three species caught in a given trap on a given date. The unit of trap-night is used to describe the number of traps over the number of nights used to survey for each site.

Trap type	**n**	*Aede albopictuss*	*Culex eraticus*	*Anopheles quadramaculatus*
BG Sentinel[1]	57	55.24	1.54	18.18
		28.10a[2]	0.47a	1.04a
Fay-Prince Omnidirectional	271	24.66	5.83	27.6
		12.15b	1.32a	3.07a
CDC Light trap	325	11.75	3.40	10.20
		2.90c	0.84a	1.24a

[1]All traps except BG Sentinel traps were baited with approximately five pounds of dry ice in a plastic cooler with a hole in the bottom. The BG Sentinel traps were baited with dry ice and a commercial attractant (BG lure).
[2]Data were transformed as the square root of (x + 0.1). The means in the lower position of each couplet are the back-transformed means of the transformed data. When means in a column are followed by the same letter, the means of transformed data are not statistically different (Tukey's mean separation test; alpha = 0.05).

Table 1.
Arithmetic mean (upper value of each couplet) and back transformed mean of transformed trapping rates of three abundant mosquito species caught in three types of trap in southern Missouri (2016–2018).

Ecoregion	**n**	*Aedes albopictus*	*Culex eraticus*	*Anopheles quadramaculatus*
Mississippi River Alluvial Basin	53	24.03	6.13	36.15
		8.54a[1]	2.79a	12.22a
Osage Plains	54	29.83	5.11	19.04
		5.66ab	1.04ab	0.96b
Ozark Highlands	207	4.60	2.35	1.68
		1.6b	0.46b	0.26b
Central Dissected Till Plains	9	0.56	0.10	0.56
		0.31ab	<0.01	0.18b

[1]Data were transformed as the square root of (x + 0.1). Statistical analysis was done on transformed data but the means reported here are the arithmetic means of the original data and the back-transformed means of the transformed data. Means of transformed data (represented by backtransformed means) within each column followed by the same lower case letter are not significantly different (Tukey's mean separation test; alpha = 0.05).

Table 2.
Arithmetic mean (upper value of each couplet) and back-transformed mean of trapping rates (mosquitoes/trap-night) for three species caught in CO_2-baited CDC traps in four ecoregions of southern Missouri (summer, 2016–2018). The value in the lower position of each couplet is the back-transformed mean of the transformed data for trapping rate.

The survey consisted of a total of 653 trap-nights. The number of trap-nights for each trap type and ecoregion combination is reported in **Tables 1–4**. The data did not display a normal distribution and many data points reflected no catch ("0"); therefore, all data were transformed by taking the square root of (x + 0.1) where x was the number of mosquitoes of a given species caught in a single trap. This transformation appeared to improve the distribution of the data. For instance, the non-transformed data for *Ae. albopictus* caught in the CDC light trap had a skewness score of 2.92 and a kurtosis score of 11.23. After transformation, those scores were reduced to 1.21 and 1.01, respectively. Both transformed scores are below the recommended maximum thresholds recommended by West [11]. We used transformed data for all subsequent analyses and reported the results with back transformed

Ecoregion	**n**	*Aedes albopictus*	*Culex eraticus*	*Anopheles quadramaculatus*
Mississippi River Alluvial Basin[1]	44	33.20	8.68	153.12
		17.54a	2.12a	51.60a
Osage Plains	43	31.79	2.55	0.28
		15.11a	0.80a	0.13b
Ozark Highlands	161	21.81	6.59	5.41
		10.70a	1.51a	0.67b
Central Dissected Till Plains	21	13.61	0.23	0.42
		8.02a	0.07a	0.16b

[1]Data were transformed as the square root of (X + 0.1). Statistical analysis was done on transformed data but the means reported here are the arithmetic means of the original data and the back-transformed means of the transformed data. Means of transformed data within each column followed by the same lower case letter are not significantly different (Tukey's mean separation test; alpha = 0.05).

Table 3.
Arithmetic mean (upper value of each couplet) and back-transformed mean of trapping rates (mosquitoes/trap-night) for three species caught in CO_2-baited fay-prince omnidirectional traps in four ecoregions of southern Missouri (summer, 2016–2018). The value in the lower position of each couplet is the back-transformed mean of the transformed data.

Ecoregion	**n**	*Aedes albopictus*	*Culex eraticus*	*Anopheles quadramaculatus*
Mississippi River Alluvial Basin[1]	17	60.7	1.29	60.41
		44.24a	0.40a	6.66a
Osage Plains	15	84.27	3.33	0.26
		45.32a	1.32a	0.14a
Ozark Highlands	25	34.12	0.64	0.16
		12.9a	0.18a	0.04a

[1]Data were transformed as the square root of (X + 0.1). Statistical analysis was done on transformed data but the means reported here are the arithmetic means of the original data and the back-transformed means of the transformed data. Means of transformed data within each column followed by the same lower case letter are not significantly different (Tukey's mean separation test; alpha = 0.05).
[2]The BG Sentinel trap was not used in the Dissected Till Plans during any of the three years of the survey.

Table 4.
Arithmetic mean (upper value of each couplet) and back-transformed mean of trapping rates (mosquitoes/trap-night) for three species caught in BG sentinel traps in three[2] ecoregions of southern Missouri (summer, 2016–2018). The value in the lower position of each couplet is the back-transformed mean of the transformed data.

means. Because trap catches for each trap type were significantly different for at least one species, we analyzed the trap catches by ecoregion separately for each trap type. We used the data for the most abundant species from each of three genera for the comparison: *Anopheles, Aedes* and *Culex*. These were also the three most abundant species in the entire survey regardless of genus. We calculated the mean trap catch for each species by ecoregion using an unbalanced analysis of variance in the PROC GLM of SAS, with mean separation using a Tukey's HSD test.

The Missouri Department of Health and Senior Services contracted this survey to the Master of Public Health Program and the Ozark Public Health Institute of Missouri State University in Springfield. (Contract #AOC16380144).

3. Results

Table 1 displays the means of trapping rates for three trap types over all three summers for the three most abundant species, one from each of three genera. Due to the non-normal distribution and the large number of traps with no adult mosquitoes (zeroes), the back-transformed means of the transformed data are also reported. Analysis indicates a statistically significant difference in the trapping rates only for *Ae. albopictus*, with the BG Sentinel using the BG Lure capturing the most mosquitoes. The Fay-Prince Omni Directional trap captured fewer than did the BG Sentinel, but more than the CDC Light trap, with all comparisons of traps for this species being statistically significant at the 0.05 probability level. The latter two traps used a carbon dioxide attractant only. For the other two species compared in this study, statistically significant differences in trap rates between traps were not detectable, though the arithmetic means for both *Cx. eraticus* and *An. quadramaculatus* were highest for the Fay-Prince trap.

Tables 2–4 display the trapping rates for all three summers by ecoregion, with each table reporting rates for one type of trap. **Table 2** reports the trap rates for the CDC miniature light trap with CO_2 bait. The CDC trap demonstrated a consistent difference in trap rates between the Mississippi River Alluvial Plain and at least one other ecoregion across all three species. The trap rate for *An. quadramaculatus* was the only one for which the Alluvial Plain was different from all other ecoregions; however, the trap rate for at least one ecoregion was different from that of the Alluvial Plain in all three species. **Table 3** displays the trapping rates for all Fay-Prince Omnidirectional traps across all three years. Unlike the CDC trap, the Fay-Prince did not demonstrate significant differences in trapping rates between ecoregions for two of the species: *Ae. albopictus* and *Cx. eraticus*. For *An. quadramaculatus*, however, a significant difference was noted in trap rates in the Mississippi Alluvial Plain and all three of the other ecoregions. **Table 4** displays the trapping rates for the BG-Sentinel trap using BG-Lure. None of these traps were used in the Central Dissected Till Plains ecoregion, so only three ecoregions were compared. No statistical differences in transformed trapping rates were detected between ecoregions for any of the three species as measured by the BG-Sentinel trap, despite very large differences in the arithmetic mean, reflecting great variation even in the transformed data. The means were somewhat similar between ecoregions for *Ae. albopictus*, as they were for the Fay-Prince trap; however, the means were widely separated for *An. quadramaculatus*. It should be noted that the number of trap-nights for Fay-Prince and CDC traps was five to six times that of BG Sentinel. The difference in findings suggests that the BG Sentinel were probably under-utilized in comparison to the other traps and sample numbers were probably insufficient.

The most productive trap for *Ae. albopictus* was the BG Sentinel. For the other two species compared here, no significant differences in average trap catch between trap types were apparent, though the Fay Prince trap demonstrated the highest arithmetic mean for both.

4. Discussion

This study suggests that the choice of traps affects conclusions about relative species abundances in different ecoregions. Though general conclusions by arithmetic mean are similar, detection of statistically significant differences in abundance may be dependent on trap type and is highly dependent on sample size. In this survey, there was an obvious difference between mosquito abundance, especially for *An. quadramaculatus*, in the Mississippi River Alluvial Plain and the other ecoregions, and this conclusion was consistent across trap types. Two trap types suggested higher abundance of *Ae. albopictus* in the Osage Plains ecoregion, though these differences were not statistically significant.

This *post hoc* analysis of trapping data confirms earlier studies demonstrating high trap effectiveness for the BG-Sentinel trap for *Ae. albopictus*, though the Fay-Prince Omnidirectional trap had somewhat similar results. This study suggests that the BG Sentinel is suitable for continued surveillance of container-inhabiting mosquitoes in Missouri, though it probably provides a disproportionate estimate of relative *Aedes* abundance. This finding will be important when interpreting survey results for *Ae. albopictus* and other vectors of Zika virus.

This survey is the largest mosquito survey in Missouri to date. It covered a much larger geographical area than any previous study and is the only one to include four different ecoregions. The survey utilized a variety of trap types. It does have several weaknesses. First, it was not originally designed as a comparison of different trap efficacies, but was instead a *post hoc* analysis of available data. In addition, due to the focus on potential vectors of Zika virus, the choice of surveillance sites emphasized habitats associated with artificial container-inhabiting species near human habitation and thus collected *Aedes* species in disproportionate numbers. Also, some of those sites were in urban habitats that may have masked some of the effect of ecoregion. Finally, the traps were not randomly assigned to sites and were at times placed in the exact same spot repeatedly over the trapping seasons. Finally, sample sizes varied greatly between the three trap types and were probably insufficient for at least one type, the BG Sentinel. Nevertheless, this survey provides consistent estimates of relative mosquito abundance by ecoregions and provides some evidence of trap type efficacy by species.

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Chapter 16

Frog Virology: Biosafety in an Experimental Farm

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Abstract

Understanding and detecting diseases of amphibians has become vitally important in conservation and ecological studies and prevent and biosecurity a determinant priority in experimental farms, mainly when related with academic and research activities. Ranavirus belongs to the family Iridoviridae, and causes an emergent infectious disease that affects different species, especially fish, reptiles and amphibians, with a significant contribution to the decline of the population. In amphibian systems, Ranaviruses transmission can occur between vertebrate classes through direct contact, by scavenging or through virus particles persisting in the environment. Subclinical infected individuals may serve as reservoirs in the most susceptible anura species. Humans play a significant role in this emergent disease and biosecurity measures are determinant to prevent the introduction of these viruses, either in commercial or experimental farms. A Biosafety Plan is a fundamental tool in the Ranaviruses prevention and include educational and training programs, relevant to the mission of a Higher Education Institution.

Keywords: amphibians, biosafety plan, infectious diseases, Ranavirus, surveillance

1. Introduction

Emerging infectious diseases are currently a threat to the conservation of global biodiversity [1]. Amphibian diseases linked to declining of amphibian populations, are a constant threat to endangered species, and are frequently a hazard in ranaculture facilities [2]. Many factors have been implicated in these declines in the wild, including introduced predators, increased ultraviolet-B radiation, chemical contaminants, habitat destruction and degradation, and emerging diseases [3]. Amphibians are susceptible to a variety of pathogens, including internal and external parasites, bacteria, fungi, and viruses. Understanding and detecting diseases of amphibians has become vitally important in conservation and ecological studies [2].

Changes in environmental conditions can be a potential driver of emerging infectious diseases [4]. Environmental influence affects the population susceptibility, with seasonal variation in response to climate (temperature) alterations, moisture availability, and their interactions' in amphibian behavior [1]. In fact, pathogens are favored for warmer ambient temperatures, that provide ideal conditions for propagation [4, 5].

The causes of the population decline are complex, but it is clear that infectious agents, either directly, or following environmentally-induced immune suppression,

play an important role in this process [6, 7]. Each of the three major life stages of amphibians (embryos, larvae, and adults) has distinct diseases [2] and at least six groups of viruses have been reported to affect amphibians, including iridoviruses, herpesviruses, and arboviruses.

Some infectious diseases of amphibians share similar pathological signs; thus, their detection, recognition, and correct diagnosis can be a challenge [2, 8]. A group of viruses belonging to the genus Ranavirus are amphibian pathogens, globally distributed, with higher morbidity and mass mortality [2, 8–11]. Ranaviruses infect at least 175 species across 52 families of ectothermic vertebrates, as fish, amphibians, and reptiles, and cause systemic diseases, compromising multiple internal organs [4–6, 12–14]. They are the second most common infectious cause of mortality in amphibians worldwide, after the fungus Batrachochytrium dendrobatidis [5], with a relevant impact in the population decline. As indicative of the ranaviruses host range and their potentially negative effects, ranaviral disease was listed by the World Organization for Animal Health (OIE) as an internationally notifiable disease [2, 8, 9, 15, 16]. Ranavirus is associated with amphibian die-offs, like many other diseases it generally does not lead to the extinction of the host [17].

Ranaviruses may function as a novel or endemic pathogen, associated with the movement of infected amphibians by humans. The infectious process involves genetics, environmental factors (pollution, temperature and other stressors) and inherent biological characteristics of the host (age, life stage, physiological aspects) that directly affect immune competence. Anthropogenic stressors also may facilitate emergence, compromising the imune system [2, 18]. Additionally, subclinical infected hosts may serve as reservoirs for more susceptible amphibian species [18].

Several authors have noted that commercial exchange of live amphibians for food, pets, and laboratory animals may be adversely influencing wild populations by direct harvesting or through the spread of disease [19, 20]. To supplement the higher demand for frogs, and to counteract the effects of over-harvesting, some countries have introduced frog farming.

Dissemination is facilitated by contact with infected individuals or contaminated water as well as inherent behaviors of amphibians such as necrophagy and cannibalism [21]. Measures that prevent or minimize the possibility of introducing potentially pathogenic infectious agents, either wild or captive amphibians, are crucial [22]. Managing ranaviral disease in captive facilities is more straightforward than in natural populations. Isolation of positive individuals and disinfection of animal enclosures are important initial steps, but similar to wild populations, it is essential to minimize possible stressors and maintain proper biosafety procedures to prevent cross contamination [23].

Vertebrate iridoviruses, specifically members of the genus Ranavirus, have become a significant cause of disease in ectothermic animals, and that from a virological, commercial and ecological point of view deserve additional study [6]. The aim of this chapter is to introduce common amphibian diseases outlining value biosafety measures in a frog farm, with production, experimentation, and research purposes, as well as academic activities, inserted in a Higher Education Institution.

2. Amphibian viruses

2.1 Ranaviruses

Amphibian ranaviruses are enveloped icosahedral DNA viruses, in the family Iridoviridae, with variable size ranging, depending on the species [5, 7, 13]. Isolates causing disease have been found in wild and cultured amphibians in Australia, the

Americas, Asia and Europe [8–10, 15]. These include Frog virus 3, Tadpole edema virus, *Rana catesbeiana* virus Z, *Bohle iridovirus*, and UK ranavirus. Other ranavirus-like were found in captive frogs (*Rana esculenta*) in Croatia, causing lethargy, edema, hemorrhages, and skin necrosis, and also in wild-caught frogs (*Bufo marinus* and *Hopodactylus* sp.) in Venezuela. In this case infected animals had no external lesions or internal symptoms [8].

The trade of amphibians for food, research [13] and as pets contributed to the dissemination of pathogens such as ranaviruses, within and among continents [8, 9]. In North America, ranaviruses are responsible for massive mortality in amphibian larvae and recent metamorphs, while die-offs rarely occur in adults. These events often occur during summer and involve hundreds to thousands of moribund and dead larvae within a few days [8].

Ranavirus epidemics seem to occur in late spring and in summer, what can be explained by the seasonal amphibian's vulnerability to ranaviral infection when the larvae of many species begin to metamorphose. In fact, many components of the amphibian immune system are down-regulated just prior to metamorphosis [8].

Infections occur mostly in amphibians that breed in standing-water habitats [3, 24], and frog farms are associated with permanent water which may increase the exposure to the pathogen, considering that water is an effective transmission route. Animals can be sublethally infected and contain the virus over a period of at least 1 year [7]. Ranaviruses can cause asymptomatic infections in resistant animals, facilitating the spread of disease with the movement of infected animals, and contributing to the prevalence of the infection in the population [8, 10].

Frog virus 3 (Ranavirus type I), was first isolated from aclinically infected leopard frogs (*Rana pipiens*) collected in the United States in 1962 [7, 8, 10, 22]. Since then, FV3-like viruses, such as Tadpole edema virus (TEV), *Rana catesbeiana* virus Z (RCVZ), and UK Ranaviruses have been study.

In laboratory, Ranavirus was shown to cause edema, necrosis, hemorrhage, and death in embryos, tadpoles, and recent metamorphs. During experimental infections, metamorphic toads developed hemorrhages and edema in the ventral skeletal musculature, stomach, and intestines [8, 10, 24]. Mortality in embryos can occur 3 to 12 days post-exposure and clinical signs include depigmentation, skin sloughing, and spinal curvature [8, 25]. Generally, the lesions caused by FV3 appear to be milder than those caused by TEV [8].

Tadpole edema virus (Ranavirus type III) is the first acutely fatal viral infection of wild tadpoles, such as the bullfrog, *Rana catesbeiana*, bufonids (*Bufo americanus*, *Bufo woodhousei fowleri*), and pelobatids (*Spea intermontana*) [22, 25]. Present gross lesions include marked edema, erythema and hemorrhages of the skin and subcutis of the body and proximal hind limbs, hydro coelom, and petechial hemorrhages in the stomach, intestines and skeletal muscles [8, 25].

Rana catesbeiana virus Z was isolated from cultured *R. catesbeiana* tadpoles in the USA. RCVZ appears to be much more pathogenic than FV3, causing massive mortality of exposed tadpoles. Similar to other ranaviruses, symptoms included edema in the abdomen, hemorrhaging in ventral regions, and lethargy [8].

The contemporary strains in the United Kingdom, in common frogs (*Rana temporaria*) and in captive-breeding facilities [12, 26] worldwide, may had origin in North America [8]. Four clinical syndromes were associated with ranavirus-like particles, in English populations of the European common frog: "ulcerative syndrome", "hemorrhagic syndrome", "ulcerative and hemorrhagic syndrome" [7, 12, 26], and "reddened skin syndrome" [25]. The ulcerative form of the disease is characterized by ulcers of the skin and the skeletal muscle, and sometimes digits necrosis, while the hemorrhagic form is described with internal hemorrhages, commonly involving the gastrointestinal and reproductive tracts [12].

The second distinct amphibian ranavirus species discovered was *Bohle Iridovirus* (BIV), isolated from metamorphosed ornate burrowing frogs (*Limnodynastes ornatus*) in Australia [8, 22]. Experimentally, BIV is highly pathogenic to tadpoles and metamorphs of *L. ornatus*, and also, to tadpoles, metamorphs and adults of the giant toad, *Bufo marinus*. Lesions produced by BIV are multifocal necroses of the liver, mesonephroid, and lungs [25].

Species within the anuran family Ranidae were generally more susceptible to ranavirus infection than other family's species (Hylidae, Bufonidae, Scaphiopodidae), as shown by phylogenetic comparative methods [5, 24].

2.2 Herpesviruses

Other viral infection of the North American leopard frog is caused by the herpesvirus, and induces a form of renal adenocarcinoma, known as Lucke's renal tumor. The tumor grows during the spring and summer, with the virus being shed in the spring to infect other frogs. Renal failure occurs with weight loss and death. There is no treatment for this disorder [27].

A herpesvirus-like dermatitis with numerous dorsal and lateral epidermal vesicle, was also detected in specimens of the spring frog, *Rana dalmatina*, in a north Italy region [24]. These enveloped viruses tend to be less stable in the environment, and transmission, from one enclosure to another by human vectors, is feasible but could be prevent by good hygiene practices [18].

2.3 Arboviruses

Arboviruses are known to infect hosts by infected arthropods. Amphibians and reptiles have been studied as potential reservoir hosts of Chikungunya virus. The possible role of ectothermic vertebrates as reservoirs or overwintering hosts has been evaluated for several arboviruses, and numerous species of mosquitoes have been described to feed on a variety of reptiles and amphibians, including mosquitoes such as *Aedes aegypti* [28].

Amphibians are infected with virus through physical contact, skin exposure to contaminated water or direct ingestion of viruses [3, 29].

In one study, a frog (*Rana ridibunda*) was found to be viremic and was able to transmit the virus to *Culex pipiens*, a bloodsucker [30]. Therefore, a frog-mosquito-frog cycle also appears to be possible under certain ecological conditions.

Since necrophagy and cannibalism are considered important forms in direct transmission of viruses in amphibians, both in the tadpole and metamorphosed phases, the ingestion of virus-carrying insects can also be a form of infection. Transmission by necrophagy and cannibalism is common in host species such as *Arnbystollla tigrinum*, *Rana sylvatica* and *R. latastei* [29, 31] and infections acquired by these routes appear to be more lethal.

Thus, the problem of transmission of arboviruses between amphibians and, as they may be carriers, must be taken in the spread of this class of viruses to insects and their transmission to other vertebrates (including humans).

3. Transmission

Ranavirus horizontal transmission can occur via direct (necrophagy, cannibalism, [13, 17] touching, biting, [8] scavenging, virus particles persisting in the environment), or indirect routes (fomites, soil, contaminated water) [8, 12, 15]. The potential for human involvement in transmission and spread of diseases, within

and among amphibian populations, is very significant [16]. Three is no evidence of ranavirus vertical transmission [32].

Rate and infection outcome vary with the route of exposure [12, 15]. Due to nutritional and energetic limitations and physiological trade-offs, host life history characteristics such as fast development, short life span, and high fecundity can be associated with increased susceptibility to pathogens [24]. Concerning nonenveloped viruses, like iridoviruses, with a tendence to be stable in the environment, prevent spread presents a greater challenge [33].

Spread of ranaviruses may be due to water movement, via fomites sedimentation, or by sublethally/aclinically infected animals [8]. Supplying several tanks with water from a single source, and allowing the water to run through successive tanks, may contribute to a serious outbreak of diseases [32]. Larvae could become infected with ranavirus when exposed to water that previously housed infected larvae [8]. Habitats, with optimal conditions for the pathogen's persistence, may form "reservoirs" [1].

Under laboratory conditions, test animals may not be exposed to the normal array of environmental conditions (diel temperature fluctuations, exposure to proper ultraviolet-B radiation), microbial communities, or other environmental elements that could influence transmission [3]. Transmission through indirect routes had been demonstrated in the laboratory [8] and, previous laboratory studies shown that ranaviruses can persist from days to years, depending on the environmental conditions [8, 34].

4. Biosecurity

Ranaviruses are emerging pathogens and a threat to global amphibian populations. Following the guidelines of the World Organization for Animal Health [35], biosafety measures, a set of management and physical measures designed to mitigate the risk of introduction of pathogenic agents into, or spread within, or release from, aquatic animal populations, should be implemented in aquaculture establishments.

Managing ranaviral disease in captive facilities is more straightforward than in natural populations, requiring surveillance, control measures and basic biosecurity conditions, namely for the purpose of international trade [21, 35]. The definition of compartment, one or more aquaculture establishments under a common biosecurity management system containing an aquatic animal population with a distinct health status, should encompass disease-specific epidemiological factors, the aquatic animal species in the compartment, production systems, biosecurity practices, infrastructural factors and surveillance [35].

4.1 Disease surveillance

Amphibian ranaviruses have been found in animals that are traded over international borders for a variety of reasons, including human consumption and the pet trade [14]. The OIE listing provides the impetus for disease surveillance and required testing of amphibians prior to transport among states or between nations [2, 35, 36].

Epidemiological and geographic factors should be taken into consideration in disease surveillance, as the disease status in adjacent areas and in areas epidemiologically linked to the compartment and the location, disease status and biosecurity of the nearest epidemiological units or other epidemiologically relevant premises [35, 36].

Disease transmission can occur between captive and free-ranging populations and a strategy of comprehensive disease surveillance in captive amphibians and

frog farm facilities, should be implemented. Captive breeding population health status must be considered when intended for release [2], and is not recommend wild amphibians, housed for any period of time, returned to their natural population unless been kept in isolation and their captive history consider as disease-free [16]. Disease emergence also may occur through geographical transport of pathogens.

Ranaviruses isolated from frog farm facilities appear to be more virulent than wild strains, emphasizing the importance for disease monitoring at these facilities. In areas with multiple endemic ranaviruses strains or species, slight variations in genetic coding can increase virulence.

Isolation of positive individuals and disinfection of animal enclosures are important steps, but similar to wild populations, it is essential to minimize possible stressors and maintain proper biosafety procedures to prevent cross contamination [23].

Simultaneous infection by multiple pathogens is possible, and some diseases become evident only after the post-metamorphic (Lucke's tumor herpesvirus). Also, the lack of gross signs of disease does not imply healthy populations, as tadpoles with no signs of illness can be infected with ranaviruses [2].

In the event of a die-off in a captive facility, freshly dead animals should be submitted for diagnostic evaluation. Live animals that are infected should be euthanized or treated, if a treatment exists, and facilities decontaminated with disinfectant [2]. To identify the causal factors for outbreaks, ideally host densities and stages of development, water and ambient temperature, and water quality should be measured during surveillance programs [36].

Testing for Ranavirus can be done with lethal and non-lethal samples. Testing liver samples for infection is a common lethal sampling technique to estimate ranavirus prevalence because the pathogen often targets this organ, especially in larval amphibians, and the liver is easy to identify and collect [11].

False negative can result from testing tail clips in [2, 11], and occur when the number of virions circulating in the host's tissues is low, or few virions are shed [11]. Lethal samples (organ tissue) will likely result in greater detection of ranavirus compared to nonlethal samples (swabs, tail-clips) [11, 36]. Non-lethal sampling techniques can be useful for ranavirus surveillance, although the prevalence of infection may be underestimated when compared to results obtained with liver samples [11].

Sample collection may include whole live or dead animals, sections of tissues, swabs of lesions or orifices or habitat samples. To prevent disease transmission between infected and uninfected individuals [17] and protect professionals from zoonotic diseases, is mandatory wearing disposable gloves when handling amphibians and, between animals, change gloves. When handling amphibians, professionals should use disposable vinyl or nitrile gloves, rinsed with distilled or sterilized water [2, 16]. Dipping gloves into disinfectant between processing animals might reduce iatrogenic pathogen transmission, however, these practices may have toxic effects on wild animals [17].

Samples can be frozen in a standard 20 °C freezer if stored for short duration (1 month); otherwise, should be stored in an 80 °C freezer. Samples can also be promptly fixed in 75% ethanol or 10% neutral buffered formalin for histology. Swabs are typically performed in the oral then cloacal regions, and the swab stored, placed on ice and frozen similar to tissues [2].

Lethal infectious diseases of amphibians may response to stressors, whether anthropogenic or natural [2], and some natural factors are host density, species composition, temperature, and host development [36]. Prevention of the spread of endemic diseases to naive populations or species is a high conservation

priority [2], thus is very important to implement appropriate strategies to minimize this risk [16].

No treatment or vaccine are currently available for ranaviruses [9, 16], but the potential for development of a Ranavirus vaccine is promising particularly considering that prior infection with a ranavirus led to enhanced immunity against subsequent exposure [37], particularly valuable in captive populations [21].

Organizations with limited knowledge about ranaviruses, in the region, supplementary efforts and time are required to document the distribution of ranaviruses, identify infection hotspots, and implement disease intervention strategies that thwart the introduction of ranavirus or reduce its prevalence [36].

4.2 Human and animal safety

Commercial exchange of live amphibians for food, pets, and laboratory animals may be adversely influencing wild populations by direct harvesting or through the spread of disease. Ranaviruses can remain viable outside of hosts for a considerable duration, and can be transported on sampling equipment, recreational gear and fomites [21, 25, 34].

Few infectious diseases of amphibians are contagious to humans, even if mandatory the decontamination of surfaces that come in contact with water bodies that contain amphibians to stop the unnecessary spread of the pathogen [21, 34]. Professionals should wear sanitary wear protection, gloves and waterproof footwear, easily disinfected, when monitoring or capturing animals. Disposable gloves should be worn whenever handling amphibians, and hands washed thoroughly after removing gloves [2].

A correct distinction between cleaning, disinfecting, and sterilizing should be considered. Cleaning refers to the action of physically removing organic and inorganic debris. Disinfecting reduces the load of contaminating organisms to a large extent, but not completely. For a well-established amphibian collection, that has had no infectious diseases or new specimens added within a year, there is little need to attempt to sterilize cages and tools. However, if a collection is experiencing disease, and/or is adding new animals, items should be sterilized [38].

Washing and disinfecting equipment is recommended whether in the presence of pathogens or not [2]. Disinfectants must be safe for use with amphibians and must inactivate a significant proportion of Ranavirus to be considered effective [9]. Common disinfectants used are chlorhexidine [2], potassium compounds [9] and sodium hypochlorite (bleach) [9, 16, 19]. Bleach is often preferred because it is cost effective, easily obtained, and effective against most bacteria and many viruses. However, bleach is not very effective at inactivating Ranavirus, requiring at least a 3% concentration [2, 9] for 10 to 15 minutes between animals [9, 16, 19] which can be toxic to amphibians. In contrast, chlorhexidine used at a dosage that is safe for amphibians (0.75% for a 1-minute exposure) can inactivate Ranavirus [2, 9]. For potassium peroxymonosulfate is recommend at 1.0% solution for disinfecting equipment for 10 minutes [9]. After disinfection, equipment may be allowed to air dry or rinsed with clean water [2].

Proper health of any aquaculture operation depends on water quality, proper nutrition, quarantine and sanitation. Warm (e.g., >25 °C) and frequently filtered water, along with low host densities, may be good preventative strategies to minimize ranavirus outbreaks in captivity [23, 39]. Sanitation can be achieved by: avoid accumulation of organic matter; disinfections of nets and other equipment used; and providing clean environment [32, 40].

Morbid animals and carcasses should not be released or discarded at the same or other sites because this may facilitate the spread or persistence of infectious

diseases. Dead amphibians that are not used for testing should be placed in double-layered plastic trash bags and disposed by burial or incineration [2, 22, 41].

4.3 Recommended procedures – biosafety plan

Health examinations to captive anuran and good biosecurity methods need to be employed because, often, little is known about the life cycles of infectious diseases, modes of transmission, and the persistence of the pathogen within and outside the amphibian host. The goal of biosecurity is to prevent mechanical transmission of pathogens and contaminants from one location to another by equipment, supplies and people, involving the safety of the humans and animals and disinfection of facilities and equipment [2].

In many cases, a pathogen will only cause disease in a host if environmental conditions are favorable. Such circumstances cause prevalence of disease in a population and leads to host mortality in frog farm facilities or wild populations. In general, the most important environmental factors affecting pathogen survival are temperature, moisture and solar, although pH, the presence of organic matter and exposure to chemicals can also be important [1].

The biosecurity program of a production unit must use healthy and disease-free breeders [32, 35, 41]; disease testing of all incoming lots [19]; treatment of water to eliminate pathogens [41]; sterilization and maintenance of materials and equipment; use of personal hygiene measures including hand, footwear, and clothing washing [16]; knowledge of potential pathogens, sources of risk and methods for their control and eradication; development and use of batches that are resistant to specific pathogens; and maintaining the environment in optimal conditions within all phases of quarantine [41].

Structural aspects should, then, be considered as the water supply (an effective transmission medium for ranaviruses) warm and frequently filtered; effective means of physical separation and facilities for people entry including access control; vehicle and vessel access [2, 23, 35]. Inadequate transportation prior to arrival at the facility, inappropriate housing and overcrowding are husbandry practices that facilitate infection diseases [18, 32].

Facilities should also consider location (isolation from other facilities); animal management and practices (unloading and loading); facilities for the introduction of material and equipment; infrastructure to store feed and veterinary products, and isolation facilities for introduced aquatic animals (quarantine) [18, 32, 35].

Quarantine is a vital component of a production-level biosecurity program, which includes a set of standard used procedures and is an essential part of good management for research facilities or farms. It is an important risk management measure and is a key activity that should be considered when developing strategies during farm production [41]. Quarantine areas can be relatively rustic for this purpose [22] and enclosures should be easily disinfected [39].

The protocol should include a detailed clinical examination that includes monitoring the animal's weight, physical posture, and changes in appearance. At least one blood smear can provide important information on the animal's health, stress, and immune status. A stool examination should be performed [22] as well as microbial culture of the oropharynx and cloaca. Diligent surveillance of the amphibian is essential for successful quarantine. Feeding time generally stimulates activity and allows to assess the amphibian's vigor and appetite [39].

A minimum length of 30 days is recommended for the quarantine period of any amphibian that arrived with a clean fecal sample, but often 60 days of quarantine is needed to process an amphibian through a prophylactic protocol. Wild-caught amphibians, whether obtained directly or indirectly, should be held for an extended

quarantine of 90 days or more [19, 22, 41]. This is also recommended for amphibians of unknown origin and those that have been exposed to especially stressful conditions during shipment or prior to shipment.

Quarantine tools and cages should be maintained well separated from established amphibians. New and ill amphibians should be serviced after the healthy and established members of a collection. Disposable vinyl or nitrile gloves are recommended [16, 17] when working with the quarantined amphibians [34]. Washing and disinfection procedures; disposal of aquatic animal waste; measures to prevent exposure to fomites or vectors; feed supply/source are hygienic or nutritional measures determent not only in the quarantine facility but in all the farm, integrating a well-designed Biosecurity Plan [21, 35, 36, 41].

The integrity of an experimental farm relies on effective biosecurity, with the implementation and monitorization of a biosecurity plan [21]. Following OIE guidelines, this plan integrate the potential pathways for introduction of identified (aquatic animal movements, wild aquatic animals, potential vectors, vehicles, people, biological products, equipment, fomites, feed, waterways); the critical control points for each pathway; measures to mitigate exposure for each critical control point; standard operating procedures; corrective actions; process verification and documentation; contingency plan; educating and training program (for workers, farmers and students) and a surveillance program [35, 36].

5. Conclusion

Amphibians are declining globally and emerging infectious diseases are one of the causes. Ranaviruses have a significant impact on diverse populations of ectothermic animals.

Interactions of amphibians with pathogenic organisms are extremely complex. Laboratory experiments, conducted on animals that are either captive-bred or have been maintained for extensive periods in captivity, are very important to understand the susceptibility of amphibians to disease.

Poor biosecurity practices can increase pathogen transmission and disease-related mortality in amphibians. Co-housing infected amphibians with uninfected individuals, even at low densities, increased disease-related mortality. Frog farm facilities should consider establishing amphibian disease surveillance programs and biosafety protocols for Ranavirus.

Biosafety measures should be implemented in aquaculture facilities, particularly in experimental/commercial farms, and a comprehensive biosecurity plan must be developed, implemented and monitored. Infrastructural factors, hygiene and disinfection, nutritional management and water supply are determinant to reduce or control risk infection. Education and training should be encourage concerning amphibian diseases and public health measures, especially when trade contributes to the spread of ranaviruses.

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The COVID-19 pandemic has reminded the world that infectious diseases are still important. The last 40 years have experienced the emergence of new or resurging viral diseases such as AIDS, ebola, MERS, SARS, Zika, and others. These diseases display diverse epidemiologies ranging from sexual transmission to vector-borne transmission (or both, in the case of Zika). This book provides an overview of recent developments in the detection, monitoring, treatment, and control of several viral diseases that have caused recent epidemics or pandemics.

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