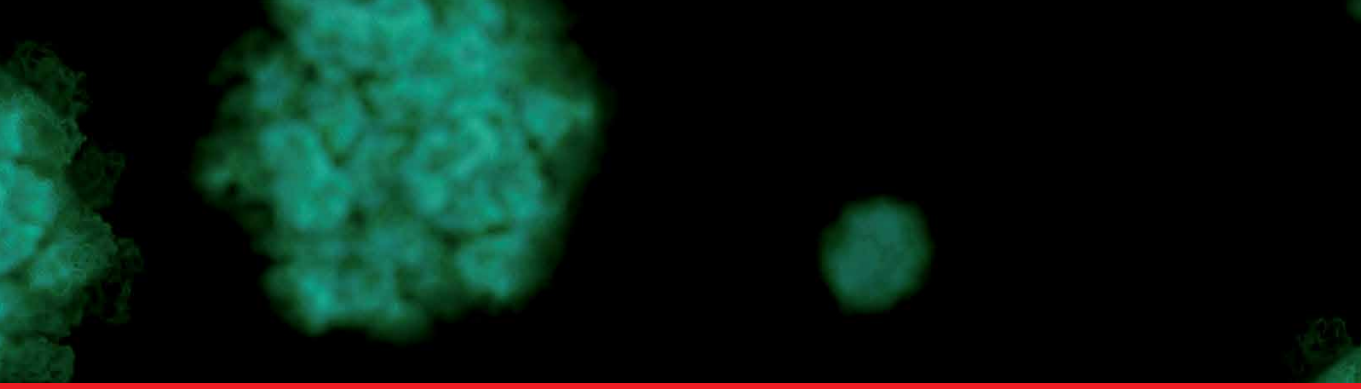

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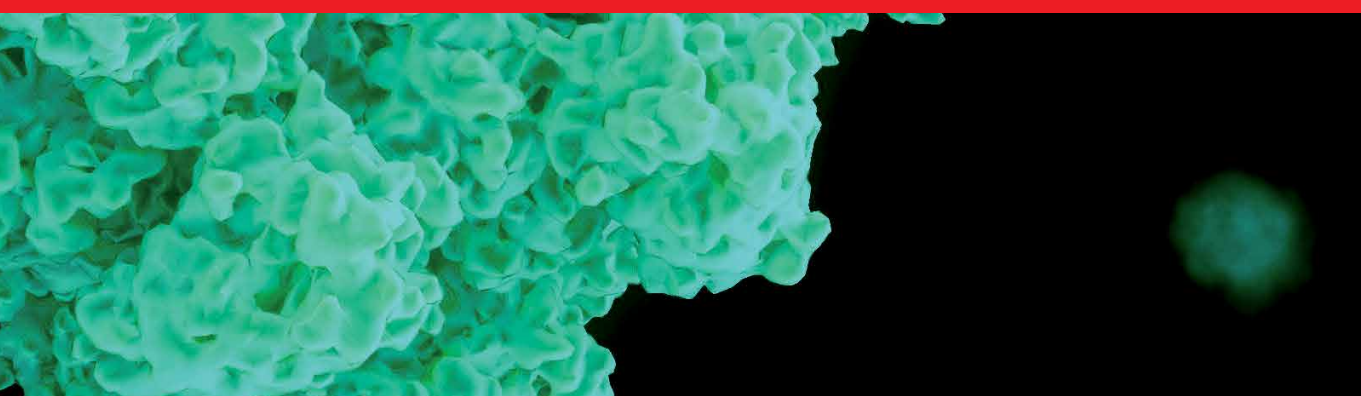


Norovirus

Edited by Gyula Mózsik

Published in London, United Kingdom



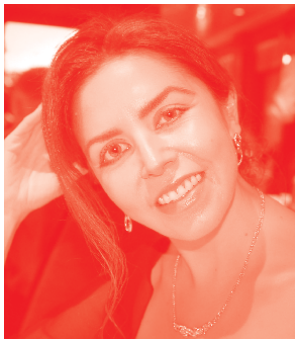




IntechOpen





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Norovirus
http://dx.doi.org/10.5772/intechopen.87659
Edited by Gyula Mózsik

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First published in London, United Kingdom, 2021 by IntechOpen
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, 5 Princes Gate Court, London, SW7 2QJ, United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

Norovirus
Edited by Gyula Mózsik
p. cm.
Print ISBN 978-1-83968-944-4
Online ISBN 978-1-83968-945-1
eBook (PDF) ISBN 978-1-83968-946-8

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Meet the editor

Gyula Mózsik MD, Ph.D., ScD (med), is an emeritus professor of Medicine at the First Department of Medicine, Univesity of Pécs, Hungary. He was head of this department from 1993 to 2003. His specializations are medicine, gastroenterology, clinical pharmacology, clinical nutrition, and dietetics. His research fields are biochemical pharmacological examinations in the human gastrointestinal (GI) mucosa, mechanisms of retinoids, drugs, capsaicin-sensitive afferent nerves, and innovative pharmacological, pharmaceutical, and nutritional (dietary) research in humans. He has published about 360 peer-reviewed papers, 197 book chapters, 692 abstracts, 19 monographs, and has edited 37 books. He has given about 1120 regular and review lectures. He has organized thirty-eight national and international congresses and symposia. He is the founder of the International Conference on Ulcer Research (ICUR); International Union of Pharmacology, Gastrointestinal Section (IUPHAR-GI); Brain-Gut Society symposiums, and gastrointestinal cytoprotective symposiums. He received the Andre Robert Award from IUPHAR-GI in 2014. Fifteen of his students have been appointed as full professors in Egypt, Cuba, and Hungary.

Contents

Preface

Different organs and systems regulate health in the human body. One such system is the gastrointestinal (GI) system/tract.

In the last 50–60 years, prevalence, morbidity, and mortality of GI inflammatory diarrheal illness has increased. The majority of deaths due to GI disease occur in developing countries. In these countries, GI disease causes 2.5 million deaths annually in children younger than 5 years.

Norovirus, a viral infection that causes adverse GI-related symptoms, results in about 685 million cases of disease and 200,000 deaths annually. Norovirus infection symptoms include nausea, vomiting, watery diarrhea, abdominal pain, and in some cases loss of taste. Headaches, fever, general lethargy, weakness, and muscle aches may also occur. Unfortunately, there is no specific treatment for norovirus. Consequently the responsibilities of virologists, epidemidologists, pediatricians, internists, gastroenterologists, geriatrists, clinical pharmacologists , pharmaceutic experts, chemists are extremely big at now and in the forthcoming time.As such, the Product Development for Vaccines Advisory Committee of the World Health Organization (WHO) has recognized norovirus as a priority disease for vaccine development.

This edited volume is a collection of reviewed and relevant research chapters about norovirus. It is divided into four sections. Section 1 includes the introductory chapter. Section 2, "Examination Methods," includes one chapter: "Optimization, Validation and Standardization of ELISA." Section 3, "Norovirus Genome Mechanism," has three chapters: "Norovirus Structure and Classification," "Molecular Mechanisms for Norovirus Genome Replication," and "Norovirus Genotypic Variability in Brazil." Section 4, "Clinical and Pharmaceutical Development," includes the final chapter: "Norovirus: Clinical Findings and Pharmaceutical Developments."

The editor thanks the contributors for their excellent works and cooperation during the preparation of the book. The editor is also especially thankful for the excellent support of Author Service Manager Ms. Maja Bozicevic at IntechOpen.

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Section 1

Introduction

Chapter 1

Introductory Chapter: Norovirus

Gyula Mózsik

1. Introduction

Acute gastroenteritis is a major global health problem and is one of the most common infectious diseases among humans [1, 2].

Despite advances in health technology and management, this diarrhoeal illness remains a common cause of morbidity and mortality [3–5] worldwide. It affects 4.5 billion people and causes 3.5–5 million deaths each year, the majority of which occur in people in developing countries [2, 6, 7]. Acute gastroenteritis leads to approximately 2.5 million deaths annually in children younger than five years in developing countries [8].

Human enteric viruses account for more than half of all cases of gastroenteritis worldwide. There are five common viral agents of gastroenteritis: norovirus, rotavirus, adenovirus (group F types 40, 41), astrovirus, and sapovirus. Norwalk virus is the prototype strain of norovirus, which was associated with an outbreak of gastroenteritis at an elementary school in Norwalk, Ohio, in 1968. Albert Kapikian discovered norovirus as the etiological agent of this outbreak in 1972 [9].

Norovirus results in about 685 million cases of disease and 200,000 deaths annually ("Norovirus Worldwide" (https//web.archiveorg/web/201812071427/https:cdc.gov/norovirus/worldwide html) CDC 15 December 2017. Achieved from the original (https:/www.cdc.gov.norovirus/worldwide.html) on 7 December 2018. Retrieved. 29 December 2017) and Nada [10].

2. A brief overview of transimission, symptoms, common treatments and prevention

Norovirus (sometimes referred to as the "winter vomiting bug") is the most common cause of gastroenteritis. Symptoms of norovirus infection include nausea, vomiting, watery diarrhea, abdominal pain, and in some cases loss of taste. Headaches, fever, general lethargy, weakness, and muscle aches may also occur. The symptoms usually develop 12–48 h after exposure, and recovery takes 1–3 days.

The disease is usually self-limiting and severe illness is rare. Although having norovirus can be unpleasant, it is not dangerous and most who contract it fully recover within a few days. In severe cases, persistent infection can lead to norovirus-associated enteropathy, intestinal villous atrophy, and absorption syndromes.

Noroviruses are transmitted directly from person to person. Norovirus infection occurs in outbreaks, especially among those living in close quarters. The virus usually spread via the fecal–oral route through contaminated food or water.

Prevention involves proper hand washing and disinfection of contaminated surfaces. Alcohol-based hand sanitizers are not effective against the norovirus, according to the National Health Service (NHS). There is no vaccine or specific treatment for norovirus. Management involves supportive treatment such as drinking sufficient fluids or receiving intravenous liquids. Oral dehydration solutions

are preferred and drinks without caffeine or alcohol can help. It must be emphasized that vaccination is the only real possibility to combat this infectious disease. This is where the help of virologists, pediatricians, internists, geriatricians, clinical pharmacologists, pharmacists, chemists, and others is desperately needed.

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References

[1] Murray CJ, Lopez AD. Mortality by cause for eight regions of the world global burden of disease study. Lancet. 1997;**349**:1269-1276

[2] WHO. Revised global burden of disease (GBD). 2002 estimated-incidence. (Online). 2002

[3] Boschi-Pinto C, Lanata CF, Mendoza W, Habte D. Diarrhoeal diseases. In: Jomison RG, Feachem RG, Makgoba ER, Bos ER, Baingana FK, Hofman KJ, Rogo KO, editors. Disease and Morality in Sub-Saharan Africa. 2nd ed. Washington, DC: The World Bank; 2006

[4] Murray CJ, Lopez AD. Evidence-based health policy: Lessons from the global burden of disease-study. Science. 1997;**274**:740-743

[5] Thapar N, Sanderson IR. Diarrhoea in children: An interface between developing and development countries. Lancet. 2004;**363**:641-653

[6] Snyder JD, Merson H. The magnitude of the global problem of acute diarrhoeal disease: A review of acute surveillance data. Bulletin of the World Health Organization. 1982;**60**:605-631

[7] Top 13: Common Communicable Disease Found in India (Online)

[8] Thapar N, Sanderson IR. Diarrhoea in children: An interface between developing and developed countries. Lancet. 2004;**363**:641-653

[9] Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM. Visualization by Immune Electron Microscopy of a 27-nm Particle Associated with Acute Infectious Nonbacterial Gastroenteritis. Journal of Virology [Internet]. American Society for Microbiology; 1972 Nov;**10**(5):1075-81. Available from: http://dx.doi.org/10.1128/jvi.10.5.1075-1081.1972

[10] Melhem NM, editor. Norovirus. Springer International Publishing; 2019; Available from: http://dx.doi.org/10.1007/978-3-030-27209-8

Section 2

Examination Methods

Chapter 2

Optimization, Validation and Standardization of ELISA

Rajna Minic and Irena Zivkovic

Abstract

The enzyme-linked immunosorbent assay (ELISA) is a commonly used analytical immunochemistry assay based on the specific bond between the antigen and the antibody. The application of this test has significantly changed the practice of medical laboratories in which it is used for detection and quantification of molecules such as hormones, peptides, antibodies, and proteins. Various technical variants of this test can detect antigen (native or foreign) or antibody, determine the intensity of the immune response whether pathological or not; the type of induced immune response as well as the innate immunity potential; and much more. These capabilities, as well as the high sensitivity and robustness of the test and a small price, make it possible to quickly and reliably diagnose diseases in most laboratories. Besides, ELISA is a test that is also used in veterinary medicine, toxicology, allergology, food industry, etc. Despite the fact that it has existed for almost 50 years, different ELISA tests with different technical solutions are still being developed, which improves and expands the application of the this exceptional test. The aim of this chapter is to empower the rider to optimize, standardize and validate an enzyme linked immunosorbent assay.

Keywords: ELISA, optimization, standardization, validation, accuracy, precision

1. Introduction

Enzyme linked immunosorbent assay (ELISA) has existed for 50 years and ELISAs with different technical solutions are still being developed, which improves and expands the range of application.

The test was first described by Engvall and Perlmann in 1971 [1–3] and was based on the work of Avrameas, who used enzyme linked antibodies in histochemistry [4, 5]. The method was quickly developed for sero-diagnosis of trichinosis [6] and antibodies to *Plasmodium vivax* and *P. falciparum* [7], to be used in epidemiological studies of malaria.

Since the discovery there have been numerous applications of ELISA, used to detect both antigens and antibodies. Besides the detection of protein antigens ELISAs that permit the determination of antibodies to native and denatured DNA [8, 9], polysaccharide antigens [10–12] and phospholipids [13] have been optimized. In fact, sometimes the name ELISA is applied to tests in which there are no antibodies, but instead specific protein–protein interactions are used. From the perspective of optimization, validation and standardization such tests can be treated in the same way. Regarding protein antigens the sensitivity of ELISA is usually in the pg/ml range [14].

When developing a diagnostic test, precise and optimal performance conditions must be found for all the steps within the test protocol. This ensures that the entire

procedure is optimal. Before routine usage in diagnostics, for example, the newly developed, or a newly modified procedure must be proven to be accurate, precise and reproducible. Also, in order to measure the values obtained with the test, it is necessary to standardize the test. Therefore, optimization, validation and standardization (OVS) of ELISA are extremely important and necessary, especially if it is to be used in clinical or veterinary medicine. This chapter will present the procedures by which ELISA is characterized in an understandable and precise way.

Reviewing the literature, we noticed that the described boundaries between optimization, standardization and validation are not clear enough. The reason for this is that in certain situations performing a single ELISA can lead to a completion of both validation and optimization characteristics, which is completely valid. Before going into more details and in order to avoid confusion it is suitable to clearly define these three terms.

According to Merriam-Webster dictionary,

Optimization is: "an (act, process, or methodology of making something such as a design, system, or decision) as fully perfect, functional, or effective as possible."

Validation is: "an act, process, or instance of validating *especially*: the determination of the degree of validity of a measuring device."

Standardization is**:** "to bring into conformity with a standard especially in order to assure consistency and regularity ... to compare with a standard: to determine the strength, value, or quality of (something) by comparison with a standard."

ELISA most often serves to measure the presence or quantity of antibodies or antigens, or biomolecules in general which can be recognized by antibodies. In biological matrices (such as serum, plasma, blood, urine and saliva) ELISA is an important diagnostic tool used to detect various antigens and antibodies. Indirect or direct ELISAs are used in medical product development, particularly for testing vaccines and new drugs. ELISA with specific antibodies can be designed to measure impurities within the medical products resulting from the production process. Antibody assays against these impurities should also be developed and validated for testing the levels of the impurities, which should be kept at a minimum in order to avoid adverse immune responses. For immunogenic substances with expected low concentrations, such as cytokines, hormones, toxins etc., sandwich ELISA is used.

Irrespective of the ELISA design (indirect, direct or sandwich), OVS principles are the same. Of paramount importance for any bioanalytical method is that it is well characterized, fully validated and documented to a satisfactory standard in order to yield reliable results.

The first step in ELISA development is optimisation, which is followed by standardization and finaly validation.

2. ELISA optimization

Optimization of an ELISA is essential to its success. Since ELISA is a multistep procedure, each component can be individually tested prior to the start of an experiment.

ELISA procedure consists of antigen or antibody coating, saturation, analyte application, detection with appropriate antibodies, primary or secondary and signal detection. Between each step the plate is washed. A variety of samples can be tested with ELISA, and the choice of assay conditions will depend upon the complexity of the sample and the expected amount of analyte present. Optimization is the establishment of ideal concentrations of each assay reagent and ideal conditions for each step and that must be done empirically. The cornersotne of any ELISA is the

selection of the protocol type: direct, indirect or sandwich; which is dependent on the type of sample, avaliable reagents and the concentration of the analyte, keeping in mind that the procedure should be as straight forward as possible.

Numerous factors should be tested, such as the concentration of antigen, or antibody used for coating, temperature, the duration of individual steps the type of coating buffer, such as phosphate-buffered saline (PBS) or carbonate buffer, sample preparation methods (with or without EDTA, decomplementation, serum or plasma or whole samples). Plate saturation is also a step which requires optimization such as different concentration of bovine serum albumine (BSA), nonfat-dried milk, or whole serum from different animals. Here we will discuss the most important steps of the optimization procedure.

2.1 Antigen coating

The first step in ELISA is coating wells with antigen or capturing antibodies. Most often this consists of applying a protein solution in PBS or carbonate buffer to microttiter plate wells. The microtiter plates for coating with proteins are special plates with modified surface, i.e. highly charged polystyrene surface with high affinity to molecules with polar or hydrophilic groups. This kind of surface has a high binding capacity for proteins, including globular antibodies and ensures proper antibody orientation. On the other hand ELISA for lipid antigens is performed on a hydrophobic surface, suited for non-protein antigens, which are not soluble in PBS or carbonate buffer, but are dissolved in an apropriate alcohol. Irrespective of the type of antigen the whole surface of the well bottom must be covered. If the whole surface is not covered the absorbance read will be lower, and if excess antibody/antigen is present, layers of antibody/antigen may form and wash away in subsequent steps, which again leads to lower signal. **Figure 1** shows the dependance of absorbance on the ammount of antibody/antigen used for coating. For the optimized protocol it is important to select that antigen/antibody concentraion that gives the highest

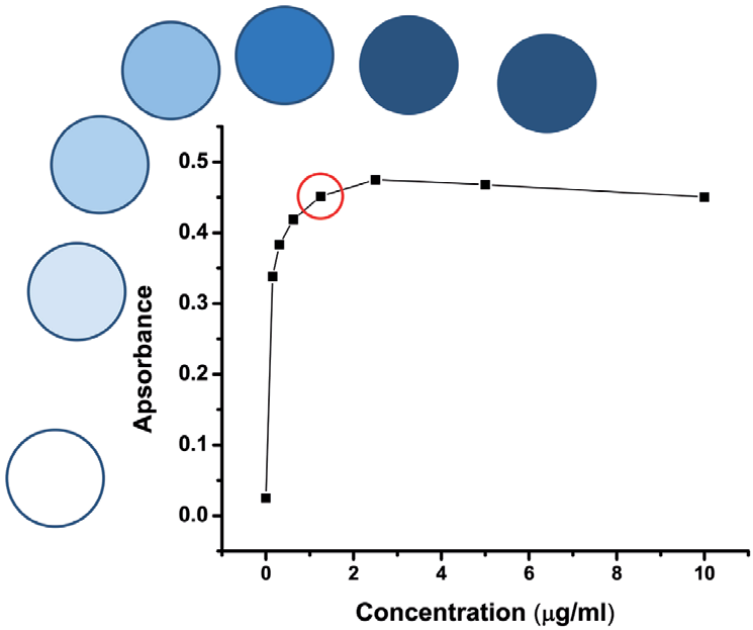

Figure 1.
Dependance of absorbance on the ammount of antibody/antigen used for well coating in ELISA.

absorbance, marked with a red circle in **Figure 1**, which ensures that the complete well surface availiable for binding is covered in a monolayer. This principle should be followed regardless of the type of antigen/antibody or the ELISA type. For axample, in sandwith ELISA the wells are covered with capture antibodies, either whole IgG or Fab fragments and in direct and indirect ELISA with the antigens.

2.2 Saturation-blocking

The process of coating an ELISA plate with antigen relies on the binding activity of the solid phase of the well, which immobilizes biomolecules on the well surface. Step after that must be blocking. During blocking free binding sites at the bottom of the wells become saturated with a blocking buffer in order to prevent the possibility of nonspecific binding and the residual binding capacity of the wells, thus greatly improving the signal-to-noise ratio and specificity. Without appropriate blocking the detection antibody could bind nonspecifically alongside the antigen, resulting in high background signal and low sensitivity.

There is a variety of blocking buffers, to choose from, not one of which is ideal for every situation. Although these buffers are called blocking buffers they usually contain a blocking component such as BSA, nonfat-dried milk, casein or whole serum. Every blocking buffer represents a compromise between reducing the background and maintaining specificity. Whole sera and serum protein albumin can cause non-specific ELISA signals in certain circumstances [15].

Even different BSA preparations show variations in the blocking activity of non-specific binding in ELISA. To prevent false positive results from cross reactive antibodies or non-specific binding of ELISA reagents to BSA, alternative blocking agents can be used and even no protein can be included in the blocking buffer [1]. These different blocking agents, (as well as their different concentration, incubation time, etc) should be tested in parallel, to discover the best way of saturation for each individual ELISA system.

2.3 Sample preparation

It is almost always necessary to dilute samples for ELISA test, so the choice of the diluent is important. Generally, standard diluent should be as similar as possible to the matrix of the sample. For example, PBS with BSA is a good serum replacement in ELISA and is most often used for biologycal samples. The next important diluent component is non-ionic detergent (Tween 20, Triton X-100, CHAPS) that, in low concentrations, prevents non-specific (hydrophobic) protein–protein interactions. The specific binding is usually more resistant to the detergent. Detergents in one step do not provide a permanent barrier to biomolecule non-specific attachment in the following steps because it washes away with water or aqueous buffer, so in certain situations, detergents should be present in all the diluents/buffers.

It may be necessary to choose a different diluent than PBS/Tween/BSA, if the analyte is not serum. In that case, it is necessary to check the standard curve and linearity of dilution for the experimantal sample. The reason for this is the influence of the components of a standard diluent or matix on antigen/antibody interactions. In such cases spike-and-recovery or linearity-of-dilution experiments should be performed.

The goal in assay development is to achieve high signal-to-noise ratio while maintaining optimal responses. The sample matrix may contain interfeering components that affect assay response to the analyte by introducing a difference in comparison to the standard diluent. In order to asses this phenomenon, spike-and-recovery experiment is designed.

The idea of spike-and-recovery is that you add (spike) a certain amount of standard into the sample buffer or the samples, and measure them in parallel with samples with no standard added. Sometimes one can compare the same amount of analyte added into the natural test sample matrix and identical spike added to the standard diluent. So it can be seen whether you can measure (recover) the exact amount again, and how much you can recover from it in percentages. If, for any reason, you can not recover the same amount in comparison to a control, this means that something in the test solution is not in favor of the assay, so one should proceed with finding the right standard diluent.

Linearity-of-dilution experiments provide information about the precision of the assay results for different diluted samples in the chosen sample diluent. These experiments are performed to demonstrate that highly concentrated samples can be accurately measured by diluting into the assay's quantitative range and the concentration can be calculated by multiplying the measured concentration by the dilution factor. Linearity-of-dilution experiment in practise means the measurement of at least three dilutions in the appropriate range in the selected diluent. There are two different ways to perform a linearity-of-dilution experiment, both with the same outcome. The usual method implies using a highly concentrated sample and then testing several different dilutions of that sample in the chosen sample diluent. Alternatively one can first prepare several different dilutions of a low concentration sample and then spike it with the same amount of the analyte before testing. If a sample does not exhibit linear dilution (i.e. linear dependence of absorbance on dilution), the situation can be that one has missed the range of linearity, as generally speaking linearity rarely or never exists over the entire range of concentrations; or that the matrix component is interfering with the measurement at the given dilution. Sometimes, matrix interference occurs if an interfering factor is present at concentrations above a certain threshold, and when the sample is diluted, interference is no longer observed. This kind of testing of a novel bioanalytical method is required by the EMA [16, 17].

When testing an experimental sample it is important to test several dilutions, all in duplicate or triplicate in conjunction with a known standard to ensure that the final results fall within the linear portion of the standard curve. This ensures the accuracy of the result. In highly concentrated samples underestimation of the concentration can occur, while in highly diluted samples overestimation can occur. Prepare different concentrations of the sample, keeping in mind the detection limit of the substrate. At this point, it is very suitable to detect maximal quantity of sample that can be detected, that is the last concentration after which there is no further absorbance increases (the same principle as for antigen coating optimization), **Figure 1**. This way the upper limit of the method is determined which enables the optimization of the next step.

At this point of optimisation, if sample is sera, high unspecific absorbance can occur, which is not related to the concentration of the sample/analyte. This can occur if the sera is not decomplemented, because active complement binds to antibody Fc. Heat-inactivation of serum for 30 minutes at 56°C eliminates complement activity, but one must keep in mind that different immunoglobulin isotypes and immunologbulins from different species show different sensitivity to heat treatment [18]. So, it is important to carefully consider or test the inactivation step.

2.4 The choise of the detecting antibody

ELISA is largely dependent on the choice of antibodies used, so antibodies should be carefully chosen. Based on the type of sample and the expected analyte

concentration, the choice of monoclonal or polyclonal antibodies, or even the combination of both, should provide optimal signal-to-noise ratio [19]. Each antibody type offers distinct advantages.

The interaction between antibodies and their antigens is described by specificity, affinity, and avidity.

Specificity is an indication of whether an antibody binds solely to a unique epitope from a single antigen in a single species, or whether it binds to similar epitopes present on several molecules from the same or a few different species, i.e. whether it is cross-reactive. Specificity is the most important quality of an antibody, and this is the principle that ELISA is based upon, so a carefull selection should be made.

Affinity describes the strength of binding of an antibody with an antigen. This binding is a reversible interaction and affinity determines how much antigen is bound by an antibody at any particular moment, which is dependent upon how quickly this binding occurs, and for how long the interaction lasts. High affinity antibodies should be used in all types of immunoassay because they rapidly produce a large number of stable interactions and provide the most sensitive detection.

Avidity is a less intuitive term than affinity as it is based on affinity, but is highly influenced by the the total number of antigen binding sites or valency, which determines the overall stability of the antibody–antigen interaction. Therefore, avidity varies with antibody isotype and whether it is intact or fragmented. Additional factors which determines avidity are the structure of the antibody, the length and motility in the hinge region and the space between the Fab fragments.

When available, one should always choose monoclonal antibodies over polyclonal antibodies, in fact, commercial ELISA kits almost always utilize monoclonal antibodies. Monoclonal antibodies have specificity for a single epitope, usually a small part of the antigens' surface. Monoclonal antibodies are therefore less likely to interact with closely-related proteins and are not generally expected to trigger non-specific signals in an immunoassay. Polyclonal antibodies are a mixture of antibodies with increased specificity to the antigen, therefore they bind different epitopes. Commercial polyclonal antibodies are often affinity purified or cross-adsorbed, but still the posibility of crossreactivity is higher. In addition, polyclonal antibody preparations can show batch to batch variations which should not be the case with monoclonal antibodies.

The advantage of using polyclonal antibodies is that they rarely fail to bind to the antigen due to a single blocked antibody binding site, antigen configuration change, or misfolding, although the latter are more important in tests other than ELISA. When combining monoclonal antibodies as in sandwich ELISA it is important to check literature or to test experimentaly the compatibility of the antibodies in terms that they do not share an epitope or for steric hinderance. Matched pairs are the basis of many sandwich ELISAs, either in kits or for in house assay set up. Matched antibody pairs means they are capable of detecting different epitopes on the same protein antigen, so they can be used together in a sandwich ELISA.

Sometimes the ELISA sensitivity can be increased by using indirect detection with polyclonal antibodies instead of direct detection with a monoclonal antibody, due to higher levels of polyclonal antibody binding to the target antigen. For cost reduction it can also be the combination of monoclonal capture with polyclonal detection.

After careful antibody selection, serial dilutions of capture antibodies should be carefully prepared for proper titration of antibody concentration. This is performed according to the previously mentioned principle of detecting maximum ammount of the component (in this case detection antibody) after which there is no further absorbance increase, **Figure 1**. Again, the ideal concentration should provide the highest signal and lowest noise.

As ELISA is a method which basicaly consists of overlaying different components which specificaly interact in each step (except washing) an optimization is required which follows the principle of titration until the complete coverage of the previous layer. Often the enzyme conjugate, i.e. enzyme responsible for color development, is already chemically bound to the detecting antibody, thereby enabling its direct use as a detection antibody in immunoassays. If this is not the case then enzyme concentration should be optimized too.

2.5 The enzyme conjugate selection

In this step, the first point is choosing the apropriate enzyme conjugate, depending on the needs of the researcher. The enzymes should be stable at typical assay temperatures: 4°C, 25°C, and 37°C; have a shelf life greater than six months when stored at 4°C; be inexpensive and commercially available. The enzymes should also survive the necessary conjugation conditions and yield productive conjugation. The enzymes should have an easily measurable activity; with high substrate turnover number. Horse radish peroxidase (HRP) and calf intestine alkalne phosphatase (ALP) are two most widely used enzymes for detection in ELISA assays [20]. HRP is usually conjugated to an antibody in a 4:1 ratio. For ALP the ratio is a little more unfavorable, 2:1, but the conjugate is more stable [21]. These enzymes are typically used because they each meet most, if not all, of the criteria necessary to produce a sensitive, inexpensive, and easily performed assay.

All enzyme-linked immunoassays, imply the usage of the enzyme substrate. Colorimetric ELISAs usually require soluble colored reaction products. The decision which substrate to choose depends on the desired sensitivity, reaction time, and the detection device. For colorimetric detection the most desirable substrates quickly produce intensely colored reaction products. When the analyte amounts span a wide range of concentrations (large dynamic range), then it is more suitable to use substrates that produce color over a longer time period (15 to 30 minutes) because then, one is able to detect the wider range of analyte-dependent color intensities. For assays with a timed endpoint, the reaction is stopped with an inhibitor suitable for the specific enzyme substrate combination after a defined time period that stops further color development. This allows detection to be performed within a reasonable time; for this, a substrate that has a "slow" reaction rate (15 to 30 minutes to completion) is optimal.

Both HRP and ALP have substrates that yield soluble colored reaction products.

The most common substrates that produce soluble reaction products with HRP are: TMB (3,3′,5,5′-Tetramethylbenzidine), ABTS (2,2′-azino-di[3-ethylbenzthiazoline] sulfonate), and OPD (o-phenylenediamine). TMB is a highly sensitive substrate, safe for laboratory workers. Due to its rapid reaction rate, it is ideally suited for on-line kinetic analysis. TMB can also be used in endpoint assays by stopping the reaction with 1 M phosphoric acid. ABTS is considered an all-purpose substrate. Although it is less sensitive than either TMB or OPD, it has the widest working range of any substrate currently available for peroxidase or alkaline phosphatase. Its reaction rate is suitable for endpoint assays and is easily stopped with 1% SDS (sodium dodecyl sulfate), which does not change the color or the absorbance of the reaction product. OPD was once the most popular substrate for peroxidase. It is slightly less sensitive than TMB, but it is cancerogenic.

The most commonly used substrate that produces a soluble reaction product with ALP is p-NPP (p-nitrophenylphosphate). pNPP is a substrate with a low reaction rate, so it usually takes 30 to 60 minutes for the dye to develop optimally. This property makes it possible to increase the sensitivity by increasing the reaction time period. At the same time, this property makes the pNPP substrate unsuitable for kinetic analysis [22].

Factors that affect the measurement of enzymatic activity are temperature, buffer composition (pH, ionic strength), build-up of product inhibitors, the increase in back-reaction as the product concentration increases, stability of the enzyme and sometimes exposure to light. As most of these facors such as pH and substrate depletion, are known, commercially available reagents are optimized for composition and concentration in order to control these parameters. For novel ELISA optimization of the most concern are reaction time and temperature.

If the antigen can clearly be detected then the substrate is appropriate. If the antigen is below the threshold for detection then one should select a more sensitive substrate.

2.6 Signal detection methods

It should be noted that the detection methodologies for ELISA are few, but the most prevalent in the laboratories is colorimetric. In addition, fluorescent and luminescent are also used.

In colorimetric detection the amount of color in each well is read by a spectrophotometer and samples are compared relative to one another or with the use of a standard curve derived from known analyte concentrations.

Fluorescent substrates [23] for ALP and HRP can potentially yield a higher signal, leading to increased sensitivity and broader dynamic range. This kind of detection requires black plates, which are also availiable with various degrees of hydrophobicity and a fluorescent plate reader is required. Fluorescence yielding substrates have a shorter half-life than colorimetric substrates, so the signal is declining over time. This kind of ELISA is useful for measuring immune responses because of broader dynamic range [19].

The same detection antibodies conjugated with ALP or HRP, can also be used for chemiluminescent assays [24]. In this type of experiment, ALP, for example, will modify a substrate, forming a chemiluminiscent product which creates light emission. ALP chemiluminiscent substrates can have pg/ml sensitivity. The signal can be read in black or white opaque ELISA plates and a luminometer is required. The advantages of this detection type are typically a higher dynamic range and lower background signal. The signal is not as stable as the colorimetric or fluorescent detection and must be read within a short time of generating the signal.

The type of substrate used depends on several factors, most notably the desired assay sensitivity and signal to bakground ratio.

3. ELISA standardization

Many laboratories have independently developed ELISA techniques for their own purposes. For results to be valid they must be comparable with results of the same ELISA test performed in different laboratories. Consistency in the assessment of ELISA results in different areas of application (diagnostics, production control, scientific research, immunogenicity assessment etc.) requires standardized and acknowledged methodological protocols. Protocol harmonization progress with respect to the international standardization and validation of this technique has been made.

Today, leading regulatory agencies for specific guidance on immunogenicity assessment of biotherapeutic products are part of EMA and WHO, [25] and there are other agencies. The National Institute for Biological Standards and Control (NIBSC), for example, part of UK Medicines and Healthcare products Regulatory Agency (MHRA), is of great importance to the field of biological standardization. It produces over 90% of the biological international standards in use around the

world. The WHOs' Biological Reference Materials are established through a standard procedure, [26] in which representative materials are tested by participating laboratories using their own methodologies and coordinated by a responsible WHO Collaborating Center [27]. Upon establishment of the reference preparation by the Expert Committee on Biological Standardization (ECBS), the material is assigned a unitage and serves as the comparator against which results from laboratories can be standardized and compared, irrespective of the location or the methods employed. This enables the results of bioanalytical methods, including ELISA, to be comparable. Based on international standards„ working standard" (i.e. in-house or secondary standards) are evaluated and compared, and subsequently adequately used.

At first glance, it is very simple to explain the process, i.e. the term of standardization in ELISA: comparing the absorbance of a sample with the absorbance of the known concentration of the standard (in-house or commercial) and based on that, determining the unknown concentration.

If the ELISA is intended for the measurement of the final detectable dilution, as in titration experiments, and not for the measurement of biomolecule quantity a reference standard may not exist.

Then the need exists for establishing a reference standard. For any ELISA, consideration must be given to the selection of standards which represent, on average, what would be expected of an immune response of the organism in question. Immunogenicity assessment relies on the measurement of antigen induced antibodies in serum or plasma. Such antibodies are heterogeneous in terms of classes, subclasses and alotypes, concentration as well as antigenic specificity. Some will neutralize the biological activity of the antigen, others will not, despite the high affinity/avidity. Irrespective of the type of ELISA system used, endpoint titration is a function of both antibody concentration and avidity. And finally, as every sample is unique with vast individual differences among humans, for example, it is not possible to make a straightforward comparison with standard antibodies. Nevertheless, although the ideal is unreachable, if wanting to produce valid and reproducible data a reference standard must be established.

The physical quantity to be measured in ELISA is absorbance. Absorbance is influenced by test parameters and photometric instrumentation, so raw, corrected or normalized OD values [28] cannot be used for inter-laboratory standardization. This is why end-point titration or determination of highest serial dilution which demonstrates a minimum of antibody activity is often used for measuring the immune response in diagnostics and vaccinology. Under some circumstances, quantitative data are not required for diagnostic purposes and sometimes end-point titration is sufficient, with an adequate semi-quantitative standard. End-point titrations are labor-intensive, costly and impractical for most routine diagnostic purposes.

In order to overcome the relativity of the measured absorbance a notion of "percent positivity" (PP) is accepted, this way the absorbance of each sample tested is expressed as a percentage of a highly positive reference standard. Although semi-quantitative, PP is expressed on a continuous scale of 0–100 and has two major advantages, first, it requires only a single dilution and second, it does not assume parallelism or uniform background activity. Therefore, it may be used for inter-laboratory standardization.

Even with measurements with qualitative standard curve, it is not correct to determine the result from a single sample dilution measurement. This can only be acceptable if there is a parallelism in dilution curves between the sample and the standard. If more quantitative data are needed, PP values can be converted to units which are directly proportional to antibody activity.

Sometimes an elegant and appropriate way to quantify samples is competitive or inhibitory ELISA. When performing competitive ELISA, one applies the

sample preincubated with the same antigen used for plate coating and measures the amount of non inhibited antibodies. There is a negative relationship between color intensity and the amount of test sample antibody inhibited by antigens. Percent inhibition (PI) of the color produced by the standard competing antibody is more widely used. The development of consistent standard curves for this kind of assay is extremely difficult, but still possible.

The specific guidance on immunogenicity assessment of biotherapeutic products has been elaborated by leading regulatory agencies such as the EMA and U.S. Food and Drug Administration (US FDA) [29–32].

4. ELISA validation

Validated analytical methods such as ELISA for quantification of biomarkers, drugs, biological products, and their metabolites in a given biological matrix (e.g. blood, plasma, serum, or urine) are critical for the successful conduct of nonclinical and clinical studies. Validating the analytical method ensures that the data are reliable [33]. Validated methods provide critical data to support the safety and effectiveness of drugs and biological products.

Although there is abundant literature relating to immunochemical methods, [34] EMEA [35, 36] and US FDA [8] have clearly defined the characteristics of the validation procedure for bioanalytical methods, which also applies to the validation of ELISAs, which are intended for use in diagnostics, toxicology, basic or applied research [37] or production control [38]. Metodology for the validation of bioanalytical methods must follow clear recomendations from reference institutions such as the EMEA [35, 39] or the WHO because that provides important measurements to be of satisfactory quality all over the world.

ELISA validation according to these recommendations means determining the following method caracteristics:

1. Specificity
2. Linearity – *Range - Limit of detection (LOD)*
3. Sensitivity
4. Accuracy
5. Precision (repetability = intra assay, inter assay, reproducibility = inter laboratory assay)
6. Robustnes

Acceptance criteria should be prospectively defined based on the intended use of the method.

4.1 Specificity

Specificity means that the method must differentiate the targeted analyte from all other matrix components. Which is why it is important to test wether *"related molecules"*, e.g. endogenous compounds, isoforms, variant forms of the analyte, or physico-chemically similar compounds interfere with the results by giving false positivity. Specificity can be confirmed by adding increasing concentrations of

available "related molecules" or drugs, into drug-naive sample matrix and measuring the amount of the macromolecule of interest within the working range. Specificity can also be tested by testing samples (serum) of unimmunized subjects (negative immunization control), or sometimes it is convinient to prove specificity with competitive (inhibitory) ELISA.

Evaluation of specificity may be conducted during optimization and validation, when more data on the behavior of the analyte become available. Specificity should be tested with quality control (QC) samples [40]. QC samples are the samples with known amounts of the analyte, in identical matrix like the sample. These are usually in-house produced samples, with a lower amount of the analyte. When the method is performed with these QC samples and satisfactory results are obtained, then the method is also good, i.e. valid. If the method does not give good enough results with the QC samples, it means that the method is not of sufficient quality, so it must be investigated why the method worked poorly. The shortcomings must be corrected, and then again checked with QC samples. Still it needs to be defined what is satisfactory. The criterion for accepting the results obtained with QC samples is that the measured value does not deviate by more than 25% from the nominal value [40].

4.2 Linearity

Linearity is the ability of the analytical method to produce results by calculating a direct proportion, within the working range. Linearity is described by range and detection limits.

Linearity is a function of values that can be graphically represented by a straight line. The linearity of an analytical method can be explained as its capability to show "results that are directly proportional to the concentration of the analyte in the sample" [39].

Unfortunately, the analytical response of a method is not always linear. Sometimes when the data are not linear they can be mathematically transformed, e.g. by applying logarithms but in some cases or some range of immunoassays transformation is not appropriate.

Linearity is important as it confirms the sensitivity of the method for the analysis of concentration within a defined range. According to the EMEA International Council for Harmonization ICH Q2(R1) guideline, linearity of a given response must be evaluated using a minimum of 5 concentrations of the analyte (multi-point calibration). Then, the collected data must be statistically analyzed, by performing regression analysis using the method of the least squares, in order to mathematically determine the line that best fits a set of data. For linearity, the results are required to be represented as linear equation (Eq. (1)).

$$y = kx + n \tag{1}$$

In a linear regression line, the regression coefficient is the constant "k" that represents the rate of change of one variable "y" as a function of change in the other "x" (thus the slope), while "n" is the Y-intercept. The correlation coefficient r, a value without units, expresses the precision of the linearity fit of the experimental data. In case of a value being less than 0.95, it may either be a result of a broad spreading during measurement or due to a non-linear correlation. Often, the coefficient of determination (R^2) is used, which is the square r. For most methods applied at $R^2 \geq 0.98$ can be achieved. If there is a perfect linear relationship, it has a value of 1 (100%). Linearity studies are important because they define the range of the method within which the results are obtained accurately and precisely.

To summarize, linearity is one major aspect in the quantitative method validation procedures. It describes the range of concentrations for which the method can function reliably. If the data are non-linear, transformation into a linear form may be performed, or the data can be accepted as is while demonstrating a clear relation between the analyte concentration and the measured absorbance [41].

Range. As mentioned previously range is determined from linearity and the data obtained which fall within the determined range should be of satisfactory accuracy and precision. The range is limited by upper and lower detection level.

Upper limit of quantification (ULOQ): is defined as a mean value of 10 duplicates of maximally achieved absorbances in the linear part of the standard curve, from which three standard deviations have been subtracted. Subtraction of the multiplied standard deviations achieves accuracy in this range from 80–120%.

Lower limit of quantification (LLOQ): is the lowest concentration of analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision. In practise this is a mean value of the smallest result measured in the linear part of the curve to which three standard deviations have been added.

4.3 Sensitivity

Senzitivity or limit of detection, (LD) for ELISA is defined in the same way as for other bionalaytical methods. At this point, it is appropriate to underline the difference between the limit of detection (LD) and lower limit of quantification nominal (LLOQ). LD is the lowest analyte concentration that can be distinguished from the assay background, while the LLOQ is the lowest concentration at which the analyte can be quantitated at defined levels for precision and accuracy. LD is determined from standard deviation of the sample blank and the slope of the linear curve (Eq. (2)).

$$L_D = 3.3(SD(b)/k) \tag{2}$$

L_D—LD (detection limit) nominal
k—slope of the linear curve Eq. (1)
SD(b)—standard deviation of the blank [39]

There are bioanalytical methods which have the same values for LD and LLOQ, but with ELISA, especially when biologycal samples are measure this is not the case, and LD is lower than LLOQ. For liretature reference of these terms one should read Armbruster and Pry [42].

4.4 Accuracy

The accuracy of an analytical method describes the closeness of the value determined by the method to the nominal concentration. In practice, as the reference material is precious and universally needed, the first step is to make a sufficient amount of the quality control (QC) samples, previously standardized against the reference material. Then the QC sample can be used for determining validation characteristics. Accuracy should be assessed on samples spiked with known amounts of the analyte, the QC samples. The accuracy can be expressed as the difference between the obtained experimnental value and the nominal value (which is acurate), using the absolute or even better the relative error.

Absolute error is the difference between the experimantal result and the nominal value, (Eq. (3)):

$$\Delta x_i = \left| \mu - x_i \right| \quad (3)$$

Δx_i—absolute error of individual measurement
μ— nominal value
x_i—measured value

It is important to perform multiple measurements for a single sample, in order to present the absolute error as the mean value of absolute errors of individual measurements (Eq. (4)).

$$\Delta x = [\left| \mu - x_1 \right| + \left| \mu - x_2 \right| + \ldots\ldots + \left| \mu - x_{n-1} \right| + \left| \mu - x_n \right|]/n \quad (4)$$

n—number of measurements
Δx—mean value of absolute or standard error

Because of the numerical nature, the absolute value of the difference does not give insight into its significance for the accuracy of measurement, so it is always important to calculate the relative error as well.

Relative error (δ) is a quotient of the absolute error and the actual (nominal) value (Eq. (5)), it is without units and can be expressed in percentages by multiplying with 100.

$$\delta = \Delta x/\mu \quad (5)$$

The level of accuracy must be determined for the whole range of the analytical procedure. Minimal requirements for this are three concentrations one close to ULOQ, one close to LLOQ and one in the middle of the range, each in triplicate.

Today it is common practise to develop an ELISA as an internal laboratory assay without the standards or the QC samples or for titration experiments for the determination of the last measurable dilution. In this situation there is no measurable quantitifier for accuracy testing. For accuracy to be calculated as % that shows how much the obtained results corresponds with the actual value, it is necessary to use concrete, absolute and measurable quantity such as analyte concentration. In practise this can be achieved [43] with inhibitory ELISA, which is based on the dependance of the absorbance on inhibitor concentration. The difference between the described calculations is in the reverse proportion, as described in the ELISA standardization section [37].

4.5 Precision

Precision is a validation characteristic which describes the reproducibility of the measurement, in other words the closeness of two measurements of the same sample. Precision is higher if the results are closer to one another. At first glance it is easy to confuse accuracy with precision, because in both cases it is about the absolute and the relative error of the obtained results. **Figure 2** shows the difference between accuracy and precicion, where accuracy describes the deviation from the actual (nominal) value, while precision describes the deviation from the mean value. Precision is determined by simply repeating the measurement.

Standard deviation, relative standard deviation (coefficient of variation) and confidence interval should be reported for each type of precision (intra, intermediate or inter) investigated [35]. The three parameters are dependent on the closeness of individual results to the mean value, and give the complete picture of the precision of the test.

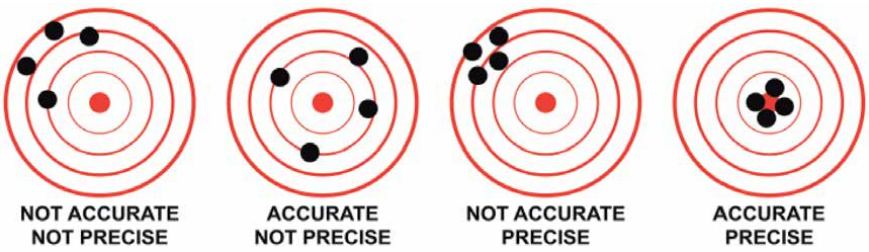

Figure 2.
Accuracy and precision defined.

A. DEVIATION is the difference between the measured value from the mean value, and has the same units as the measured value (Eq. (6)).

$$d_i = \left| \bar{x} - x_i \right| \tag{6}$$

$\bar{x}$—mean value of repeated measurements of the same sample
x_i—one measured value
d_i—deviation, the difference between the mean value and one measurement
Standard deviation is the mean value of all measurement deviations Eq. (7).

$$SD = \sqrt{\frac{1}{N}\sum_{i=0}^{n}(di)} \tag{7}$$

B. COEFFICIENT OF VARIATION CV (relative standard deviation) is standard deviation expressed in percentages and is calculated based on the measured mean value $\bar{x}$ (Eq. (8)).

$$CV(\%) = (SD: \bar{x}) * 100 \tag{8}$$

C. CONFIDENCE INTERVAL (CI) is the range of values within which the "actual" result is found. A CI of 95% means that if the measurement was to be repeated an infinite number of times, 95% of the results would fall within this range of values. For validation purpose, higher CI, 95% or 99% is needed, with optimal performance within the middle part of the range. A wide CI can be caused by small number of samples or by a large variance between sample measurements. Range of values for the given CI shows precision. This parameter is easily calculated by statistical programmes, or by a profesional statistician.

4.5.1 Intra-assay precision (repetability)

Intra-assay validation shows the reproducibility between wells within an assay plate. Data resulting from intra-assay validation helps ensure that repeated measurement of the same sample on a single plate gives comparable results. Repeatability should be assessed using a minimum of 6 determinations covering the specified range for the procedure (e.g. 3 concentrations, 2 replicates each), or a minimum of 6 determinations at 100% of the test concentration [39].

The % CV for each sample is calculated by finding the standard deviation of multiplicate results dividing that by the multiplicate mean, and multiplying the

result by 100 (Eq. (8)). The average of the individual CVs is reported as the intra-assay CV ($CV_{intra\text{-}assay}$).

Usually, $CV_{intra\text{-}assay}$ of 10% or less is considered satisfactory [44].

4.5.2 Intermediate precision

Intermediate precision (sometimes called within-lab reproducibility) shows the reproducibility between assays done on different days, or different plates. Satisfactory inter-assay precision is typically <10% [44].

For example, to monitor plate-to-plate variation the same samples are analyzed in quadruplicate on ten different plates. The plate means are calculated and then used to calculate the overall mean, standard deviation, and % CV. Overall % CV is calculated by dividing the SD of the plate means with mean of the plate means and multiplying by 100 (Eq. (8)). The average of the all plates % CV represents the inter-assay CV ($CV_{intermediate}$). In order to monitor daily variation quadruplicate samples are analyzed in ten different days and analyzed in the same way.

4.5.3 Reproducibility (inter-laboratory assay precision)

Reproducibility is assessed by means of an inter-laboratory trial. The outcome of the cross validation is critical in determining whether the obtained data are reliable and whether they can be compared and used. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopeias.

Satisfactory value for $CV_{inter\text{-}assay}$ is 10–15% [43].

Analyzing the literature it can be seen that the term inter assay is sometimes used for precision assessment on different days or on different plates, and sometimes for testing in different laboratories. Acording to EMEA, the term inter assay precision describes precision of the measurement assessment in different laboratories. If it is to be used in a different context it shold be described.

4.6 Robustness

Robustness testing involves monitoring the effects of small unintentional errors on the quantitative and qualitative characteristics of the method, where the errors relate to the internal parameters described in the method prescription. For example, buffer temperature, incubation temperature, sample incubation time, secondary antibody incubation time, number of washes before color development, color development time, and the like. This feature shows the reliability of the method despite minor deviations in performance.

There is also the notion of rigidity - as a sub-notion of robustness - which monitors the effects of changes in external parameters such as other lots of chemicals, other people working, other instruments used and the like.

Practically, this property is not measured or calculated in a certain way, but is established during the development of the method (optimization). Data on this can also be collected during operation.

This guideline describes full validation methodology. In case when method is already validated, when a smaller change to the protocol is instated, a full validation may not be necessary. It is possible to perform partial validation, and the nature of the modification will determine the extent of validation required. All modifications should be reported and the scope of revalidation or partial validation justified [34].

5. Conclusion

In our experience ELISA is an excellent analytical method which can be used for the detection and quantification of numerous biomolecules. No matter what this specific biomolecule is, the basis of ELISA is the antigen–antibody interaction. The existence of this specific interaction usually enables the construction of different ELISA protocols, dependent on your prior knowledge and imagination. After careful protocol optimization, determination of validation characteristics and the acquirement of an appropriate standard you can get a reliable and inexpensive analytical method useful in diagnostics, research or biomedicine in general.

Acknowledgements

Work presented in this paper was supported by the Institute of Virology, Vaccines and Sera "Torlak" funds and by Ministry of Education, Science and Technological Development, Republic of Serbia.

Conflict of interest

The authors declare that they have no known competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

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References

[1] Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry. 1971 Sep;**8**(9):871-874. DOI: 10.1016/0019-2791(71)90454-x PMID: 5135623

[2] Engvall E, Jonsson K, Perlmann P. Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes. Biochimica et Biophysica Acta. 1971 Dec 28;**251**(3):427-434. DOI: 10.1016/0005-2795(71)90132-2

[3] Engvall E, Perlmann P. Enzyme-linked immunosorbent assay, Elisa. 3. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. Journal of Immunology. 1972 Jul;**109**(1):129-135 PMID: 4113792

[4] Avrameas S. Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. Immunochemistry. 1969 Jan;**6**(1):43-52. DOI: 10.1016/0019-2791(69)90177-3 PMID: 4975324

[5] Avrameas S. Immunoenzyme techniques: Enzymes as markers for the localization of antigens and antibodies. International Review of Cytology. 1970;**27**:349-385. DOI: 10.1016/s0074-7696(08)61250-4 PMID: 4190535

[6] Ljungström I, Engvall E, Ruitenberg EJ. Proceedings: ELISA, enzyme linked immunosorbent assay - a new technique for sero-diagnosis of trichinosis. Parasitology. 1974 Oct;**69**(2):xxiv PMID: 4419374

[7] Voller A, Huldt G, Thors C, Engvall E. New serological test for malaria antibodies. Br Med J. 1975 Mar 22;**1**(5959):659-661. DOI: 10.1136/bmj.1.5959.659 PMID: 1092412; PMCID: PMC1672875

[8] Klotz JL, Minami RM, Teplitz RL. An enzyme-linked immunosorbent assay for antibodies to native and denatured DNA. Journal of Immunological Methods. 1979;**29**(2):155-165. DOI: 10.1016/0022-1759(79)90065-6 PMID: 479614

[9] Dimitrijević LA, Radulović MI, Cirić BP, Petrićević MM, Inić AB, Nikolić DN, et al. Human monoclonal IgM DJ binds to ssDNA and human commensal bacteria. Human Antibodies. 1999;**9**(1):37-45 PMID: 10331184

[10] Petrušić V, Todorović N, Živković I, Dimitrijević R, Muhandes L, Rajnpreht I, et al. Autoantibody response and pregnancy-related pathology induced by combined LPS and tetanus toxoid hyperimmunization in BALB/c and C57BL/6 mice. Autoimmunity. 2015 Mar;**48**(2):87-99. DOI: 10.3109/08916934.2014.961061 Epub 2014 Nov 28. PMID: 25430703

[11] Chiani P, Bromuro C, Cassone A, Torosantucci A. Anti-beta-glucan antibodies in healthy human subjects. Vaccine. 2009 Jan 22;**27**(4):513-519. DOI: 10.1016/j.vaccine.2008.11.030. Epub 2008 Nov 27 PMID: 19041357

[12] Yoneda A, Kurokawa T. A sensitive sandwich ELISA to measure (1→3)-β-d-glucan levels in blood. Journal of Immunological Methods. 2011 Feb 28;**365**(1-2):158-165. DOI: 10.1016/j.jim.2010.12.011 Epub 2010 Dec 22. PMID: 21184758

[13] Zivkovic IP, Stojanovic MM, Petrusic VZ, Inic-Kanada AB, Micic MV, Dimitrijevic LA. Network connectivity is shown to change in C57BL/6 mice during a continuing immune response subsequent to tetanus toxoid hyperimmunization. Biological Research. 2010;**43**(4):393-402 Epub 2011 Feb 1. PMID: 21526265

[14] Zhang S, Garcia-D'Angeli A, Brennan JP, Huo Q. Predicting detection limits of enzyme-linked immunosorbent assay (ELISA) and bioanalytical techniques in general.

[15] Xiao Y, Isaacs SN. Enzyme-linked Immunosorbent assay (ELISA) and blocking with bovine serum albumin (BSA) - not all BSAs are alike. Journal of Immunological Methods. 2012;**384**(1-2):148-151

[16] EMEA, editor. E.M.A. Guideline on Bioanalytical Method Validation. London, England: E.M.A. (EMEA); 2011. p. 8.

[17] EMEA, editor. E.M.A. Guideline on Bioanalytical Method Validation. London, England: E.M.A. (EMEA); 2011. p. 17.

[18] Akazawa-Ogawa Y, Nagai H, Hagihara Y. Heat denaturation of the antibody, a multi-domain protein. Biophysical Reviews. 2018;**10**(2):255-258

[19] Cell Signal Technology. Overview of Enzyme-Linked Immunosorbent Assay (ELISA). Available from: https://www.cellsignal.com/contents/_/overview-of-enzyme-linked-immunosorbent-assay-(elisa)/elisa-educational [Accessed: 23rd August 2020].

[20] Lequin RM. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). Clinical Chemistry. 2005;**51**(12):2415-2418

[21] Winston SE, Fuller SA, Evelegh MJ, Hurrell JGR. Conjugation of enzymes to antibodies. Current protocols in molecular biology. 2001;**50**(1):11.1.1-11.1.7

[22] ThermoFisher Scientific. Enzyme Substrates for ELISA. Available from: https://www.thermofisher.com/rs/en/home/life-science/protein-biology/protein-assays-analysis/elisa/elisa-reagents-buffers/enzyme-substrates-elisa.html [Accessed 21st August 2020]

[23] Hemmila IA. Applications of Fluorescence in Immunoassays. New York: John Wiley & Sons, Inc; 1991

[24] Van Dyke K, Van Dyke R. Luminescence Immunoassay and Molecular Applications. Boston: CRC Press, Inc.; 1990

[25] Wadhwa M, Thorpe R. Harmonization and standardization of immunogenicity assessment of biotherapeutic products. Bioanalysis. 2019;**11**(17):1593-1604

[26] WHO e. Recommendations for the preparation, characterization and establishment of international and other biological reference standards. WHO T.R.S. 2006;**932**(2):73-131

[27] WHO, editor. Providing International biological reference preparations. Available from: https://www.who.int/activities/providing-international-biological-reference-preparations. [Accessed: 23rd August 2020].

[28] Wright PF, Nilsson E, Van Rooij EM, Lelenta M, Jeggo MH. Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. Revue Scientifique et Technique 1993,12 (2):435-450.

[29] EMEA, editor. E.M.A. Guideline on immunogenicity assessment of therapeutic proteins. London, England:E.M.A. (EMEA); 2017. p. 1-24.

[30] EMEA, editor. E.M.A. Guideline on immunogenicity assessment of monoclonal antibodies intended for in vivo clinical use. London, England: (EMEA). 2012:1-10

[31] FDA, editor. Immunogenicity assessment for therapeutic protein

products. U.S.D.o.H.a.H. Services. Rockville, Maryland: Food and Drug Administration (FDA); 2014.

[32] FDA, editor. Guideline on immunogenicity testing of therapeutic protein products – developing and validating assays for anti-drug antibody detection. U.S.D.o.H.a.H. Services. Rockville, Maryland: Food and Drug Administration (FDA); 2019.

[33] FDA, editor. Guidance for Industry Bioanalytical Method Validation. U.S.D.o.H.a.H. Services. Rockville, Maryland: Food and Drug Administration (FDA); 2001.

[34] Andreasson U, Perret-Liaudet A, van Waalwijk van Doorn L, Blennow K, Chiasserini D, Engelborghs S, et al. A practical guide to immunoassay method validation. Frontiers in Neurology 2015, 6:179.

[35] EMEA, editor. E.M.A. Note for Guidance on Validation of Analytical Procedures: Text and Methodology. London, England:E.M.A. (EMEA); 1995. p. 1-15.

[36] EMEA, editor. E.M.A. Guideline on bioanalytical method validation. London, England:E.M.A. (EMEA); 2011. p. 1-23.

[37] Mitic K, Muhandes L, Minic R, Petrusic V, Zivkovic I. Optimization and Validation of ELISA for pre-clinical trials of influenza vaccine. Folia Biol (Praha). 2016;62(6):241-249.

[38] Milovanović V, Lj D, Petrušić V, Kadrić J, Minić R, Živković I. Application of the 3R concept in the production of European antiviperinum on horses – Multisite, low volumes immunization protocol and ELISA. Acta Vet (Belgrade). 2018;**68**(4):401-419

[39] EMEA, editor. E.M.A. Validation of Analytical Procedures: Text and Methodology Q2(R1). London, England:E.M.A. (EMEA); 2005. p.1-13.

[40] Azadeh M, Sondag P, Wang Y, Raines M, Sailstad J. Quality controls in ligand binding assays: Recommendations and best practices for preparation, qualification, maintenance of lot to lot consistency, and prevention of assay drift. The AAPS Journal. 2019;**21**(5):89

[41] Moosavi SM, Ghassabian S. Linearity of calibration curves for analytical methods: A review of criteria for assessment of method reliability. In: Stauffer M, editor. Calibration and Validation of Analytical Methods - a Sampling of Current Approaches. London, UK: IntechOpen; 2018. pp. 109-127

[42] Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. Clinical Biochemist Reviews. 2008;**29**:S49-S52

[43] Biddlecombe RA, Law B. Validation of an immunoassay. In: Law B, editor. Immunoassay a Practical Guide. London, UK: Taylor&Francis Group; 1996. pp. 176-178

[44] Murray A, Lawrence GP. How should the repeatability of clinical measurements be analysed? An assessment of analysis techniques with data from cardiovascular autonomic function tests. The Quarterly Journal of Medicine. 1993;**86**(12):831-836

Section 3

Norovirus Genome Mechanism

Chapter 3

Norovirus Structure and Classification

Manisha Rani, Sushma Rajyalakshmi, Sunitha Pakalapaty and Nagamani Kammilli

Abstract

Norovirus are a major cause of acute gastroenteritis worldwide. Diarrheal disease is now the fourth common cause of mortality children under the age of 5 years but remain the 2nd most cause of morbidity. NoV are associated with 18% diarrheal diseases worldwide where rotavirus vaccinations has been successfully introduced. NoV has become major cause of gastroenteritis in children. NoV belong to family caliciviridae. They are non-enveloped, single stranded positive sense RNA Viruses. The genome consists of 3 Open reading frames, ORF-1 codes for non-structural protein, ORF-2 codes for major capsid protein VP1 and ORF-3 for minor capsid protein VP2. Based on sequence difference of the capsid gene (VP1), NoV have been classified in to seven genogroup GI-GVII with over 30 genotypes. Genogroups I, II, IV are associated with human infection. Despite this extensive diversity a single genotype GII.4 has been alone to be the more prevalent. Basic epidemiological disease burden data are generated from developing countries. NoV are considered fast evolving viruses and present an extensive diversity that is driven by acquisition of point mutations and recombinations. Immunity is strain or genotype specific with little or no protection conferred across genogroups. Majority of outbreaks and sporadic norovirus cases worldwide are associated with a single genotype, GII.4 which was responsible for 62% of reported NoV outbreaks in 5 continents from 2001 to 2007. GII.4 variants have been reported as major cause of global gastroenteritis pandemics starting in 1995 frequent emergence of novel GII.4 variants is known to be due to rapid evolution and antigenic variation in response to herd immunity. Novel GII.4 variants appear almost every 2 years. Recent GII.4 variant reported include Lordsdale 1996, Farmington Hills 2002, Hunter 2004, Yerseke 2006a, Den Haag 2006b, Apeldoon 2007, New Orleans 2009,most recently Sydney 2012. Detailed molecular epidemiologic investigation of NoV is associated for understanding the genetic diversity of NoV strain and emergence of novel NoV variants. However, reports have revealed that not all individuals develop symptoms and a significant proportion remains asymptomatic after NoV infections.

Keywords: Acute gastroenteritis, ORF, Genogroups, Immunity

1. Introduction

The acute gastroenteritis is a major health problems, one of the most common infectious diseases among humans [1, 2]. The annual incidence of diarrheal disease is estimated of annual number is over 4.5 billion cases worldwide [3]

The global estimated of annual number of mortalities with gastroenteritis vary between 3.5–5 million cases in majority of deaths occurring among people in developing countries [4]. Diarrhoea remains into 10 most common communicable diseases found in India [5] (annual mortality is 2.5 million deaths each year in children less than five years of age in developing countries) [6].

In human enteric viruses account for more than half of all cases of gastroenteritis worldwide [7, 8]. Viral causes of gastroenteritis are follow: norovirus, rotavirus, adenovirus (group F- type 40/41), astrovirus and sapovirus [9–11].

2. Norovirus

Norwalk virus is the prototype strain of Norovirus and was associated with an outbreak of gastroenteritis at an elementary school in Norwalk, Ohio, in 1968. The discovery of Norovirus as the aetiological agent of the outbreak was made by Albert Kapikian in 1972 [12].

Using immune electron microscopy (IEM), stool samples were examined from a volunteer who had been experimentally inoculated with a faecal filtrate from the original outbreak. From these studies, Kapikian proposed the name "Norwalk virus" as the causative agent of the outbreak [12]. This was the first human virus specifically associated with gastroenteritis.

2.1 Classification

Norovirus, previously known as Norwalk-like viruses, belongs to the family, Caliciviridae [13]. The Caliciviridae family is comprised of four genera, Norovirus, Sapovirus, Lagovirus and Vesivirus [14]. Norovirus and Sapovirus are found in the genera Norovirus and Sapovirus, respectively, whilst other caliciviruses of veterinary importance, rabbit hemorrhagic disease virus and feline calicivirus, are found in Lagovirus and Vesivirus, respectively. Recently, two additional generas have been proposed within the Caliciviridae family, provisionally named Becovirus or Nabovirus, a bovine enteric calicivirus [15–17]. All six genera infect animals, but only Norovirus and Sapovirus contain strains that infect both humans and animals.

Based on phylogenetic analysis of the full length nucleotide sequence of the capsid gene [VP1], the Norovirus genus is divided into five genogroups (GI, GII, GIII, GIV and GV). Norovirus GI, GII and GIV are associated with human gastroenteritis. Norovirus GII includes porcine, as well as human strains, GIII contains only bovine strains, and GV contains only murine strains [18].

2.2 Structure

Norovirus is a small virion of 27 to 32 nm in diameter and has a buoyant density of 1.33 to 1.41 g/cm3 in caesium chloride [19, 20]. It is a non-enveloped, single-stranded, positive-sense, RNA virus with a genome of 7.4 to 7.7 kb [21, 22]. The RNA is polyadenylated at the 3′ end. All calicivirus genomes begin with a GU [nucleotide sequence] at the 5′ end terminal. A 5′ end sequence, of between 16 and 28 nucleotides depending on the genus is repeated internally in the genome and corresponds to the start of the subgenomic RNA [located at the start of the capsid gene, VP1]. This sequence is thought to be part of an RNA-dependent RNA polymerase [RdRp] promoter [23].

The Norovirus genome contains three ORFs: ORF1, ORF2 and ORF3.

The initial characterisation of the genome was based on the sequence homology of ORF1 in human calicivirus to characterised proteins of picornaviruses [24]. These conserved motifs included a "2C-like" helicase [a nucleoside triphosphatase,

NTPase, in Norovirus genome], a "3C-like" protease [3CLpro] and a "3D-like" RdRp (RdRp in Norovirus genome). Proteolytic mapping and enzymatic studies using site directed mutagenesis and recombinant expression systems have revealed the presence of three other non-structural proteins within the Norovirus polyprotein, including a 3A-like protein, a viral protein-genome linked (VpG) and a N-terminal protein of unknown function [25–29]. All six non-structural proteins proceed N to C terminus in the Norovirus polyprotein (**Figure 1**).

ORF1 encodes a 200 kDa polyprotein which undergoes proteolytic cleavage mediated by a virus-encoded 3CLpro, located upstream of the RdRp. Proteolytic processing is rapid, co-translational and results in the production of six non-structural proteins [30, 31]. ORF2 encodes the major structural protein, VP1 [60 kDa], which is responsible for capsid-related functions, including self-assembly and capsid formation, host interactions and immunogenicity of the virus [32–34].

The ORF3 region encodes a small basic protein of 20 to 30 kDa involved in expression and stability of the VP1 capsid protein [34]. Downstream from ORF3, a 42 to 78 nucleotide non-translated region is present and attached to a polyadenylated tail [35].

2.3 Non-structural proteins

2.3.1 N terminal protein

Expression of the Norovirus N terminal protein demonstrated that the N terminal protein was localised to the golgi apparatus and led to its disassembly into discrete aggregates [36]. In addition, the N terminal protein interacts with the vesicle-associated membrane protein–associated protein A [VAP-A], which plays a role in regulated vesicle transport [37, 38]. Therefore, the N terminal protein is predicated to interact with intracellular membranes and may act as an anchor to membrane-bound replication complexes of Norovirus [39].

2.3.2 NTPase

NTPase protein (alternatively designated p41) of the Norovirus GI strain, Southampton virus, has NTPase activity and a helicase domain. The protein

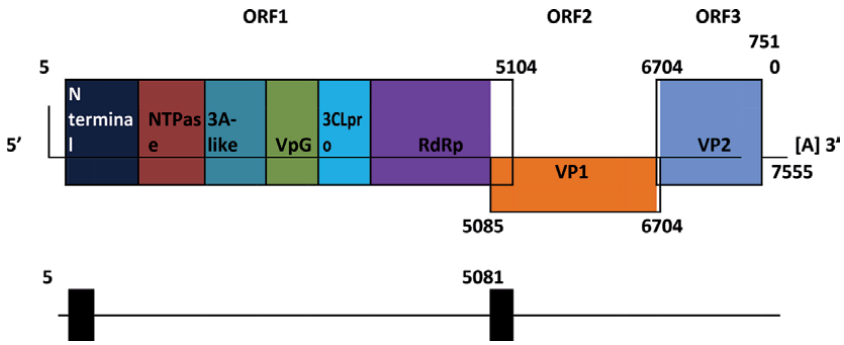

Figure 1.
Genomic organisation of NoV. The genomic organisation and nucleotide positions are shown with reference to human NoV/Lordsdale virus/1993/UK, GenBank accession number X86557. The NoV genome is organised into three ORFs, with the 3′ end of ORF1 overlapping the 5′ end of ORF2 by 20 bp, and the 3'end of ORF2 overlaps the 5′ end of ORF3 by one bp. ORF1 encodes for six non-structural proteins: N terminal protein, nucleoside triphosphate (NTPase), a 3A-like protein, viral protein-genome linked (VpG), "3C-like" protease (3CLpro) and an RdRp. ORF2 encodes for the major structural protein, VP1, which self assembles into the viral capsid. ORF3 encodes for a minor structural protein, VP2, involved in stabilisation of VP1. The polyadenylated tail at the 3'end of the genome is indicated by [a]. The two putative RdRp promoter sites are shown below the image as black boxes.

sequence of the Norovirus p41 protein showed regions of high similarity to the 2C protein of enteroviruses. Norovirus may hydrolyse NTPs for a function distinct from nucleic acid unwinding [40]. The specific role of p41 in the viral replicative cycle has not yet been determined.

2.3.3 3A-like protein

A parallel between picornaviruses and caliciviruses have been demonstrated for the 3A and 3A-like protein, respectively [41–43]. The 3A-like protein [alternatively designated p22 or p20 for Norovirus GI and GII, respectively] in the Norovirus genome occupies a position similar to that of the 3A protein in picornavirus. The specific function of the Norovirus 3A-like protein is unknown, but it has been suggested to be involved in cellular membrane trafficking and replication complexes [44].

2.3.4 VpG

VpG is essential for the production of infectious caliciviruses [45]. Human VpG has been shown to bind to translational initiation factors in vitro and may also be involved in the recruitment of ribosomes to viral RNA. Recently, VpG has been suggested to play a role in RNA replication [46, 47]. VpG was uridylylated in vitro by the RdRp, suggesting it may function as a protein-primer during RNA replication. Another study by Belliot and colleagues demonstrated that Norovirus VpG was nucleotidylylated by the proteinase-polymerase form of the human Norovirus RdRp. This occurred in a template-independent manner in the presence of Mn2+; furthermore, the linkage between RNA to VpG was covalent. Mutational analysis identified tyrosine 27 of the Norovirus VpG as the target amino acid for this linkage, which was susceptible to phosphodiesterase treatment. Thus, the linkage of RNA to VpG via a phosphodiester bond was confirmed. In addition, there was evidence for the presence of an RNA element in the 3′ end of the polyadenylated genome which enhanced nucleotidylylation of the VpG by the RdRp in the presence of Mg2+ [48].

2.3.5 "3C-like" protease

Norovirus 3CLpro (19 kDa) is crucial to the proteolytic processing of ORF1 polyprotein into six non-structural proteins. Characterisation of Norovirus 3CLpro has revealed an active nucleophilic residue in the conserved GDCG motif, common to all chymotrypsin-like 3Cpro. The motif contains amino acid residues essential to formation of an active site. The amino acid residues exists as a catalytic triad in Norovirus, and include cysteine (Cys139), histidine (His 30), and glutamate (Glu 54), which function as a nucleophile, general base, and anion, respectively. All three amino acid residues are important to the enzymatic activity for proteolysis [49, 50].

It has also been suggested that the Norovirus 3CLpro can cleave the host encoded poly [A]-binding protein, and as a result, cellular translation is inhibited. This suggests an important mechanism of host cell modulation during viral replication [51].

2.3.6 RNA-dependent RNA polymerase

The Norovirus RdRp is a non-structural protein involved in the replication of the Norovirus genome. It has been proposed that Norovirus proteinase-polymerase precursor is a bifunctional enzyme with protease and RdRp activity both exhibited during viral replication [52].

2.4 Structural proteins

2.4.1 VP1

ORF2 encodes the major capsid protein, VP1, of the Norovirus genome. The VP1 capsid protein can be divided into three domains, the N terminal domain, shell [S] domain, which is buried inside the capsid, and a protruding [P]domain. A flexible hinge connects the S and P domains. The S domain is highly conserved and is essential for the formation of the icosahedral capsid shell. The P domain comprises of two subdomains: P1, a moderately conserved subdomain and P2, which is hypervariable in its nucleotide sequence. The P2 subdomain of the norovirus genome is the most exposed region of the capsid structure, hence, it contains immune and cellular recognition binding sites [53–56].

2.4.2 VP2

ORF3 encodes a small minor structural protein, VP2, of the Norovirus genome. VP2 is highly variable in sequence between strains and varies in length from 208 to 268 amino acids. The function of VP2 involves the upregulation of VP1 expression and stabilisation of the VP1 in the virus structure. Furthermore, VP2 protects VP1 from disassembly and protease degradation [57]. The role of VP2 in viral replication is unknown, but it may interact with RNA, due to its highly basic, and therefore be involved in packaging of the viral genome. In addition, the VP2 protein is reported to be involved in the formation of infectious viral particles [58, 59].

2.4.3 Transmission

A highly infectious agent, Norovirus is primarily transmitted through person-to person and commonly via the faecal oral route. Aerosolised vomitus containing Norovirus is another transmission mode by which the virus disseminates in outbreaks of gastroenteritis [60, 61]. A study by Marks et al. reported attack rates of Norovirus infections of up to 60% in individuals in close proximity (who were seated next to and on the adjacent table in a restaurant) to the index person who vomited. The attack rate of infection was directly proportional to the distance from the vomiter. Other sources of transmission include the consumption of contaminated food (oysters, vegetables, fresh and frozen produce) [62–65] or water (drinking, ice or recreational) [66–68]. In addition, fomite contamination in an outbreak setting has been demonstrated as an alternative transmission route [69, 70].

3. Clinical features and pathogenesis of norovirus

3.1 Clinical manifestation

Norovirus infection is characterised by an onset of vomiting, diarrhoea, nausea, and may also be accompanied by variable systemic symptoms including, fever, headache, chills or myalgia [71–74]. Diarrhoeal stool is non-bloody, lacks mucus and may be loose or watery. Following an incubation period of 1 to 2 days, the illness is usually mild and self-limited, which generally persists for a short duration of 1 to 3 days. A 68% sensitivity and 99% specificity was determined when the criteria was used in conjunction with laboratory detection techniques, including ELISA and nucleic acid amplification assays [75]. Norovirus infection affects all age groups and is often more severe in the elderly, the young, and in transplant and

immunocompromised patients [76–79]. Studies have shown that symptoms can persist for up to five days or longer and infection may progress to chronic disease [80]. Prolonged viral shedding can occur in the presence or absence of clinical symptoms and death may occur [81–86].

3.2 Pathogenesis

The pathogenicity of NoV was studied in human volunteers inoculated with the prototype strain, Norwalk virus [NoV GI]. Acute infection with NoV resulted in a histopathological lesion in the jejunum and correlated with a broadening and blunting of the villi and crypt cell hyperplasia of the small intestinal tract. These observations provided suggestive evidence that NoV replication is restricted to the small intestine.

Additional studies in volunteers who developed an illness or characteristic lesion, showed the levels of the small intestinal brush border enzymatic activities [alkaline phosphatase, sucrase and trehalase] were significantly reduced, resulting in transient carbohydrate malabsorption [87]. Furthermore, there was a marked delay in gastric emptying. It has been suggested that the reduced gastric motility is responsible for symptoms, specifically nausea and vomiting associated with gastroenteritis.

3.3 Immunity

The immunogenicity associated with Norovirus disease is not well defined. Early studies on host immune responses to Norovirus infection were based on human challenge studies by oral immunisation with either infectious virus or recombinant VLPs [88–93]. Challenge studies have shown that short-term immunity lasts for six to 14 weeks, and is strain specific [94]. Thus, infection is induced following challenge to a serologically distinct strain. Interestingly, individuals with high levels of pre-existing antibodies against Norwalk virus were reportedly more susceptible to infection than individuals who had a non-detectable or had low levels of serum antibodies after challenge with the same strain.

More recently, the structural recognition site of HBGAs by Norovirus has been determined by mutagenesis and crystallographic studies [95]. Based on crystallographic structures, the receptor site involved in host-cell recognition was the P domain, more specifically the outermost P2 surface on the Norovirus capsid gene [96, 97]. Such findings will provide an understanding into the complex interaction between HBGAs and Norovirus, and could lead to intervention strategies to block attachment of virus to host recognition sites. The study of the role of genetic mechanisms in Norovirus infection is a new area in Norovirus immunology, and further studies are required to understand the complex interactions between specific Norovirus genotypes (particularly, newly emergent Norovirus strains) and susceptibility to infection.

3.4 Replication

Little is known about human Norovirus biology, in particular, human Norovirus replication, immunogenicity and pathogenicity due to the lack of an in vitro cell culture and small animal model systems [98]. However, in recent times our understanding of calicivirus replication has come from other studies, including the animal calicivirus, Feline calicivirus [99], and the use of a gnotobiotic pig as an animal model for the study of human Norovirus pathogenesis [100]. However, a significant advancement in the study of Norovirus biology was the development

of the first in vitro cell culture system for the cultivation of murine norovirus 1 (MNV-1) [101]. MNV-1 was used to study immunity and pathogenesis of Norovirus in a mouse model. Subsequently, MNV-1 was successfully propagated in the murine macrophage cell line RAW 264.7 and revealed a tropism for cells of the haematopoietic lineage, specifically the macrophages and dendritic cells. It was proposed that macrophages could contribute to the spread of Norovirus through the host. Norovirus infection of dendritic cells in the lumen of the intestine also provides a point of infection for Norovirus; however, it remains unclear if human Norovirus targets such cells. Regardless, MNV share many molecular and biological properties with human Norovirus, and therefore, provides an important animal model to understand the biology and pathogenesis of human Norovirus infection. Other systems which have provided significant information regarding Norovirus replication are the replicon and reverse genetics systems.

Molecular advances have led to the development of a Norovirus replicon and a recombinant T7 vaccinia virus expressed Norovirus [102–105]. Studies have shown Norovirus RNA is infectious and capable of replication in three cell types: human hepatoma Huh7-cells, hamster BHK21 cells and human embryonic kidney [HEK] 293 T/17 cells. However, the main limitation of these systems was the inability for virions to spread to other neighbouring cells in the culture system. The inability to culture human Norovirus has been suggested to occur at the level of attachment and entry into the cells. Another in vitro cell culture system for human Norovirus was recently reported based on a rotating wall vessel bioreactor technology to engineer a 3D model of the human small intestinal epithelium (**Figure 2**).

However, the model may not provide direct evidence of in vitro propagation of human Norovirus and needs further investigation [107]. Nevertheless, the system can offer an insight into host-cell interaction in Norovirus infection.

Recently, an infectious reverse genetics system for MNV that generates.

infectious virus from a genomic complementary DNA [cDNA] clone under the control of an RNA polymerase II promoter was described. The principle of the Norovirus reverse genetics system was demonstrated by mutagenesis of the protease polymerase cleavage site to show that the protease-polymerase cleavage was essential for the recovery of infectious MNV [108]. Overall, the development of such systems provides an approach to perform functional analyses of the Norovirus genome, as well as the study of the molecular biology and replication of Norovirus.

3.5 RNA recombination

RNA recombination is an important mechanism in the evolution of RNA viruses. Recombination in viruses can affect phylogenetic groupings, increase the

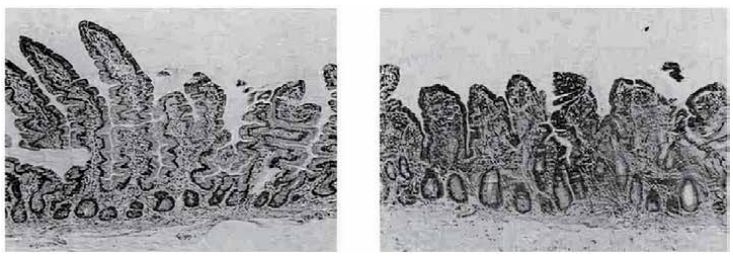

Figure 2.
Intestinal biopsy of jejunal tissue from a human volunteer infected with Norwalk virus. (A-left fig) Normal jejunal biopsy before administration of Norwalk virus. Villi and cellularmorphology appear normal. (B-right fig) Jejunal biopsy after administration with the viral agent. Villi are broadened and flattened; epithelial lining cells appear disorganised. Image taken from [106].

virulence and pathogenicity of the virus, and affect anti-viral drug design. By exchange of genetic material through recombination, a new variant of the virus is produced [109]. In recent years, human Norovirus recombinants have been detected frequently in cases of gastroenteritis worldwide. This increase in prevalence of Norovirus recombinants suggests that infection with at least two virus strains is common. The proposed site of recombination in Norovirus is within the highly conserved ORF1/ORF2 overlap [110–112].

3.6 Treatment and prevention

Norovirus associated gastroenteritis is mild and self-limiting, and generally resolves without complications. However, death from Norovirus associated gastroenteritis has been previously reported [113–115]. In severe cases of Norovirus infection, hospitalisation is required and the administration of an oral fluid and electrolyte treatment is often required to replace the loss of fluids. The oral administration of bismuth subsalicylate after the onset of symptoms has been demonstrated to reduce the duration of abdominal cramps and gastrointestinal symptoms during experimentally induced Norovirus illness in adults [116]. The best control measure for the prevention of Norovirus infection is with good hygiene practices. These include, thorough and frequent hand washing, and the disposal or disinfection of contaminated materials. In addition, extra measures should be implemented in healthcare facilities to prevent large-scale outbreaks, such as restriction of staff movement between wards containing infected patients, the isolation of symptomatic patients, the exclusion of affected staff from work until 48 h after the cessation of symptoms, and the closure of affected units to limit the spread of infection. However, the impact of preventive measures in affected institutions is reduced due to the environmental stability of Norovirus outside the host. This is due to the fact that Norovirus has a non-enveloped structure, is acid stable, persists in the environment and resistant to chlorination of up to 300 ng/ml. Furthermore, quaternary ammonium disinfectants are ineffective in the disinfection of Norovirus [117, 118]. Although a combination of detergent and sodium hypochlorite solution has been reported to be effective in the decontamination of surfaces [119]. Therefore, to prevent and control the spread of Norovirus disease, strict hand hygiene and use of effective disinfectants should be enforced during outbreaks. Importantly, for the efficient implementation of precautionary measures in an outbreak setting, a rapid detection system for the diagnosis of a Norovirus infection would be ideal.

4. Laboratory diagnosis

4.1 Detection of norovirus

Detection of the aetiological agent of gastroenteritis is important as only bacterial and parasitic agents are treatable by current therapeutic agents.

Furthermore, for clinical and epidemiological studies the availability of detection methods for viral nucleic acid, viral antigen, or antibody responses is valuable. Various methods have been used for the diagnosis of Norovirus infection, including electron microscopy [EM], IEM, radioimmunoassays, ELISAs and viral RNA based nucleic acid amplification assays. Of the available detection methods, the most commonly used assays for Norovirus diagnosis include ELISAs and RT-PCR [120].

4.1.1 Electron microscopy

The detection of Norovirus has traditionally relied on EM. It enables the identification of Norovirus by their characteristic morphology. However, the sensitivity of EM detection is low, requiring at least 106 viral particles/g of stool for visualisation. Therefore, this technique is useful only for specimens collected immediately upon the onset of illness when substantial quantities of viral shedding occur. Furthermore, EM is a robust tool but time consuming, requires a high level of technical skill, is labour intensive and not available to all clinical laboratories. Thus, EM is not feasible for large epidemiological studies. Modifications of the EM method, such as, or solid phase IEM [121–125] have also been used to aid in virus identification. Both these methods are based on antigen–antibody reactions. However, like EM, the application of IEM is rarely applied to epidemiological investigations.

4.1.2 Elisa

An ELISA offers an efficient diagnostic method for the identification of Norovirus infection. The rapid turnover and simplicity for screening a large number of samples makes ELISAs an ideal system for use in a diagnostic laboratory. Norovirus are antigenically diverse and therefore assays may be limited in the detection of a broad range of Norovirus strains in circulation. This has probably contributed to the poor performance assessments [sensitivity and specificity] of commercially available ELISAs in different countries when compared to sensitive molecular methods, such as RT-PCR [126–128]. The potential for ELISAs to give false negatives and false-positives due to poor sensitivity and poor specificity, respectively, has limited their use for diagnosing outbreaks where large numbers of samples are being tested.

4.1.3 Reverse transcription-polymerase chain reaction

RT-PCR has remained the most reliable means of diagnosing Norovirus infection as it is the most sensitive routine method used compared to EM and ELISA [129, 130]. The availability of RT-PCR amplification has greatly facilitated sequencing and genome characterisation of Norovirus strains [131–133]. The RT-PCR assay employs primers that target conserved regions of the Norovirus genome, such as the RdRp and/or the VP1 gene. Until recently, Norovirus RT-PCR assays have used primers that targeted the RdRp [3′ end of ORF1 of the Norovirus genome], which is highly conserved among Norovirus. By sequence analysis of the capsid gene [VP1] in the Norovirus genome, another conserved region located at the 5′ end of the capsid gene was identified. This region offered better segregation of Norovirus genotypes by phylogenetic analysis. Moreover, analysis of both regions, RdRp and VP1 is necessary for the detection of Norovirus recombinant strains. These technical advances have improved detection and enhanced epidemiologic surveillance by molecular genotyping and sequence analysis. However, conventional RT-PCR assays have progressively been replaced by real-time RT-PCR, which is more sensitive, faster and offers quantification of RNA viruses. This technology is not only quicker but enables quantitation using the Ct of the unknown target RNA sample compared directly to the Ct of a standard curve, which contains a defined number of copies of the target virus. The Ct value is the basis for accurate and reproducible quantitation using real-time RT-PCR. The application of a standard curve in a real-time RT-PCR assay also enables the determination of viral kinetic parameters associated with Norovirus infection, such as the number of viruses excreted [that is, a measure of viral load in a sample], duration of viral excretion and the viral decay rate.

Several real-time nucleic acid amplification assays have been developed for the detection and quantitation of Norovirus RNA in clinical specimens, by the use of SYBR Green dye chemistry, and probes, including taq-man probes and hybridization probes [134, 135].

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References

[1] Murray, C. J. L., and A. D. Lopez. 1997. Mortality by cause for eight regions of he world: global burden of disease study. Lancet 349:1269-1276.

[2] WHO 2002, posting date. Revised global burden of disease (GBD) 2002estimates- incidence. [Online.]

[3] WHO 2002, posting date. Revised global burden of disease (GBD) 2002 estimates- incidence. [Online.]

[4] Snyder, J. D., and M. H. Merson. 1982. The magnitude of the global problem of acute diarrhoeal disease: a review of active surveillance data. Bull World Health Organ 60:605-631.

[5] Top 13 Common Communicable Diseases Found in India. [Online.]

[6] Kosek, M., C. Bern, and R. L. Guerrant. 2003. The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. Bull World Health Organ 81:197-204.

[7] Hansman, G. S., L. T. Doan, T. A. Kguyen, S. Okitsu, K. Katayama, S. Ogawa, K. Natori, N. Takeda, Y. Kato, O. Nishio, M. Noda, and H. Ushijima. 2004. Detection of norovirus and sapovirus infection among children with gastroenteritis in Ho Chi Minh City, Vietnam. Arch Virol 149:1673-1688.

[8] Chen, S. Y., Y. C. Chang, Y. S. Lee, H. C. Chao, K. C. Tsao, T. Y. Lin, T. Y. Ko, C. N. Tsai, and C. H. Chiu. 2007. Molecular epidemiology and clinical manifestations of viral gastroenteritis in hospitalized pediatric patients in Northern Taiwan. J Clin MicrobioI 45:2054-2057

[9] Harrington, P. R., J. Vinje, C. L. Moe, and R. S. Baric. 2004. Norovirus capture with histo-blood group antigens reveals novel virus-ligand interactions. J Virol 78:3035-3045.

[10] Clark, B., and M. McKendrick. 2004. A review of viral gastroenteritis. Curr Opin Infect Dis 17:461-469.

[11] Wilhelmi, I., E. Roman, and A. Sanchez-Fauquier. 2003. Review: Viruses causing gastroenteritis. Clin Infect Dis 9:247-262.

[12] Kapikian, A. Z., R. G. Wyatt, R. Dolin, T. S. Thornhill, A. R. Kalica, and R.M. Chanock. 1972. Visualization by immune electron microscopy of a27-nm particle associated with acute infectious nonbacterial gastroenteritis. J Virol 10:1075-1081.

[13] Mayo, M. A. 2002. A summary of taxonomic changes recently approved by ICTV. Arch Virol 147:1655-1656.

[14] Green, K. Y., T. Ando, M. S. Balayan, T. Berke, I. N. Clarke, M. K. Estes, D. O. Matson, S. Nakata, J. D. Neill, M. J. Studdert, and H. J. Thiel. 2000. Taxonomy of the caliciviruses. J Infect Dis 181 Suppl 2:S322-S330.

[15] Bridger, J. C., G. A. Hall, and J. F. Brown. 1984. Characterization of a calici-like virus (Newbury agent) found in association with astrovirus in bovine diarrhea. Infect Immun 43:133-138.

[16] Oliver, S. L., E. Asobayire, A. M. Dastjerdi, and J. C. Bridger. 2006. Genomic characterization of the unclassified bovine enteric virus Newbury agent-1 (Newbury1) endorses a new genus in the family Caliciviridae. Virology 350:240-250.

[17] Smiley, J. R., K. O. Chang, J. Hayes, J. Vinje, and L. J. Saif. 2002. Characterization of an entero pathogenic bovine calicivirus representing a potentially new calicivirus genus. J Virol 76:10089-10098.

[18] Zheng, D. P., T. Ando, R. L. Fankhauser, R. S. Beard, R. I. Glass, and S. Monroe. 2006. Norovirus classification and proposed strain nomenclature. Virology 346:312-323.

[19] Kapikian, A. Z., M. K. Estes, and R. M. Chanock. 1996. Norwalk group ofviruses, p. 783-810. *In* B. N. Knipe and P. M. Howley (ed.), Fields. Lippincott, Raven, Philadelphia.

[20] Kapikian, A. Z., J. L. Gerin, R. G. Wyatt, T. S. Thornhill, and R. M. Chanock. 1973. Density in cesium chloride of the 27 nm 8FIIa particle associated with acute infectious nonbacterial gastroenteritis: determination by ultracentrifugation and immune electron microscopy Proc Soc ExpBiol Med 142:874-877.

[21] Green, K. Y., T. Ando, M. S. Balayan, T. Berke, I. N. Clarke, M. K. Estes, D. O. Matson, S. Nakata, J. D. Neill, M. J. Studdert, and H. J. Thiel. 2000. Taxonomy of the caliciviruses. J Infect Dis 181 Suppl 2:S322-S330.

[22] Jiang, X., D. Y. Graham, K. Wang, and M. K. Estes. 1990. Norwalk virus genome cloning and characterisation. Science 250:1580-1583.

[23] Meyers, G., C. Wirblich, and H. J. Thiel. 1991. Genomic and subgenomic RNAs of rabbit hemorrhagic disease virus are both protein-linked and packaged into particles. Virology 184: 677-686.

[24] Neill, J. D. 1990. Nucleotide sequence of a region of the feline calicivirus genome which encodes picornavirus-like RNA-dependent RNA polymerase, cysteine protease and 2C polypeptides. Virus Res 17:145-160.

[25] Dunham, D. M., X. Jiang, T. Berke, A. W. Smith, and D. O. Matson. 1998. Genomic mapping of a calicivirus VPg. Arch Virol 143:2421-2430

[26] Liu, B., I. N. Clarke, and P. R. Lambden. 1996. Polyprotein processing in Southampton virus: identification of 3C-like protease cleavage sites by in vitro mutagenesis. J Virol 70:2605-2610.

[27] Liu, B. L., G. J. Viljoen, I. N. Clarke, and P. R. Lambden. 1999.Identification of further proteolytic cleavage sites in the Southampton calicivirus polyprotein by expression of the viral protease in E. coli. J Gen Virol 80 (Pt 2):291-296.

[28] Pfister, T., and E. Wimmer. 2001. Polypeptide p41 of a Norwalk-like virus is a nucleic acid-independent nucleoside triphosphatase. J Virol 75:1611-1619.

[29] Seah, E. L., J. A. Marshall, and P. J. Wright. 1999. Open reading frame 1of the Norwalk-like virus Camberwell: completion of sequence and expression in mammalian cells. J Virol 73:10531-10535.

[30] Liu, B., I. N. Clarke, and P. R. Lambden. 1996. Polyprotein processing in Southampton virus: identification of 3C-like protease cleavage sites by in vitro mutagenesis. J Virol70:2605-2610.

[31] Sosnovtseva, S. A., S. V. Sosnovtsev, and K. Y. Green. 1999. Mapping of the feline calicivirus proteinase responsible for autocatalytic processing of the nonstructural polyprotein and identification of a stable proteinase-polymerase precursor protein. J Virol 73:6626-6633.

[32] Chen, S. Y., Y. C. Chang, Y. S. Lee, H. C. Chao, K. C. Tsao, T. Y. Lin, T. Y.Ko, C. N. Tsai, and C. H. Chiu. 2007. Molecular epidemiology and clinical manifestations of viral gastroenteritis in hospitalized pediatric patients in Northern Taiwan. J Clin Microbiol 45:2054-2057.

[33] Jiang, X., M. Wang, D. Y. Graham, and M. K. Estes. 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J Virol 66:6527-6532.

[34] Bertolotti-Ciarlet, A., S. E. Crawford, A. M. Hutson, and M. K. Estes. 2003. The 3' End of Norwalk virus mRNA contains determinants that regulate the expression and stability of the viral capsid protein VP1: a novel function for the VP2 protein. J Virol 77:11603-11615.

[35] Green, K. Y., R. M. Chanock, and A. Z. Kapikan. 2001. Human Calicivirus, p. 841-874. *In* D. M. Knipe and P. M. Howley (ed.), Fields Virology, 4 ed, vol. 1. Lippincott Williams & Wilkins, Philadelphia.

[36] Fernandez-Vega, V., S. V. Sosnovtsev, G. Belliot, A. D. King, T. Mitra, A. Gorbalenya, and K. Y. Green. 2004. Norwalk virus N-terminal nonstructural protein is associated with disassembly of the Golgi complex in transfected cells. J Virol 78:4827-4837.

[37] Ettayebi, K., and M. E. Hardy. 2003. Norwalk virus nonstructural proteinp48 forms a complex with the SNARE regulator VAP-A and prevents cell surface expression of vesicular stomatitis virus G protein. J Virol77:11790-11797.

[38] Weir, M. L., H. Xie, A. Klip, and W. S. Trimble. 2001. VAP-A binds promiscuously to both v- and tSNAREs. Biochem Biophys Res Commun 286:616-621.

[39] Ettayebi, K., and M. E. Hardy. 2003. Norwalk virus nonstructural proteinp48 forms a complex with the SNARE regulator VAP-A and prevents cell surface expression of vesicular stomatitis virus G protein. J Virol 77:11790-11797.

[40] Pfister, T., and E. Wimmer. 2001. Polypeptide p41 of a Norwalk-like virus is a nucleic acid-independent nucleoside triphosphatase. J Virol75:1611-1619

[41] Blakeney, S. J., A. Cahill, and P. A. Reilly. 2003. Processing of Norwalk virus nonstructural proteins by a 3C-like cysteine proteinase. Virology 308:216-224

[42] Konig, M., H. J. Thiel, and G. Meyers. 1998. Detection of viral proteins after infection of cultured hepatocytes with rabbit hemorrhagic disease virus. J Virol 72:4492-4497.

[43] Sosnovtsev, S. V., M. Garfield, and K. Y. Green. 2002. Processing map and essential cleavage sites of the nonstructural polyprotein encoded by ORF1 of the feline calicivirus genome. J Virol 76:7060-7072.

[44] Belliot, G., S. V. Sosnovtsev, T. Mitra, C. Hammer, M. Garfield, and K. Y.Green. 2003. In vitro proteolytic processing of the MD145 norovirus ORF1 nonstructural polyprotein yields stable precursors and products similar to those detected in calicivirus-infected cells. J Virol 77:10957-10974.

[45] Burroughs, J. N., and F. Brown. 1978. Presence of a covalently linked protein on calicivirus RNA. J Gen Virol 41:443-446.

[46] Belliot, G., S. V. Sosnovtsev, K. O. Chang, P. McPhie, and K. Y. Green. 2008. Nucleotidylylation of the VPg protein of a human norovirus by its proteinase-polymerase precursor protein. Virology 374:33-49.

[47] Rohayem, J., I. Robel, K. Jager, U. Scheffler, and W. Rudolph. 2006. Protein-primed and de novo initiation of RNA synthesis by Norovirus 3Dpol. J Virol 80: 7060-7069.

[48] Belliot, G., S. V. Sosnovtsev, K. O. Chang, P. McPhie, and K. Y. Green. 2008. Nucleotidylylation of the VPg protein of a human norovirus by its proteinase-polymerase precursor protein. Virology 374:33-49

[49] Someya, Y., N. Takeda, and T. Miyamura. 2002. Identification of

active-site amino acid residues in the Chiba virus 3C-like protease. J Virol 76:5949-5958.

[50] Zeitler, C. E., M. K. Estes, and B. V. Venkataram Prasad. 2006. X-raycrystallographic structure of the Norwalk virus protease at 1.5-a resolution. J Virol 80:5050-5058.

[51] Kuyumcu-Martinez, M., G. Belliot, S. V. Sosnovtsev, K. O. Chang, K. Y. Green, and R. E. Lloyd. 2004. Calicivirus 3C-like proteinase inhibits cellular translation by cleavage of poly(A)-binding protein. J Virol 78:8172-8182.

[52] Belliot, G., H. Laveran, and S. S. Monroe. 1997. Detection and genetic differentiation of human astroviruses: phylogenetic grouping varies by coding region. Arch Virol 142:1323-1334.

[53] Hale, A. D., T. N. Tanaka, N. Kitamoto, M. Ciarlet, X. Jiang, N. Takeda, D.W. Brown, and M. K. Estes. 2000. Identification of an epitope common to genogroup1 "norwalk-like viruses". J Clin Microbiol 38:1656-1660.

[54] Hardy, M. E., T. N. Tanaka, N. Kitamoto, L. J. White, J. M. Ball, X. Jiang, and M. K. Estes. 1996. Antigenic mapping of the recombinant Norwalk virus capsid protein using monoclonal antibodies. Virology 217:252-261.

[55] Lochridge, V. P., K. L. Jutila, J. W. Graff, and M. E. Hardy. 2005.Epitopes in the P2 domain of norovirus VP1 recognized by monoclonal antibodies that block cell interactions. J Gen Virol 86:2799-2806.

[56] Tan, M., P. Huang, J. Meller, W. Zhong, T. Farkas, and X. Jiang. 2003. Mutations within the P2 domain of norovirus capsid affect binding to human histo-blood group antigens: evidence for a binding pocket. J Virol 77:12562-12571.

[57] Bertolotti-Ciarlet, A., S. E. Crawford, A. M. Hutson, and M. K. Estes. 2003. The 3' End of Norwalk virus mRNA contains determinants that regulate the expression and stability of the viral capsid protein VP1: a novel function for the VP2 protein. J Virol 77:11603-11615.

[58] Glass, P. J., L. J. White, J. M. Ball, I. Leparc-Goffart, M. E. Hardy, and M.K. Estes. 2000. Norwalk virus open reading frame 3 encodes a minor structural protein. J Virol 74:6581-6591.

[59] Asanaka, M., R. L. Atmar, V. Ruvolo, S. E. Crawford, F. H. Neill, and M.K. Estes. 2005. Replication and packaging of Norwalk virus RNA incultured mammalian cells. Proc Natl AcadSci U S A 102:10327-10332.

[60] Marks, P. J., I. B. Vipond, D. Carlisle, D. Deakin, R. E. Fey, and E. O. Caul. 2000. Evidence for airborne transmission of Norwalk-like virus (NLV) in a hotel restaurant. Epidemiol Infect 124:481-487.

[61] Marks, P. J., I. B. Vipond, F. M. Regan, K. Wedgwood, R. E. Fey, and E.O. Caul. 2003. A school outbreak of Norwalk-like virus: evidence for airborne transmission. Epidemiol Infect 131:727-736.

[62] Cheng, P. K., D. K. Wong, T. W. Chung, and W. W. Lim. 2005. Norovirus contamination found in oysters worldwide. J Med Virol 76:593-597.

[63] Herwaldt, B. L., J. F. Lew, C. L. Moe, D. C. Lewis, C. D. Humphrey, S. Monroe, E. W. Pon, and R. I. Glass. 1994. Characterization of a variant strain of Norwalk virus from a food-borne outbreak of gastroenteritis on a cruise ship in Hawaii. J ClinMicrobiol 32:861-866.

[64] Long, S. M., G. K. Adak, S. J. O'Brien, and I. A. Gillespie. 2002. General outbreaks of infectious

intestinal disease linked with salad vegetables and fruit, England and Wales, 1992-2000. Commun Dis Public Health 5:101-105.

[65] Martinez, A., A. Dominguez, N. Torner, L. Ruiz, N. Camps, I. Barrabeig, Arias, J. Alvarez, P. Godoy, P. Balana, A. Pumares, R. Bartolome, D. Ferrer, U. Perez, R. Pinto, J. Buesa, and C. V. Gastroenteritis Study Group. 2008. Epidemiology of food borne Norovirus outbreaks in Catalonia, Spain. BMC Infect Dis 8:47.

[66] Maunula, L., I. T. Miettinen, and C. H. von Bonsdorff. 2005. Norovirus outbreaks from drinking water. Emerg Infect Dis 11:1716-1721.

[67] Podewils, L. J., L. Zanardi Blevins, M. Hagenbuch, D. Itani, A. Burns, C. Otto, L. Blanton, S. Adams, S. S. Monroe, M. J. Beach, and M. Widdowson. 2007. Outbreak of norovirus illness associated with a swimming pool. Epidemiol Infect 135:827-833.

[68] Webby, R. J., K. S. Carville, M. D. Kirk, G. Greening, R. M. Ratcliff, S. Crerar, K. Dempsey, M. Sarna, R. Stafford, M. Patel, and G. Hall. 2007. Internationally distributed frozen oyster meat causing multiple outbreaks of norovirus infection in Australia. Clin Infect Dis 44:1026-1031.

[69] Jones, E. L., A. Kramer, M. Gaither, and C. P. Gerba. 2007. Role of fomite contamination during an outbreak of norovirus on houseboats. Int J Environ Health Res 17:123-131.

[70] Wu, H. M., M. Fornek, K. J. Schwab, A. R. Chapin, K. Gibson, E. Schwab, C. Spencer, and K. Henning. 2005. A norovirus outbreak at along-term-care facility: the role of environmental surface contamination. Infect Control Hosp Epidemiol 26:802-810.

[71] Agus, S. G., R. Dolin, R. G. Wyatt, A. J. Tousimis, and R. S. Northrup. 1973. Acute infectious nonbacterial gastroenteritis: intestinal histopathology. Histologic and enzymatic alterations during illness produced by the Norwalk agent in man. Ann Intern Med 79:18-25.

[72] Dolin, R., N. R. Blacklow, H. DuPont, S. Formal, R. F. Buscho, J. A. Kasel, R. P. Chames, R. Hornick, and R. M. Chanock. 1971. Transmission of acute infectious nonbacterial gastroenteritis to volunteers by oral administration of stool filtrates. J Infect Dis 123:307-312.

[73] Turcios, R. M., M. A. Widdowson, A. C. Sulka, P. S. Mead, and R. I. Glass. 2006. Reevaluation of epidemiological criteria for identifying outbreaks of acute gastroenteritis due to norovirus: United States, 1998-2000. Clin Infect Dis 42:964-969.

[74] Gallimore, C. I., D. Lewis, C. Taylor, A. Cant, A. Gennery, and J. J. Gray. 2004. Chronic excretion of a norovirus in a child with cartilage hairhypoplasia (CHH). J Clin Virol 30:196-204.

[75] Goller, J. L., A. Dimitriadis, A. Tan, H. Kelly, and J. A. Marshall. 2004. Long-term features of norovirus gastroenteritis in the elderly. J Hosp Infect 58:286-291.

[76] Murata, T., N. Katsushima, K. Mizuta, Y. Muraki, S. Hongo, and Y. Matsuzaki. 2007. Prolonged norovirus shedding in infants <or=6 months of age with gastroenteritis. Pediatr Infect Dis J 26:46-49.

[77] Rockx, B., M. de Wit, H. Vennema, J. Vinje, E. De Bruin, Y. Van Duynhoven, and M. Koopmans. 2002. Natural history of human calicivirus infection: a prospective cohort study. Clin Infect Dis 35:246-253.

[78] Lee, B. E., X. L. Pang, J. L. Robinson, D. Bigam, S. S. Monroe, and J. K. Preiksaitis. 2008. Chronic Norovirus

and Adenovirus infection in a solid organ transplant recipient. Pediatr Infect Dis J 27:360-362.

[79] Parashar, U. D., L. Dow, R. L. Fankhauser, C. D. Humphrey, J. Miller, T. Ando, K. S. Williams, C. R. Eddy, J. S. Noel, T. Ingram, J. S. Bresee, S.S. Monroe, and R. I. Glass. 1998. An outbreak of viral gastroenteritis associated with consumption of sandwiches: implications for the control of transmission by food handlers. Epidemiol Infect 121:615-621.

[80] Ozawa, K., T. Oka, N. Takeda, and G. S. Hansman. 2007. Norovirus infections in symptomatic and asymptomatic food handlers in Japan. J ClinMicrobiol 45:3996-4005.

[81] Lee, B. E., X. L. Pang, J. L. Robinson, D. Bigam, S. S. Monroe, and J. K. Preiksaitis. 2008. Chronic Norovirus and Adenovirus infection in a solid organ transplant recipient. Pediatr Infect Dis J 27:360-362.

[82] Jenkins, P., S. R. Greene, J. R. Archer, D. Hoang-Johnson, M. Quinn, P. Duncan, G. Johnson, B. I. Rosen, P. Smith, V. Reddy, J. Schlegelmilch, J. Pendarvis, M. Donovan, J. E. Gunn, M. A. Barry, M. Davies, J. Vinje, M.-A. Widdowson, Z. Moore, J. K. Schaffin, and J. E. Tate. 2007.Norovirus activity-United States, 2006-2007. MMWR Morb Mortal Wkly Rep 56:842-846.

[83] Graham, D. Y., X. Jiang, T. Tanaka, A. R. Opekun, H. P. Madore, and M.K. Estes. 1994. Norwalk virus infection of volunteers: new insights basedon improved assays. J Infect Dis 170:34-43.

[84] Gallimore, C. I., D. Cubitt, N. du Plessis, and J. J. Gray. 2004. Asymptomatic and symptomatic excretion of noroviruses during a hospital outbreak of gastroenteritis. J ClinMicrobiol 42:2271-2274.

[85] Schreiber, D. S., N. R. Blacklow, and J. S. Trier. 1973. The mucosal lesion of the proximal small intestine in acute infectious nonbacterial gastroenteritis. N Engl J Med 288:1318-1323.

[86] Agus, S. G., R. Dolin, R. G. Wyatt, A. J. Tousimis, and R. S. Northrup. 1973. Acute infectious nonbacterial gastroenteritis: intestinal histopathology. Histologic and enzymatic alterations during illness produced by the Norwalk agent in man. Ann Intern Med 79:18-25.

[87] Baron, R. C., H. B. Greenberg, G. Cukor, and N. R. Blacklow. 1984. Serological responses among teenagers after natural exposure to Norwalk virus. J Infect Dis 150:531-534.

[88] Greenberg, H. B., J. Valdesuso, A. Z. Kapikian, R. M. Chanock, R. G. Wyatt, W. Szmuness, J. Larrick, J. Kaplan, R. H. Gilman, and D. A. Sack. 1979. Prevalence of antibody to the Norwalk virus in various countries. Infect Immun 26:270-273.

[89] Johnson, P. C., J. J. Mathewson, H. L. DuPont, and H. B. Greenberg. 1990. Multiple-challenge study of host susceptibility to Norwalk gastroenteritis in US adults. J Infect Dis 161:18-21.

[90] Wolf, S., J. Hewitt, M. Rivera-Aban, and G. E. Greening. 2008. Detection and characterisation of F+ RNA bacteriophages in water and shellfish: Application of a multiplex real-time reverse transcription PCR. J Virol Methods 149:123-128.

[91] Matsui, S. M., and H. B. Greenberg. 2000. Immunity to calicivirus infection. J Infect Dis 181:S331-S335.

[92] Parrino, T. A., D. S. Schreiber, J. S. Trier, A. Z. Kapikian, and N. R. Blacklow. 1977. Clinical immunity in acute gastroenteritis caused by Norwalk agent. N Engl J Med 297:86-89.

[93] Bu, W., A. Mamedova, M. Tan, M. Xia, X. Jiang, and R. S. Hegde. 2008.

Structural basis for the receptor binding specificity of Norwalk virus. J Virol 82:5340-5347.

[94] Cao, S., Z. Lou, M. Tan, Y. Chen, Y. Liu, Z. Zhang, X. C. Zhang, X. Jiang, X. Li, and Z. Rao. 2007. Structural basis for the recognition of blood group trisaccharides by Norovirus. J Virol8:5949-5957.

[95] Choi, J.-M., A. M. Huston, M. K. Estes, and B. V. V. Prasad. 2008.Atomic resolution structural characterisation of recognition of histo-blood group antigens by Norwalk virus. Proc Natl AcadSci U S A 105:9175-9180.

[96] Duizer, E., K. J. Schwab, F. H. Neill, R. L. Atmar, M. P. Koopmans, and M.K. Estes. 2004. Laboratory efforts to cultivate noroviruses. J Gen Virol 85:79-87.

[97] Chang, K. O., D. W. George, J. B. Patton, K. Y. Green, and S. V. Sosnovtsev. 2008. Leader of the capsid protein in feline calicivirus promotes the replication of Norwalk virus in cell culture. J Virol 82:9206-9217.

[98] Cheetham, S., M. Souza, T. Meulia, S. Grimes, M. G. Han, and L. J. Saif. 2006. Pathogensis of a genogroup II human norovirus in gnotobiotic pigs. J Virol 80:10372-10381.

[99] Karst, S. M., C. E. Wobus, M. Lay, J. Davidson, and H. W. t. Virgin. 2003. STAT1-dependent innate immunity to a Norwalk-like virus. Science 299:1575-1578.

[100] Asanaka, M., R. L. Atmar, V. Ruvolo, S. E. Crawford, F. H. Neill, and M.K. Estes. 2005. Replication and packaging of Norwalk virus RNA incultured mammalian cells. Proc Natl AcadSci U S A102:10327-10332.

[101] Chang, K. O., S. V. Sosnovtsev, G. Belliot, A. D. King, and K. Y. Green. 2006. Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line. Virology 353:463-473.

[102] Guix, S., M. Asanaka, K. Katayama, S. E. Crawford, F. H. Neill, R. L. Atmar, and M. K. Estes. 2007. Norwalk virus RNA is infectious inmammalian cells. J Virol 81:12238-12248.

[103] Katayama, K., G. S. Hansman, T. Oka, S. Ogawa, and N. Takeda. 2006. Investigation of norovirus replication in a human cell line. Arch Virol 151:1291-1308

[104] Chan, M. C., Y. P. Wong, and W. K. Leung. 2007. Cell culture assay for human noroviruses. Emerg Infect Dis 13:1117; author reply 1117-8.

[105] Ward, V. K., C. J. McCormick, I. N. Clarke, O. Salim, C. E. Wobus, L. Thackray, H. W. t. Virgin, and P. R. Lambden. 2007. Recovery ofinfectious murine norovirus using pol II-driven expression of full-length cDNA. Proc Natl Acad Sci U S A104:11050-11055.

[106] Blacklow, N. R., J. E. Herrmann, and W. D. Cubitt. 1987. Immunobiology of Norwalk virus. Ciba Found Symp 128:144-161.

[107] Worobey, M., and E. C. Holmes. 1999. Evolutionary aspects ofrecombination in RNA viruses. J Gen Virol 80:2535-2543.

[108] Ambert-Balay, K., F. Bon, F. Le Guyader, P. Pothier, and E. Kohli. 2005. Characterization of new recombinant noroviruses. J ClinMicrobiol 43: 5179-5186.

[109] Bull, R. A., G. S. Hansman, L. E. Clancy, M. M. Tanaka, W. D. Rawlinson, and P. A. White. 2005. Norovirus recombination in ORF1/ORF2 overlap. Emerg Infect Dis 11:1079-1085.

[110] Phan, T. G., K. Kaneshi, Y. Ueda, S. Nakaya, S. Nishimura, A. Yamamoto, K.

Sugita, S. Takanashi, S. Okitsu, and H. Ushijima. 2007.Genetic heterogeneity, evolution, and recombination in noroviruses. J Med Virol 79:1388-1400.

[111] Green, K. Y., G. Belliot, J. L. Taylor, J. Valdesuso, J. F. Lew, A. Z. Kapikian, and F. Y. Lin. 2002. A predominant role for Norwalk-like virusesas agents of epidemic gastroenteritis in Maryland nursing homes for the elderly. J Infect Dis 185:133-146.

[112] Jenkins, P., S. R. Greene, J. R. Archer, D. Hoang-Johnson, M. Quinn, P. Duncan, G. Johnson, B. I. Rosen, P. Smith, V. Reddy, J. Schlegelmilch, J. Pendarvis, M. Donovan, J. E. Gunn, M. A. Barry, M. Davies, J. Vinje, M.-A. Widdowson, Z. Moore, J. K. Schaffin, and J. E. Tate. 2007.Norovirus activity--United States, 2006-2007. MMWR Morb Mortal Wkly Rep 56:842-846.

[113] Verhoef, L., E. Duizer, H. Vennema, J. Siebenga, C. Swaan, L. Isken, M. Koopmans, K. Balay, P. Pothier, P. McKeown, G. van Dijk, P. Capdepon, and G. Delmas. 2008. Import of norovirus infections in the Netherlands and Ireland following pilgrimages to Lourdes, 2008- preliminary report. Euro Surveill 13:pii=19025.

[114] Steinhoff, M. C., R. G. Douglas, Jr., H. B. Greenberg, and D. R. Callahan. 1980. Bismuth subsalicylate therapy of viral gastroenteritis. Gastroenterology 78:1495-1499

[115] Doultree, J. C., J. D. Druce, C. J. Birch, D. S. Bowden, and J. A. Marshall. 1999. Inactivation of feline calicivirus, a Norwalk virus surrogate. J Hosp Infect 41:51-57.

[116] Duizer, E., P. Bijkerk, B. Rockx, A. De Groot, F. Twisk, and M. Koopmans. 2004. Inactivation of caliciviruses. Appl Environ Microbiol 70:4538-4543.

[117] Barker, J., I. B. Vipond, and S. F. Bloomfield. 2004. Effects of cleaning and disinfection in reducing the spread of Norovirus contamination via environmental surfaces. J Hosp Infect 58:42-49.

[118] Rabenau, H. F., M. Sturmer, S. Buxbaum, A. Walczok, W. Preiser, and H.W. Doerr. 2003. Laboratory diagnosis of norovirus: which method is thebest? Intervirology 46:232-238.

[119] Dolin, R., R. C. Reichman, K. D. Roessner, T. S. Tralka, R. T. Schooley, W. Gary, and D. Morens. 1982. Detection by immune electron microscopy of the Snow Mountain agent of acute viral gastroenteritis. J Infect Dis 146:184-189.

[120] Kapikian, A. Z., R. G. Wyatt, R. Dolin, T. S. Thornhill, A. R. Kalica, and R.M. Chanock. 1972. Visualization by immune electron microscopy of a27-nm particle associated with acute infectious nonbacterial gastroenteritis. J Virol 10:1075-1081.

[121] Thornhill, T. S., R. G. Wyatt, A. R. Kalica, R. Dolin, R. M. Chanock, and A.Z. Kapikian. 1977. Detection by immune electron microscopy of 26- to 27-nm viruslike particles associated with two family outbreaks of gastroenteritis. J Infect Dis 135:20-27.

[122] Lewis, D. C. 1990. Three serotypes of Norwalk-like virus demonstrated by solid-phase immune electron microscopy. J Med Virol 30:77-81.

[123] Lewis, D. C., N. F. Lightfoot, and J. V. Pether. 1988. Solid-phase immune electron microscopy with human immunoglobulin M for serotyping of Norwalk-like viruses. J ClinMicrobiol 26:938-942.

[124] Burton-MacLeod, J. A., E. M. Kane, R. S. Beard, L. A. Hadley, R. I. Glass, and T. Ando. 2004. Evaluation and comparison of two commercial enzyme-linked immunosorbent assay kits for detection of antigenically diverse

human noroviruses in stool samples. J ClinMicrobiol 42:2587-2595.

[125] De Bruin, E., E. Duizer, H. Vennema, and M. P. Koopmans. 2006. Diagnosis of Norovirus outbreaks by commercial ELISA or RT-PCR. J Virol Methods.

[126] Dimitriadis, A., L. D. Bruggink, and J. A. Marshall. 2006. Evaluation ofthe Dako IDEIA norovirus EIA assay for detection of norovirus using faecal specimens from Australian gastroenteritis outbreaks. Pathology 38:157-165.

[127] Rabenau, H. F., M. Sturmer, S. Buxbaum, A. Walczok, W. Preiser, and H.W. Doerr. 2003. Laboratory diagnosis of norovirus: which method is thebest? Intervirology 46:232-238.

[128] Richards, A. F., B. Lopman, A. Gunn, A. Curry, D. Ellis, H. Cotterill, S. Ratcliffe, M. Jenkins, H. Appleton, C. I. Gallimore, J. J. Gray, and D. W. Brown. 2003. Evaluation of a commercial ELISA for detecting Norwalk-likevirus antigen in faeces. J Clin Virol 26:109-115.

[129] Jiang, X., D. Y. Graham, K. Wang, and M. K. Estes. 1990. Norwalk virus genome cloning and characterisation. Science 250:1580-1583.

[130] Katayama, K., H. Shirato-Horikoshi, S. Kojima, T. Kageyama, T. Oka, F. Hoshino, S. Fukushi, M. Shinohara, K. Uchida, Y. Suzuki, T. Gojobori, and N. Takeda. 2002. Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. Virology 299:225-239.

[131] Lambden, P. R., E. O. Caul, C. R. Ashley, and I. N. Clarke. 1993.Sequence and genome organization of a human small round-structured (Norwalk-like) virus. Science 259:516-519.

[132] Ferreira, M. S., M. P. Xavier, T. M. Fumian, M. Victoria, S. A. Oliveira, L.H. Pena, J. P. Leite, and M. P. Miagostovich. 2008. Acutegastroenteritis cases associated with noroviruses infection in the state of Rio de Janeiro. J Med Virol 80:338-344.

[133] Fukuda, S., S. Takao, M. Kuwayama, Y. Shimazu, and K. Miyazaki. 2006. Rapid detection of norovirus from fecal specimens by real-time reverse transcription-loop-mediated isothermal amplification assay. J ClinMicrobiol 44:1376-1381

[134] Hohne, M., and E. Schreier. 2004. Detection and characterization ofnorovirus outbreaks in Germany: application of a one-tube RT-PCR using a fluorogenic real-time detection system. J Med Virol 72:312-319.

[135] Kageyama, T., S. Kojima, M. Shinohara, K. Uchida, S. Fukushi, F. B. Hoshino, N. Takeda, and K. Katayama. 2003. Broadly reactive and highlysensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. J ClinMicrobiol 41:1548-1557.

Chapter 4

Molecular Mechanisms for Norovirus Genome Replication

Muhammad Amir Yunus

Abstract

The genomes of positive strand RNA viruses often contain more than one open reading frame. Some of these viruses have evolved novel mechanisms to regulate the synthesis of the other open reading frames that in some cases involved the production of a subgenomic RNA or RNAs. Very often, the presence of the subgenomic RNA is used as indicator for active viral genome replication. Norovirus, a major cause for gastroenteritis as well as with all other caliciviruses follow a typical positive strand RNA viruses genome replication strategy. In addition, noroviruses also produce a subgenomic RNA during their replication in infected cells. Efficient and adequate synthesis of norovirus subgenomic RNA is crucial for successful viral replication and productive infection leading to the generation of infectious viral progeny. This chapter will dissect the significant findings on mechanisms involved in norovirus genome replication as well as focusing on subgenomic RNA production.

Keywords: RNA-dependent RNA polymerase, subgenomic RNA, replication, internal initiation, core promoter

1. Introduction

Noroviruses are often associated with outbreaks of gastroenteritis in hospitals, on cruise ships, schools, nursing homes and military camps where a close person to person contact cannot be avoided [1]. Infection is typically followed by a 24 to 48 hour incubation period before emergence of the clinical disease, symptoms of which include acute diarrhea and projectile vomiting, usually accompanied by several signs/symptoms such as abdominal cramps, myalgia, malaise, headache, nausea and low grade fever [2, 3]. Noroviruses are the most common cause of gastroenteritis infections due to their stability, low infectious dose, large host reservoir (humans), short term immunity, multiple transmission routes and large genetic diversity between strain [4]. The human norovirus (HuNv) infection is self-limiting and the symptoms typically last between 12 and 60 hours [3]. However, viral shedding appears to be prolonged up to several weeks after the symptoms are resolved, especially in persons with impaired immunity where persistent infection often occur by reinfection [5, 6]. More importantly, illness among the elderly and immunocompromised patient can be fatal due to the severe dehydration. The main transmission route for noroviruses is by fecal-oral, through the contaminated food, water or surfaces especially [1, 7]. Consumption of contaminated fresh produce food such as salads, fruits and sandwiches that requires no prior heating have also

been linked as a possible source of food-borne infections [8]. Furthermore, a high concentration of norovirus was also found within the gastrointestinal tissue of contaminated bivalves such as oysters and mussels that are filter feeders. Therefore, these contaminated bivalves are also considered as another important foodborne source of norovirus infection [9]. In addition, airborne transmission that involves the aerosolized vomit from an infected person has also been demonstrated [10, 11]. These findings are supported by the low infectious dose required for norovirus infection; less than 10 viral particles are sufficient enough to establish infection with Norwalk virus [12]. First described as Norwalk virus, which was responsible for a gastroenteritis outbreak at a school in Norwalk, Ohio US, in 1968 [13], human noroviruses (HuNv) are today recognized as the leading cause of viral gastroenteritis infections in human population. The United States Centre for Disease Control and Prevention (CDC) has estimated that noroviruses are responsible for at least 23 million cases of food borne illness each year in the United States with approximately 50 thousands hospitalization and 300 death [4, 14]. However, in one of the reviews which involves a period of study from 1996 to 2007, it is estimated to be nearly 110,000 hospitalization per epidemic years with the cost of approximately 500 million US dollar per year [15]. The recorded surveillance data from the Food Borne Viruses in Europe Network also indicates that more than 85% of viral gastroenteritis outbreaks that occurred between 1995 and 2000 could be attributed to these viruses [4, 16]. The cost to the United Kingdom National Health Service (NHS) in England and Wales as a direct result of the outbreaks occurring in hospitals has been estimated to be approximately 115 million pounds in 2002–2003 [17]. However, due to the acute nature of the infection, it is difficult to identify all the norovirus infection cases and therefore the real cost can be considered higher. Furthermore, the global impact of gastroenteritis caused by HuNv is hard to be estimated since most of the annually 3.5 to 5 million deaths are from developing world with inadequate healthcare, surveillance and diagnostic systems [1]. There is still no licensed vaccine against norovirus made available. However, there are few promising candidates in the pipeline with one already in phase 2 [18]. In addition, efforts in developing norovirus-specific antiviral drugs are also ongoing. To enable these efforts, our fundamental knowledge on norovirus biology needs to be enhanced especially with regards to norovirus genome replication. This chapter will emphasize on subgenomic RNA replication aspect of norovirus particularly focusing on works with MNV.

2. Building of norovirus particle

The first norovirus virion to be observed by immune-electron microscopy was Norwalk virus in 1972 by Albert Kapikian. The virions are icosahedral, with a diameter ranging from 27 to 39 nm and a buoyant density of 1.36 ± 0.04 g/cm^3 [4, 13, 19]. The virus's capsids are composed of 180 copies of a major protein VP1 (formed into 90 dimers) and one or two copies of the minor capsid protein VP2 [20, 21]. Studies using Norwalk virus-like particles (VLPs) revealed that the major protein VP1 is structurally divided into two domains referred to as the 'shell' (S) and 'protruding' (P) domains, with the P domain being further divided into P1 and P2 subdomains [21]. The inner S domain sub-units interact each other to form a continuous 'shell' structure for capsid while the P domain emanates from the S domain surface and forming cup-like structure. Furthermore, the outer P2 subdomain has been recognized as the most variable region of the calicivirus capsid and the region that determines the species-specific binding of these viruses to the respective cell receptor [22, 23]. Molecularly, norovirus particle capsid encloses the viral genome,

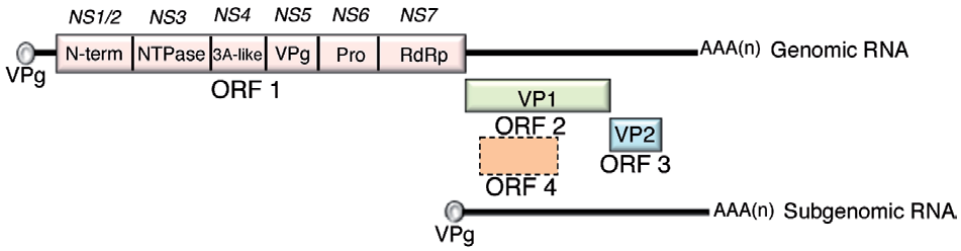

Figure 1.
Diagrammatic representation of the norovirus genome. The length of the genomic RNA (G RNA) is approximately 7.3 to 8.3 kb with subgenomic RNA (SG RNA) about 2.4 kb. The genome normally contains three ORFs with an additional ORF4 in MNV. ORF1 is translated into a large polyprotein which is post-translationally cleaved into non-structural proteins (NS1-NS7) at the position indicated. These NS proteins have alternative nomenclature (indicated in the diagram). ORF2 and ORF3 (and ORF4 of MNV) are thought to be translated from the SG RNA template and produce structural proteins; the major capsid (VP1) and minor capsid (VP2) protein.

a positive-sense single stranded RNA molecule of about 7.4 to 8.3 kb in size. The genome has a virus encoded protein covalently linked to the 5′ end (VPg) and a poly A tail at the 3′ end (**Figure 1**). This genomic RNA (G RNA) encodes three open reading frames (ORFs) flanked by two short untranslated regions (UTRs) and with a small degree of overlap at the 5′ and 3′ junctions between ORF1 and ORF2 [24, 25]. In addition, within the *Norovirus* genus, only murine norovirus (MNV) contains a fourth alternative open reading frame (ORF4) which overlaps ORF2 in a + 1 frameshift.

3. The norovirus life cycle

Like all viruses, the life cycle of noroviruses begins with the attachment of the viral particles to their specific receptor on the membrane of the host cell. Susceptibility to norovirus infections in humans, specifically Norwalk virus, is associated with ABO histo-blood group antigens (HBGA) and individual secretor status [26]. HBGA are carbohydrates found on the surface of gut epithelial cells [4, 20, 27]. These carbohydrate molecules are involved in the attachment of noroviruses but are unlikely to be the main receptor as co-receptor may also be involved [27]. In addition, the secretor status of individuals also determines the susceptibility to norovirus infections [28]. Individuals who are non-secretors of H type 1 were found to be resistant to norovirus infections due to a mutation in the α-(1,2)-fucosyltransferase (*FUT2*) gene, involved in the production of H-type 1 antigen in saliva and mucosa [29]. Study using murine macrophages which support the propagation of the MNV had found that terminal sialic acid moieties present on gangliosides can act as a receptor for MNV attachment [30]. However, MNV entry into permissive cells has been shown to be pH independent [31]. Relatively recently, genome-wide CRISPR screens have identified CD300lf as the receptor for MNV attachment to host cells [32]. CD300lf is a type I integral membrane protein with a single extracellular Ig-like domain. CD300lf is part of a larger family of CD300 molecules that function as cell death sensors, as they recognize phospholipids typically found on the inner leaflet of cells [33]. After a successful attachment, the norovirus particles are believed to get internalized via endocytosis mechanisms such as cholesterol- and dynamin-dependent [34, 35]. At this stage, the viral genome is released from the capsid and translocated to the endosomal membrane in order to enter the host cell cytoplasm. However, relatively little is known about detailed mechanisms of norovirus entry into cell's cytoplasm to date.

After entry of the positive sense viral genome into the host cell cytoplasm, it can immediately act as mRNA for protein synthesis. The subsequent event of norovirus life cyle is a pioneer round of viral proteins translation from the positive strand viral genomic RNA. The norovirus VPg (viral protein genome link) protein is a 13–15 kDa non-structural protein covalently linked to the 5′ end of the viral genomic (G RNA) and subgenomic RNA (SG RNA) and acts as a cap substitute (**Figure 1**). The VPg protein recruits host cells translation initiation factors in initiating the translation process to produce viral proteins [36, 37]. This mechanism is a unique strategy employed by noroviruses to ensure the preferential translation of their RNA over host cell mRNA which possess a classical 5′ cap structure. In fact, all caliciviruses use this translational strategy since their 5'UTR is relatively short (only 5 nucleotides in MNV) compared to the closely related picornavirus genome which contains a much longer 5'UTR. Even though the picornavirus genome also possesses a VPg at the 5'end, this smaller protein (~22 amino acids) does not have any sequence homology with the calicivirus VPg and is not involved in picornavirus translation. Indeed, picornavirus translation is driven by the presence of an internal ribosomal entry site (IRES) structure within its 5'UTR [38]. Translation of the first open reading frame of noroviruses typically yields a large polyprotein, representing the non-structural proteins. This large polyprotein is subjected to further processing by the virus encoded 3C-like (3CL) protease at five specific protease cleavage sites yielding six mature forms of the non-structural proteins [39, 40]. Sosnovtsev *et al.* have demonstrated that the proteolytic processing of MNV non-structural proteins in an *in vitro* system closely correlates to the products observed in infected RAW264.7 cells [25]. Uncleaved precursor proteins like NS6/NS7 (Pro-Pol) and NS1/NS2 can also be detected [25]. However, unlike the FCV protease and polymerase that functions as a fusion protein called p76 in infected cells, these proteins in noroviruses must be separated in order to be functionally active [25, 41].

The NS1/2 protein is the first non-structural protein in noroviruses (**Figure 1**) and is predicted to have a similar function to the picornavirus 2B protein, which is involved in membrane rearrangement and results in a modification of membrane permeability [42]. The enterovirus 2B protein which is a member of the *Picornaviridae* family is localized to the endoplasmic reticulum (ER) and the Golgi complex, reduces ER and Golgi complex calcium ion levels, and further inhibits protein trafficking through the Golgi complex [43]. Studies using Norwalk virus revealed that expression of the NS1/2 protein, also referred to as the N-Term protein, leads to Golgi disassembly, indicating a potential role for this protein in replication complex formation [42, 44].

The norovirus NS3 protein is a nucleotide triphosphatase (NTPase) (**Figure 1**). A study using a human norovirus (Southampton virus) showed that NS3 has NTPase activity that functions to hydrolyse nucleotide triphosphate [45]. In MNV infected cells, the NS3 has been shown to associate with the viral replication complex [46]. In addition, the equivalent protein in FCV called p39, was found to co-localize with viral replication complexes suggesting a possible role in replication [41, 47].

Little is known about the NS4 protein. However, it is thought that NS4 may play a role in tissue culture adaptation of MNV since repeated passage of MNV-1 in RAW264.7 cells give rise to attenuated viruses in part caused by sequence changes in NS4 [48]. Furthermore, NS4 is also thought to recruit VPg to membranous replication complexes during replication [46]. Targeted mutations in poliovirus 3A, the NS4 equivalent, resulted in viruses defective in RNA synthesis [49] indicating that by analogy, the norovirus NS4 may also contribute to viral RNA synthesis.

The NS5 encodes the viral VPg protein that plays a multifunctional role in the viral life cycle. The main role of VPg has been identified to be in translation initiation. This 13–15 kDa protein is covalently linked to the 5′ end of the G RNA and

SG RNA of caliciviruses [50]. VPg has been shown to be essential for viral RNA infectivity as treatment of viral RNA with proteinase K rendered the viral RNA non-infectious [51]. *In vitro* translation and infectivity of RNA are also abolished upon the removal of FCV and MNV VPg from viral RNA [50, 52]. However, *in vitro* transcribed capped FCV and MNV RNA generated from cDNA clone were infectious when transfected into cells [53, 54]. These observations indicate that the VPg plays a role as a cap substitute during the typical mRNA translation process. Using *in vitro* assays, MNV, FCV and Lordsdale virus VPg have been shown to bind the cap-binding eIF4F component, eIF4E [37, 52, 55]. Glutathione S-transferase (GST) pulldowns using Norwalk virus VPg demonstrated that other eIF4F components such as the eIF4A helicase and the scaffold protein eIF4G also associate with the translation complex [36]. Although both the FCV and MNV VPg proteins bind to eIF4E, only this interaction in FCV is essential as inhibition of eIF4E activity was found to severely affect FCV VPg linked RNA [37]. The same inhibition in MNV did not affect *in vitro* translation of MNV VPg linked RNA [52]. Differences were also observed in the requirement for eIF4A *in vitro,* where an increased requirement of MNV translation for eIF4A had been demonstrated [52]. Encoded by NS5 in the ORF 1 of the viral G RNA, the calicivirus VPg protein has also been shown to interact with the viral polymerase and capsid protein indicating a multifunctional role for this protein in the calicivirus life cycle [56].

The NS6 encodes the viral 3C-like protease and is thought to play a role in inhibition of cellular protein synthesis in infected cells. *In vitro* studies using recombinant norovirus $3CL^{pro}$ demonstrated that it cleaved polyA binding protein (PABP) [57] and the eukaryotic initiation factor eIF4G [58], both of which are required in mRNA translation of host cells. In FCV, the protease is present only in its active state when fused to the polymerase. The calicivirus 3C-like protease is released from the ORF1 polyprotein by autocatalytic cleavage, subsequently cleaving the other proteins in ORF1 with high specificity [41].

The NS7 protein, located at the C-terminus the norovirus ORF 1, encodes the RNA-dependent RNA polymerase (RdRp), which is a key enzyme in viral replication. This protein will be elaborated further in the subsequent subsection of this chapter because it plays a major role in viral G RNA and SG RNA replication.

The ORF2 and ORF3 of noroviruses code the structural proteins VP1 and VP2 respectively. Both of these proteins are expressed from the viral VPg-linked SG RNA that is 3′ co-terminal with the G RNA (**Figure 1**). However, in lagoviruses, sapoviruses and neboviruses, the capsid protein may also be produced from the G RNA as the capsid genes for these viruses are in frame with ORF1 giving rise to a polyprotein that contains both the non-structural proteins and the major capsid protein [59, 60]. ORF2 of norovirus encodes the 58.9 kDa major capsid protein (VP1) and ORF3 encodes the 22.1 kDa minor capsid protein (VP2) [61]. The expression of VP1 protein with or without co-expression of VP2 allows dimer formation that can be further assembled to produce VLPs in the absence of RNA genome [62–64]. Since the HuNv is currently not efficiently propagated in tissue culture, VLPs have been used to study a variety of virus-host interactions as they are morphologically and antigenically indistinguishable from real virus particles [4]. In FCV, the capsid protein contains a leader peptide (leader capsid or LC) at its N terminus that is cleaved by p76 to give rise to the mature capsid protein VP1. The VP2 protein has been shown to stabilize and protect VLPs from proteolytic degradation when this protein is co-expressed with VP1 in the baculovirus system [65]. The very basic character of VP2 suggests an interaction with nucleic acid and it may contribute to the encapsidation of the viral RNA. However, this hypothesis has yet to be examined and confirmed. Furthermore, at least for FCV, the VP2 protein is essential for the production of infectious particles and for virus replication [66]. In addition to

the ORF2 and ORF3, there is another alternative ORF, namely ORF4, which was found in MNV, overlapping with the VP1 coding region and encoding the virulence factor 1 protein (VF1) [67]. This VF1 protein has been demonstrated to play a role in infection and virulence *in vivo*. Infection of $STAT1^{-/-}$ mice with a mutant virus lacking the ability to express ORF4 resulted in a delayed onset of clinical signs compared with WT virus infected mice. Using a reverse genetics system, VF1 has been shown to function as a classical viral accessory protein that is not required for replication in tissue culture [67].

The pioneer round of viral proteins production is proceeded with G and SG RNA replication once the viral replication-related proteins are made available in the infected cell's cytoplasm. This particular process will be further elaborated in separate section below. When all the viral proteins become available and the replication has occurred, the viral RNA progeny is then packaged into viral particles. As mentioned earlier, the VP2 protein may contribute to this event. The mechanism of calicivirus encapsidation has yet to be studied in great detail. Present evidence suggests that the SG RNA could be encapsidated separately in the case of RHDV as well as in FCV [68, 69]. However, little is known about the mechanisms of viral release, but since norovirus infections induce apoptosis, it is speculated that apoptosis-induced membrane collapse releases the virus particles from the infected cells [70–72].

4. The norovirus genome replication

Once the translation of the norovirus non-structural proteins has begun, their presence in infected cells induces the formation of cytoplasmic membrane-bound replication complexes, enabling the viral genome replication process to take place [73]. These replication complexes, which contain the viral RdRp, viral RNA (single and double-stranded intermediates) and other viral enzymes and host cell factors, act as a surface or platform for the viral replication. The rearrangement of intracellular membranes (particularly the endoplasmic reticulum and Golgi apparatus) of MNV-1 infected RAW264.7 cells has been observed whereby membrane vesicles start to appear at twelve hours post infection [74]. The elaboration of norovirus genome replication in this chapter will be done interchangeably with the function of the central replication enzyme, RdRp.

The RdRp, also known as the RNA replicase, is an enzyme that catalyzes the synthesis of RNA from RNA templates. This particular virus enzyme is therefore distinct from the typical eukaryotic DNA-dependent RNA polymerase that catalyzes transcription of mRNA from a DNA template. All RNA viruses carry an RdRp gene in their RNA genome since this viral replication enzyme is pivotal for genome replication in infected cells. In addition, the virions of negative strand and double-stranded genome viruses must contain the RdRp as a ribonucleoprotein component since the incoming RNA genome cannot be translated or copied directly by the cellular machinery. The first viral RdRp was discovered in the early 1960's from poliovirus (PV). The poliovirus RNA polymerase (PV3D) is one of the best-studied viral RdRp and is often used as a reference for other newly identified RdRps. Studies including structural, RNA binding, nucleoside triphosphate (NTP) binding, polymerization of nucleotides, RNA strand displacement, and interactions with other viral proteins have been thoroughly investigated for PV3D [75–78].

Most of our understanding on the properties of viral RdRps comes from *in vitro* studies using purified proteins. This includes the initiation of RNA synthesis that is driven by RdRps. The mechanism of RNA synthesis initiation is divergent between RdRps from different viruses. However, common mechanisms have been

determined to be the *de novo* and the primer-dependent initiation. The presence of an RdRp, an RNA template, the initiating NTP (NTPi) and a second NTP is required in order to achieve the *de novo* initiation. The initiating NTPi, sometimes known as the one-nucleotide primer provides the 3′-hydroxyl (OH) group for the addition of the next nucleotide and elongation usually follows immediately [79]. The *de novo* initiation normally occurs at 3'end of viral RNA. However, internal initiation may also appear as in the case of SG RNA synthesis. For RdRps that employ a primer for primer-dependent initiation, the primer can be a protein-linked oligonucleotide (i.e; VPg-pU-pU, as in the case of picornavirus) or oligonucleotides with a 5'end capped structure that is cleaved from the cellular mRNA in a process called 'cap-snatching' (as used by many segmented negative strand RNA viruses such as influenza virus) [79]. Some viral RdRps also exhibit the terminal transferase activity that confers an ability to incorporate NTPs at the 3′ end of viral RNA template. RdRps with this property can initiate RNA replication by 'copy-back' or 'template-primed' synthesis mechanism. Incorporation of NTPs at the 3′ end of RNA template forms a loop structure able to fall back onto the RNA template and eventually serve as a primer for the RdRp to carry on with elongation. Terminal transferase activity for hepatitis C virus [80], poliovirus [81] and more significantly for HuNv RdRp [82] has been reported *in vitro* whereby the detection of double length RNA as a predominant product compared to the template RNA used in the reaction is often observed. However, this 'copy-back' synthesis by RdRp could theoretically be an artifact of *in vitro* reactions [79].

The RdRp gene of noroviruses is located at the C-terminal of non-structural polyprotein. With an approximate size of 57.5 kDa (in MNV), this virally encoded non-structural (NS7 in MNV) protein plays a key role in norovirus G RNA and SG RNA replication. Generally, the replication of G RNA is achieved through a negative sense RNA intermediate which serves as a template for the production of nascent positive sense viral G RNA. This general mechanism also applies to the caliciviruses where the presence of negative sense G RNA as well as SG RNA has been shown by Northern blot analysis during the infection of FCV in tissue culture. Currently, four main mechanisms for the initiation of RNA synthesis by recombinant calicivirus (including norovirus) RdRps have been demonstrated *in vitro*. They are: a *de novo* initiation and primer-independent initiation [82–84], back-priming base initiation [79, 85, 86] and a protein-primed initiation via VPg nucleotidylylation [84, 87]. The biochemical features of bacterially expressed recombinant RdRp noroviruses (HuNV and MNV) have been well characterized and *in vitro* enzymatic activity has been described [87–90]. Out of these four established mechanisms however, the *de novo* initiation is the proposed model to be employed by norovirus for the synthesis of both the G RNA and SG RNA by direct interaction between viral RdRp with its' VP1 (at the shell domain). A cell-based assay supported this proposed model through indirect measurement of 5′-triphosphorylated RNA production by the RdRp [91].

5. Production of subgenomic RNA in other viruses relative to norovirus

The genome organization and strategies for gene expression of positive strand RNA viruses are diverse. In addition to the occurrence of specific proteolytic cleavage sites which mediate the translational processing of the large polyprotein and give rise to several mature proteins encoded by one large ORF, many viruses often express their downstream ORFs through the transcription and translation of a SG RNA. Generally, SG RNAs of positive strand RNA viruses are identical to the 3′ ends of their parental G RNA. However, they vary in length where the 5′ end of

these SG RNAs are in proximity with the start codon of respective ORF. In most cases, these viral SG RNAs carry the ORFs that code for proteins required in the intermediate and late stages of infection, such as the structural proteins. Animal positive stranded RNA viruses that produce SG RNA include the *Coronaviridae* and *Arteriviridae* family of *Nidovirales* order, *Togaviridae*, *Nodaviridae*, *Astrovoridae* and *Caliciviridae* families. However, the vast majority of plant viruses have been demonstrated to produce SG RNAs. These viruses are from the *Luteoviridae*, *Bromoviridae*, *Tombusviridae* and *Closteroviridae* families and the *Tobravirus*, *Carlavirus*, *Tymovirus*, *Potexvirus*, *Hordeivirus*, *Tobamovirus*, *Sobemovirus* and *Furovirus* genera [92]. The mechanism of SG RNA synthesis has been studied in more detail in plant viruses than in animal viruses. Therefore, most of our understanding of the mechanisms of how SG RNA synthesis is achieved comes from established models for plant viruses.

There are currently two well-characterized and one additional mechanism for positive strand RNA virus SG RNA synthesis. The first described mechanism and the most widely recognized model is internal initiation, which has been clearly demonstrated in studies involving brome mosaic virus (BMV) (*Bromoviridae* family). In this instance the viral RdRp initiates (+) strand SG RNA4 transcription internally at a specific promoter region on the full-length (−) strand template of G RNA3 [93]. The BMV genome is composed of three positive sense, capped RNAs. RNA1 (monicistronic) encodes protein 1a with capping and putative RNA helicase activities. RNA2 (monocistronic) encodes protein 2a, a putative RNA-dependent RNA polymerase. RNA3 (bicistronic) encodes for two proteins: 3a, which is required for cell-to-cell movement, and the capsid protein. The capsid is translated from a subgenomic RNA, RNA4 [94]. The transcription of SG RNA4 is driven by the interaction of the replicase with the promoter sequence which functions on the minus-strand RNA3 and is situated directly upstream of the SG RNA4 initiation site. Initial studies showed that at least four key nucleotides in the core promoter are recognized by the viral replicase prior to the initiation of SG RNA4 synthesis highlighting the importance of primary RNA sequences in the SG RNA promoter [95]. Subsequent studies however, showed that a short RNA hairpin in the core promoter serves as the replicase binding site and that some of the key nucleotides help to form a stable hairpin structure in this core promoter region [96, 97]. Eventually, Sivakumaran et al. [98] concluded that the key nucleotides in the core promoter as reported previously act by directing replicase recognition. Whilst the formation of stem-loop is only required at a step after the binding of replicase to this promoter region [98].

Animal viruses such as Sindbis virus (alphavirus) and Rubella virus (rubivirus) from the *Togaviridae* family have also been extensively studied as models for internal initiation of SG RNA synthesis [99]. The Sindbis virus genome consists of an 11.7 kb positive strand RNA which is capped at its 5'end and is polyadenylated at the 3'end (**Figure 2**) [100]. The four alphavirus non-structural proteins (nsP1234; which involve in catalysis the genome replication) are translated from the 5′ ORF, and are synthesized as a polyprotein, which are subsequently processed into individual proteins. The 3' ORF codes for the three structural proteins; capsid (C) and envelope proteins (E123) are translated from the SG RNA. Synthesis of SG RNA is mediated via an internal promoter on the (−) strand viral RNA. The minimal sequence on the (−) strand RNA which has SG promoter activity *in vivo* corresponds to a region from −19 to +5 on the viral genome, using the initiation nucleotide of the SG RNA (nucleotide 7598 of the viral genome) as +1 [101]. Further studies have shown that a longer nucleotide sequence from −98 to +14 is required to obtain a more efficient SG RNA transcription [102]. On the other hand, the *in vitro* synthesis of SG RNA using a cell-free system proved that the internal initiation

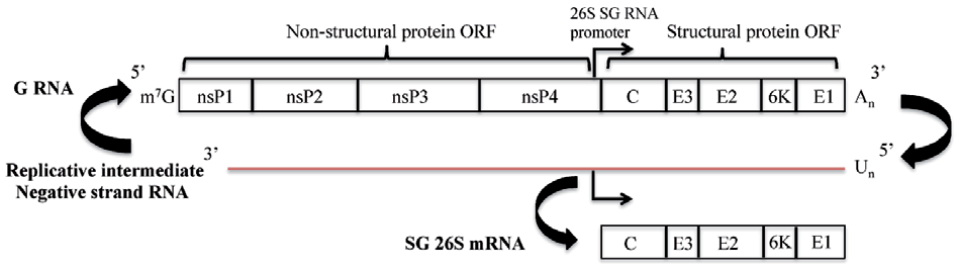

Figure 2.
Schematic representations of alphavirus virus genome organization. The genomic RNA (G RNA) has a methyl guanine cap structure (m^7G) at the 5'end and a polyadenylated tail (an) at the 3'end. The non-structural proteins are translated from non-structural protein ORF from the G RNA while the structural proteins are translated from SG 26S RNA that is transcribed from a replication intermediate negative strand RNA and the 26S subgenomic RNA promoter. The figure is adapted from Spurgers and Glass [100].

mechanism is employed, where the critical component which is a minus-strand promoter-template corresponding to the region of the Sindbis virus genome from nucleotide 7441 to nucleotide 7772 (−157 to +175 relative to the SG RNA transcription initiation site at nucleotide 7598) [103]. Therefore, it could be concluded that sometimes, the minimal promoter requirements *in vitro* are generally insufficient *in vivo*. Additional sequences are required *in vivo* to allow the replicase complex to come into proximity with the core promoter. Such requirements may not be critical in highly purified *in vitro* systems [92].

The second mechanism for SG RNA synthesis is termed as a premature termination and occurs during the (−) strand template synthesis from the full length (+) strand G RNA. This premature termination gives rise to a subgenomic-length (−) strand RNA that then serves as a template for subsequent end-to-end (+) strand SG RNA synthesis. The generation of this smaller subgenomic-length (−) strand complementary RNA is due to the early disengagement of the RdRp when it reaches a RNA secondary structure in the (+) strand viral genome template (known as a termination signal). These RNA structures are normally comprised of either local secondary structures or long-distance RNA interactions that form a highly ordered structure. The plant virus tomato bushy stunt virus (TBSV), the prototype member of the *Tombusviridae* family, provides the best-studied and complex example for premature during SG RNA synthesis. This virus was first isolated from tomato plants in 1935 where it causes stunting of growth, leaf mottling, and deformed or absent fruit. The size of the (+) strand TBSV genome is 4.8 kb in length and it contains five functional ORFs [104]. The 5′-terminal ORF encodes p33 and a read through product p92. These two proteins are the only viral proteins required for viral RNA synthesis, and both are translated directly from the viral G RNA [105]. The translation of the other three proteins (p41, p22 and p19) is supported by the production of two SG RNAs. The coat protein, p41 is translated from the SG RNA1 while the p22 (cell to cell movement) and the p19 (suppression of host defense mechanism) proteins are translated from SG RNA2 via overlapping ORFs [106]. The employment of a premature termination mechanism for TBSV SG RNAs synthesis is mediated by the formation of two different sets of long-distance RNA–RNA interactions, both present in the positive strand genomic RNA. The first one involves an RNA sequence located immediately 5′ to the site of transcriptional initiation of SG RNA1 called receptor sequence (RS1) and partner segments positioned ~1000 nucleotides upstream called activator sequence (AS1) which mediate the transcription of SG RNA1 [107]. The second interaction which mediates the synthesis of SG RNA2 involves the distal element (DE) which is located ~1100 nucleotides upstream from the initiation site of SG RNA2 transcription. This DE must base pair with a

portion of the core element (CE) located just 5′ to the SG RNA2 initiation site [108]. Furthermore, another long distance interaction between AS2/RS2 has been identified and is essential (along with DE/CE) for regulating the production of SG RNA2 [104]. It is possible that the AS/RS structure could be bound by a protein factor that stabilizes them, therefore facilitating the premature termination step of RNA copying by the viral replicase. In another plant virus that utilizes the premature termination mechanism for SG RNA synthesis, a more complex RNA–RNA interaction has been demonstrated. In red clover necrotic mosaic virus (RCNMV), an AS/RS-like interaction is also essential for SG RNA transcription from RNA1. However, this interaction forms in *trans* between the two G RNA segments called RNA1 and RNA2 [109].

In addition to the two well-characterized SG RNA synthesis mechanisms described above, there is another more unusual mechanism employed by members of the families *Coronaviridae* and *Arteriviridae* from the *Nidovirales* order, known as discontinuous transcription. Viruses from these families contain a very large positive sense RNA genome (between 15 and 31 kb) and produce a nested set of seven 3′ co-terminal SG RNAs. Uniquely, all these SG RNAs contain a 90 nucleotide leader sequence derived from the 5′ end of the G RNA. These SG RNAs are synthesized from non-contiguous sequences at the 5′ and 3′ ends, respectively, of the viral (+) strand genome. The leader and body of SG RNAs are separated by a conserved sequence found in the intergenic regions (IG) that can be found at the 3'end of the leader and at the 5′ end of the SG RNA body. Discontinuous transcription occurs during (−) strand RNA synthesis. Most of the (+) strand RNA template is not copied, perhaps because it loops out as the polymerase completes the synthesis of leader RNA. The resulting (−) strand RNAs with leader sequences at the 3′ ends, are then copied to form the various length SG RNAs.

6. The norovirus subgenomic RNA transcript and its translational products

All noroviruses produce a SG RNA during their replication cycle in infected cells. This SG RNA is 3′ co-terminal with the full-length G RNA, has VPg linked at the 5′ end and carries a poly-A tail at the 3′ end. Typically, the SG RNAs of noroviruses contains ORF2 and ORF3 (and ORF4 in the case of MNV and some sapoviruses) which code for viral structural proteins (VP1 and VP2). The production of a SG RNA message may act to delay the production of structural proteins until the initial rounds of viral replication have taken place. Both positive and negative sense SG RNA intermediates (~2.5 kb in length) can be detected by northern blot analysis of purified FCV replication complexes [73].

Following the transcription of MNV SG RNA, the expression of this messenger transcript via VPg-dependent translation initiation is achieved as described for the G RNA. The 5′ proximal ORF2, which encodes the major capsid protein is first translated when the scanning ribosomal complex encounters the first AUG codon, a typical strategy for translation. However, in viruses with polycistronic SG RNAs, the translation of their 3′ terminal ORF is not as efficient as the preceding ORF. Therefore, many viruses employ several strategies to provide sufficient access for ribosomes to downstream ORFs. These strategies include leaky scanning of 40S subunits past the start codon of the first ORF, the possession of intercistronic internal ribosome entry signal, programmed ribosomal frame-shifting during elongation and stop codon suppression at the termination step [110]. All noroviruses SG RNA are bicistronic messages. The translation of the 3′ proximal ORF in this case is achieved by a unique mechanism called termination reinitiation. In this

mechanism, a proportion of the 40S ribosomal subunit remains associated with the mRNA following the translational termination at the preceding stop codon. This enables reinitiation at the AUG of a downstream ORF, which is in close proximity. This characteristic has been observed for different caliciviruses where the initiation codon of VP2 (overlapped with VP1) is only 2 nucleotides away from the stop codon of VP1 for RHDV. Meanwhile for Norwalk virus, FCV and MNV, the start codon of VP2 is overlapped with the stop codon of VP1 [111]. Other than the close proximity between the stop and start codon, the efficiency of termination-reinitiation translation is also determined by a stretch of 70 to 80 nucleotides upstream of the stop-start window which facilitates the transit of the ribosome through the stop codon of VP1. This region of conserved sequence is termed TURBS (termination upstream ribosome binding site motif). The translation of VP2 from the FCV, RHDV and MNV SG RNA is dependent on this TURBS region, which is located immediately upstream of the VP1 stop codon [110–112]. The TURBS contain two important sequences; the 5′ sequence (termed as Motif 1) is proposed to function in binding the 18S rRNA (through complementary sequence) whilst the other sequence is thought to be important in tethering the ribosome to enable translation of VP2 at the correct site [110, 113]. Alternatively, the TURBS may also act by interacting with eIF3 or eIF3/40S ribosome complexes preventing disassembly of the ribosome following VP1 translation termination. This alternative mechanism is supported by the fact that purified eIF3 is able to stimulate translational re-initiation [114].

7. The replication of norovirus subgenomic RNA

The presence of SG RNA of norovirus in infected cells is often used as indicator for active viral genome replication. Importantly, the mechanism that is used by noroviruses to achieve their SG RNA transcription is poorly understood until very relatively recently. Initial evidence from *in vitro* studies using RHDV RNA transcripts suggests that the internal initiation mechanism is employed [115]. *In vitro* promoter mapping analysis using a panel of nested negative sense RNA templates that included the region before the start of ORF2, demonstrated that the RHDV RdRp requires 60 bases upstream of the start of the SG RNA transcription start site in order to produce SG RNA [115]. At this point, this finding indicates the existence of a promoter site upstream of the SG RNA start site that enables the binding of RdRp and internally initiates the SG RNA synthesis on the negative strand G RNA. Subsequently, in another study involving MNV, mutational analysis of an evolutionarily conserved RNA stem loop structures using the available reverse genetics system has highlighted that the stability of a specific RNA structure is critical for MNV replication [116]. This specific RNA stem loop structure was consistently detected exactly 6 nucleotides upstream of the SG RNA start site in all caliciviruses on both the positive (SL5018) and negative strand RNA (SLa5045) (**Figure 3**) [116]. This observation implies that these RNA secondary structures may accommodate a functional role in viral SG RNA synthesis. Furthermore, the stem loop structure was generally found to be more stable on the negative strand genome (SLa5045) than the positive strand. Thus, such a structure was initially hypothesized to play a role as the putative SG RNA promoter for the synthesis of MNV SG RNA via internal initiation mechanism. It is also possible that after internal initiation has occurred to produce newly synthesized VPg-linked SG RNA, this RNA may then be picked up by the viral replication machinery and replicated in a similar manner to the G RNA, effectively producing a negative strand SG RNA molecule.

As reported by Simmonds et al. [116], a mutant cDNA clone containing a series of non-coding mutations called m53 that destabilized the RNA structure was

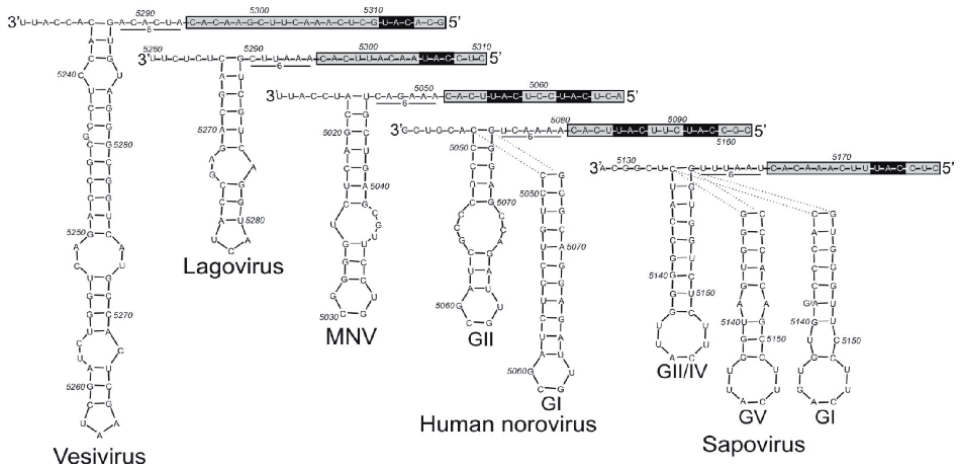

Figure 3.
Conserved RNA secondary structures upstream of the subgenomic transcript predicted by Alifold programme for the 5 calicivirus genera. The stable small secondary stem loop structure was consistently found 6 nucleotides upstream of the MNV SG RNA initiation site. The stem loop is shown in its antisense orientation (SLa5045). Gray filled boxes represents the SG RNA start site and black boxes represents the ORF2 initiation codon. The unpaired 6 nucleotides sequences between the predicted structure and the subgenomic start site are underlined (figure is taken from Simmonds et al. [116]).

generated. These mutations were designed to destabilize the stem loop structure by weakening the base pairing without affecting the NS7 coding sequence. This mutated cDNA clone was used in the DNA-based reverse genetics system and reported to cause a lethal phenotype effect, whereby no infectious virus can be detected by TCID50 in the recoveries. However, by compensating the initial m53 mutations to restore the base pairing within the stem loop structure (called m53r), a viable virus was recovered with a titer close to that of the wild type virus. This series of experiments concluded that RNA stem loop structure is important for viral replication and might function as part of the SG RNA promoter. Even though the m53 mutation disrupting SLa5045 caused a lethal phenotype, serial "blind" passage of the recoveries (from the DNA based reverse genetics system) in RAW264.7 cells often produced viable viruses. Sequence analysis revealed that these viable viruses contained two types or classes of mutation. The first class were phenotypic-revertant viruses where nucleotide changes were identified that resulted in partial reformation of the SLa5045 stem loop structure. The ability to isolate phenotypic revertant viruses that repaired the defective RNA structure was not unexpected as the stem loop structure is predicted to play an important role in viral replication. This observation indicates that m53 mutation in the viral genome results in poor viral genome replication in tissue culture. Phenotypic-revertant mutations arise in tissue culture and those that promote replication are favored and amplified during the serial "blind" passage until they become dominant. Another type of mutation observed were suppressor mutations, whereby the m53 mutation in the SLa5045 was still present, but changes outside the stem loop structure, within the NS7 coding region, were also identified [117]. Further characterization of this suppressor mutant viruses in cell culture revealed that they possess a slower growth kinetics, lower-level proteins production and lower-level of G RNA and SG RNA transcripts synthesis compared to WT virus [117]. More importantly, these data indicate that nucleotide changes were responsible for the suppression phenotype rather than any amino acid change, suggesting the potential involvement of long-range RNA–RNA interactions between SLa5045 and a region ~100 nucleotides upstream of this Sla5045 stem loop structure [117]. However, this hypothesis is yet to be proven with

scientific experimental and the current readily available bioinformatics tools are not adequate to accurately predict such long range RNA–RNA interaction. On the other note, such long range RNA–RNA interactions between promoter regions are well established with some sequences being up to ~1500 nucleotides apart e.g. the nodavirus Flock House virus (FHV) and tombusvirus tomato bushy stunt virus (TBSV) have been documented to contain such interactions even though these viruses employ a premature termination mechanism for their SG RNA synthesis [118, 119]. In the case of MNV however, it is worth to note that this long-range RNA–RNA interaction presumably occurs on the negative strand RNA to produce a suppression effect on m53 mutation of the SLa5045.

Utilizing the MNV reverse genetics system, virus recoveries using series of modified cDNA with additional copy of SLa5045 in *cis* within the noncoding region upstream the capsid gene (SLa5045Dup) showed that only m53r mutation and two nucleotide changes at the terminal loop in the second copy stem loop of m53 backbone construct (m53/m53r and m53TL-Dis) produced detectable viable virus [117]. The other construct namely SLa5045Dup m53/SLa WT + 8, whereby the nucleotide spacing of the stem loop and initiation site of the SG RNA was increased from 6 to 8 nucleotides, failed to produce any viable virus. In addition, total sequence modification that retained or totally disrupt the stem structure in the additional SLa5045 also caused a debilitating effect to the virus [117]. These constructs were designed with the aim to introduce a more synonymous mutations since the second copy of SLa5045 is located at the noncoding region compared to the first stem loop which positioned at the NS7 coding region. Any extensive mutations introduced in the first copy of the structure would affect the NS7 coding capacity. These set of data demonstrate that the sequence, exact location in the norovirus genome and stability of SLa5045 are mandatory for virus replication [117].

Even though the presence of low levels of negative sense SG RNA have been argued for the premature termination of negative sense G RNA during elongation by RdRp that produces SG-length negative sense RNA transcript (act as template for positive sense SG RNA) [73, 120], a more detailed study suggests that norovirus SG RNA replication follows the internal initiation mechanism. Employing genetics and biochemical tools, a recent study demonstrates that accurate norovirus SG RNA synthesis is depend on a sequence and genotype-specific interaction of the viral RdRp with a stem-loop sequence (SLa5045) on the minus-strand RNA [117]. In that study, the investigators performed an *in vitro* RNA synthesis assay involving series of chemically synthesized RNA templates containing the SLa5045 sequence (from MNV and human GII.4 norovirus) that called proscripts and recombinant MNV RdRp. The outcomes of that specific experiment indicate that the norovirus RdRp is capable of recognizing the stem loop sequence and subsequently direct the RNA synthesis. Therefore, the role of stem loop structure as core promoter for norovirus SG RNA synthesis has been established. However, whether there was any direct interaction between the RNA stem loop with RdRp remained unclear until a more detailed biochemical study came out later in 2015. Using a reversible crosslinking peptide fingerprinting analysis (RCAP) in one of the mapping studies, the investigators identified that 17 peptides originating from MNV RdRp were associated with RNA proscripts that contained the noroviruses SG RNA core promoter sequences (from MNV and HuNv GII.4) [121]. Based on the MNV-1 crystal structure, most of these cross-linked peptides are precisely located in the central cavity of the enzyme which is critical for RNA synthesis [121]. A more detailed mutational and functional analysis also revealed that residues R411 (arginine at position 411) and R416 (arginine at position 416) of amino acid sequence in MNV RdRp contributed to the binding towards subgenomic promoter hairpin [121]. These series of studies concluded that the noroviruses are highly likely employing an internal initiation mechanism for their SG RNA synthesis.

8. Conclusion

The synthesis of norovirus SG RNA is a clear signal for the existence of genome replication since the production of this smaller RNA (that is 3′ co-terminal with the full length viral genome) is dependent on efficient genome replication in infected cells. Furthermore, the transcription of SG RNA at the middle and latter stages of infection is also thought to regulate the production of infectious virions. Since the capsid proteins of noroviruses are translated from the SG RNA messenger, the encapsidation process is initiated once the viral RNA replication begins. Investigations on the involvement of functional RNA elements in regulating the synthesis of the MNV SG RNA were carried out extensively to determine the mechanism employed by noroviruses in their genome replication accurately. Based on the establised data available recently, now clear that we could confidently presume that norovirus follows the internal initiation mechanism for the synthesis of SG RNA. The studies also proved the crucial role of small stem loop/hairpin structure within the coding region of NS7 in the viral replication.

Acknowledgements

The author would like to acknowledge Research University Grant (RUI), Universiti Sains Malaysia (1001/CIPPT/8012205).

Conflict of interest

The author declares no conflict of interest.

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References

[1] D. Bailey and I. Goodfellow, "Noroviruses," *eLS*. 15-Mar-2009, doi: https://doi.org/10.1002/9780470015902.a0000420.

[2] R. Dolin *et al.*, "Transmission of acute infectious nonbacterial gastroenteritis to volunteers by oral administration of stool filtrates.," J. Infect. Dis., vol. 123, no. 3, pp. 307-312, Mar. 1971, doi: 10.1093/infdis/123.3.307.

[3] M. E. Hardy, "Norwalk and 'Norwalk-Like Viruses' in Epidemic Gastroenteritis," *Clin. Lab. Med.*, vol. 19, no. 3, pp. 675-690, 1999, doi: https://doi.org/10.1016/S0272-2712(18)30110-0.

[4] M. K. Estes, B. V Prasad, and R. L. Atmar, "Noroviruses everywhere: has something changed?," Curr Opin Infect Dis, vol. 19, no. 5, pp. 467-474, 2006.

[5] R. L. Atmar *et al.*, "Norwalk virus shedding after experimental human infection," Emerg. Infect. Dis., vol. 14, no. 10, pp. 1553-1557, 2008.

[6] C. I. Gallimore *et al.*, "Environmental monitoring for gastroenteric viruses in a pediatric primary immunodeficiency unit," J. Clin. Microbiol., vol. 44, no. 2, pp. 395-399, 2006, doi: 10.1128/JCM.44.2.395-399.2006.

[7] I. Boxman *et al.*, "Norovirus on swabs taken from hands illustrate route of transmission: a case study," J. Food Prot., vol. 72, no. 8, pp. 1753-1755, 2009.

[8] M. A. Widdowson *et al.*, "Norovirus and foodborne disease, United States, 1991-2000," Emerg. Infect. Dis., vol. 11, no. 1, pp. 95-102, 2005.

[9] P. Tian, A. L. Engelbrektson, X. Jiang, W. Zhong, and R. E. Mandrell, "Norovirus recognizes histo-blood group antigens on gastrointestinal cells of clams, mussels, and oysters: a possible mechanism of bioaccumulation," J. Food Prot., vol. 70, no. 9, pp. 2140-2147, 2007.

[10] P. J. Marks, I. B. Vipond, D. Carlisle, D. Deakin, R. E. Fey, and E. O. Caul, "Evidence for airborne transmission of Norwalk-like virus (NLV) in a hotel restaurant," Epidemiol. Infect., vol. 124, no. 3, pp. 481-487, 2000.

[11] M. A. Widdowson *et al.*, "Probable transmission of norovirus on an airplane," JAMA, vol. 293, no. 15, pp. 1859-1860, 2005, doi: 10.1001/jama.293.15.1859.

[12] P. F. Teunis *et al.*, "Norwalk virus: how infectious is it?," J. Med. Virol., vol. 80, no. 8, pp. 1468-1476, 2008, doi: 10.1002/jmv.21237.

[13] A. Z. Kapikian, "The discovery of the 27-nm Norwalk virus: an historic perspective," *J. Infect. Dis.*, vol. 181 Suppl, pp. S295-302, 2000, doi: 10.1086/315584.

[14] P. S. Mead *et al.*, "Food-related illness and death in the United States," Emerg Infect Dis, vol. 5, no. 5, pp. 607-625, 1999.

[15] B. A. Lopman, A. J. Hall, A. T. Curns, and U. D. Parashar, "Increasing rates of gastroenteritis hospital discharges in US adults and the contribution of norovirus, 1996-2007," Clin. Infect. Dis., vol. 52, no. 4, pp. 466-474, 2011, doi: 10.1093/cid/ciq163.

[16] B. A. Lopman, M. H. Reacher, Y. Van Duijnhoven, F. X. Hanon, D. Brown, and M. Koopmans, "Viral gastroenteritis outbreaks in Europe, 1995-2000," Emerg Infect Dis, vol. 9, no. 1, pp. 90-96, 2003.

[17] B. A. Lopman *et al.*, "Epidemiology and cost of nosocomial gastroenteritis, Avon, England, 2002-2003," Emerg. Infect. Dis., vol. 10, no. 10, pp. 1827-1834, 2004.

[18] J. Treanor *et al.*, "A phase 2 study of the bivalent VLP norovirus vaccine candidate in older adults; impact of MPL adjuvant or a second dose," *Vaccine*, vol. 38, no. 36, pp. 5842-5850, 2020, doi: https://doi.org/10.1016/j.vaccine.2020.06.011.

[19] S. M. Karst, C. E. Wobus, M. Lay, J. Davidson, and H. W. Virgin IV, "STAT1-dependent innate immunity to a norwalk-like virus," *Science (80-.).*, 2003, doi: 10.1126/science.1077905.

[20] A. M. Hutson, R. L. Atmar, and M. K. Estes, "Norovirus disease: changing epidemiology and host susceptibility factors," Trends Microbiol., vol. 12, no. 6, pp. 279-287, 2004, doi: 10.1016/j.tim.2004.04.005.

[21] B. V Prasad, M. E. Hardy, T. Dokland, J. Bella, M. G. Rossmann, and M. K. Estes, "X-ray crystallographic structure of the Norwalk virus capsid," Science (80-.)., vol. 286, no. 5438, pp. 287-290, 1999.

[22] R. Chen *et al.*, "Inter- and intragenus structural variations in caliciviruses and their functional implications," J. Virol., vol. 78, no. 12, pp. 6469-6479, 2004, doi: 10.1128/JVI.78.12.6469-6479.2004.

[23] M. Tan, R. S. Hegde, and X. Jiang, "The P domain of norovirus capsid protein forms dimer and binds to histo-blood group antigen receptors," J. Virol., vol. 78, no. 12, pp. 6233-6242, 2004, doi: 10.1128/JVI.78.12.6233-6242.2004.

[24] M. J. Carter, I. D. Milton, J. Meanger, M. Bennett, R. M. Gaskell, and P. C. Turner, "The complete nucleotide sequence of a feline calicivirus," Virology, vol. 190, no. 1, pp. 443-448, 1992.

[25] S. V Sosnovtsev *et al.*, "Cleavage Map and Proteolytic Processing of the Murine Norovirus Nonstructural Polyprotein in Infected Cells," J Virol, vol. 80, no. 16, pp. 7816-7831, 2006.

[26] P. Huang *et al.*, "Noroviruses bind to human ABO, Lewis, and secretor histo-blood group antigens: identification of 4 distinct strain-specific patterns," J Infect Dis, vol. 188, no. 1, pp. 19-31, 2003.

[27] R. Dolin, "Noroviruses--challenges to control," N Engl J Med, vol. 357, no. 11, pp. 1072-1073, 2007.

[28] A. M. Hutson, F. Airaud, J. LePendu, M. K. Estes, and R. L. Atmar, "Norwalk virus infection associates with secretor status genotyped from sera," J Med Virol, vol. 77, no. 1, pp. 116-120, 2005.

[29] M. Thorven *et al.*, "A homozygous nonsense mutation (428G-->A) in the human secretor (FUT2) gene provides resistance to symptomatic norovirus (GGII) infections," J Virol, vol. 79, no. 24, pp. 15351-15355, 2005.

[30] S. Taube *et al.*, "Ganglioside-linked terminal sialic acid moieties on murine macrophages function as attachment receptors for murine noroviruses," J. Virol., vol. 83, no. 9, pp. 4092-4101, 2009, doi: 10.1128/JVI.02245-08.

[31] J. W. Perry, S. Taube, and C. E. Wobus, "Murine norovirus-1 entry into permissive macrophages and dendritic cells is pH-independent," Virus Res., vol. 143, no. 1, pp. 125-129, 2009, doi: 10.1016/j.virusres.2009.03.002.

[32] K. Haga *et al.*, "Functional receptor molecules CD300lf and CD300ld within the CD300 family enable murine noroviruses to infect cells," Proc. Natl. Acad. Sci. U. S. A., vol. 113, no. 41, pp. E6248–E6255, Oct. 2016, doi: 10.1073/pnas.1605575113.

[33] F. Borrego, "The CD300 molecules: an emerging family of regulators of the immune system," Blood, vol. 121, no. 11, pp. 1951-1960, Mar. 2013, doi: 10.1182/blood-2012-09-435057.

[34] J. W. Perry and C. E. Wobus, "Endocytosis of murine norovirus 1 into

murine macrophages is dependent on dynamin II and cholesterol.," J. Virol., vol. 84, no. 12, pp. 6163-6176, Jun. 2010, doi: 10.1128/JVI.00331-10.

[35] A. Gerondopoulos, T. Jackson, P. Monaghan, N. Doyle, and L. O. Roberts, "Murine norovirus-1 cell entry is mediated through a non-clathrin-, non-caveolae-, dynamin- and cholesterol-dependent pathway.," J. Gen. Virol., vol. 91, no. Pt 6, pp. 1428-1438, Jun. 2010, doi: 10.1099/vir.0.016717-0.

[36] K. F. Daughenbaugh, C. S. Fraser, J. W. Hershey, and M. E. Hardy, "The genome-linked protein VPg of the Norwalk virus binds eIF3, suggesting its role in translation initiation complex recruitment," Embo J, vol. 22, no. 11, pp. 2852-2859, 2003.

[37] I. Goodfellow *et al.*, "Calicivirus translation initiation requires an interaction between VPg and eIF4E.," EMBO Rep., vol. 6, no. 10, pp. 968-972, 2005.

[38] Y. Liu, E. Wimmer, and A. V Paul, "Cis-acting RNA elements in human and animal plus-strand RNA viruses," Biochim. Biophys. Acta, vol. 1789, no. 9-10, pp. 495-517, 2009, doi: 10.1016/j.bbagrm.2009.09.007.

[39] S. J. Blakeney, A. Cahill, and P. A. Reilly, "Processing of Norwalk virus nonstructural proteins by a 3C-like cysteine proteinase," Virology, vol. 308, no. 2, pp. 216-224, 2003.

[40] B. Liu, I. N. Clarke, and P. R. Lambden, "Polyprotein processing in Southampton virus: identification of 3C-like protease cleavage sites by in vitro mutagenesis," J. Virol., vol. 70, no. 4, pp. 2605-2610, 1996.

[41] S. V Sosnovtsev, M. Garfield, and K. Y. Green, "Processing map and essential cleavage sites of the nonstructural polyprotein encoded by ORF1 of the feline calicivirus genome," J Virol, vol. 76, no. 14, pp. 7060-7072, 2002.

[42] V. Fernandez-Vega *et al.*, "Norwalk virus N-terminal nonstructural protein is associated with disassembly of the Golgi complex in transfected cells," J. Virol., vol. 78, no. 9, pp. 4827-4837, 2004.

[43] A. S. de Jong *et al.*, "Functional analysis of picornavirus 2B proteins: effects on calcium homeostasis and intracellular protein trafficking," J Virol, vol. 82, no. 7, pp. 3782-3790, 2008.

[44] K. Ettayebi and M. E. Hardy, "Norwalk virus nonstructural protein p48 forms a complex with the SNARE regulator VAP-A and prevents cell surface expression of vesicular stomatitis virus G protein," J Virol, vol. 77, no. 21, pp. 11790-11797, 2003.

[45] T. Pfister and E. Wimmer, "Polypeptide p41 of a Norwalk-like virus is a nucleic acid-independent nucleoside triphosphatase," J Virol, vol. 75, no. 4, pp. 1611-1619, 2001.

[46] J. L. Hyde, S. V Sosnovtsev, K. Y. Green, C. Wobus, H. W. Virgin, and J. M. Mackenzie, "Mouse norovirus replication is associated with virus-induced vesicle clusters originating from membranes derived from the secretory pathway," J. Virol., vol. 83, no. 19, pp. 9709-9719, 2009, doi: 10.1128/JVI.00600-09.

[47] D. Bailey *et al.*, "Feline calicivirus p32, p39 and p30 proteins localize to the endoplasmic reticulum to initiate replication complex formation," J. Gen. Virol., vol. 91, no. Pt 3, pp. 739-749, 2010, doi: 10.1099/vir.0.016279-0.

[48] D. Bailey, L. B. Thackray, and I. G. Goodfellow, "A single amino acid substitution in the murine norovirus capsid protein is sufficient for attenuation in vivo," J. Virol., vol. 82, no. 15, pp. 7725-7728, 2008, doi: 10.1128/JVI.00237-08.

[49] H. D. Bernstein and D. Baltimore, "Poliovirus mutant that contains a

cold-sensitive defect in viral RNA synthesis," J. Virol., vol. 62, pp. 2922-2928, 1988.

[50] T. P. Herbert, I. Brierley, and T. D. Brown, "Identification of a protein linked to the genomic and subgenomic mRNAs of feline calicivirus and its role in translation," J Gen Virol, vol. 78, no. Pt 5, pp. 1033-1040, 1997.

[51] J. N. Burroughs and F. Brown, "Presence of a covalently linked protein on calicivirus RNA," J Gen Virol, vol. 41, no. 2, pp. 443-446, 1978.

[52] Y. Chaudhry *et al.*, "Caliciviruses differ in their functional requirements for eIF4F components," J Biol Chem, vol. 281, pp. 25315-25325, 2006.

[53] S. Sosnovtsev and K. Y. Green, "RNA transcripts derived from a cloned full-length copy of the feline calicivirus genome do not require VPg for infectivity," Virology, vol. 210, no. 2, pp. 383-390, 1995.

[54] M. A. Yunus, L. M. W. Chung, Y. Chaudhry, D. Bailey, and I. Goodfellow, "Development of an optimized RNA-based murine norovirus reverse genetics system.," J. Virol. Methods, vol. 169, no. 1, pp. 112-118, Oct. 2010, doi: 10.1016/j.jviromet.2010.07.006.

[55] K. F. Daughenbaugh, C. E. Wobus, and M. E. Hardy, "VPg of murine norovirus binds translation initiation factors in infected cells," Virol J, vol. 3, p. 33, 2006.

[56] W. J. Kaiser, Y. Chaudhry, S. V Sosnovtsev, and I. G. Goodfellow, "Analysis of protein-protein interactions in the feline calicivirus replication complex," J Gen Virol, vol. 87, no. Pt 2, pp. 363-368, 2006.

[57] M. Kuyumcu-Martinez, G. Belliot, S. V Sosnovtsev, K. O. Chang, K. Y. Green, and R. E. Lloyd, "Calicivirus 3C-like proteinase inhibits cellular translation by cleavage of poly(A)-binding protein," J. Virol., vol. 78, no. 15, pp. 8172-8182, 2004, doi: 10.1128/JVI.78.15.8172-8182.2004.

[58] M. M. Willcocks, M. J. Carter, and L. O. Roberts, "Cleavage of eukaryotic initiation factor eIF4G and inhibition of host-cell protein synthesis during feline calicivirus infection," J Gen Virol, vol. 85, no. Pt 5, pp. 1125-1130, 2004.

[59] R. L. Atmar and M. K. Estes, "Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses," Clin. Microbiol. Rev., vol. 14, no. 1, pp. 15-37, 2001, doi: 10.1128/CMR.14.1.15-37.2001.

[60] S. L. Oliver, E. Asobayire, A. M. Dastjerdi, and J. C. Bridger, "Genomic characterization of the unclassified bovine enteric virus Newbury agent-1 (Newbury1) endorses a new genus in the family Caliciviridae," Virology, vol. 350, no. 1, pp. 240-250, 2006, doi: 10.1016/j.virol.2006.02.027.

[61] I. N. Clarke and P. R. Lambden, "Organization and expression of calicivirus genes," *J Infect Dis*, vol. 181 Suppl, pp. S309-16., 2000.

[62] A. Bertolotti-Ciarlet, L. J. White, R. Chen, B. V Prasad, and M. K. Estes, "Structural requirements for the assembly of Norwalk virus-like particles," J. Virol., vol. 76, no. 8, pp. 4044-4055, 2002.

[63] K. Y. Green, A. Z. Kapikian, J. Valdesuso, S. Sosnovtsev, J. J. Treanor, and J. F. Lew, "Expression and self-assembly of recombinant capsid protein from the antigenically distinct Hawaii human calicivirus," J. Clin. Microbiol., vol. 35, no. 7, pp. 1909-1914, 1997.

[64] X. Jiang, M. Wang, D. Y. Graham, and M. K. Estes, "Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein," J. Virol., vol. 66, no. 11, pp. 6527-6532, 1992.

[65] A. Bertolotti-Ciarlet, S. E. Crawford, A. M. Hutson, and M. K. Estes, "The 3′ end of Norwalk virus mRNA contains determinants that regulate the expression and stability of the viral capsid protein VP1: a novel function for the VP2 protein," J Virol, vol. 77, no. 21, pp. 11603-11615, 2003.

[66] S. V Sosnovtsev, G. Belliot, K. O. Chang, O. Onwudiwe, and K. Y. Green, "Feline calicivirus VP2 is essential for the production of infectious virions," J. Virol., vol. 79, no. 7, pp. 4012-4024, 2005, doi: 10.1128/JVI.79.7.4012-4024.2005.

[67] N. McFadden *et al.*, "Norovirus Regulation of the Innate Immune Response and Apoptosis Occurs via the Product of the Alternative Open Reading Frame 4," *PLoS Pathog.*, vol. 7, no. 12, p. e1002413, 2011, doi: 10.1371/journal.ppat.1002413.

[68] G. Meyers, N. Tautz, E. J. Dubovi, and H. J. Thiel, "Viral cytopathogenicity correlated with integration of ubiquitin-coding sequences," Virology, vol. 180, pp. 602-616, 1991.

[69] J. D. Neill, "The subgenomic RNA of feline calicivirus is packaged into viral particles during infection," Virus Res, vol. 87, no. 1, pp. 89-93, 2002.

[70] C. Alonso, J. M. Oviedo, J. M. Martin-Alonso, E. Diaz, J. A. Boga, and F. Parra, "Programmed cell death in the pathogenesis of rabbit hemorrhagic disease," Arch Virol, vol. 143, no. 2, pp. 321-332., 1998.

[71] K. Bok, V. G. Prikhodko, K. Y. Green, and S. V Sosnovtsev, "Apoptosis in murine norovirus-infected RAW264.7 cells is associated with downregulation of survivin," J. Virol., vol. 83, no. 8, pp. 3647-3656, 2009, doi: 10.1128/JVI.02028-08.

[72] S. V Sosnovtsev, E. A. Prikhod'ko, G. Belliot, J. I. Cohen, and K. Y. Green, "Feline calicivirus replication induces apoptosis in cultured cells," Virus Res., vol. 94, no. 1, pp. 1-10, 2003.

[73] K. Y. Green *et al.*, "Isolation of enzymatically active replication complexes from feline calicivirus-infected cells," J Virol, vol. 76, no. 17, pp. 8582-8595, 2002.

[74] C. E. Wobus *et al.*, "Replication of Norovirus in cell culture reveals a tropism for dendritic cells and macrophages.," *PLoS Biol.*, vol. 2, no. 12, p. e432, Dec. 2004, doi: 10.1371/journal.pbio.0020432.

[75] D. W. Gohara, S. Crotty, J. J. Arnold, J. D. Yoder, R. Andino, and C. E. Cameron, "Poliovirus RNA-dependent RNA polymerase (3Dpol): structural, biochemical, and biological analysis of conserved structural motifs A and B," J Biol Chem, vol. 275, no. 33, pp. 25523-25532, 2000.

[76] J. L. Hansen, A. M. Long, and S. C. Schultz, "Structure of the RNA-dependent RNA polymerase of poliovirus," Structure, vol. 5, no. 8, pp. 1109-1122, 1997.

[77] O. C. Richards and E. Ehrenfeld, "Poliovirus RNA replication," Curr. Top. Microbiol. Immunol., vol. 161, pp. 89-119, 1990.

[78] E. Wimmer, C. U. T. Hellen, and X. M. Cao, "Genetics of Poliovirus," Annu. Rev. Genet., vol. 27, pp. 353-436, 1993.

[79] C. C. Kao, P. Singh, and D. J. Ecker, "De Novo Initiation of Viral RNA-Dependent RNA Synthesis," *Virology*, vol. 287, no. 2, p. 251, 2001.

[80] C. T. Ranjith-Kumar, J. Gajewski, L. Gutshall, D. Maley, R. T. Sarisky, and C. C. Kao, "Terminal nucleotidyl transferase activity of recombinant Flaviviridae RNA-dependent RNA polymerases: implication for viral RNA synthesis," J. Virol., vol. 75, no. 18, pp. 8615-8623, 2001.

[81] J. J. Arnold and C. E. Cameron, "Poliovirus RNA-dependent RNA polymerase (3Dpol) is sufficient for template switching in vitro," J Biol Chem, vol. 274, no. 5, pp. 2706-2716, 1999.

[82] J. Rohayem, K. Jager, I. Robel, U. Scheffler, A. Temme, and W. Rudolph, "Characterization of norovirus 3Dpol RNA-dependent RNA polymerase activity and initiation of RNA synthesis," J Gen Virol, vol. 87, no. 9, pp. 2621-2630, 2006, doi: 10.1099/vir.0.81802-0.

[83] S. Fukushi *et al.*, "Poly(A)- and primer-independent RNA polymerase of Norovirus," J Virol, vol. 78, no. 8, pp. 3889-3896, 2004.

[84] J. Rohayem, I. Robel, K. Jager, U. Scheffler, and W. Rudolph, "Protein-primed and de novo initiation of RNA synthesis by norovirus 3Dpol," J. Virol., vol. 80, no. 14, pp. 7060-7069, 2006, doi: 10.1128/JVI.02195-05.

[85] A. L. Lopez Vazquez, J. M. Martin Alonso, and F. Parra, "Characterisation of the RNA-dependent RNA polymerase from Rabbit hemorrhagic disease virus produced in Escherichia coli," Arch Virol, vol. 146, no. 1, pp. 59-69., 2001.

[86] L. Wei *et al.*, "Proteinase-polymerase precursor as the active form of feline calicivirus RNA-dependent RNA polymerase," J Virol, vol. 75, no. 3, pp. 1211-1219, 2001.

[87] K. R. Han *et al.*, "Murine norovirus-1 3Dpol exhibits RNA-dependent RNA polymerase activity and nucleotidylylates on Tyr of the VPg," J. Gen. Virol., vol. 91, no. Pt 7, pp. 1713-1722, 2010, doi: 10.1099/vir.0.020461-0.

[88] G. Belliot *et al.*, "Norovirus proteinase-polymerase and polymerase are both active forms of RNA-dependent RNA polymerase," J. Virol., vol. 79, no. 4, pp. 2393-2403, 2005, doi: 10.1128/JVI.79.4.2393-2403.2005.

[89] M. Hogbom, K. Jager, I. Robel, T. Unge, and J. Rohayem, "The active form of the norovirus RNA-dependent RNA polymerase is a homodimer with cooperative activity," J Gen Virol, vol. 90, no. Pt 2, pp. 281-291, 2009.

[90] J. H. Lee *et al.*, "Crystal structures of murine norovirus-1 RNA-dependent RNA polymerase," J. Gen. Virol., vol. 92, no. Pt 7, pp. 1607-1616, 2011, doi: 10.1099/vir.0.031104-0.

[91] C. V Subba-Reddy, I. Goodfellow, and C. C. Kao, "VPg-primed RNA synthesis of norovirus RNA-dependent RNA polymerases by using a novel cell-based assay," J. Virol., vol. 85, no. 24, pp. 13027-13037, 2011, doi: 10.1128/JVI.06191-11.

[92] W. A. Miller and G. Koev, "Synthesis of subgenomic RNAs by positive-strand RNA viruses," Virology, vol. 273, no. 1, pp. 1-8, 2000.

[93] S. Adkins, R. W. Siegel, J. H. Sun, and C. C. Kao, "Minimal templates directing accurate initiation of subgenomic RNA synthesis in vitro by the brome mosaic virus RNA-dependent RNA polymerase," RNA, vol. 3, no. 6, pp. 634-647, 1997.

[94] C. C. Kao and K. Sivakumaran, "Brome mosaic virus, good for an RNA virologist's basic needs," Mol. Plant Pathol., vol. 1, no. 2, pp. 91-97, 2000, doi: 10.1046/j.1364-3703.2000.00017.x.

[95] S. Adkins and C. C. Kao, "Subgenomic RNA promoters dictate the mode of recognition by bromoviral RNA-dependent RNA polymerases," Virology, vol. 252, no. 1, pp. 1-8, 1998, doi: 10.1006/viro.1998.9449.

[96] P. C. Haasnoot, F. T. Brederode, R. C. Olsthoorn, and J. F. Bol, "A conserved

hairpin structure in Alfamovirus and Bromovirus subgenomic promoters is required for efficient RNA synthesis in vitro," RNA, vol. 6, no. 5, pp. 708-716, 2000.

[97] P. C. Haasnoot, R. C. Olsthoorn, and J. F. Bol, "The Brome mosaic virus subgenomic promoter hairpin is structurally similar to the iron-responsive element and functionally equivalent to the minus-strand core promoter stem-loop C," RNA, vol. 8, no. 1, pp. 110-122, 2002.

[98] K. Sivakumaran, S. K. Choi, M. Hema, and C. C. Kao, "Requirements for brome mosaic virus subgenomic RNA synthesis in vivo and replicase-core promoter interactions in vitro," J. Virol., vol. 78, no. 12, pp. 6091-6101, 2004, doi: 10.1128/JVI.78.12.6091-6101.2004.

[99] W. P. Tzeng and T. K. Frey, "Mapping the rubella virus subgenomic promoter," J. Virol., vol. 76, no. 7, pp. 3189-3201, 2002.

[100] K. B. Spurgers and P. J. Glass, "Vaccine Development for Biothreat Alpha viruses," *J. Bioterror. Biodef.*, vol. 01, no. 01, 2011, doi: 10.4172/2157-2526.s1-001.

[101] R. Levis, S. Schlesinger, and H. V Huang, "Promoter for Sindbis virus RNA-dependent subgenomic RNA transcription," J. Virol., vol. 64, no. 4, pp. 1726-1733, 1990.

[102] M. M. Wielgosz, R. Raju, and H. V Huang, "Sequence requirements for Sindbis virus subgenomic mRNA promoter function in cultured cells," J. Virol., vol. 75, no. 8, pp. 3509-3519, 2001, doi: 10.1128/JVI.75.8.3509-3519.2001.

[103] M. L. Li, Y. H. Lin, and V. Stollar, "A cell-free system for the synthesis of Sindbis virus subgenomic RNA: importance of the concentration of the initiating NTP," Virology, vol. 341, no. 1, pp. 24-33, 2005, doi: 10.1016/j.virol.2005.07.007.

[104] S. Wang, L. Mortazavi, and K. A. White, "Higher-order RNA structural requirements and small-molecule induction of tombusvirus subgenomic mRNA transcription," J. Virol., vol. 82, no. 8, pp. 3864-3871, 2008, doi: 10.1128/JVI.02416-07.

[105] S. K. Oster, B. Wu, and K. A. White, "Uncoupled expression of p33 and p92 permits amplification of tomato bushy stunt virus RNAs," J. Virol., vol. 72, no. 7, pp. 5845-5851, 1998.

[106] K. A. White and P. D. Nagy, "Advances in the molecular biology of tombusviruses: gene expression, genome replication, and recombination," Prog. Nucleic Acid Res. Mol. Biol., vol. 78, pp. 187-226, 2004, doi: 10.1016/S0079-6603(04)78005-8.

[107] H. X. Lin and K. A. White, "A complex network of RNA-RNA interactions controls subgenomic mRNA transcription in a tombusvirus," EMBO J., vol. 23, no. 16, pp. 3365-3374, 2004, doi: 10.1038/sj.emboj.7600336.

[108] G. Zhang, V. Slowinski, and K. A. White, "Subgenomic mRNA regulation by a distal RNA element in a (+)-strand RNA virus," RNA, vol. 5, no. 4, pp. 550-561, 1999.

[109] T. L. Sit, A. A. Vaewhongs, and S. A. Lommel, "RNA-mediated trans-activation of transcription from a viral RNA," Science (80-.)., vol. 281, no. 5378, pp. 829-832, 1998.

[110] S. Napthine, R. A. Lever, M. L. Powell, R. J. Jackson, T. D. Brown, and I. Brierley, "Expression of the VP2 protein of murine norovirus by a translation termination-reinitiation strategy," *PLoS One*, vol. 4, no. 12, p. e8390, 2009, doi: 10.1371/journal.pone.0008390.

[111] G. Meyers, "Translation of the minor capsid protein of a calicivirus is initiated by a novel termination-dependent reinitiation mechanism," J Biol Chem, vol. 278, no. 36, pp. 34051-34060, 2003.

[112] G. Meyers, "Characterization of the Sequence Element Directing Translation Reinitiation in RNA of the Calicivirus Rabbit Hemorrhagic Disease Virus," J. Virol., vol. 81, no. 18, pp. 9623-9632, 2007, doi: 10.1128/jvi.00771-07.

[113] C. Luttermann and G. Meyers, "A bipartite sequence motif induces translation reinitiation in feline calicivirus RNA," *J. Biol. Chem.*, p. M608948200, 2007, doi: 10.1074/jbc.M608948200.

[114] T. A. Poyry, A. Kaminski, E. J. Connell, C. S. Fraser, and R. J. Jackson, "The mechanism of an exceptional case of reinitiation after translation of a long ORF reveals why such events do not generally occur in mammalian mRNA translation," Genes Dev., vol. 21, no. 23, pp. 3149-3162, 2007, doi: 10.1101/gad.439507.

[115] M. Morales, J. Barcena, M. A. Ramirez, J. A. Boga, F. Parra, and J. M. Torres, "Synthesis in vitro of rabbit hemorrhagic disease virus subgenomic RNA by internal initiation on (−) sense genomic RNA: mapping of a subgenomic promoter," J Biol Chem, vol. 279, no. 17, pp. 17013-17018, 2004.

[116] P. Simmonds, I. Karakasiliotis, D. Bailey, Y. Chaudhry, D. J. Evans, and I. G. Goodfellow, "Bioinformatic and functional analysis of RNA secondary structure elements among different genera of human and animal caliciviruses," Nucleic Acids Res., vol. 36, no. 8, pp. 2530-2546, 2008, doi: 10.1093/nar/gkn096.

[117] M. A. Yunus *et al.*, "The murine norovirus core subgenomic RNA promoter consists of a stable stem-loop that can direct accurate initiation of RNA synthesis," J. Virol., vol. 89, no. 2, pp. 1218-1229, 2015.

[118] I. R. Choi, M. Ostrovsky, G. Zhang, and K. A. White, "Regulatory activity of distal and core RNA elements in Tombusvirus subgenomic mRNA2 transcription," J. Biol. Chem., vol. 276, no. 45, pp. 41761-41768, 2001, doi: 10.1074/jbc.M106727200.

[119] B. D. Lindenbach, J. Y. Sgro, and P. Ahlquist, "Long-distance base pairing in flock house virus RNA1 regulates subgenomic RNA3 synthesis and RNA2 replication," J. Virol., vol. 76, no. 8, pp. 3905-3919, 2002.

[120] K. O. Chang, S. V Sosnovtsev, G. Belliot, A. D. King, and K. Y. Green, "Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line," Virology, vol. 353, no. 2, pp. 463-473, 2006, doi: 10.1016/j.virol.2006.06.006.

[121] X. Lin *et al.*, "Subgenomic promoter recognition by the norovirus RNA-dependent RNA polymerases," Nucleic Acids Res., vol. 43, no. 1, pp. 446-460, Jan. 2015, doi: 10.1093/nar/gku1292.

Chapter 5

Norovirus Genotypic Variability in Brazil

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Abstract

Norovirus (NoV) has been recognized as the most common etiological agent of acute gastroenteritis (AGE) in various epidemiological settings worldwide. The virus displays a high genetic diversity that can be classified into genogroups, genotypes, and recombinant strains. Only genogroups I, II, and IV have been found to infect humans. Variants of genogroup II genotype 4 are the most widely circulating strains and have been responsible for all NoV outbreaks globally since the mid-1990s. Several studies from different Brazilian regions have been conducted to detect and genetically characterize NoV from sporadic AGE cases and outbreaks. In this chapter, we have summarized the data that focused on the genetic variabilities of NoVs and thus highlight the value of a surveillance system in assessing not only the true burden of the disease, but also the detection and characterization of emerging novel variants.

Keywords: norovirus, gastroenteritis, genetic diversity, recombinant variants

1. Introduction

Noroviruses (NoVs) are small, non-enveloped viruses with icosahedral symmetry and diameters ranging between 27 and 40 nm [1] belonging to the family Caliciviridae and the genus Norovirus. The virus genome consists of simple, positive-sense RNA strands of approximately 7.4 to 7.7 kb and contains three open reading frames (ORFs) [2, 3]. ORF1 encodes non-structural proteins, such as RNA-dependent RNA polymerase (RdRp), ORF2 encodes the major capsid VP1 protein, and ORF3 encodes a minor structural protein VP2 [4]. The VP1 protein has the N-terminal (N), shell (S), and protruding (P) domains, and the current classification of NoVs into 10 genogroups (Gl to GX) has been based on these gene sequences. These viruses have been further classified into 49 and 60 confirmed genotypes and types based on amino acids of the complete VP1 and partial nucleotide sequences of RNA-dependent RNA polymerase regions, respectively [5]. Among the genogroups, only GI, GII, and GIV contain strains that infect humans. GII genotype 4 (GII.4) is the predominant NoV genotype causing gastroenteritis outbreaks worldwide, with periodic emergence and pandemic spread of novel lineages of the NoV GII.4 variants [6]. Mutations and recombination frequently occur within NoV genomes, and these evolutionary forces contribute to the emergence of new GII.4 variants every two to three years capable of re-infecting individuals already exposed to the virus [4].

Recently, a new variant of the virus (GII.17) that has never been reported before was detected in several countries in Asia, Europe, and North and South America, including Brazil, during outbreaks of acute gastroenteritis (AGE) [4].

NoVs are strongly associated with acute non-bacterial gastroenteritis; they are highly infectious and can affect all ages. The main routes of transmission of these viruses vary between fecal-oral, contact with infected people, ingestion of contaminated food and/or water, and aerosol produced by vomiting [2, 7]. Infection with these viruses is usually acute and limited: vomiting, diarrhea, and nausea are the main symptoms. However, in immunocompromised and hospitalized patients, there may be prolonged viral excretion and clinical complications due to virus infection [8].

2. Norovirus variants in Brazil

It is well known that NoVs are responsible for outbreaks, sporadic cases, and hospitalizations in Brazil [9]. However, generally in Brazil, the investigations of NoVs in hospital studies have been primarily focused on epidemiological surveys of diarrheal diseases caused by Rotavirus as a cause of severe gastroenteritis [8]. The first molecular detection of NoVs in Brazil occurred in the early 1990s and, since then, the presence of GI and GII has been reported in several regions of the country [10]. The tremendous diversity of NoV GII.4 variants with recombinant genotypes has been reported throughout the five geopolitical regions (North, Northeast, Central-West, South, and Southeast) of Brazil [11–18] as depicted in **Figure 1**. It has been suggested that the GII.17 variant detected in 2015 might have been introduced during the soccer World Cup event held in Brazil in 2014 [4].

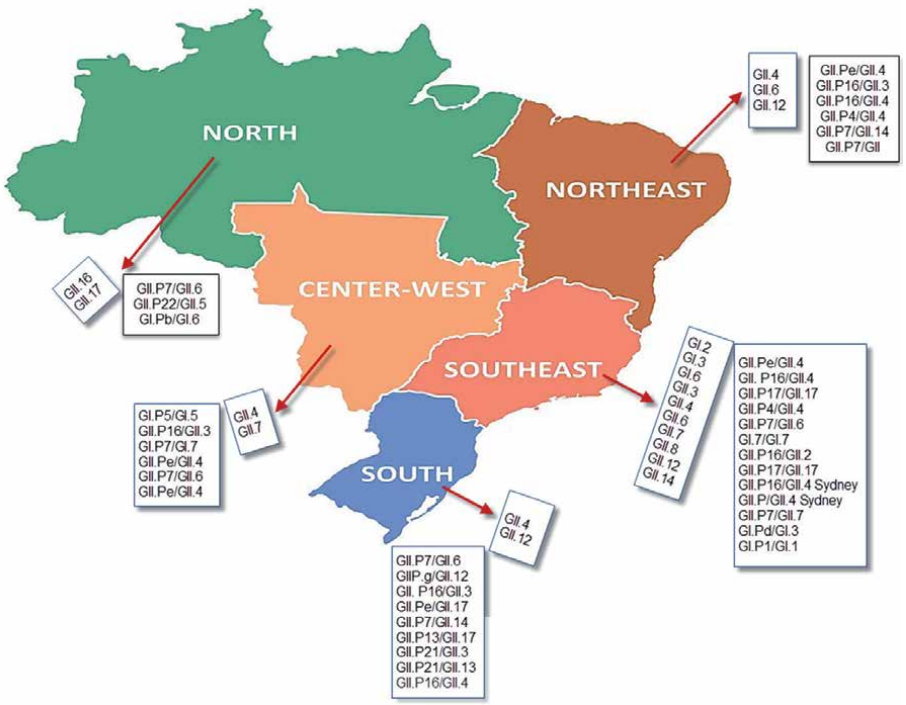

Figure 1.
Distribution of NoVs genotypes and recombinants detected in the five geopolitical regions of Brazil.

2.1 South region

The South region of Brazil is made up of three states: the Rio Grande do Sul, Santa Catarina, and Paraná. The region covers an area of 576,409.6 km2, making it the smallest region in the country. It is considered the third most populated and second most densely populated region in Brazil, as it has a population of 29.4 million people and a population density of 50 individuals per km2. Several studies have been conducted to determine the prevalence of NoV-associated gastroenteritis as well as the genotypic characterization of the virus circulating in the southern region of Brazil [9, 19]. documented the circulation of eight different NoV recombinant strains responsible for AGE outbreaks in the southern region from 2004 to 2011. These strains were identified by the recombination tools as GII.P7/GII.6, GIIP.g/GII.12, GII.P16/GII.3, GII.Pe/GII.17, GII.P7/GII.14, GII.P13/GII.17, GII.P21/GII.3, and GII.P21/GII.13. Among these strains, the GII.P7/GII.6 was the strain with the most frequent recombination, circulating from 2004 to 2010, followed by GII.Pg/GII.12, which was only detected in 2009. In 2016, Débora Maria Pires Gonçalves Barreira and colleagues [11] reported for the first time on the circulation and predominance of the newly emergent GII.P16-GII.4 Sydney strain along the southeastern coast of Brazil. Other studies confirmed the circulation of the pathogenic GIV genotype in both clinical and environmental samples [20, 21].

2.2 Southeast region

This region is made up of four states: Rio de Janeiro, São Paulo, Espírito Santo, and Minas Gerais. It encompasses an area of 924,511.3 km2 and has a population of 86.3 million people, which ranks as the fourth largest region by area and the most populated. It is also the most densely populated region, with a population density of 87 individuals per km2. Various studies have been conducted in this region to highlight the diversity of the circulating NoV genotypes. For example, an earlier report on sporadic infections in Brazilian children in the state of São Paulo revealed a high prevalence of GII and that the majority of the sequenced strains were phylogenetically clustered with GII.4. The same study also reported different potential recombinant strains [13]. In the state of Espírito Santo, an emerging recombinant NoV genotype was detected between January 2015 and July 2016; in the first year, the study reported a predominance of GII.Pe/GII.4 Sydney 2012 and, in the second year, a high prevalence of the GII. P16/GII.4 recombinant strain was observed [11]. The same study also reported the detection of GII.P17/GII.17 in three samples in 2016, which had already been detected in the country in 2015. A recent study by Cantelli et al. [22] aimed to scrutinize the genetic divergence of noroviruses in fecal specimens obtained from children with or without acute diarrheic episodes from a low-income urban area, the Manguinhos community, in the state of Rio de Janeiro. The results of this study showed 10 different genotypes circulating in this community between November 2014 and April 2018: GII.P4/GII.4, GII.P7/GII.6, and GI.7/GI.7 were the most frequent, followed by GII.P16/GII.2, GII.P17/GII.17, GII.P16/GII.4 Sydney, GII.P and GII.4 Sydney, GII.P7/GII.7, GI.Pd/GI.3, and GI.P1/GI.1. In addition, the frequent detection and genetic diversity of NoV observed in children who did not have episodes of acute diarrhea may mean frequent exposure to the virus.

2.3 Center-west region

The Brazilian Central-West region includes the following states: Mato Grosso, Mato Grosso do Sul, Goiás, and the Federal District (which includes Brasília, the

capital of Brazil). Few studies on the variability of NoVs from human clinical samples have been performed in this region. In the State of Goiás, feces were collected from children with or without symptoms of AGE in the periods 2009–2011 and 2014–2015 to characterize the ORF1 - RdRp and ORF2 - VP1 regions of the NoV genome. This study identified GII.P7-GII.6 (the most frequent recombination), GII. Pe/GII.4 (the second most frequent recombination in this study and characterized as Sydney 2012 variant), and GII. P16-GII.3 [23]. The data from the same study indicate that the distribution of NoV genotypes circulating in this region varies over time and that some recombinant strains had different recombinant breakpoints in samples obtained at different periods.

2.4 North region

The Brazilian North region includes the following states: Tocantins, Pará, Amapá, Amazonas, Roraima, Acre, and Rondônia. This is the largest region in Brazil, corresponding to 45.27% of the national territory. It is the least inhabited region of the country, with only 3.8 inhabitants per km^2. Recombinant strains of NoVs have been previously detected in a few clinical cases or in outbreaks that occurred in this region. However, in the Amazon states, between 2011 and 2014, the circulation of a recombinant strain GII.P7 / GII.6 was documented, which may indicate that this strain is already established in the population [2]. A recent study on samples collected during 2015 and 2016 from children under 5 years of age in Pará and Amazonas states demonstrated an increase in the circulation of the emerging GII.17_2014 strain in the Amazon region, and their phylogenetic approach suggests a single introduction of this genotype to the Amazon region [7]. Another strain that has also been detected in Amazonas is Gll.4, including variants GII.4 New Orleans_2009, and Sydney_2012 [2]. The same genotype detected in the Amazon region (GII.17_2014) was also detected in children with gastroenteritis in Belém, the capital of the state of Pará (neighboring the state of Amazonas) in 2016 [24]. However, a few years prior in this same region, more precisely during the period 2012–2015, stools and blood samples were collected from children hospitalized with acute gastroenteritis, and the viral load in the serum was very low when compared to the stools. In this study, a recombinant strain considered unusual was detected (GII.P13 / GII.17). However, while the main strain detected was GII.4 Sydney 2012, strains GII.P7 / GII.6, GII.P22 / GII.5, and GI.Pb /GI.6 were also reported [8]. An older study, by Siqueira et al. [25], also conducted in Belém, which lasted for almost 30 years (1982–2011), had followed both children in the community and outpatients and patients in hospitals to identify NoV genotypes related to cases of acute gastroenteritis. The Gll.4 (or GII.P4) genotype was the unique variant detected in all collected samples during the period 1998 to 2011. However, between 1982 and 1986, the GII genotypes were highlighted. P6 and GII.P7 were prevalent during the period 1990–1992, and GII.P3, GII.6, and GII.7 were reported during the period 1992–1994, with GII.P3 noted as the most prevalent variant. The GII.P21 genotype also had a wide circulation and was detected more frequently from the end of September 1998 until 2011, and was widely detected between the years 2001–2002 and 2008–2011.

2.5 Northeast region

The Brazilian Northeast region includes the following states: Bahia, Sergipe, Alagoas, Pernambuco, Paraíba, Rio Grande do Norte, Ceará, Piauí, and Maranhão. In several cities in the state of Pernambuco, between 2014 and 2017, stool samples were collected from individuals with acute gastroenteritis, with children up to

3 years old being the most affected by the virus. After sequencing the ORF1–ORF2 regions of 20 strains circulating in the state, four different GII genotypes were found: GII.Pe-GII.4, GII.P16-GII.3, GII.P16-GII.4, and GII.P4-GII.4, of which GII. Pe-GII.4 was the most prevalent in the region In the semi-arid region of North-eastern Brazil, which has the lowest income and the largest income disparity in the country, a study was conducted between 2009 and 2012 to determine the distribution of NoV genotypes in children ranging in age from 2 to 36 months with diarrhea and living in the cities in the states of Paraíba, Ceará, Pernambuco, or Piauí. In the study subjects, 45.2% of the individuals were positive for NoV, with genogroups GII and Gl being detected in 94.6% and 5.3% of positive samples, respectively. Based on the polymerase region, the most frequent genotypes were GII.P7 and GII.P16, while based on the capsid region, the main genotypes were GII.3, GII.14, and GII.4 New Orleans_2009. However, when both regions were analyzed, the authors observed a high frequency of recombinant strains classified as GII.P16-GII.3, GII.P7-GII.14, and GII.P7-GII, of which GII.P16-GII.3 had the highest prevalence [26].

3. Conclusions

Overall, this chapter has shown the circulation of multiple NoV strains in Brazil, which may lead to the occurrence of novel recombinant strains. Therefore, efforts to improve the national surveillance system are warranted to facilitate early detection of novel emergent variants, preparedness for upcoming epidemics, and the development and production of vaccines.

Acknowledgements

This work was supported by grant 2018/08631-3 from the Fundação de Amparo à Pesquisa do Estado de São Paulo..

Conflict of interest

The authors declare that there is no conflict of interest.

Notes/thanks/other declarations

The authors would like to acknowledge the laboratory of medical investigation 56 (LIM 56) for support.

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References

[1] KY G. The noroviruses In: Knipe DM, Howl ey PM, editors. 5 th edition. Fields virology. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 949-979.

[2] Hernandez JM, Silva LD, Junior ECS, Bandeira RS, Rodrigues EAM, Lucena MSS, et al. Molecular epidemiology and temporal evolution of norovirus associated with acute gastroenteritis in Amazonas state, Brazil. BMC Infect Dis. 2018;18(1):147.

[3] Guarines KM, Mendes RPG, de Magalhães JJF, Pena L. Norovirus-associated gastroenteritis, Pernambuco, Northeast Brazil, 2014-2017. J Med Virol. 2020;92(8):1093-101.

[4] Andrade JSR, Fumian TM, Leite JPG, Assis MR, Bello G, Mir D, et al. Detection and molecular characterization of emergent GII.P17/GII.17 Norovirus in Brazil, 2015. Infect Genet Evol. 2017;51:28-32.

[5] Chhabra P, de Graaf M, Parra GI, Chan MC, Green K, Martella V, et al. Updated classification of norovirus genogroups and genotypes. J Gen Virol. 2019;100(10):1393-406.

[6] Siebenga JJ, Vennema H, Renckens B, de Bruin E, van der Veer B, Siezen RJ, et al. Epochal evolution of GGII.4 norovirus capsid proteins from 1995 to 2006. J Virol. 2007;81(18):9932-41.

[7] das Neves Costa LCP, Teixeira DM, Portela ACR, de Lima ICG, da Silva Bandeira R, Sousa Júnior EC, et al. Molecular and evolutionary characterization of norovirus GII.17 in the northern region of Brazil. BMC Infect Dis. 2019;19(1):1021.

[8] Reymão TKA, Fumian TM, Justino MCA, Hernandez JM, Bandeira RS, Lucena MSS, et al. Norovirus RNA in serum associated with increased fecal viral load in children: Detection, quantification and molecular analysis. PLoS One. 2018;13(7):e0199763.

[9] Fumian TM, da Silva Ribeiro de Andrade J, Leite JP, Miagostovich MP. Norovirus Recombinant Strains Isolated from Gastroenteritis Outbreaks in Southern Brazil, 2004-2011. PLoS One. 2016;11(4):e0145391.

[10] Paula FL, Sardi SI, Tigre DM, Fernandes FMC, Campos GS. ACUTE GASTROENTERITIS ASSOCIATED WITH NOROVIRUS GII.4 VARIANTS. Arq Gastroenterol. 2018;55(3):264-6.

[11] Barreira DMPG, Fumian TM, Tonini MAL, Volpini LPB, Santos RP, Ribeiro ALC, et al. Detection and molecular characterization of the novel recombinant norovirus GII.P16-GII.4 Sydney in southeastern Brazil in 2016. PLoS One. 2017;12(12):e0189504.

[12] Gallimore CI, Lewis D, Taylor C, Cant A, Gennery A, Gray JJ. Chronic excretion of a norovirus in a child with cartilage hair hypoplasia (CHH). J Clin Virol. 2004;30(2):196-204.

[13] Castilho JG, Munford V, Resque HR, Fagundes-Neto U, Vinjé J, Rácz ML. Genetic diversity of norovirus among children with gastroenteritis in São Paulo State, Brazil. J Clin Microbiol. 2006;44(11):3947-53.

[14] Soares CC, Santos N, Beard RS, Albuquerque MC, Maranhão AG, Rocha LN, et al. Norovirus detection and genotyping for children with gastroenteritis, Brazil. Emerg Infect Dis. 2007;13(8):1244-6.

[15] Victoria M, Carvalho-Costa FA, Heinemann MB, Leite JP, Miagostovich M. Prevalence and molecular epidemiology of noroviruses in hospitalized children with acute gastroenteritis in Rio de

Janeiro, Brazil, 2004. Pediatr Infect Dis J. 2007;26(7):602-6.

[16] Xavier MP, Oliveira SA, Ferreira MS, Victoria M, Miranda V, Silva MF, et al. Detection of caliciviruses associated with acute infantile gastroenteritis in Salvador, an urban center in Northeast Brazil. Braz J Med Biol Res. 2009;42(5):438-44.

[17] Ferreira MS, Xavier MaP, Tinga AC, Rose TL, Fumian TM, Fialho AM, et al. Assessment of gastroenteric viruses frequency in a children's day care center in Rio De Janeiro, Brazil: a fifteen year study (1994-2008). PLoS One. 2012;7(3):e33754.

[18] Fioretti JM, Ferreira MS, Victoria M, Vieira CB, Xavier MaP, Leite JP, et al. Genetic diversity of noroviruses in Brazil. Mem Inst Oswaldo Cruz. 2011;106(8):942-7.

[19] de Andrade JaS, Rocha MS, Carvalho-Costa FA, Fioretti JM, Xavier MaP, Nunes ZM, et al. Noroviruses associated with outbreaks of acute gastroenteritis in the State of Rio Grande do Sul, Brazil, 2004-2011. J Clin Virol. 2014;61(3):345-52.

[20] Teixeira DM, Hernandez JM, Silva LD, Oliveira DeS, Spada PK, Gurjão TC, et al. Occurrence of Norovirus GIV in Environmental Water Samples from Belém City, Amazon Region, Brazil. Food Environ Virol. 2016;8(1):101-4.

[21] Fioretti JM, Fumian TM, Rocha MS, Dos Santos IAL, Carvalho-Costa FA, de Assis MR, et al. Surveillance of Noroviruses in Rio De Janeiro, Brazil: Occurrence of New GIV Genotype in Clinical and Wastewater Samples. Food Environ Virol. 2018;10(1):1-6.

[22] Cantelli CP, da Silva MFM, Fumian TM, da Cunha DC, Andrade JDSR, Malta FC, et al. High genetic diversity of noroviruses in children from a community-based study in Rio de Janeiro, Brazil, 2014-2018. Arch Virol. 2019;164(5):1427-32.

[23] Dábilla N, Almeida TNV, Franco FC, Cunha MDP, Fiaccadori FS, Souza M. Recombinant noroviruses detected in Mid-West region of Brazil in two different periods 2009-2011 and 2014-2015: Atypical breakpoints of recombination and detection of distinct GII.P7-GII.6 lineages. Infect Genet Evol. 2019;68:47-53.

[24] Silva LD, Bandeira RD, Junior EC, Lima IC, da Penha Júnior ET, Teixeira DM, et al. Detection and genetic characterization of the emergent GII.17_2014 norovirus genotype among children with gastroenteritis from Northern Brazil. Infect Genet Evol. 2017;48:1-3.

[25] Siqueira JAM, Bandeira RDS, Oliveira DS, Dos Santos LFP, Gabbay YB. Genotype diversity and molecular evolution of noroviruses: A 30-year (1982-2011) comprehensive study with children from Northern Brazil. PLoS One. 2017;12(6):e0178909.

[26] Gondim RDG, Pankov RC, Prata MMG, Medeiros PHQS, Veras HN, Santos AKS, et al. Genetic Diversity of Norovirus Infections, Coinfections, and Undernutrition in Children From Brazilian Semiarid Region. J Pediatr Gastroenterol Nutr. 2018;67(6):e117-e22.

Section 4

Clinical and Pharmaceutical Development

Chapter 6

Norovirus: Clinical Findings and Pharmaceutical Developments

Ying-Fei Yang and Chung-Min Liao

Abstract

Norovirus (NoV) is one of the most ubiquitous factors contributing to acute gastroenteritis that causes widespread outbreaks in travel industry, military, or healthcare facilities. NoV could lead to serious symptoms and result in severe societal costs worldwide. Surprisingly, there has been no available licensed vaccines, albeit there are ongoing pre-clinical or clinical trials of several candidate vaccines. Development of effective universal vaccines has been found difficult clinically due to the frequent point mutations and a lack of robust animal model and cell culture system. Preclinical studies showed that vaccines with virus-like particles (VLPs) have high immunogenicity and efficacies and were demonstrated to be protective and safe. Recent *in vitro* research also suggests that human intestinal enteroids can enhance our understanding of protection mechanism and give guidance for vaccine development. Overall, this chapter will give a comprehensive review of the current challenge and progress of clinical findings, efficacy/safety of the developing vaccines, and antiviral drug developments for NoV in clinical trials or preclinical investigations.

Keywords: norovirus, gastroenteritis, vaccine, immunogenicity, clinical findings

1. Introduction

Norovirus (NoV) has been the leading cause contributing to acute gastroenteritis worldwide [1]. The Product Development for Vaccines Advisory Committee of the World Health Organization (WHO) also recognized NoV as a priority disease for vaccine development [1]. It was estimated that the global burden of NoV disease was more than 677 million cases and 213515 deaths annually [1]. Surveillance studies in the United States suggested that NoV leads to ~20% of acute gastroenteritis in children under 5 years old [2–4]. It was also demonstrated that rate of NoV gastroenteritis in this group of age exceeds that of rotavirus gastroenteritis [2–4]. Particularly high rates of mortality and illness requiring medical care present at age > 65 years and under 5 year olds, respectively [4, 5]. Sudden onset of symptoms including watery diarrhea, vomiting, abdominal cramps, malaise, and fever are commonly seen and usually more serious occur in immunocompromised or elderly individuals [1].

NoVs are small (diameter, 35–40 mm), non-enveloped, positive, and single-stranded RNA viruses belonging to the *Caliciviridae* family [1, 6]. With Norovirus being one of the genera of the family, it could be classified into seven genogroups based on the phylogenetic analysis of the virus genome and capsid sequence, where GI, GII, and GIV infect humans and GI and GII are major groups causing human infections (**Table 1**) [1]. GI and GII genogroups could be respectively subdivided into 9 and 25 genotypes based on the viral capsid protein (VP1) sequence.

Genus	Genogroup	Genotypes	Species infected
Lagovirus	GI	GI.1–9	Human
	GII	GII.1–23	Human and pig
Nebovirus	GIII	GIII.1–2	Cow and sheep
Norovirus	GIV	GIV.1–2	Human, cat, dog, and lion
Sapovirus	GV		Mouse and rat
Vesivirus	GVI	GVI.1–2	Dog
	GVII		Dog

Table 1.
Classification of the Caliciviridae *family, among which GI and GII are the most prevalent genogroups for NoV infections in human (adapted from Melhem [1] and Cortes-Penfield [33]).*

Development of NoV vaccines has been in progress for more than a decade. There has been no licensed vaccines for prevention of NoV infections [1]. Although there are several candidates that have gone through clinical trials such as the Adenovirus type 5 vaccine expressing the GI.1 capsid protein (Vaxart) and the bivalent GI and GII.4 VLP vaccine (Takeda), development of a vaccine with broad-spectrum antiviral effects against various or newly emerging viral strains are of increasingly needed [1].

2. Clinical observations

It was estimated that one in six patients with acute diarrhea has NoV as the causative pathogen [7, 8]. Nov is highly contagious and was estimated to release more than 30 million virus particles from vomiting [8–10]. The infecting dose are only 10–100 virus particles to induce related symptoms, and the attack rate is more than 50% [8–12]. Generally, the incubation period of NoV is from 24 to 48 hours. There's no prodromal illness before the onset of the NoV, and the symptoms are usually explosive [8, 12, 13]. Predominant symptoms include fever, vomiting, diarrhea and clinically lasts for 2 to 3 days [8, 14]. Vomiting and diarrhea are the most common symptoms that occurs in 70% and 90% infected patients, respectively.

In a 3-year multicentered study presenting clinical features of Norovirus in Taiwan children, fever and vomiting are found to stop in approximately 3 and 2 days, respectively [8, 14]. However, diarrhea lasts as long as around 6 days [8, 14]. Malnutrition, dehydration, and dysfunctional intestinal barrier will also worsen the illness [7, 8].

Arias et al. [15] also performed a prospective study provided evidence that symptoms such as abdominal pain, vomiting, and myalgia are common in both children and adults, while myalgia and diarrhea are more common seen in adults. There was no difference found between male and female subjects (**Figure 1**). However, fever and abdominal pain were observed to be more frequent in male than in female individuals (34.5% vs. 28.9%, $p = 0.022$; 70.8% vs. 64.4%, $p = 0.013$, respectively) (**Figure 1**) [15]. In another stratified factor, age may play as an influential role in symptoms of subjects. It was noticed that risk of diarrhea was higher in elderly (> 65 years) group (OR; 2.61; 95% CI: 1.93–3.55) (**Figure 2**) [15]. Vomiting and abdominal pain were found to be more frequent in children at age < 5 years old (**Figure 2**) [15].

It was reported that there are 3 to 10 episodes of diarrhea per day in patients with NoV gastroenteritis [16]. Moreover, NoV gastroenteritis could be a major threat to

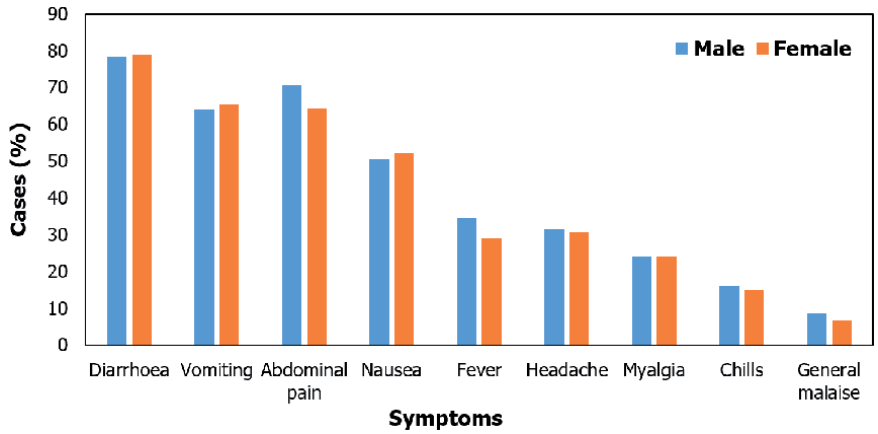

Figure 1.
Distribution of symptoms of NoV based on male and female among 1544 cases from 2004 to 2005 in Catalonia, Spain (adapted from arias et al. [15]).

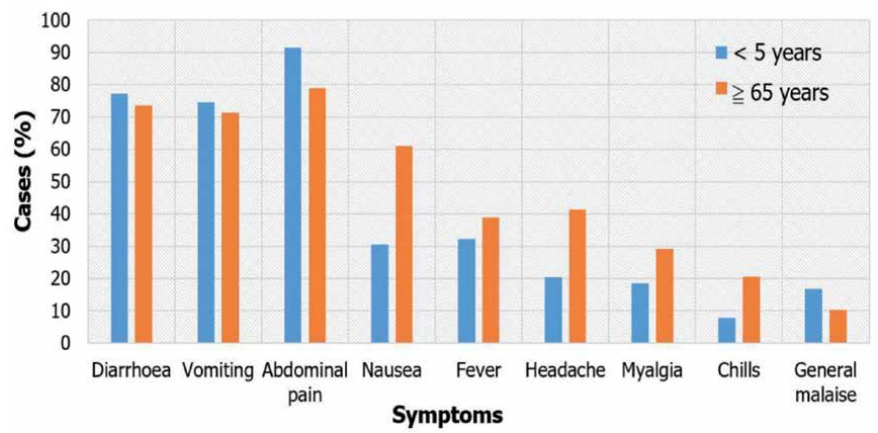

Figure 2.
NoV symptoms at two different age groups of children (< 5 years) and elderly (> 65 years) among 1544 cases from 2004 to 2005 in Catalonia, Spain (adapted from arias et al. [15]).

patients especially with hematopoietic stem cell transplant or chemotherapy [17]. It was also reported that NoV could infect individuals many times or with more than one NoV strain during the course of an outbreak [7, 8].

2.1 NoV vaccines in development

Candidate NoV vaccines in development are either in clinical trials or still in preclinical stages (**Table 2**). There have been two groups of virus-like particles (VLP)-based vaccines in development. One is a combination of GI.1 and GII.4 VLPs that is in human clinical trials, whereas the other is a mixture of GI.3 and GII.4 VLPs, which is in preclinical developments [4] (**Table 2**). In addition, there are two other groups of vaccines based on recombinant adenovirus serotype 5 vectors expressing NoV VP1. One is currently in Phase I and is based on GI.1 NoV sequence, and the other is based on GI.4 sequence and developed my Chinese Center for Disease Control and Prevention [4] (**Table 2**). Vaccines in development will be described in the following sections in this chapter.

Stage of trial	Leading Investigators	Candidate vaccine	NoV genotype	Administration route	References
Phase I	Vaxart	Recombinant Adenovirus expressing NoV VP1	GI.1	Oral	[28]
Phase IIb	Takeda	NoV VLP	GI.1, GII.4	Intramuscular	[25, 26]

Table 2.
NoV vaccines under clinical trials (adapted from Nicolas et al. [4]).

2.1.1 GI.1/Gll.4 VLP vaccines

Initially, the VLP-type NoV vaccine in human had oral administration of unadjuvanted GI.1 NoV VLPs. Results showed that there were 4-fold increments of virus-specific serum IgG level and no adverse events in 83% of the recipients [18]. In light of this finding, there's development of a VLP vaccine adjuvanted with mucoadhesin and monophosphoryl lipid A for intranasal delivery [19, 20]. The vaccine was a Phase I study with double-blind and placebo-controlled design. Results showed that it could induce NoV-specific IgG and IgA memory B cells, IgG, and IgA. No occurrence of serious adverse events [4, 19, 20].

Another randomized, double-blind, and placebo-controlled study that assessed efficacy of intranasally delivered vaccine gave subjects challenged with ~10 human-infectious doses of Norwalk virus 2 doses of vaccine/placebo [4, 21]. Results showed that a 32% absolute reduction of the risk (37% vs. 69%; $P = 0.06$) for gastroenteritis in the vaccine recipients. The result was associated with the increase of NoV-specific antibody levels including IgA and serum HBGA-blocking antibodies [4, 21]. However, there was no significant reduction of the duration of illness or symptoms. Moreover, local nasal symptoms including sneezing, itching, stuffiness, and nasal discharge were more common after second dose of vaccination in vaccine arm, regardless that there were no occurrence of serious adverse events [4, 21].

On the other hand, the prevalence of GII.4 NoV resulted in the addition of a GII.4 VLP component to GI.1 VLPs to generate bivalent vaccines [4, 22]. Previous preclinical study showed that the addition of GII.4 VLP, based on 3 different sequences of GII.4 NoV strain variants, led to induction of reactive antibodies to heterologous GI.3, GII.1, GII.3, and GIV.1 NoVs [4, 22]. Also, a randomized and placebo-controlled clinical trial of a bivalent GI.1 and consensus GII.4 VLP vaccine adjuvanted with aluminum hydroxide and monophosphoryl lipid A was delivered to subjects with a series of 2 intramuscular injections [4, 22, 23]. Developments of GI.1- and GII.4-specific serum antibody peaked at day 7 were observed after vaccination. No adverse events were distinguished. NoV-specific antibody-secreting cells, plasmablasts, and memory B cells were also evidenced. However, it was found that dose escalation did not lead to higher levels of NoV-specific antibodies. After first vaccine dose, HBGA-blocking antibodies were developed at high levels in all age groups (18–49, 50–64, and 65–85 years), whereas there was little additional increments in levels after second vaccination [4, 22, 23].

A bivalent vaccine targeting GII.4 NoV was also assessed through a randomized, double-blind, and placebo-controlled trial with 63 and 64 subjects received NoV vaccine and placebo vaccine, respectively [4, 24]. Among the participants, 56 and 53 individuals were challenged with 4400 reverse-transcription polymerase chain reaction units of a GII.4 NoV variant, which was not included in the consensus GII.4 sequence. Although there were no statistical significance in the decrease of prevalence in gastroenteritis, milder symptoms were observed after NoV challenge. No reported adverse events. No reductions of duration of NoV illness and time from challenge to onset of symptoms were discovered after vaccination [4, 24].

Another study for investigation of a bivalent GI.1/GII.4 vaccine is in Phase II clinical trials that prevention of NoV-related illness and infection after intranasally or intramuscularly administration were also demonstrated. The Phase IIb, double-blind, randomized, placebo-controlled, efficacy trial with participants from the US Navy was just performed (NCT02669121) [4, 25]. Results showed that increases of GI.1 and GII.4 HBGA-blocking antibodies in vaccines and in some placebo cases infected with GII.2 revealed the cross-activity in the immune responses to different genotypes [26].

Due to the lack of *in vitro* culture systems for human NoV, there has been no inactivated or live attenuated NoV particle vaccines developed [4]. Although there are 2 NoV candidate vaccines in human trials, the immediate development of vaccines based on NoV particles seems difficult [4].

2.1.2 Adenovirus vector-based GI.1 VP1 vaccines

It was evidenced that a recombinant serotype 5 adenovirus vector with expression of a GII.4 NoV VP1 was immunogenic in mice intranasally vaccinated [4, 27]. NoV-specific IgG and IgA in feces, respiratory mucosa, and serum were observed [4, 27]. It was also found by the same group that combination of booster vaccination using NoV VKPs and adenovirus vector could enhance immune responses [4, 27].

Another adenovirus vector-based NoV vaccine (VXA-GI.1-NN) expressing NoV major capsid protein VP1 from the GI.1 Norwalk virus that is orally administered is also in development [4, 28]. The immunogenicity and tolerability of the vaccine against H1N1 influenza expressing the same adenovirus-vectored platforms were reported [4, 29]. The VXA-GI.1-NN was announced to go through the completion of a Phase I trial (NCT02868073). Recent results showed that the vaccine had no dose-limiting toxicities and only mild/moderate adverse events presented. Immunogenicity and tolerability end points of the vaccine were also reached [30]. Serum NoV-blocking antibody levels significantly increased in recipients. No adverse events observed.

In addition, a newest phase I (NCT03721549) GI.1 study (Lot 001-09NV) sponsored by the WCCT Global was also conducted to explore its efficacy with a controlled human infection model (CHIM). Results indicated that Lot 001-09NV could be a useful challenge strain for vaccine studies aiming at the establishment of immune correlates [31]. However, development of a multivalent vaccine is in need since the vaccine did not show consistently robust immune responses to heterotypic NoV strains [4, 29].

3. Drugs in clinical development

Candidate drugs for NoV treatments are still in the process of developments although there has been intensive need for effective NoV antivirals. Most candidates are still in the early stages of preclinical trials. Human NoV culture in enteroid system are frequently applied in the preclinical research to assess safety or efficacy of candidate drugs. Although the landscape for antiviral developments is frequently changed, this chapter will give a comprehensive review of the drugs that have been approved by regulatory agencies, previously or in the process of clinical trials.

3.1 Polymerase inhibitors

3.1.1 Nucleoside analogs

Among all NoV antiviral targets, the RNA-dependent RNA polymerase (RdRp) is one of the candidates identified to be effective in inhibition of NoV activity in preclinical stages. The RdRp acts as an attractive antiviral therapy since it has the advantage

of lacking host homologs to minimize the chance of off-target adverse effects [32, 33]. The RdRp-targeting antivirals could be divided into two major classes: the nucleoside analogs (NAs) and the non-nucleoside inhibitors (NNIs). Typically, NAs inhibit RNA syntheses through mimicry of the generation of nucleoside triphosphate (NTPs) that lead to chain termination after incorporation [33, 34]. On the contrary, NNIs exert lower antiviral activity and bind allosterically to block rearrangements of conformation of the viral polymerase to form active replication complex [33, 35].

3.1.1.1 2CMC

Some of the NA-drugs were repurposed to treat human NoV since they have the mechanism of RNA synthesis inhibition for various kinds of viruses. One of the drugs is called 2CMC (2'-C-methylcytidine), which was originally served as an antiviral therapy for HCV. It also exhibited inhibition activities against flaviviruses such as West Nile virus, DENV, yellow fever virus [33, 36]. Valopicitabine, one of the oral 2CMC prodrug used against for HCV, was halted for development due to undesirable gastrointestinal effects reported [33, 37]. However, although the 2CMC was discontinued for clinical treatment, it has been widely served as a potential NoV-treating drug. It was found RNA synthesis of Norwalk replicon was dose-dependently reduced by the 2CMC [33, 38]. Murine NoV (MNV) plaque and RNA synthesis were also demonstrated to be inhibited [33, 39].

3.1.1.2 T705 (Flavipiravir)

T705 is a purine analog that showed to induce lethal mutagenesis against MNV [33, 40, 41]. It has also been approved as an influenza treatment in Japan [42]. Although it was evidenced to inhibit replication of several viruses such as Ebola virus, hantaviruses, flaviviruses, and arena viruses with *in vitro* and mouse models, it showed poor antiviral activity for MNV replication in cell cultures [33, 43].

3.1.1.3 Ribavirin (RBV)

The RBV (1-α-D-ribofuranosyl-1,2,4-triazole-3- carboxamide) is a guanosine analog and the first drug found to inhibit MNV and human NoV replication in 2007. It was originally used as clinical treatments for HCV, Lassa fever, respiratory syncytial virus, and hepatitis E viruses (Snell, 2001). The EC50 was 40 μM for Norwalk replicon and had 82% reduction of replicon genome at 100 μM [33, 44]. However, since some studies found RBV had poor inhibition of MNV and Norwalk replicon, coupled with numerous adverse effects, it is not a desirable NoV antivirals [45].

3.1.1.4 CMX521

CMX521 is a novel antiviral drug discovered by the Chimerix. The press release reported that it is at phase I clinical trial and has potent antiviral activity against NoV. Pharmacokinetics, safety, tolerability were evaluated in approximately 50 healthy adults [33, 46].

3.1.1.5 Non-nucleoside inhibitors

3.1.1.5.1 Suramin and related derivative

Suramin is a sleeping sickness medication that was found to effectively inhibit in vitro activities of MNV and human polymerases [47]. NF03, which is the smaller

derivative of suramin, could inhibit mouse and human NoV RdRp activities with IC50s of 200 and 71.5 nM, respectively [47]. NF023 was also a smaller suramin derivative that could inhibit both human and mouse NoV RdRp activities. In addition, naphthalene disulfonate (NAF2) and tetrasodium (PPNDS) were also found to be capable of inhibiting human NoV RdRp [33, 48, 49]. However, despite of the promising effectiveness, the antiviral efficacy of these compounds are reduced due to the poor bioavailability and cell permeability [33, 48, 50, 51] Another study also identified some compounds (NIC02 [2.2.11], NIC04, NIC10, and NIC12) by using viral enzyme activity assays. MNV replication and Norwalk replicon were both found to be inhibited [33, 52].

3.1.2 Protease inhibitors

Rupintrivir is one of the protease inhibitors (PIs) discovered for NoV. In addition to the potential efficacy of this compound, it also possesses broad-spectrum antiviral effects against coronaviruses, picornaviruses, and caliciviruses such as FCV, MNV, and Norwalk replicon [33, 53]. However, it was also found that Rupintrivir had limited bioavailability and pharmacokinetics due to the low potency *in vivo*. As a result, the Rupintrivir may not be an ideal therapy for the NoV infection [33, 47].

3.1.3 Protein targets

Deubiquitinase (DUB) inhibitor is one of the recently discovered NoV antivirals that can regulate ubiquitin-ubiquitin-proteasome system [33, 54–57]. WP1130 [2.4.1] is a small synthetic DUB inhibitor found to inhibit NoV and MNV replication through unfolded protein response. However, due to the low bioavailability, show that MNV inhibition was limited to small intestine in mice [33, 56].

3.1.4 Immunomodulators

Immunomodulators are found to be a potent therapeutic antiviral for NoV since they could induce powerful host response. Interferons are one of the best immunomodulators and many studies have shown that type I, type II IFNs, and their receptors could provide protection against human and murine NoV infections [33, 58–66]. The type III IFNs (IFN-λ) has been recently explored and was shown to be capable of preventing persistent MNV infection in mice system [33, 67, 68].

3.1.5 Compounds with unknown targets

Currently, the only NoV antiviral candidate that completed clinical trials is called NTZ. The NTZ was found to have a good antimicrobial activity against various bacterial, viral, and protozoan infections. It is also an FDA-approved drug for the treatment of *Giardia* and *Crytosporidium* infections [33, 69]. Treatment of NTV was found to reduce durations of gastroenteritis symptoms when compared to the placebo ($P = 0.0295$) in the phase II randomized double-blind trial [33, 70]. Another study also evidenced that NTZ was capable of curing immunosuppressed transplant patient with NoV infection and 10 consecutive days of gastroenteritis symptoms [33, 71]. It was also recently found that NTZ could potently inhibit GI NoV replicon at a clinically relevant concentration (5 ug mL^{-1}) [33, 72].

NoV in stool samples from a pediatric patient with chronic NoV following kidney transplantation was also shown to be cleared [73]. There have been some discrepancy regarding the effectiveness of NTZ for NoV treatment [33, 74–77].

However, since NTZ is currently the only therapeutic method except from RBV and immunoglobulins, it is still supportive to NoV infected-patients.

3.2 Remarks on gastroenteritis symptoms in coronavirus

Acute gastroenteritis symptoms including diarrhea, vomiting and abdominal pain are not only limited to NoV infections. Viruses such as rotavirus, astrovirus, calicivirus are common etiologic agents for acute gastroenteritis [78]. Coronaviruses, which is the genus of the most concerned issue of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic in the world, is also identified as causative agent of diarrhea [78]. It was reported that approximately 12% of COVID-19 patients experienced gastrointestinal symptoms based on a systematic review and meta-analysis of published studies from November 1, 2019 to March 30, 2020 [79]. One study observed mild initial gastrointestinal symptoms (e.g., diarrhea, abdominal pain, nausea, and vomiting) preceded the fever and respiratory problems. The study also found the fecal test remained positive post 12 day of disease onset. Longer durations between symptom onset and fecal virus-positive and viral clearance were also observed [80, 81]. Another study also found gastrointestinal symptoms such as diarrhea (24.2%), anorexia (17.9%), and nausea (17.9%) occurred in COVID-19 patients [80, 82]. Results from a systematic meta-analysis study also showed the incidence rate of diarrhea from 2–50% of the COVID-19 positive cases [80, 83]. Furthermore, according to clinical cases, intestinal damages were found to be manifested after respiratory symptoms [80, 84].

Taken together, since gastroenteritis symptoms could commonly occur in various virus-infected individuals. It is not easy to differentiate if it is caused by the SARS-CoV-2 by simply observing the symptoms of patients. It was also indicated that patients with gastroenteritis symptoms also take a longer time to present to healthcare systems and to be confirmed after diagnosis [85]. Further detection of SARS-CoV-2 RNA or diagnosis of other symptoms correlated with SARS-CoV-2 disease onset are required. Precautions should also be taken carefully for the infectiveness and transmissibility of SARS-CoV-2 in COVID-19 positive feces in stool [80].

4. Conclusions

NoV has been a major cause leading to acute gastroenteritis and hundreds of thousands mortalities annually. Also, significant economic and social impacts have been resulted from this pathogen despite preclinical or clinical research are intensively ongoing. Technical issues such as the limitations in the current used human culture systems need to be overcome for the development of effective vaccines or drugs. Epidemiological studies also suggest that development of multivalent vaccines for both GI and GII NoV are the only solution for broad-spectrum and effective protection. As mentioned previously in this chapter, it is promising that several vaccines have gone through clinical trials and many drugs are currently in clinical use. However, it is of note that since clinical trials mainly enrolled adults, it would be necessary to evaluate the safety and effectiveness of the candidate vaccines in all age groups since NoV poses greater threats to children and elderly groups. To facilitate the progress of vaccine/drug developments for NoV antivirals, exploration of the relationship between viral strains and host human immunogenicity and antigen types at clinical practices would also be helpful. Improvements in diagnostic methods and outbreak containment or management may help to alleviate the epidemics.

Acknowledgements

We thank Ministry of Science and Technology of Republic of China for financial support under Grant MOST 107-2313-B-002-034-MY3.

Conflict of interest

The authors declare no conflict of interest.

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References

[1] Nada M. Norovirus. 1st ed. Springer; 2019.

[2] Payne DC, Vinjé J, Szilagyi PG, et al. Norovirus and medically attended gastroenteritis in U.S. children. N Engl J Med. 2013;368:1121-1130. DOI: 10.1056/NEJMsa1206589

[3] Koo HL, Neill FH, Estes MK, et al. Noroviruses: The Most common pediatric viral enteric pathogen at a large university hospital after introduction of rotavirus vaccination. J Pediatric Infect Dis Soc. 2013;2:57-60. doi: 10.1093/jpids/pis070

[4] Nicolas WC, Sasirekha R, Mary KE, et al. Prospects and challenges in the development of a Norovirus vaccine. Clin Ther. 2017; 39(8):1537-1549. DOI: 10.1016/j.clinthera.2017.07.002

[5] Hall AJ, Lopman BA, Payne DC, et al. Norovirus disease in the United States. Emerging Infect Dis. 2013;19:1198-1205. DOI: 10.3201/eid1908.130465

[6] Caul EO, Appleton H. The electron microscopical and physical characteristics of small round human fecal viruses: an interim scheme for classification. J Med Virol 1982; 9:257-265. DOI: 10.1002/jmv.1890090403

[7] Karin B, Kim YG. Norovirus gastroenteritis in immunocompromised patients. Review N Engl J Med 2012;367(22):2126-32. DOI: 10.1056/NEJMra1207742

[8] Way-Seah L, Edmond ASN. Clinical manifestation. In: Paul KSC, Hoi SK, Martin CWC, editors. The Norovirus. 1st ed. Elsevier; 2017.

[9] Patterson W, Haswell P, Fryers PT, et al. Outbreak of small round structured virus gastroenteritis arose after kitchen assistant vomited. Commun Dis Rep CDR Rev 1997; 7(7):R101–R103.

[10] Debbie W. Infection Prevention and Control Theory and Clinical Practice for Healthcare Professionals. 1st ed. John Wiley & Sons; 2008.

[11] Vipond IB, Caul EO, Lambden PR, et al. 'Hyperemesis hiemis': new light on an old symptom. Microbiol Today 1999; 26:110-111.

[12] Caul ED. Small round structured viruses – airborne transmission and hospital control. The Lancet 1994; 343(8098):1240-1242. DOI: 10.1016/s0140-6736(94)92146-6.

[13] CDC. Norovirus Illness: Key Facts [Internet]. 2015. Available from: https://www.cdc.gov/norovirus/downloads/keyfacts.pdf [Accessed: 2021-03-03]

[14] Chen CJ, Wu FT, Huang YC, et al. Clinical and epidemiologic features of severe viral gastroenteritis in children: a 3-year surveillance, multicentered study in Taiwan with partial rotavirus immunization. Medicine 2015; 94 (33):e1372. DOI: 10.1097/MD.0000000000001372

[15] Arias C, Sala MR, Domínguez A, et al. Epidemiological and clinical features of norovirus gastroenteritis in outbreaks: a population-based study. Clin Microbiol Infect 2010; 16(1):39-44. DOI: 10.1111/j.1469-0691.2009.02831.x.

[16] Tian G, Jin M, Li H, et al. Clinical characteristics and genetic diversity of noroviruses in adults with acute gastroenteritis in Beijing, China in 2008-2009. J Med Virol 2014; 86 (7):1235-1242. DOI: 10.1002/jmv.23802

[17] Schwartz S, Vergoulidou M, Schreier E, Loddenkemper C, Reinwald M, Schmidt-Hieber M,

Flegel WA, Thiel E, Schneider T. Norovirus gastroenteritis causes severe and lethal complications after chemo- therapy and hematopoietic stem cell transplantation. Blood 2011; 117:5850-5856. DOI: 10.1182/blood-2010-12-325886

[18] Ball JM, Graham DY, Opekun AR, et al. Recombinant Norwalk virus-like particles given orally to volunteers: phase I study. Gastroenterology. 1999;117:40-48. DOI: 10.1016/s0016-5085(99)70548-2

[19] El-Kamary SS, Pasetti MF, Mendelman PM, et al. Adjuvanted intranasal Norwalk virus-like particle vaccine elicits antibodies and antibody-secreting cells that express homing receptors for mucosal and peripheral lymphoid tissues. J Infect Dis. 2010;202(11):1649-58. DOI: 10.1086/657087

[20] Ramirez K, Wahid R, Richardson C, et al. Intranasal vaccination with an adjuvanted Norwalk virus like particle vaccine elicits antigen specific B memory responses in human adult volunteers. Clin Immunol 2012;144:98-108. DOI: 10.1016/j.clim.2012.05.006

[21] Atmar RL, Bernstein DI, Harro CD, et al. Norovirus vaccine against experimental human Norwalk Virus illness. N Engl J Med. 2011; 365:2178-2187. DOI: 10.1056/NEJMoa1101245

[22] Parra GI, Bok K, Taylor R, et al. Immunogenicity and specificity of norovirus Consensus GII.4 virus- like particles in monovalent and bivalent vaccine formulations. Vaccine. 2012;30:3580-3586. DOI: 10.1016/j.vaccine.2012.03.050.

[23] Sundararajan A, Sangster MY, Frey S, et al. Robust mucosal-homing antibody-secreting B cell responses induced by intramuscular administration of adjuvanted bivalent human norovirus-like particle vaccine. Vaccine. 2015;33:568-576. DOI: 10.1016/j.vaccine.2014.09.073

[24] Bernstein DI, Atmar RL, Lyon GM, et al. Norovirus vaccine against experimental human GII.4 virus illness: a challenge study in healthy adults. J Infect Dis. 2015;211: 870-878. DOI: 10.1093/infdis/jiu497

[25] Atmar RL, Baehner F, Cramer JP, et al. Rapid responses to 2 virus- like particle Norovirus vaccine candidate formulations in healthy adults: A randomized controlled trial. J Infect Dis. 2016; 214:845-853. DOI: 10.1093/infdis/jiw259

[26] James S, Paul MM, Eric L. Efficacy of an intramuscular bivalent norovirus GI.1/GII.4 virus-like particle vaccine candidate in healthy US adults. Vaccine. 2020; 38(41):6442-6449. DOI: 10.1016/j.vaccine.2020.07.069

[27] Guo L, Wang J, Zhou H, et al. Intranasal administration of a re-combinant adenovirus expressing the norovirus capsid protein stimulates specific humoral, mucosal, and cellular immune responses in mice. Vaccine. 2008;26:460-468. DOI: 10.1016/j.vaccine.2007.11.039

[28] http://www.vaxart.com/NRfiles/VaxartAnnouncesNorovirusPhaseIResults021417.pdf

[29] Liebowitz D, Lindbloom JD, Brandl JR, et al. High titre neutral- ising antibodies to influenza after oral tablet immunisation: a phase 1, randomised, placebo-controlled trial. Lancet Infect Dis 2015;15:1041-1048. DOI: 10.1016/S1473-3099(15)00266-2

[30] Leesun K, David L, Karen L, et al. Safety and immunogenicity of an oral tablet norovirus vaccine, a phase I randomized, placebo-controlled trial. JCI Insight 2018; 3(13):e121077. DOI: 10.1172/jci.insight.121077

[31] Roberto M, Lisa CL, Shaily JG, et al. Production and Clinical Evaluation of Norwalk GI.1 Virus Lot 001-09NV in Norovirus Vaccine Development. J Infect Dis 2020; 221(6):919-926. DOI: 10.1093/infdis/jiz540

[32] Malet H, Massé N, Selisko B, et al. The flavivirus polymerase as a target for drug discovery. Antiviral Res. 2008;80(1):23-35. DOI: 10.1016/j.antiviral.2008.06.007

[33] Natalie EN, Daniel ET, Peter AW. Norovirus antivirals: Where are we now? Review Med Res Rev. 2019; 39(3):860-886. DOI: 10.1002/med.21545

[34] Galmarini C, Mackey J, Dumontet C. Nucleoside analogues: mechanisms of drug resistance and reversal strategies. Leukemia. 2001;15(6):875-890.

[35] Caillet-Saguy C, Simister PC, Bressanelli S. An objective assessment of conformational variability in complexes of hepatitis C virus polymerase with non-nucleoside inhibitors. J Mol Biol. 2011;414(3):370-384. DOI: 10.1016/j.jmb.2011.10.001

[36] Claire P, Amador A, Samira B, et al. Synthesis and pharmacokinetics of valopicitabine (NM283), an efficient prodrug of the potent anti-HCV agent 2'-C-methylcytidine. J Med Chem. 2006;49(22):6614-6620. DOI: 10.1021/jm0603623

[37] Gardelli C, Attenni B, Donghi M, et al. Phosphoramidate prodrugs of 2'-C-methylcytidine for therapy of hepatitis C virus infection. J Med Chem. 2009;52(17):5394-5407. DOI: 10.1021/jm900447q

[38] Rocha-Pereira J, Jochmans D, Debing Y, et al. The viral polymerase inhibitor 2'-C-methylcytidine inhibits Norwalk virus replication and protects against norovirus-induced diarrhea and mortality in a mouse model. J Virol 2013;87(21):11798-11805. DOI: 10.1128/JVI.02064-13

[39] Rocha-Pereira J, Jochmans D, Dallmeier K, et al. Inhibition of norovirus replication by the nucleoside analogue 2'-C-methylcytidine. Biochem Biophys Res Commun 2012;427(4):796-800. DOI: 10.1016/j.bbrc.2012.10.003

[40] Yousuke F, Brian BG, Kazumi T, et al. Favipiravir (T-705), a novel viral RNA polymerase inhibitor. Antiviral Res 2013;100(2):446-54. DOI: 10.1016/j.antiviral.2013.09.015

[41] Armando A, Lucy T, Ian G. Favipiravir elicits antiviral mutagenesis during virus replication in vivo. Elife 3:e03679. DOI: 10.7554/eLife.03679

[42] Kiso M, Takahashi K, Sakai-Tagawa Y, et al. T-705 (favipiravir) activity against lethal H5N1 influenza A viruses. Proc Natl Acad Sci USA. 2010;107(2):882-887. DOI: 10.1073/pnas.0909603107

[43] Lisa O, Anja L, Stephanie W. Successful treatment of advanced Ebola virus infection with T-705 (favipiravir) in a small animal model. Antiviral Res 2014;105:17-21. DOI: 10.1016/j.antiviral.2014.02.014

[44] Kyeong-Ok C, David WG. Interferons and ribavirin effectively inhibit Norwalk virus replication in replicon-bearing cells. J Virol 2007;81(22):12111-12118. DOI: 10.1128/JVI.00560-07

[45] Costantini VP, Whitaker T, Barclay L, et al. Antiviral activity of nucleoside analogues against norovirus. Antivir Ther 2012;17(6):981-991.

[46] Chimerix. Chimerix announces discovery and demonstrated preclinical activity supporting ongoing phase 1 study of new antiviral for treatment and prevention of norovirus. 2018. http://ir.chimerix.com/

news-releases/news-release- details/chimerix-announces-discovery-and-demonstrated-preclinical [Accessed: 2021-03-03]

[47] Eloise M, Margherita P, Delia T, et al. Structure-based inhibition of norovirus RNA-dependent RNA polymerases. J Mol Biol 2012;419(3-4):198-210. DOI: 10.1016/j.jmb.2012.03.008

[48] Croci R, Pezzullo M, Tarantino D, et al. Structural bases of norovirus RNA dependent RNA polymerase inhibition by novel suramin-related compounds. PLOS One. 2014;9(3):e91765. DOI: 10.1371/journal.pone.0091765

[49] Tarantino D, Pezzullo M, Mastrangelo E, et al. Naphthalene-sulfonate inhibitors of human norovirus RNA-dependent RNA-polymerase. Antiviral Res 2014;102:23-28. DOI: 10.1016/j.antiviral.2013.11.016

[50] Beindl W, Mitterauer T, Hohenegger M, Ijzerman AP, Nanoff C, Freissmuth M. Inhibition of receptor/G protein coupling by suramin analogues. Mol Pharmacol. 1996;50(2):415-423.

[51] Mastrangelo E, Mazzitelli S, Fabbri J, et al. Delivery of suramin as an antiviral agent through liposomal systems. ChemMedChem 2014;9(5):933-939. DOI: 10.1002/cmdc.201300563

[52] Eltahla AA, Lim KL, Eden JS, Kelly AG, Mackenzie JM, White PA. Nonnucleoside inhibitors of norovirus RNA polymerase: scaffolds for rational drug design. Antimicrob Agents Chemother. 2014;58(6):3115-3123. DOI: 10.1128/AAC.02799-13

[53] Kim Y, Lovell S, Tiew KC, et al. Broad-spectrum antivirals against 3C or 3C-like proteases of picornaviruses, noroviruses, and coronaviruses. J Virol. 2012;86(21):11754-11762. DOI: 10.1128/JVI.01348-12.

[54] Charbonneau M-E, Gonzalez-Hernandez MJ, Showalter HD, Donato NJ, Wobus CE, O'Riordan MXD. Small molecule deubiquitinase inhibitors promote macrophage anti-infective capacity. PLOS One. 2014;9(8):e104096. DOI: 10.1371/journal.pone.0104096

[55] Marta GH, Anupama P, Kofi EG. Chemical derivatives of a small molecule deubiquitinase inhibitor have antiviral activity against several RNA viruses. PLOS One. 2014;9(4):e94491. DOI:10.1371/journal.pone.0094491

[56] Perry JW, Ahmed M, Chang KO, Donato NJ, Showalter HD, Wobus CE. Antiviral activity of a small molecule deubiquitinase inhibitor occurs via induction of the unfolded protein response. PLOS Pathog 2012;8(7):e1002783. DOI: 10.1371/journal.ppat.1002783

[57] Luo H. Interplay between the virus and the ubiquitin–proteasome system: molecular mechanism of viral pathogenesis. Curr Opin Virol. 2016;17:1-10. DOI: 10.1016/j.coviro.2015.09.005

[58] Chang KO, Sosnovtsev SV, Belliot G, King AD, Green KY. Stable expression of a Norwalk virus RNA replicon in a human hepatoma cell line. Virology. 2006;353(2):463-473. DOI: 10.1016/j.virol.2006.06.006

[59] Karst SM, Wobus CE, Lay M, Davidson J, Virgin HWt. STAT1-dependent innate immunity to a Norwalk-like virus. Science. 2003;299(5612):1575-1578. DOI: 10.1126/science.1077905

[60] Wobus CE, Karst SM, Thackray LB, et al. Replication of norovirus in cell culture reveals a tropism for dendritic cells and macrophages. PLOS Biol. 2004;2(12):e432. DOI: 10.1371/journal.pbio.0020432

[61] Seungmin H, Nicole SM, Monique WB, et al. Nondegradative role of Atg5-Atg12/ Atg16L1 autophagy protein complex in antiviral activity of interferon gamma. Cell Host Microbe. 2012;11(4):397-409. DOI:10.1016/j.chom.2012.03.002

[62] Maloney NS, Thackray LB, Goel G, et al. Essential cell-autonomous role for interferon (IFN) regulatory factor 1 in IFN-γ-mediated inhibition of norovirus replication in macrophages. J Virol. 2012;86(23):12655-12664. DOI: 10.1128/JVI.01564-12

[63] Thackray LB, Duan E, Lazear HM, et al. Critical role for interferon regulatory factor 3 (IRF-3) and IRF-7 in type I interferon-mediated control of murine norovirus replication. J Virol. 2012;86(24):13515-13523. DOI: 10.1128/JVI.01824-12

[64] Changotra H, Jia Y, Moore TN, et al. Type I and type II interferons inhibit the translation of murine norovirus proteins. J Virol. 2009;83(11):5683-5692. DOI: 10.1128/JVI.00231-09

[65] Chang KO, George DW. Interferons and ribavirin effectively inhibit Norwalk virus replication in replicon-bearing cells. J Virol. 2007;81(22):12111-12118. DOI: 10.1128/JVI.00560-07

[66] McCartney SA, Thackray LB, Gitlin L, Gilfillan S, Virgin iv HW, Colonna M. MDA-5 recognition of a murine norovirus. PLOS Pathog. 2008;4(7):e1000108. DOI: 10.1371/journal.ppat.1000108

[67] Nice TJ, Baldridge MT, McCune BT, et al. Interferon-λ cures persistent murine norovirus infection in the absence of adaptive immunity. Science. 2015;347(6219):269-273. DOI:10.1126/science.1258100

[68] Nice TJ, Robinson BA, Van Winkle JA. The role of interferon in persistent viral infection: insights from murine norovirus. Trends Microbiol. 2017;26:510-524. DOI: 10.1016/j.tim.2017.10.010.

[69] Fox LM, Saravolatz LD. Nitazoxanide: a new thiazolide antiparasitic agent. Clin Infect Dis. 2005;40(8):1173-1180.

[70] Rossignol JF, El-Gohary YM. Nitazoxanide in the treatment of viral gastroenteritis: a randomized double-blind placebo-controlled clinical trial. Aliment Pharmacol Ther. 2006;24(10):1423-1430. DOI: 10.1111/j.1365-2036.2006.03128.x.

[71] Siddiq DM, Koo HL, Adachi JA, Viola GM. Norovirus gastroenteritis successfully treated with nitazoxanide. J Infect. 2011;63(5):394-397. DOI: 10.1016/j.jinf.2011.08.002

[72] Wen D, Yuebang Y, Maikel PP. Opposing effects of Nitazoxanide on murine and human Norovirus. J Infect Dis. 2017;216(6):780-782. DOI: 10.1093/infdis/jix377

[73] Kathryn H, Soren G, Tom BH. Successful treatment of chronic norovirus gastroenteritis with nitazoxanide in a pediatric kidney transplant recipient. Pediatr Transplant. 2018;22(4):e13186. DOI: 10.1111/petr.13186

[74] Capizzi T, Makari-Judson G, Steingart R, Mertens WC. Chronic diarrhea associated with persistent norovirus excretion in patients with chronic lymphocytic leukemia: report of two cases. BMC Infect Dis. 2011;11(1):131. DOI: 10.1186/1471-2334-11-131

[75] Echenique IA, Stosor V, Gallon L, Kaufman D, Qi C, Zembower TR. Prolonged norovirus infection after pancreas transplantation: a case report and review of chronic norovirus. Tranpl Infect Dis 2016;18(1):98-104. DOI: 10.1111/tid.12472

[76] Jurgens PT, Allen LA, Ambardekar AV, McIlvennan CK. Chronic norovirus infections in cardiac transplant patients: considerations for evaluation and management. Prog Transplant 2017;27(1):69-72. DOI: 10.1177/1526924816679843

[77] Kempf B, Edgar JD, Mc Caughey C, Devlin LA. Nitazoxanide is an ineffective treatment of chronic norovirus in patients with x-linked agammaglobulinemia and may yield false-negative polymerase chain reaction findings in stool specimens. J Infect Dis 2017;215(3):486-487. DOI: 10.1093/infdis/jiw497

[78] Wilhelmi I, Roman E, Sánchez-Fauquier A. Viruses causing gastroenteritis. Review Clin Microbiol Infect 2003;9(4):247-262. DOI: 10.1046/j.1469-0691.2003.00560.x.

[79] Sravanthi P, MD, Madhav D, Viveksandeep Thoguluva Chandrasekar, Prevalence of Gastrointestinal Symptoms and Fecal Viral Shedding in Patients With Coronavirus Disease 2019. A Systematic Review and Meta-analysis. JAMA Netw Open 2020;3(6):e2011335. DOI: 10.1001/jamanetworkopen.2020.11335

[80] Sonia Villapol. Gastrointestinal symptoms associated with COVID-19: impact on the gut microbiome. Transl Res 226:57-69. DOI: 10.1016/j.trsl.2020.08.004

[81] Han C, Duan C, Zhang S. Digestive symptoms in COVID-19 patients with mild disease severity: clinical presentation, stool viral RNA testing, and outcomes. Am J Gastroenterol 2020;115:916-923. DOI: 10.14309/ajg.0000000000000664

[82] Lin L, Jiang X, Zhang Z. Gastrointestinal symptoms of 95 cases with SARS-CoV-2 infection. Gut. 2020;69:997-1001. DOI: 10.3410/f.737675327.793573160

[83] D'Amico F, Baumgart DC, Danese S, Peyrin-Biroulet L. Diarrhea during COVID-19 infection: pathogenesis, epidemiology, prevention, and management. Clin Gastroenterol Hepatol 2020;18:1663-1672. DOI: 10.1016/j.cgh.2020.04.001

[84] Meini S., Zini C., Passaleva M.T. Pneumatosis intestinalis in COVID-19. *BMJ Open Gastroenterol.* 2020;7. DOI: 10.1136/bmjgast-2020-000434

[85] Ping A, Hongbin C, Haixia R. Gastrointestinal Symptoms Onset in COVID-19 Patients in Wuhan, China. Dig Dis Sci 2020: 1-10. DOI: 10.1007/s10620-020-06693-6

Edited by Gyula Mózsik

This book provides an overview of norovirus, a viral infection that adversely affects the gastrointestinal system. Unfortunately, there is no specific treatment available for this illness. As such, the World Health Organization (WHO) has identified norovirus as a priority disease for vaccine development. Chapters in this edited volume cover such topics as examination methods and genome mechanisms of norovirus, and clinical and pharmaceutical developments in managing this illness.

Published in London, UK
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ISBN 978-1-83968-946-8
9 781839 689468