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Polymerase Chain Reaction for Biomedical Applications

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POLYMERASE CHAIN REACTION FOR BIOMEDICAL APPLICATIONS

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Notice

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Meet the editor

Dr. Samadikuchaksaraei is an associate professor and head of the Institute for Regenerative Medicine at Iran University of Medical Sciences (IUMS) in Tehran. He received his MD from IUMS and PhD from Imperial College London, United Kingdom. After 22 years of clinical practice and biomedical research, he is a well-known figure who routinely applies molecular biology techniques, including PCR, in his lab. He has more than 120 publications, serves on editorial boards of 6 journals, and regularly reviews for 25 journals and 10 granting bodies and patenting organizations. Additionally, Dr. Samadikuchaksaraei is consulted by the National Science and Technology Development Council, Academy of Medicine and Ministries of Health and Science. His achievements have been recognized by several awards and honors.

Contents

Preface

Polymerase chain reaction (PCR) has been invented in the mid-1980s by Kary Mullis, who was later awarded half a Nobel Prize in chemistry in 1993. It is the most important methodology that revolutionized the fields of molecular biology and molecular diagnosis. It has rapidly become an essential technique for identification or manipulation of genetic material.

Polymerase Chain Reaction for Biomedical Applications aims to provide an up-to-date compilation of chapters on how to perform PCR for different applications and how to obtain an optimized result from the protocols presented in this book. The basis of the protocols is described in each chapter in order to enable the readers to effectively employ the methods that fit to their needs.

The chapters are authored by known experts in the field who collaborated to provide practically feasible protocols for both novice and experienced scientists and technologists. The book begins with the chapter (1) that provides guidelines to perform an optimized real-time PCR with the highest sensitivity and specificity. The next chapter (2) on high-throughput platforms in real-time PCR describes how a high number of samples could be simultaneously processed and how a large number of assays could be performed on a single sample. Chapter 3 on hot cell-direct PCR is about performing PCR and RT-PCR directly on cell lysate without the need of extraction of DNA or RNA. This method reduces the time needed for sample preparation and keeps sample loss to the minimum, which is very important when limited amount of sample is available.

Chapter 4 discusses the significance of contamination of PCR reactions with previously performed PCR products, which leads to false-positive results. This is of particular importance in regulatory agencies and diagnostic laboratories and should be properly taken into account and prevented. Chapters 5 through 7 describe specifically designed and easy-to-perform PCR assays for detection of viral infections in immunocompromised patients, detection of sexually transmitted diseases, and detection of respiratory tract infections. Chapter 8 presents the detection of pathogenic, including nonculturable, organisms in hospital environment. Chapter 9 describes how polymerase chain reaction could be used in different aspects of food safety including detection of food-borne pathogenic and toxicogenic organisms, detection of genetically modified organisms, and detection of different species in food products. Chapter 10 details the methods commonly used for introduction of mutations using polymerase chain reaction.

I hope that *Polymerase Chain Reaction for Biomedical Applications* provides readily reproducible protocols, which could be followed in the laboratory. I would like to thank all the authors for their contributions and the long time they spent to prepare this valuable collection.

Also, I would like to extend my thanks to Ms. Romina Rovan for her great help during the editing of this book and to InTechOpen for their commitment and support.

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Chapter 1

Guidelines for Successful Quantitative Gene Expression in Real-Time qPCR Assays

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Additional information is available at the end of the chapter

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Abstract

This chapter was developed to provide some important guidelines for studies with quantitative PCR (qPCR) using either dyes or probes, citing several essential components necessary for a good PCR assay. The efficiency and specificity of quantitative PCR (qPCR) depend on several parameters related to mRNA quantification that must be controlled to avoid mistakes in data interpretation. Avoiding contamination with proteins, carbohydrate and phenolic compounds during RNA extraction and purification processes will improve RNA quality and provide reliable results. Specific primers and sensible probes are also crucial to intensify efficiency, specificity and fluorescence. Other parameters such as the optimization of primer concentrations and efficiency primer curves must be done. During gene-expression profile quantification, qPCR assays using reference genes are required to normalize the target gene expression data. These reference genes are checked for stability to identify the most stable genes among a group of candidate genes that will be used to normalize the qPCR data, using programs such as geNorm, BestKeeper and NormFinder. Additionally, the choice of appropriate reference genes for a specific experimental condition is fundamental. The main aim of this chapter is to provide guidelines and highlight precautions to obtain a successful qPCR assays.

Keywords: gene expression, real-time qPCR, reference genes

1. Introduction

The polymerase chain reaction (PCR) technique was first introduced by Kary Mullis [1]. Thereafter, progresses in PCR reactions raised a more sensitive PCR technique, a quantitative PCR (qPCR: quantitative real-time polymerase reaction) that employs cDNA as template. cDNA is a complementary DNA from RNA molecules synthesized from reverse transcriptase reactions. During the qPCR reactions, a dye or probe binds to and is incorporated into amplified double-stranded DNA (dsDNA), acting as a fluorescent reporter during amplification. Thus, the enhancement of fluorescent signal is directly proportional to the number of PCR products synthesized in the reactions.

qPCR is widely known as the most effective method to analyze modulations in gene expression because of its efficiency to detect and precisely quantify the target genes, even at low expression levels [2]. The reactions of qPCR enable us to measure the mRNA expression levels in numerous kinds of samples. Nonetheless, a successful qPCR assay requires an appropriate normalization approach, avoiding nonspecific variations among cDNA samples. Thus, employing qPCR with target genes coupled to reference genes is determinant to avoid probable mistakes in either RNA extraction or contamination in the course of sample manipulation.

Summarily, several precautions must be considered in order to obtain consistent results and avoid mistakes in the data interpretation in qPCR assays [3], including: (i) an accurate primer design with respect to specificity and efficiency; (ii) a purified mRNA free of contaminants, such as carbohydrates, proteins and phenols; and (iii) a rigorous choice of reference gene, which must be stable for analyzed experimental condition.

1.1. Quality of RNA

High quality of RNA is an essential requirement for qPCR. There are several probable contaminants that may interfere in PCR reactions by inhibiting mainly transcriptase reverse and DNA polymerases enzymes, such as DNA genomic, excess of proteins and carbohydrates, as well as phenolic compounds [3]. RNA can be quantified at 260 nm in spectrophotometer and readings of absorbance at 280 and 230 are used to detect proteins and carbohydrates, respectively. According to Sambrook et al. [4], in order to verify the RNA purity, the values of ratio $A_{260/280}$ between 1.8 and 2.0 denote a low contamination with proteins, whereas a ratio $A_{260/230} > 2.0$ indicates very low contamination with carbohydrates. These arguments are corroborated by the reports of Ref. [5].

The integrity of the RNA must be also analyzed trough electrophoresis gel. In this sense, the RNA reliability is investigated by analyzing the 28S and 18S ribosomal bands; and their absence individual or dual suggests the RNA degradation. In general, an electrophoretic approach employing agarose gel at 0.8–1.0% is useful to detect the integrity of ribosomal RNA subunits [4].

An additional purification step must be performed before starting qPCR reactions, digesting the genomic DNA; on the contrary, the DNA can act as template during qPCR and produce

unreliable results. It may be avoided employing RNase-free DNase enzymes directly in the samples of RNA or without treatments but using specific primers designed in the exon-exon boundary of gene coding region.

1.2. Primer design and probe considerations

Designing specific primers and adopting appropriate probes are crucial requirements for amplification efficiency, specificity and fluorescence in qPCR assays. The primer should be designed in junction exon-exon of genic sequence to avoid amplification of contaminant genomic DNA, amplifying specifically the target cDNA sequence [6]. The primer efficiency might be analyzed employing serial dilutions or standard curves, defining the ideal primer concentration and/or assessing the reaction efficiency. In this case, the log of each used concentration in standard curve is plotted against a C_q value for that concentration, eluding the reaction performance and other reaction parameters (including y-intercept, slope and coefficient of correlation). In the literature, the researches have typically used the formula:

$$\text{Primer efficiency} = 10^{(-1/\text{slope})} - 1 \quad (1)$$

Slope is the C_q value of first dilution subtracted of the $C_q$value of last dilution, divided by the number of dilutions (**Figure 1**). Therefore, considering that in a 100% qPCR efficiency the total PCR products will double after each cycle, the standard curve slope must be −3.33 (100 = 100% = $10^{(-1/-3.33)}$ − 1). In the large majority of cases, an acceptable slope is around −3.33 cycles, although, a slope between –3.9 and –3.0 (80–110% efficiency) is commonly suitable [7, 8]. An example of inefficient primer by analyzing serial dilutions is provided in **Figure 1A**, whereas a perfect efficiency curve is shown in **Figure 1B**.

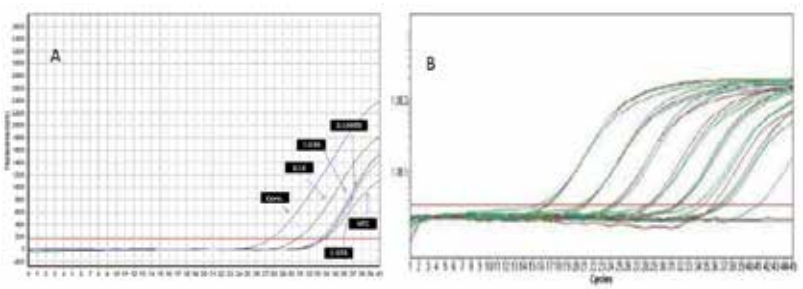

Figure 1. Efficiency curve from inefficient (**A**) and efficient (**B**) primers using serial dilutions in real-time PCR assays.

Inefficient reactions always provide inaccurate calculated levels of target input. Thus, the researcher may either (i) optimize primer concentrations or (ii) design alternative primers to improve reaction efficiency. Several programs are available to perform primer design, including PerlPrimer [9], primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Primer3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). In the

large majority of them, a previous annotation of gene sequence instituting the introns and exons is necessary to input the program.

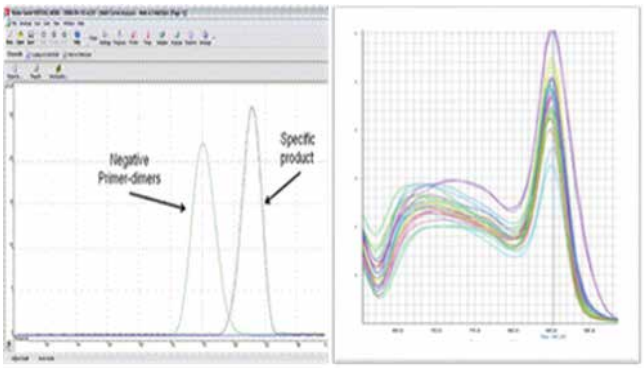

Figure 2. Melting curve after qPCR cycle showing a homodimer and/or heterodimer formation from samples using ineffective primers in qPCR reactions.

Highly effective primers for qPCR assays must not form neither primer-dimer nor nonspecific amplification. Some online software such as https://www.idtdna.com/calc/analyser are accessible to analyze the primers with respect to the formation of homodimers, heterodimers and self-dimers, as well as a harpin formation between the forward and reverse primer [10, 11]. At the end of qPCR cycles, elaborating a melting curve is fundamental to assess the primer specificity, in which a peak lower than 78°C most likely corresponds to the formation of dimmers and/or alternatively unspecific amplifications (**Figures 2** and **3**).

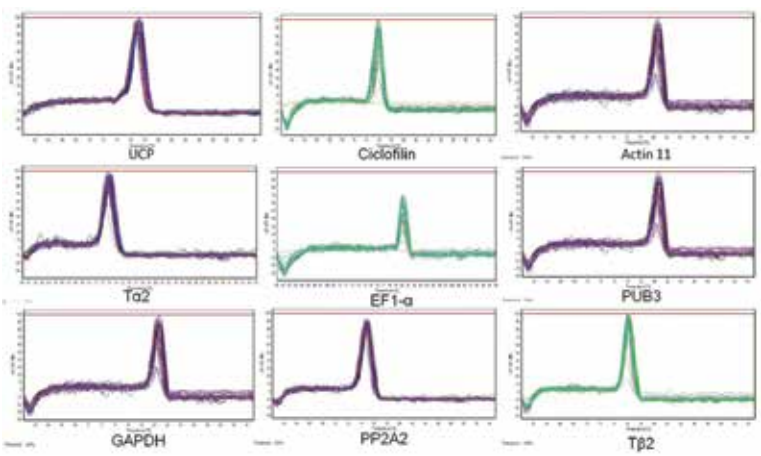

Figure 3. Melting curve of nine candidate for reference genes showing a good specificity with a single peak. UCP – Ubiquitin conjugation protein; Ciclofilin; ACT11 – actin-11; Tα2 – tubulin alpha-2; EF1-α – elongation factor 1-alpha; PP2A2 – protein phosphatase 2A-2; PUB3 – polyubiquitin-3; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; Tβ2 – tubulin beta-2. Adapted with permission from Rocha et al. [6].

Probe, or dye, is a fluorescent marker capable to incorporate inside the qPCR amplification product, into the double-stranded DNA (dsDNA) [12]. It is widely used to measure the amount of amplified DNA during qPCR reactions, taking into account that the fluorescent signal is directly proportional to the amount of PCR products (amplicons) produced in the exponential phase of the reaction. During the reactions, the amplicon accumulation rate improves the fluorescence level, it being directly proportional to the amount of DNA amplified in the sample [12, 13]. SYBR Green is perhaps the best known fluorescent dye that binds to dsDNA and fluoresces upon excitation (**Figure 4A**); whereas TaqMan®, Molecular Beacon and Scorpions are probes designed to react with specific DNA sequences.

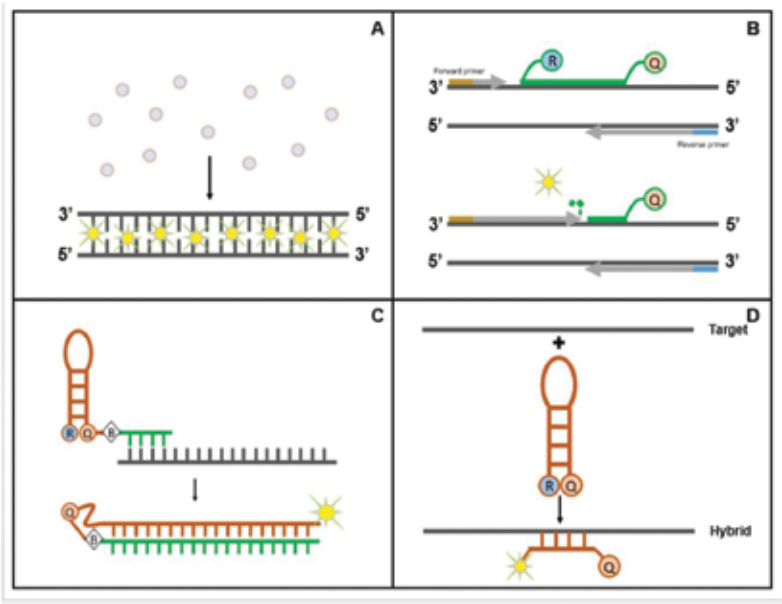

Figure 4. Dyes (SYBR Green, A) and probes (TaqMan, B; Molecular Beacon, **C**; Scorpions, **D**) employed in real-time PCR assays. Adapted with permission from Rocha et al. [3].

The TaqMan® probe is generally an oligonucleotide sequence complementary to specific regions of target DNA, in which a quencher and a reporter fluorophore dye binds to its 3′ and 5′ regions, respectively [14]. TaqMan® interacts to complementary target DNA during the amplification reactions and, thereafter, is cleaved by *Taq* DNA polymerase 5′-3′ exonuclease activity (**Figure 4B**). During cleavage, the reporter dye is released and a fluorescent signal is generated, increasing cycle-by-cycle [12]. Yet, the Molecular Beacon probe remained in a hairpin structure (composed by a quencher and a reporter dye) when free in the solution; thus, no fluorescence is emitted because of the fluorescent reporter and the quencher are extremely closed (**Figure 4C**) [14].

Scorpions are single-stranded oligonucleotide probes consisting of nearly 20–25 nt, composed by a reporter fluorophore at 5′ region and a quencher at 3′ region, resembling a stem and loop

structure where the primer is attached (**Figure 4D**). The stem-and-loop structure acts as a blocker to prevent DNA polymerase activity during the interaction of the probe with the target DNA [12, 15]. In the absence of reaction, reporter and quencher are closely near, occurring at a continuous suppression of fluorescence by the reporter. In general, dyes are less specific than probes; whereas the dyes may bind to any regions of DNA double-stranded during a PCR amplification reaction, the probes are specific for binding in particular regions that allow to emit fluorescence. SYBR Green has been widely used because it has low cost and high efficiency.

1.3. Importance of the reference genes to normalize qPCR data

Reference or constitutive genes are required to normalize the target gene-expression quantification in qPCR assays. The normalization of expression levels is pivotal once it avoids misinterpretation of data obtained in qPCR reactions. Thus, a group of constitutive genes should be analyzed by stability, choosing the most stable ones as reference to be used in the data normalization. The assays of normalization must be conducted using at least eight stable reference genes because a single reference one as proposed by Livak et al. [7]) is not always constitutively expressed in all cell types [16, 17].

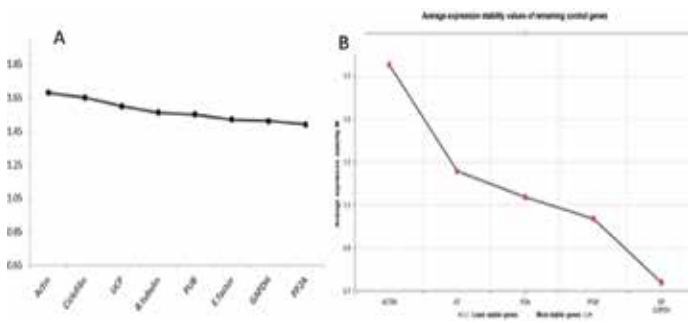

Figure 5. *M* values exported from GeNorm for select stable reference genes in all tissues (embryo axis + endosperm + inner integument) (**A**) and inner integument (**B**) of developing *Jatropha curcas* seeds. Genes with *M* values >1.5 are not appropriate to normalize data as suggested by the GeNorm program. Candidate genes: Actin; Ciclofilin; UCP; B-tubulin; PUB; E. factor or EF; GAPDH or G3PDH; PP2A2 or P2A; A-tubulin (AT).

Accuracy of relative gene expression can be severely affected by a wrong choice of reference genes to normalize and validate the final results; consequently, employing inappropriate genes as reference for data normalization may lead to erroneous results and data misinterpretation [18]. Thus, the expression stability of a reference gene must be confirmed in each experimental condition before the qPCR assays and it should be taking into account that a unique gene is generally not suitable for normalization [16, 17].

In the last decade, several tools have been developed to identify genes for normalization purposes and ensure a reliable normalized gene expression, including BestKeeper, geNorm and NorFinder [8, 16, 19, 20]. These programs are available online for free download and are

widely used to calculate a normalization factor over multiple reference genes, improving the robustness of the normalization even further [21].

GeNorm program has been cited as the best statistical method to choose stable reference genes for qPCR reactions. Summarily, the principle is that the expression level of reference genes must be equal in all samples, regardless of experimental condition or cell type. The *M* values below cut-off (<1.5) are considered the most stable genes among all candidate reference genes (**Figures 5** and **6**) [16]. Thus, highly stable genes are recognized by the lowest *M* values and genes presenting the highest *M* values should be disregarded and not be used as reference [10, 16, 20].

All genes expressed cutoff values for M of <1.0, as suggested by GeNorm. The most stable reference gene for samples of inner integument of developing *Jatropha curcas* seeds were : GAPDH, UCP,ACTIN, PP2A2 and ciclofilin as showed in **Figure 6**. However, the less stable genes were: EF1-α and tubulin alpha-2. This **Figure 6** show that 5 genes are necessary to narmalization the qPCR assay in developing seeds as cited above (**Figure 6**). In to Under stress conditions leaves exposure, different gene combinations were also necessary for accurate normalization. For total (a mix of all conditions) and SA stress treatments in leaves exposure, three and four genes were respectively required to normalize gene expression in leaves (**Figure 6**). Nevertheless, for PEG and NaCl stress treatments, four and five genes were respectively necessary to normalize gene expression (**Figure 7a** and **7b**). The best combinations of stable genes in each tissue under stress conditions were as follows: for total stress, the following two genes were used for normalisation: E. factor, PP2A and GAPDH (**Figure 7a** and **7b**). However, in SA stress, four genes were required: PP2A, E. factor, GAPDH and PUB. In PEG stress, two genes were identified as the best genes for normalisation: PP2A and E. factor, while in NaCl stress, five genes were necessary: PP2A, GAPDH, E. factor, PUB and B. tubulin (**Figure 7a** and **7b**) as suggested by geNorm with cutoff values for M of <1.0.

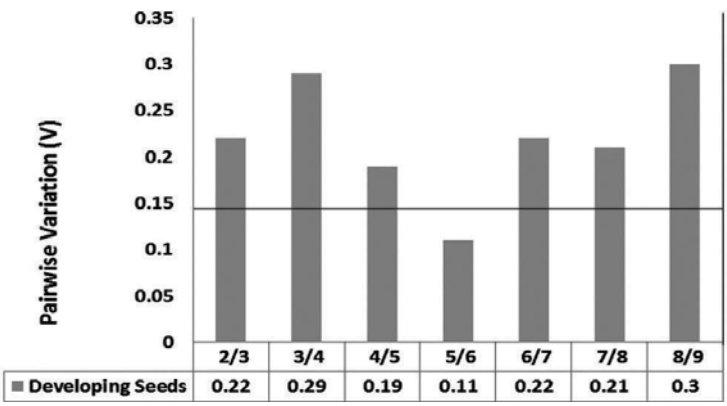

Figure 6. Optimal number of reference genes in inner integument of developing *Jatropha curcas* seeds, according to GeNorm program. A combination of pairwise variation (*V* value) with cut-off <0.15 is considered ideal for select the number of genes for normalization data.

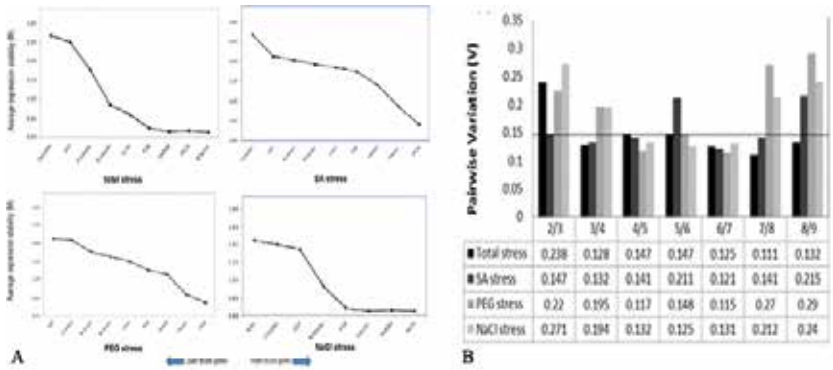

Figure 7. In leaves exposure to total stress were needed three genes were used to normalisation: E. factor, PP2A and GAPDH; however, in SA stress, four genes were required: PP2A, E. factor, GAPDH and PUB (Figs 7a and 67b). In PEG stress, were necessary four genes: PP2A, E. factor, GAPDH and PUB. In NaCl stress, five genes were necessary: PP2A, GAPDH, E. factor, PUB and B. tubulin. Normally, 0.15 has been recommended as a cut-off value for the pair-wise variation, below which the inclusion of additional reference genes is not required, although this should not be an absolute rule.

The GeNorm also indicates the optimal number of genes to be used as reference in normalization by evaluating the variation in pairs (*V* values) and analyzing the disparity of expression in pairwise gene combinations. In this sense, the ideal number of genes is also influenced by experimental condition, it being selected by calculating *V* values as a pairwise variation (V_n/V_{n+1}) between two consecutively ranked normalization factors (NF), followed by an addition of the subsequent more stable reference gene (NF_n and NF_{n+1}).

Currently, the GeNorm is integrated to qBASEPlus (Biogazelle) software, constituting a pivotal tool to offer the more stable reference genes (*M* value) coupled to the number of genes appropriate for normalization (*V* value). qBASEPlus is widely employed to determine the relative expression of qPCR assays based on normalization factor (NF), requiring at least eight reference genes and 2 samples (control and treatment).

The values of *V* used for selecting the number of reference genes for qPCR assays in *J. curcas* plants exposed to abiotic stresses are shown in **Figure 7**. The evaluations were performed in leaves of plants submitted to isolate and combined [salicilic acid (SA) + polyethylene glycol (PEG) + NaCl] stress. Considering cut-off *V* value <0.15, the genes appropriate for normalize data in each condition stress were (**Figure 7**):

a. **Combined stress**: E. factor, PP2A and GAPDH.

b. **SA stress**: PP2A and E. factor.

c. **PEG stress**: PP2A, E. factor, GAPDH, PUB, actin and B. tubulin.

d. **NaCl stress**: PP2A, GAPDH, E. factor, PUB, B. tubulin and UCP.

NormFinder is an algorithm capable of determining gene normalization among a series of candidate genes. The normalization is done according to the candidate gene expression stability in both specific sample set and experimental design. The NormFinder employs a

mathematical model coupled to a solid statistical framework to determine an overall expression variation of candidate normalization genes, as well as a variation among a subgroup form the set of samples [19]. Markedly, the NormFinder also provides a stability value for each gene that is an estimative for variation in the expression, enabling the operator to evaluate the occurrence of normalization gene-introduced systematic error [21, 22].

Rank	Developing seeds	
	Gene name	**Stability value**
1	GAPDH	0.035
2	UCP	0.052
3	PP2A	0.148
4	CICLOFILIN	0.262
5	PUB	0.315
6	ACTIN	0.334
7	B.TUBULIN	0.429
8	E.FACTOR	0.508
9	A.TUBULIN	0.532

Genes with lowest stability values are considered appropriate to normalize data.

Table 1. Ranking of stable reference genes for qPCR experiments using developing *Jatropha curcas* seeds according to the NormFinder software.

Rank	Combined stress		SA stress		PEG stress		NaCl stress	
	Gene name	**Stability value**	**Gene name**	**Stability value**	**Gene name**	**Stability value**	**Gene name**	**Stability value**
1	PP2A	0.112	PP2A	0.201	GAPDH	0.304	GAPDH	0.123
2	GAPDH	0.254	E.FACTOR	0.423	E.FACTOR	0.374	E.FACTOR	0.298
3	E.FACTOR	0.448	GAPDH	0.544	PP2A	0.458	PP2A	0.301
4	B.TUBULIN	0.672	PUB	0.723	ACTIN	0.490	PUB	0.457
5	ACTIN	0.680	ACTIN	0.812	PUB	0.520	B.TUBULIN	0.910
6	PUB	0.829	CICLOFILIN	0.878	UCP	0.610	UCP	1.423
7	UCP	1.050	A.TUBULIN	1.201	CICLOFILIN	0.820	CICLOFILIN	1.460
8	A.TUBULIN	1.321	B.TUBULIN	1.422	A.TUBULIN	1.331	ACTIN	1.480
9	CICLOFILIN	1.420	UCP	1.497	B.TUBULIN	1.489	A.TUBULIN	1.501

Genes with lowest stability values are considered appropriate to normalize data.

Table 2. Ranking of stable reference genes for qPCR experiments using *Jatropha curcas* plants exposed to abiotic stresses isolate and combined [Salicilic acid (SA) + Polyethylene Glycol (PEG) + NaCl], according to the NormFinder software.

The ranking of candidate reference genes for normalize data of qPCR reactions of *J. curcas* using the NormFinder software is presented in **Tables 1** and **2**. In developing seeds, most stable genes were GAPDH, UCP, PP2A and ciclofilin with stability values of 0.035, 0.052, 0.148 and 0.262, respectively (**Table 1**). Under abiotic stress conditions, PP2A, GAPDH and EF1-α were considered as the most stable genes with respect to stability values, regardless of stress condition (combined, PEG, SA and NaCl stress) (**Table 2**).

Rank	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	ACTIN	**CICLOFILIN**	**GAPDH**	**PP2A**	**PUB**	**UCP**	**A.TUBULIN**	**B.TUBULIN**	**E.FACTOR**
N	20	20	20	20	20	20	20	20	20
GM [CP]	26.79	31.08	22.54	29.90	31.61	22.96	30.85	31.66	26.26
AM [CP]	26.85	31.14	22.60	29.96	31.72	23.08	31.01	31.77	26.41
Min [CP]	23.41	27.45	19.64	26.17	27.19	20.16	25.51	26.00	21.51
Max [CP]	30.92	34.69	27.96	34.87	36.71	29.89	37.96	38.04	33.56
CV [%CP]	4.91	5.04	5.87	4.62	4.74	8.02	8.55	6.34	8.54
SD [±CP]	1.32	1.57	1.33	1.39	2.14	1.85	2.65	2.01	2.25
Coefficient of correlation [*r*]	0.656	0.548	0.861	0.957	0.666	0.579	0.894	0.843	0.819
p-Value	0.002	0.012	0.001	0.001	0.001	0.008	0.001	0.001	0.001

N = number of cDNA samples; GM = geometric mean of C_q value; AM = arithmetic mean of C_q value; min and max = extreme values of C_q; CV [% CP] = coefficient of variance expressed as percentage of C_q value; SD [± CP] = standard deviation of C_q value; *r* = Pearson coefficient of correlation; *p*-value = *p*-value associated with the Pearson coefficient of correlation.

Table 3. Descriptive statistics of reference gene expression in black-grass based on the BestKeeper analysis.

Unlike the NormFinder software, the algorithm of BestKeeper assesses the variability of reference gene by analyzing the quantification cycle ("C_q") value, which takes into account the coefficient of correlation ("*r*") and standard deviation ("SD") values. Values of SD [$\pm C_q$] < 2 are considered acceptable. According to this software, the most stable genes commonly present the highest *r*- and lowest SD-values; and the less stability of candidate genes is denoted by the highest SD values (**Table 3**) [8, 12, 23, 24].

According to the BestKeeper software, the PP2A (r = 0.958; SD = 1.38), GAPDH (r = 0.887; SD = 1.29), beta tubulin (r = 0.843; SD = 1.02) and alpha tubulin (r = 0.889; SD = 1.04) genes showed the best correlations (**Table 3**) and were considered ideal for normalization data. Although

actin has presented a SD value = 1.32 and showed an *r* value = 0.65, becoming inappropriate for normalization of data.

GeNorm, NormFinder and BestKeeper are determinant for reference gene evaluation and normalization data in qPCR assay. GeNorm is considered the best software since it does not only provide the best reference genes by *M* value but also supply the *V* value that indicates an ideal number of genes necessary for normalization purposes; whereas the NormFinder and BestKeeper algorithms specifically identify the most stable candidate genes. Nonetheless, all three algorithms are employed together in order to provide reliable normalization results.

In general, two kinds of qPCR are widely employed in studies of gene expression, absolute and relative qPCR, in which references genes (i.e., constitutive genes expressed in all cells) must be used to quantify the results. The relative mRNA quantification by real-time PCR has been the most frequently reported, as initially described by Livak et al [7].

Relative qPCR has several advantages, excluding the need for standard curves. It uses mathematical equations to calculate the relative expression level from target gene as compared (relative) to reference control and/or calibration. By using both calibrator and reference gene, the amount of target gene transcripts in a sample is first normalized with the reference genes and their expression is relativized to normalized calibrator, according to the following formula:

$$\text{Relative gene expression} = 2^{-\Delta\Delta C_q} \tag{2}$$

where $\Delta\Delta C_q = \Delta C_q$ (sample) − ΔC_q (calibrator); and ΔC_q = target gene C_q − reference gene C_q; note: C_q = cycle quantification is usual known as C_t = cycle threshold [7]. It is very important to mention that biological and technical sample replications must be carried out to conduct a statistical analysis, evaluating significantly gene expression and validating the results.

In the last years, the use of a single reference gene proposed by Livak et al. [7] has been not advised for qPCR data normalization since it may vary depending on specific tissues. Thus, Vandesompele et al. [16] suggests employing at least eight candidate reference genes in geNorm built-in qBASE plus (biogazelle) software for obtaining reliable results in qPCR assays.

2. Conclusion remarks

qPCR is an efficient power tool to ensure the mRNA expression in several kinds of samples. To obtain reliable results, numerous parameters should be considered, including: (a) a good quality RNA; (b) specific and efficient primers; (c) appropriate dyes or probes according to the analysis; (d) stable reference genes with respect to analyzed condition; (e) normalization of expression; and (f) a combined approach of available software. Finally, we highlight that adopting all described guidelines, the possible errors and wrong procedures will be decreased, thus rendering successfully the results in real-time qPCR assays.

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Chapter 2

High-Throughput Platforms in Real-Time PCR and Applications

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Additional information is available at the end of the chapter

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Abstract

The miniaturization of reactions by designing nanoliter-scale PCR platforms, as Taqman® OpenArray®, Dynamic Array™, or SmartChip, has been a big step forward in real-time PCR. Each platform has some particular characteristics that differentiate them. These nanoliter-scale PCR platforms enable substantial savings in the amount of reagents and sample because the reaction volumes are at nanoliter levels. In addition, it is possible to perform thousands of reactions in a few hours. Therefore, high-throughput real-time PCR platforms result in promising systems that are capable of processing a large number of samples simultaneously and also to perform a large number of assays per sample. All of this can be translated in the amazing applicability of this technology in all kinds of analytical fields, such as medical research, animal science, and food safety, among others.

Keywords: real-time PCR, high-throughput, SNPs, microRNA, gene expression

1. Introduction

In the decade of the nineties, real-time PCR became popular and meant an important step in molecular biology because of the great advantages over conventional PCR biology and its quantitative abilities. This revolution in PCR methods was achieved, thanks to the use of intercalating fluorophores, specific labeling probes with fluorescence emission, as well as equipment with the ability to detect this fluorescence during the reaction [1]. In this way,

measurement of gene expression, absolute quantification of pathogens, and genotyping analysis became routine analysis in life science research.

Until now, the limiting factor for real-time PCR compared to other molecular techniques was the number of tests that could be performed simultaneously. For example, hybridization techniques using microarrays, as those designed by Illumina® and Affimetrix®, allow the analysis of thousands of genetic markers in one single array [2, 3]. The realization of this type of screening by real-time PCR in standard formats (384 and 96 microwell plate) is infeasible due to both operational and economic reasons. In this context, in the few last years, some companies started to develop real-time PCR methods using a smaller reaction volume. As a beginning, some options for sample miniaturization such as the TaqMan® array microfluidic cards designed by Applied Biosystems® or the arrays designed by Qiagen® became available in the market. These formats use small volume that minimizes sample and reagent consumption. Their use is nowadays very popular in the field of gene expression, where it is possible to find predesigned array cards for certain diseases and metabolic pathways [4, 5]. They have also applications in other fields as microRNA screening or detection of foodborne pathogens [6, 7]. However, this improvement in the number of assays and samples that can be analyzed at the same time is still far away from the capabilities of microarray techniques.

The development of real-time high-throughput platforms was a breakthrough that changed the perception about real-time PCR and their possibilities. From the very beginning, the use of nanoliter reaction volumes and the possibility of custom designs converted this technology into an attractive tool for research purposes. Thus, this nanoliter-scale PCR became very common in fields such as medicine and pharmacogenomics research, allowing the inclusion of large number of assays and samples in one run. All the previous features have positioned real-time PCR in direct competition with microarrays and next-generation sequencing, also because it is a more sensitive and specific technique.

2. Principles and available high-throughput real-time PCR platforms

Currently, there are three commercially available high-throughput real-time PCR platforms: Dynamic Array™ chip (Fluidigm®, South San Francisco, CA, USA) TaqMan® OpenArray® (Applied Biosystems®, Carlsbad, CA, USA), and SmartChip (Wafergen Bio-systems Inc., Fremont, CA, USA). The operating principle of the three high-throughput platforms is the use of a nano-scale approach with thousands of reactions in a single run, reducing the sample and reagents consumption to the minimum. However, there are very important differences depending on the platform used.

2.1. Dynamic array

Microfluidic Dynamic Array™ with the integrated fluidic circuit (IFC) along with Biomark™ System from Fluidigm was the first real-time high-throughput platform available in the market [8]. The design of Dynamic Array™ is based on an integrated network of channels, chambers and valves that automatically combine the reactions. Thus, the dynamic plate is

formed mainly by three components. On the one side, there are separate sample inlets, one for each sample. On the other side, there are separate primer-probe inlets, one for each probe. The IFC is placed in the middle, and it is here where the reaction chambers are located. This mechanism uses manual pipetting to load samples and primers-probes, and avoids the use of robotic liquid-handling to set up microwell plates. However, this kind of plates needs another instrument (IFC controller) controlled by a software to pressure load the assay components in the wells of the IFC in a process of 55 min. These wells, called reaction chambers, are formed by two containment valves and one interface valve. Thus, pressure is applied to the fluids contained in sample and detector inlets simultaneously. In this way, the fluids are transported into reaction chamber and fluid lines, respectively. The interface valve prevents sample and detector fluids from mixing. Then, the two containment valves are closed and interface valve is opened, pushing the detector fluid into the reaction chamber for mixing with the sample. Once the components are properly mixed, the interface valve is closed again and the chip is ready for cycling [9]. The real-time PCR cycling is performed in the Biomark™ System. At the end of each cycle, the chip is imaged and at the end of the whole process, the software generates PCR curves and a heat map, where each square represents a reaction chamber and the color indicates the CT value.

IFC	**48.48 Dynamic Array IFC**	**96.96 Dynamic Array IFC**	**192.24 Dynamic Array IFC**	**FLEXsix IFC**
Application	Genotyping/gene expression	Genotyping/gene expression	Genotyping/gene expression	Genotyping/gene expression
Pipetting steps	96	192	216	Variable
Assay inlets	48	96	24	6×12
Sample inlets	48	96	192	6×12
Reactions chambers	2304	9616	4608	864
Reaction volume (nl)	9	7	8	9

Table 1. Dynamic array chips commercially available and their principal characteristics.

The company has a web tool [10] to customize assays for genotyping and gene expression with the specific conditions in which works, Dynamic Array™. There are different designs of Dynamic Array™ chips (**Table 1**) with reaction volumes ranging from 7 to 9 nl, depending on the array [11]. Of all the available nanoliter-scale platforms, Dynamic Array™ uses the smallest reaction volumes, while the other platforms use volumes of 33 and 100 nl. FLEXsix IFC is the model with the least number of reaction chambers (864). However, it can be considered as a model to select targets and to optimize assay. It has 6 partitions that allow 12 performing samples and 12 assays per sample in independent runs. The next designs in terms of number of chambers are 48.48 Dynamic Array™ and 96.96 Dynamic Array™ that analyze 48 and 96 samples and assays per sample, respectively. These three models can be used both for genotyping and gene expression assays. The last model was called 192.24 Dynamic Array™ and it

allows performing 24 assays per sample and 192 samples. As an example of their great processing capability and taking into account the whole workflow, the 96.96 Dynamic Array™ enables 9216 reactions in less than 4 h and it is capable to generate 36,000 data points per person and day. Despite the clear potential of these four designs available to the customer, Dynamic Array™ systems lack intermediate formats or even bigger dynamic chips that would allow the realization of a greater number of assays per sample.

One of the main advantages of Dynamic Array™ platforms compared to their direct competitors is the flexibility derived from the inexistence of preprinted assays. Dynamic Array™ has the possibility to choose the analyses that are going to be performed in each run. Although this flexibility is a clear advantage, the platform has problems of compatibility with TaqMan® assays. While in OpenArray® platform the same company designs and preprints the assays, in Dynamic Array™ another company provides the primers and probes, resulting in the subsequent problems of compatibility. These problems have been observed already in some intercomparative studies. For instance, Fedick et al. [12] used Dynamic Array™ and OpenArray® platforms for genotyping members of the Ashkenazi Jewish community. They observed problems of compatibility with the design of TaqMan® probes for genotypic analysis with Dynamic Array™. This incompatibility reduced the sensibility and the specificity of the assay in comparison with OpenArray®. Also, Farr et al. [13] detected problems of compatibility between RT primers and TaqMan® assays when Dynamic Array™ was employed for microRNA screening. These problems of compatibility could be solved simply by implementing a meticulous design of custom primers, which are adapted to Dynamic Array™ conditions.

2.2. TaqMan® OpenArray®

TaqMan® OpenArray® (Applied Biosystems) was the second high-throughput platforms released in the market. In the year 2006, Morrison et al. [14] published the Biotrove Inc (MA, USA) design of a through-hole array. It was composed of 3072 holes of 33 nl reaction volume in 48 groups of 64 holes each one. These plates have a hydrophobic exterior surface and hydrophilic interior (the inside surface of the 33 nl holes). This intelligent design allows fluid deposited on the hydrophobic surface to move into the hydrophobic holes. These arrays require the use of the OpenArray® NT autoloader for loading plates and the OpenArray® NT cycler for running the array [15]. In 2009, this technology was acquired by Applied Biosystems®, which now is a part of Thermo Fisher Scientific (MA, USA). For this reason, presently the OpenArray® technology works with OpenArray® AccuFill™ System for loading samples and the QuantStudioTM 12K Flex real-time PCR system that allows running four OpenArray® plates at the same time. The main applications described for this instrument up to now are gene expression, genotyping, microRNA screening, or drug metabolism analysis.

The first difference with the Dynamic Array™ is that in the OpenArray® design, the primers and probes are preloaded in the holes by the company. As a result, the user only needs to load the samples and the PCR mix onto the plate. However, the primers and probes cannot be changed while with Dynamic Array™ it is possible to choose which primers are used in the reaction if we have a library of them. On the other hand, the number of plate designs available for the customer with OpenArray® technology is higher.

The first step in an experiment with OpenArray® is to decide the right plate design that is convenient for the number of samples and assays per sample we want to perform. The division of the plate in 48 subarrays of 64 holes each allows variable designs, which are different for gene expression and genotyping assays (**Table 2**). The customers place the order for the desired design through an application on the web page of Thermo Fisher Scientific. It is possible to find millions of predesign TaqMan® assays in the database of the company, all of them successfully tested by the manufacturer. In addition to them, the costumer can order his custom assays by loading the target sequence in the web page and the company would design then the primers and the probes according to the real-time PCR conditions in which OpenArray® technology works. It is also possible to obtain some predesigned OpenArray® plates commercially available for specific genotyping and gene expression purposes. For example, there are OpenArray® plates for human identification and for expression of inflammation, cell-stem, or cancer-related genes [16]. Also, there is an OpenArray® plate for screening of microRNA in human samples [17].

Format	Gene expression		Genotyping		
	Number of assays	Number of samples	Format	Number of assays	Number of samples
18	18 × 3*	48	16	16	144
56	56	48	32	32	96
112	112	24	64	64	48
168	168	16	128	128	24
224	224	12	192	192	16
			256	256	12

* In format 18×3 the assays are performed in triplicate.

Table 2. OpenArray® formats for genotyping and gene expression available for the costumers.

The two most widely used approaches to generate fluorescence in real-time PCRs are SYBR® Green and labeled probes. SYBR® Green is a chemical reagent that is introduced into the secondary structure of the DNA double helix. Consequently, the more the PCR products formed the more the fluorescence emitted. This molecule is commonly used in gene expression, mainly due to their affordable price. SYBR® Green allows the obtainment of a larger number of tests at a relatively low price. With regard to labeled probes, these nucleotide sequences were designed with the purpose of increasing the specificity of quantitative PCR. These probes bind to the middle of the target sequence, which is going to be amplified. They are labeled in the 5′ end of their structure with a fluorophore that will be released when the target section is amplified, resulting in fluorescence emission during amplification. Labeled probes are usually preferred in genotyping assays because using two labeled probes with two different fluorophores in the same reaction is possible to differentiate the two alleles. In the early development

of OpenArray®, most of the experiments were performed using SYBR® Green [14, 15, 18] instead of TaqMan® Probes. However, nowadays this technology works mostly with TaqMan® probes both for genotyping and gene expression, because they increase the specificity of the assays and data processing is easier.

A characteristic trait of OpenArray® plates is that they need to be loaded with the help of OpenArray® AccuFill™ System, while Dynamic Arrays™ are loaded manually. This does not mean a saving in equipment, because Dynamic Arrays™ needs another apparatus to transfer the samples and the primers to the reaction chambers. Moreover, this transfer process takes 55 min, while loading plates with OpenArray® AccuFill™ System only takes a few minutes. With OpenArray® AccuFill™ the process is very simple. First, the samples are mixed with the Master Mix in a 384-well plate. The order of the samples on this plate depends of the OpenArray® format that is going to be used. Afterward, the tip system of the OpenArray® AccuFill™ equipment transfers each sample to the corresponding subarray, showing a specific movement according the chosen array format. The sample is deposited in the hydrophobic surfaces of the array and the liquid is naturally transferred to the hydrophilic 33 nl holes. Once the array is filled, it has to be properly sealed and it is ready for the real-time PCR analysis. One of the great advantages of the OpenArray® system is that is possible to run four plates at the same time. Since the whole real-time PCR process takes about 4 h, in just one run 12,288 data points can be obtained.

The huge amount of data originated during an OpenArray® experiment resulted in the need for specific software that would enable to handle the results. For this, there are two options to process OpenArray® data. One is the software that controls the equipment and the other option is using the analytical platform available on the net. Thermo Fisher Scientific has created a Cloud where the user can upload the experiment files and analyze them with no need of installing anything on the computer. The Cloud also offers the possibility of sharing the files with another person, so that several people from different sites can work on the same files.

TaqMan® OpenArray® plates have a characteristic that may be considered as an advantage and a disadvantage at the same time, the preprinted primers. When custom plates are ordered, the company designs the primers and probes for the target sequence according the OpenArray® working conditions, and preprints them in each hole of the plate. The advantage is that the company performs quality control assays, but the problem is that the minimum order in the custom mode is 10 plates per order. Thus, if the researchers are not satisfied with the results obtained for some assays or even if they want to change the design, they have to order another 10 plates, with the subsequent economic costs.

2.3. SmartChip

In 2010, the last high-throughput platform was released to the market under the commercial name of SmartChip real-time PCR system (WaferGen Biosystems, CA, USA). This technology is based on a chip of 5184 individual wells. Within the three available technologies described until now, this is the one that uses the largest volume of reaction: 100 nl per reaction [95]. SmartChip has some characteristics that make it stand out from Dynamic Array™ and OpenArray®. A clear advantage of SmartChip systems is that they allow profiling of more

than 1000 genes in a single run and in quadruplicate. These platforms would therefore enable the user to perform a screening of genes closer to the amplitude of microarrays technologies than those obtained with the other two systems. However, the number of samples that can be analyzed in this case is only one per run. In fact, the company describes the SmartChip technology in three basic steps: (1) discovery: analyzing hundreds to thousands of genes, (2) validation: analyzing tens to hundreds of genes and (3) screening: analyzing tens of genes as informative genes of a disease signature. In addition to the top model >1000 genes format of SmartChip, the company offers also customized formats for specific hypothesis-based purposes. These formats permit the inclusion of 384 to 12 samples in a single chip (**Table 3**).

Assay configuration	Samples analyzed at the same time
1243	1 (in quadruplicate)
12	384
24	216
36	144
48	108
54	96
72	72
80	64
96	54
120	42
144	36
216	24
248	20
296	16
384	12

Table 3. Possible configuration of SmartChip panel for custom analysis.

The company offers three ready-to-use predesigned panels: the SmartChip Human Oncology Panel with 968 gene-specific assays in quadruplicate, the SmartChip Human microRNA Panel with 778 microRNA-specific assays in quadruplicate, and a SmartChip Bacterial Vaginosis Panel that includes 19 pathogens common in woman vaginosis. For custom assays, it is necessary to send the sequences to the company service. With this information, they design the primers according to the specific conditions of the SmartChip real-time PCR and preload them into the chip. Until now, this technology has been applied mainly in gene expression studies, working only with SYBR® Green chemistry.

The SmartChip complete system is composed of a cycler and a dispenser, with the corresponding required software. For loading or dispensing the samples on the chip, the user has two options. The SmartChip nanodispenser, to dispense only one sample in the entire chip, and designed specifically for the >1000 panels, and the SmartChip multisample nanodispenser, created for loading up to 384 samples on a single chip. In both options, the loading time is less than 10 min. Afterward, the chips are run in a SmartChip cycler, in a process that lasts 2 h. The obtained data would be analyzed with the SmartChip qPCR software.

3. Application fields of high-throughput real-time PCR platforms

3.1. Genotyping

3.1.1. Basic concepts of genotyping

One of the most promising applications of high-throughput real-time PCR in science is genotyping. This technique is based on the analysis of single nucleotide polymorphisms (SNPs) of the DNA. SNPs are single base pairs in a specific position of genomic DNA where different sequence alternatives, the alleles, exist in normal individuals in the population and the minor allele has an abundance of 1% at least [19]. The DNA is composed of four different nucleotides: adenine, guanine, thymine, and cytosine. Thus, in theory the SNPs could be tetra-allelic but in practice, this is a rare situation and normally SNPs are bi-allelic. As such, this type of genetic polymorphism is very common in genomic DNA of mammals. For example, around 1.4 million of single-nucleotide polymorphisms have been identified in the initial sequencing of the human genome, of which 60,000 are located in the coding region of genes [20]. The importance of SNPs is huge because these nucleotide modifications can be silent but they can also be responsible for the predisposition to certain diseases or be the direct cause of them. For instance, there are some studies that have related the presence of certain SNPs in individuals with diabetes, obesity, hypertension, or even cancer [21–24].

The development of next-generation sequencing (NGS) and microarrays have allowed the discovery and analysis of hundreds of thousands of SNPs [25]. However, broad screening approach has also a few drawbacks. For example, to study the presence of certain SNPs associated with a disease in a population the number of SNPs that are going to be analyzed is as important as the number of samples that can be processed simultaneously. This is exactly where real-time PCR gains importance over other technologies. In this context, there are two common techniques to detect single nucleotide polymorphisms with real-time PCR. The first option is using SYBR® Green and melting curve analysis. This method is based on detecting small differences in PCR melting (dissociation) curves after real-time PCR cycles. These differences in the dissociation curve allow to detect the differences in a single nucleotide [26]. The other option is based on the use of probes, applying two probes with different 5′-end labeling in the same reaction. Thereby, if both the fluorophores are detected it means that the sample is a heterozygote for the target DNA region. On the contrary, if the equipment only detects one of the fluorophores the sample is a homozygote. The major problem of selecting

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real-time PCR for genotyping analysis is the high costs involved in analyzing a wide range of samples and SNPs, mainly due to prices of probes and master mix. High-throughput real-time PCR platforms made PCR an ideal tool to analyze SNPs, thanks to their nanoscale working capacity and the consequent saving of reagents and samples. One of the research areas that have more directly benefited from this technology is human medicine. The previous fact is based on the possibility of including a large number of samples and assays in one day of work. This can be translated into the tracking of a large number of SNPs per sample, in order to assess their influence on the development of certain diseases.

3.1.2. High-throughput real-time PCR in genotyping

In 2011, Chan et al. [27] evaluated the use of the Fluidigm 48.48 Dynamic Array biochip for genotyping purposes in clinical setting, using peripheral blood and buccal wash samples, obtaining promising results. PCR has found its place also in cancer research and it has been widely used already. In a recent study, Henríquez-Hernández et al. [28] compared the classical method of PCR restriction fragment length polymorphism (RFLP) and the high-throughput automated assay Biotrove OpenArray(®) NT cycler, using them for genotyping 118 patients with cancer. Their results suggest that the modern PCR technology is more viable and reproducible than the traditional RFLP method. They concluded that OpenArray® technology can be considered as a robust, simple handling, and user-friendly tool for genotyping purposes in the field of oncology. Henríquez-Hernández et al. [29] also used the potential of this tool to evaluate the influence of the SNPs present in a population with prostate cancer in radiation-induction toxicity. In a different study, Henríquez-Hernández [30] evaluated the influence in prostate cancer of genetic variations in genes involved in testosterone metabolism and found one polymorphism characteristic of Spanish patients. Another example is the research performed by Julin et al. [31] using TaqMan® OpenArray® genotyping assay to correlate the leukocyte telomere length and the overall risk of aggressive prostate cancer. In a similar approach, Zhao et al. [32] used genotyping assay with OpenArray® technology to evaluate the influence of SNPs of 16 antioxidant genes in the increased risk of glioma.

Apart from oncology, this technology has demonstrated its applicability in other fields of medicine. For instance, some authors have reported the influence of SNPs in the risk of developing Type 2 diabetes [33], their relationship with male fertility, or even the existence of a link between polymorphisms and smoking in male fertility [34, 35]. In a very recent work of Araujo and coworkers [36], the association of 39 genes and the etiology of nonsyndromic cleft lip and palate in Brazilian population was evaluated. In the field of dermatology, Villarreal Martínez et al. [37] found an association between six SNPs and the risk of developing psoriasis. Apart from the clear role of high-throughput real-time PCR in experimental science, another possibility of this technique in human medicine is its application in molecular diagnostic testing in a hypothesis-based manner. For instance, Arrojo Martins et al. [38] used OpenArray® plates for screening mutations involved in nonsyndromic deafness. Dionisio Tavares Niewiadonski et al. [39] applied the same approach for genetic testing of blood donors to assess the genotype frequencies of nucleotide-polymorphisms (SNPs) associated with venous thrombosis, hyperhomocysteinemia and hereditary hemochromatosis. High-throughput platforms

have also managed to find their way in pharmacogenomics research. They are very useful in this kind of studies because of the possibility of testing a wide range of SNPs with implications in drug metabolism in a fast and cheap way. In this sense, Iskakova et al. [40] studied SNPs involved in the absorption, distribution, metabolism, and excretion of multiple drugs in Kazakhs. Also, it makes possible the development of personalized treatments according to individual genetic characteristics.

Another promising application of this nanoliter-scale PCR in combination with SNPs is individual identification and paternity test. Until a few years ago, short tandem repeats (STRs) were the most common genetic markers used for individual identification, paternity, and kinship testing [41]. STR consists of a unit of 2–13 nucleotides repeated hundreds of times in a row on the DNA strand. STR analysis measures the exact number of repeating units. This fact makes STRs more informative than SNPs in genetic analysis because SNPs remain only in a nucleotide variation [42]. However, this lack of information can be solved by simply including more SNPs in the assay. As an indicative example, a number of 2–2.25 SNPs give the same information than 1 STRs [43]. In comparison to STR assays, high-throughput real-time platforms make feasible the use of SNPs for individual and paternity analysis, both for humans and animals, because of its relatively affordable price, the number of samples that can be analyzed in a run and the easier analysis of the obtained results. In a similar context, Pomeroy et al. [44] developed an OpenArray® to be used for forensic human DNA applications, with only seven SNPs for individual identification. In the same year, Wang et al. [45] showed the possibilities of other nanoliter-scale platform, the Fluidigm 48.48 Dynamic array, for genotyping purposes in human. In an interesting work by Kerr et al. [46], OpenArray® panel with 32 SNPs was used to create a pedigree of the Scottish by the analysis of 10,000 blood samples. It is also possible to find commercial arrays for human identification. In this sense, Thermo Fisher Scientific has predesigned OpenArray® for human individual identification with 32 and 64 SNPs.

Individual identification is also important in food safety to ensure traceability according to Commission Regulation (EC) 178/2002 [47]. Molecular genetic analysis and more precisely SNPs are a useful tool for this purpose. Capoferri et al. [48] used 16 SNPs to verify traceability of the meat production chain with successful results. However, this study used a 96-well plate real-time PCR assay with the consequent high consumption of reagents and had the limitation of not being capable of analyzing a large number of samples at the same time. High-throughput real-time PCR platforms solved these problems, as demonstrated by Fernández et al. [49] and Pozzi et al. [50] who developed a OpenArray® panel with 32 SNPs that allow individual and cattle rustling identification. For an analytical target of 32 SNPs, this nanoscale technology permits to analyze 96 samples per array. Besides, QuantStudio™ 12 K flex is capable of running four OpenArray® plates at the same time, in a process of only 4 h. Thus, in a working day of 8 h, 768 samples can be analyzed. This number reflects the enormous possibilities of high-through real-time PCR.

Many other areas of life science research, such as animal and plant sciences, can benefit as well from the diverse advantages of nanoliter-scale platforms. Recently, Catanese et al. [51] developed a panel of 96 SNPs with the aim of genotyping anchovy populations. It is notewor-

thy that they selected these best 96 markers from a panel of 424 SNPs using an OpenArray® platform, and then they selected Fluidigm 96.96 Dynamic Array™ for routine analysis due to economic reasons. One of the important points in agriculture is the breeding of crops with certain characteristics of productive interest. In different studies, a panel of 192 SNPs was used for the study of genetic diversity in sugar beet, to study the vigor of the genotypes, and to assess the resistance of these plants to pathogens [52–55].

To sum up, the existing literature clearly indicates the great potential of high-throughput real-time platforms for genotyping. In the future, it could become routine technique, for example, in assessing individual patients in order to test their predisposition to certain diseases through the use of predetermined SNP panels. These panels would allow an early change of habits, reducing the possibility of suffering from certain diseases such as Type 2 diabetes or obesity. In this way, nutrigenetics has become an important research field, studying how the different genetic variants of people influence their metabolism of nutrients, diet, and the diseases associated therewith [96]. The development and commercialization of SNP panels for which their influence in nutrition-related diseases has been demonstrated would be a big step forward in personalized nutrition.

3.2. Transcriptomics

3.2.1. Basic concepts in transcriptomics

Transcriptomics, in its many forms, refers to the study of the expression profiles of genes through the complete set of RNA transcripts (transcriptome). This revolutionary ``omic'' technology comprises different techniques as microarrays, RNA sequencing, and real-time PCR. Until now, only microarrays and RNA-NGS allowed genome-wide surveys. However, these two methods have some drawbacks. Microarrays can only detect sequences homologous to the one that is on the array, and RNA-NGS is costly and the analysis of results required highly specialized technicians. Also, RNA-NGS is perfect for blind studies but not sufficiently adequate for targeted analysis. Real-time PCR is a highly quantitative and sensitive technique suitable for different transcriptomics aims. However, 384- and 96-well plates only allow the analysis of a small number of transcripts and samples at the same time. In this context, high-throughput real-time PCR platforms represented a step forward and placed real-time PCR closer to microarrays and RNA-NGS.

3.2.2. High-throughput real-time PCR in mRNA-based transcriptomics

The most classic part of transcriptomics is the study of messenger RNA (mRNA). It has great interest in life sciences because mRNA is the intermediate step between the genes and the proteins. In this sense, the analysis of the modifications in gene expression levels through mRNA is an important tool which gives us an idea of the influence of internal and external factors in gene expression. For the quantification of mRNA levels by real-time PCR, a previous conversion of mRNA to cDNA, the template of PCR, is needed. This quantification could absolute or relative to a control gene. Although probes are more specific than SYBR® Green, this last chemistry is normally the option chosen for gene expression, mainly due to economic

reasons. The first studies evaluating the possibilities of high-throughput real time PCR platforms were performed for gene expression assays by Morrison et al. [14], who evaluated the possibilities of OpenArray® technology. Although nowadays OpenArray® technology works with TaqMan® probes, the first study used SYBR® Green chemistry. These researchers reported already the need of increasing sample concentration through volume miniaturization, in order to maintain a constant number of target molecules in the reaction. In 2008, Spurgeon et al. [9] evaluated the 48.48 Dynamic Array™ chip for gene expression purposes. Thanks to their studies, a preamplification step of cDNA samples was added for these dynamic platforms before real-time PCR analysis, with the aim of maintaining a high level of target molecules in the reaction. However, this preamplification reaction means that a new step needs to be included in the experimental high-throughput workflow, increasing the total processing time and costs.

As in the case of genotyping applications, in transcriptomics human medicine was the first field benefited with the development of nanoliter-scale real-time PCR. In 2012, Chen et al. [56] used a SmartChip real-time PCR platform to evaluate gene expression in colon cancer, while Javelaud et al. [57] used OpenArray® technology to evaluate the expression of hundreds of genes in melanoma cell-lines. An interesting application was carried out by Li et al. [58] using OpenArray® to quantify gene expression from degraded RNA of formalin-fixed paraffin-embedded tumors. They concluded that this platform could be optimized for gene expression in this kind of preserved samples, opening an interesting avenue of research in cancer. In breast cancer, Song et al. [59] evaluated the expression of 1243 mRNA in breast tissue using SmartChip, showing the potential of this technology to analyze the transcriptome in a similar level of microarrays. However, the SmartChip of 1243 only allows the analysis of one sample in quadruplicate (**Table 3**). The results obtained with OpenArray® plates led the team of Ciarloni et al. [60] to validate a panel of 29 genes with interest in colorectal cancer, starting with an initial panel of 667 candidate genes. In a similar way, Kim et al. [61] validated a panel of eight promising genes in patients with metastatic renal cell carcinoma from an initial panel of 424 genes. Other examples of the application of high-throughput platforms in medicine studies include the work from Patel et al. [62], who employed an OpenArray® panel of 631 genes to evaluate gene expression in human embryonic and induced pluripotent stem cells. Koh et al. [63] employed Fluidigm 48.48 Dynamic Array™ for monitoring the tissue-specific global gene expression in humans thought circulating cell-free RNA in the blood. Thus, this real-time method could be employed for transcriptome analysis of humans and evaluate their global health without the need for invasive tissue sampling.

The work carried out by Forreryd et al. in 2014 [64] deserves special mention. They assessed different high-throughput gene expression platforms to predict skin sensitization without the use of animals. The study used Fluidigm® 96.96 Dynamic Array™ and OpenArray® with nCounter®. The OpenArray® system demonstrated the easier protocol and the less time required for analysis, obtaining the results in 3 h, in contrast to the 7 h of Fluidigm® 96.96 Dynamic Array™ and the 22 h of nCounter®. However, Fluidigm® 96.96 Dynamic Array™ had a superior sensitivity due to the additional cDNA preamplification step implemented prior to PCR and it was also the cheapest option. Likewise, the authors suggested the importance

of selecting appropriate reference genes because one of the two reference genes employed in the study did not amplified well. The final authors' conclusions reflected the great potential of nonarray-based platforms for reducing assay costs and increasing sample throughput. Finally, the results of this comparative study highlight the importance of establishing clear priorities in terms of sensibility, analytical time, and costs when acquiring a high-throughput platform.

Besides medicine, other research fields have also benefited from the capacities of high-throughput real-time PCR. For instance, de Boer et al. [65] used Fluidigm 48.48 Dynamic Array™ to evaluate the potential of gene expression as a biomarker of chemical contamination, using a species of arthropod often employed for ecotoxicological testing. They concluded that the combination of these analytical platforms and multivariate analysis could be a valuable tool in ecotoxicology, combining high throughput capacity with analytical sensitivity. In a similar approach, Harty et al. [66] employed a Fluidigm 96.96 Dynamic Array™ to evaluate expression profiles of adhesion G protein coupled receptor in zebrafish and their possibilities as models in human medicine. In another animal model, Tosches et al. [97] employed a Fluidigm 48.48 Dynamic Array™ to evaluate the pathway of melatonin using a zooplankton. These platforms also found their applicability in animal production as demonstrated by Robic et al. [67], who evaluated the influence of an SNP in the transcripts of quantitative trait loci (QTL), responsible for the accumulation of androsterone in boar fat. In aquaculture, Bonacic et al. [68] employed OpenArray® plates to evaluate gene expression in lipid metabolism with diets rich on omega-3 and omega-6 in fish. Other applications include immunological studies and virology. The work from Rosa et al. [69] reported the usefulness of Fluidigm 96.96 Dynamic Array™ to evaluate different transcriptional patterns in the cattle tick *Rhipicephalus microplus* in response to microbial challenge. Their findings demonstrated that certain pathogens cause downregulation of immune-related genes, favoring their survival and vector colonization. In parallel, Tierney et al. [70] used a Fluidigm 48.48 Dynamic Array™ to analyze the transcript patterns of Epstein-Barr virus with the aim of know more about the first phases of infection.

3.2.3. High-throughput real-time PCR in microRNA-based transcriptomics

The discovery of microRNAs (miRNAs) has changed the previous concepts of gene expression regulation. These small noncoding RNA molecules with a length between 21 and 25 nucleotides and they are found in plants, animals, and some viruses [71]. In human, miRNA are found in plasma, urine, cerebrospinal fluid, and saliva and have an important function as post-transcriptional regulators of gene expression. Their different levels have been associated with a wide range of human diseases [72] and have potential as biomarkers [73]. There are many technologies for the measurement of miRNAs such as microarrays, NGS, and real-time PCR, being the last one and the most sensitive and reproducible method. Also, the releasing of high-throughput real-time PCR platforms have allowed a reduction of costs and minimization of the time required for detection of broad miRNA signatures.

Some studies have demonstrated the applicability of nanoliter-scale PCR in the analysis of microRNA. Jang et al. [74] made an interplatform comparative study using conventional real-time PCR, microarrays and Fluidigm 48.48/96.96 Dynamic Array™, concluding that these

nanoscale platforms could be used to develop cost-effective and customized assays, with rapid turnaround for profiling and validating of miRNA expression. Farr et al. [13] performed a similar study comparing the usefulness of high-throughput platforms for validation of a circulation microRNA signature in diabetic retinopathy. An interesting point of this work is the direct comparison of the two principal high-throughput real-time PCR platforms commercially available: OpenArray® and Dynamic Array™. Authors found that OpenArray® system was the most reproducible platform with less inter- and intrarun variations and had a more user-friendly software for further analyses. One of the main problems of Dynamic Array™ is the replicate variability, caused by their low-volume assay. OpenArray® use the double of the volume used in Dynamic Array™, and in this sense researchers found that variability increased in parallel with volume reductions.

This technology was rapidly introduced in human medicine because of their potential uses in this field. Keller et al. [75] evaluated the expression profiles of 863 microRNAs in 454 blood samples using SmartChip real-time PCR system and found a disease association with microRNAs profiles. Their potential as biomarkers was shown by Mooney et al. [76], who found minimal variations in miRNA profiles of healthy volunteers according to sex and sample timing using OpenArray® plates. Given their value as biological markers, the field of oncology research soon adopted this methodology to screen the microRNA profiles of this disease. Thus, Hudson et al. [77] used the commercially available TaqMan® OpenArray® Human MicroRNA Panel and found overexpression of miR-10a and miR-375 in medullary thyroid carcinoma. Using the same panel, others researchers achieve great results for lung cancer detection [78], for rectal cancer [79], and brain metastasis in mutant lung cancers [80]. With Fluidigm 96.96 Dynamic Array™, Kara et al. [81] discovered the downregulation of 18 miRNAs in patients with colorectal cancer. Research on other human diseases has also used miRNA molecules as biomarkers. The microRNA profiles have been related to human late-onset Fuchs' dystrophy [82], Alzheimer [83] or Crohn disease [84]. It is remarkable that most of these studies used the TaqMan® OpenArray® Human microRNA Panel.

The results of the studies cited before show the great importance of miRNA in human diseases and how their variation could be a good biomarker for diagnostic purposes. Most of these studies used the TaqMan® OpenArray® Human microRNA Panel for the screening of microRNA, showing its potential. However, this panel only allows the analysis of three samples simultaneously, eliminating one of the mains advantages of high-throughput real-time PCR platforms. So, the next step would be the validation of panels with fewer microRNA targets focused on specific diseases, giving the possibility of introducing more samples at the same time. Apart from diagnostic of diseases, microRNA also has potential in other fields. Benson et al. [85] observed that rifampin treatment modifies the microRNA profiles. Thus, it is possible that the administration of determinate substances causes a variation of miRNA profile in humans allowing their use as a new-generation anti-doping biomarkers [86]. This knowledge can be transferred to food safety and by employing miRNA as biomarkers in the administration of banned substances in animal production, thus developing, for instance, a panel of miRNA for each drug [71].

3.3. Detection of pathogens and antimicrobial and virulence genes

One of the great applications of high-throughput real-time PCR platforms is the detection of pathogen for medical and food safety purposes. The development of real-time PCR was a major breakthrough in microbiological analyses. The classical microbiology used large incubation times with the need of multistep procedures for the detection of some bacteria. Real-time PCR avoids the waiting time caused for this incubation. Likewise, real-time PCR allows quantification of genes with great importance, such as resistance or virulence genes.

The introduction of the technology described in this chapter allows performing a large number of assays in a large number of samples. These achievements have a great importance in hospital and food safety microbiology labs. In 2008, Stedtfeld et al. [18] developed an OpenArray® plate to detect human pathogens based on the detection and quantification of virulence factors of these pathogens. Another step forward in microbiological analysis was the OpenArray® panel developed by Gonzalez et al. [87], which allow the identification, including virulotype, and subtypes of O157 and non-O157 enterohemorrhagic *Escherichia coli* with a panel of 28 genes in a single anaylisis. All of this will make the characterization of Enterohemorrhagic *Escherichia coli* possible in less than 4 h, avoiding the use of large number of conventional PCR assays, saving time and money. In a similar study, Dhoubhadel et al. [88] used the Fluidigm 48.48 Dynamic Array™ for molecular serotyping and serotype-specific quantification of *Streptococcus pneumoniae*. Researchers found a good correlation between conventional serotyping and serotype by nanofluidic PCR system. The use of this high-throughput technology brings great saving of time but it also has the drawback of needing a large number of samples for the analysis. In this context, it is important to mention that sometimes microbiology labs do not have a large number of samples for a specific assay.

In the work of Ishi et al. [89], Dynamic Array™ platform and TaqMan® Probes were used for the simultaneous quantification of food and waterborne pathogens as *Salmonella* Typhimurium, *Listeria monocitogenes, Vibrio parahaemolyticus, Clostridium perfringens* or even viruses. One of the advantages of use Dynamic Array™ for pathogen detection is the possibility of changing which microorganism to detect according to the current needs. The existing results show the possibility of routine monitoring of multiple pathogens in large number of food and water samples. Grigorenko et al. [90] developed an OpenArray® panel to detect bloodborne pathogens as viruses, bacteria, or parasites in humans. The aim was to detect these pathogens in blood donors. With this technology they were able to detect at least 10 cell/ml blood of *Trypanosoma cruzi* and 10,000 cell/ml blood of *Escherichia coli*, a detection limit that seems too high for *Escherichia coli*.

On certain occasions, instead of detecting pathogenic microorganism, it is interesting to detect virulence and resistance genes. With regard to this, Looft et al. [91] used OpenArray® to evaluate the change in resistance genes in swine intestinal microbiome in pigs fed with feed supplemented with antibiotics. Tseng et al. [92] evaluated the virulence gene content of Shiga Toxin-Producing *Escherichia coli* from finishing swine using Fluidigm 96.96 Dynamic Array™. The antibiotic resistoma in sewage sludge composting was evaluated by Su et al. [93] using the Smart-Chip real-time PCR system and a chip containing almost 300 antibiotic resistance genes. Similarly, Xu et al. [94] screened 285 antibiotic resistance genes in drinking water

treatment plants and distribution systems. These studies give an idea of the potential of these platforms for wide screening, as it is possible to evaluate most of the important antibiotic resistance genes in one run. All of this permits to establish a relationship between this resistoma and other factors such as microbial community or water treatments.

4. Conclusions

The development of high-throughput real-time PCR platforms was a big step in real-time PCR. There was an important development from the 384 reactions that could be performed at the same time in conventional systems to the 12,288 reactions that can be performed in one run in nanoliter-scale platforms. However, this nanoliter real-time PCR has yet some drawbacks in comparison to other techniques. While in NGS it is possible to analyze unknown sequences, with real-time it is necessary to know beforehand the target sequence to design the primers and the probes. While in microarray technology it is possible to analyze thousands of sequences at the same time, the highest target with these nanoliter platforms is 1200 different sequences. However high-throughput real-time PCR platforms have great advantages, achieving operational improvements that the other platforms are not capable of reaching. They have a great facility for processing large number of samples, the sample consumption is less than in other platforms and the time necessary to get the results is at most 7 h with easy protocols. Also, the analysis of the results is relatively easy and the price is relatively cheap. But without any doubt the great advantage of these platforms is their specificity and their sensibility, which makes them the gold standard in quantification analysis. Although, fast mode is common in real-time PCR, high-throughput platforms do not work in this mode. Therefore, the introduction of fast mode would be another big step because it would increase the number of assays that can be performed in one day. Another clear challenge is to increase the number of assays and samples that can be analyzed at the same time. Even though one platform can perform 1200 assays in one run, this is only for one sample. Being capable of performing this number of assays for a large number of samples would place real-time PCR at the same level than others platforms for genotyping and transcriptomics analyses. The future of these platforms goes through the design of plates for routine applications in medicine, nutrition, or food safety. However, in food safety and microbiology this technique has been under used until now. Thus, the development of plates that could analyze the microbiome of multiple types of samples could be a great advance approaching this technique to NGS for microbiome analysis.

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Chapter 3

Hot Cell-Direct PCR Aimed at Specific Cell Detection

Izumi Kubo, Yuko H. Itoh and Shunsuke Furutani

Additional information is available at the end of the chapter

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Abstract

Since the polymerase chain reaction (PCR) was proposed, it has become an essential method in the field of biological gene analysis, providing a method to amplify DNA sequences of interest. To detect and/or analyze genes in cells, the gene or expressed gene must first be extracted before PCR. This procedure takes time and may result in the loss of samples. In order to avoid such drawbacks, two methods, hot cell-direct PCR and reverse transcription-PCR (RT-PCR), were invented, to detect genes in cells. Using hot cell-direct PCR, specific genes in microbial cells such as *invA* in *Salmonella enterica* have been easily detected and applied to discriminate Archaea from bacteria. As hot cell-direct PCR and RT-PCR are fairly simple processes, they can be applied to detect genes in single cells. We developed an original compact disc (CD)-shaped microfluidic device with microchambers for single-cell isolation and a detection system for expressed genes in isolated single cells in a microchamber on the device. We succeeded in the detection of PCR and RT-PCR products in individual cells and successfully detected *S. enterica* cells by hot cell-direct PCR. Expressed genes in Jurkat cells—human leukemia T cells—were analyzed by this method.

Keywords: hot cell-direct PCR, RT-PCR, cell, gene, expressed gene

1. Introduction

Almost three decades ago, in 1987, the polymerase chain reaction (PCR) was first proposed by Mullis and Faloona [1]. In PCR, specific DNA sequences are amplified through repeated cycles of temperature changes that denature the DNA sequence, bind primer sequences for the target sequence (annealing) and elongate the reaction catalyzed by DNA polymerase [1]. Since non–heat-resistant DNA polymerase was used initially, polymerase had to be added after each

reaction cycle because the enzyme denatured and became inactivated. However, after the use of heat-resistant DNA polymerase, such as Taq polymerase, PCR prevailed widely, allowing for the amplification of various genes [2].

Since1987, PCR has been applied to various biological fields of study. In the medical field, it was used to detect and identify pathogenic bacteria [3–7], in diagnostics of genetic or familial diseases [8–10], in prenatal diagnosis of fetal genes [11–13] and in risk assessment of hypertension [14, 15]. In forensic medicine, it was utilized to identify humans [16–20].

In the field of food science, PCR was used to detect food-borne bacteria and viruses and to discriminate beef, pork and chicken [21–23]. It was also useful to discriminate genetically engineered crops. More recently, in the field of environmental sciences, it was utilized to conduct a metagenomic analysis of soil, water and other samples to estimate microbes in the environment [24–26].

Usually genes in a living cell must be extracted from cell lysates in order to perform PCR. In the extraction process, DNA from cells is condensed, thus avoiding the negative effect of substances in cells on PCR. However, it takes time to lyse cells and extract DNA from the lysates, and these procedures are troublesome. Moreover, a small amount of DNA might be lost in the procedure. Recently, it was shown that PCR of a crude sample of lysate could be performed without extraction [27]. This implies that the substances in the lysate did not seriously affect PCR.

To establish an easy and rapid PCR procedure, we attempted to execute PCR in a single container in which target cells were lysed. As the simplest method, we proposed that in the PCR reagent mixture, that cells be lysed by heat treatment and that subsequent PCR heat cycles be repeated without the addition of any reagent to the same container (**Figure 1**). This method was performed with only a heat-changing procedure after placing cells and the PCR reagent mixture together in one container. We named the process hot cell-direct PCR [28]. This method can avoid contamination during extraction [29].

On the other hand, the detection of an expressed gene (m-RNA) in cells is important in gene detection as well as genomic DNA, because biological phenomena are closely related to the expression level and phase of a vast number of gene expression events. Conventional detection

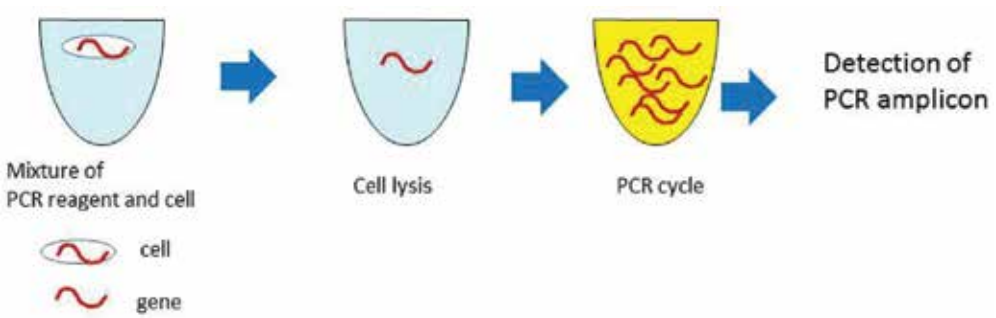

Figure 1. Scheme of hot cell-direct PCR.

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of m-RNA was performed as follows. After the lysis of target cells, total m-RNA was extracted from the obtained lysate. The m-RNA was reverse-transcribed to complementary DNA (c-DNA) from total RNA by using reverse transcriptase and the obtained c-DNA was amplified by PCR. This procedure is referred to as reverse transcription PCR (RT-PCR), which has been applied to analyze gene expression in biological phenomena. RT-PCR has to be performed very carefully, and experience and skills are necessary because RNA molecules are easily degraded by RNase.

In our study, we examined whether it is possible or not for PCR to express an m-RNA gene without extracting m-RNA from cells, as is done for genomic DNA. This would avoid contamination of RNase by researchers and allow RT-PCR to be performed without any trained skills. To perform such a procedure, every reagent necessary for RT-PCR should be initially mixed with a suspension of target cells, which must be lysed by heat treatment. Reverse transcription and PCR should be performed in the same tube. This method needs heat-stable reverse transcriptase different from conventional RT-PCR. We developed a method that utilized heat-stable reverse transcriptase and demonstrated that without extracting m-RNA from the cell lysate, that RT-PCR could be performed efficiently compared to conventional RT-PCR [30]. We named the method hot cell-direct RT-PCR. This method allowed for the amplification of genes from a small number of copies both by hot cell-direct PCR and RT-PCR and even from the lysate of a single cell.

In this chapter, we review these methods and describe some examples, including application of hot cell-direct PCR to detect pathogenic bacteria by amplification of a specific bacterial gene and the discrimination of microbial species. Using hot cell-direct RT-PCR, it may be possible to detect expressed genes in eukaryotic cells.

2. Hot cell-direct PCR of microbial cells

In general, conventional methods to detect and identify bacteria require multiple subculture steps, such as enrichment culture, and selective culture. Collected samples are first multiplied in nutrition-rich medium, then cultured in selective medium, taking several days to detect and identify bacterial species in samples [31]. This needs not only time but also a lot of culture medium and labor. If the target bacterium has a specific gene, amplification of that gene by PCR and simultaneous detection of the PCR product will reduce the time and medium required to detect and identify bacteria from the sample. Using hot cell-direct PCR, bacteria will be easily detected in the lysate without the need to extract DNA from the cell lysate and allowing for the easy detection of bacterial species [29]. We applied hot cell-direct PCR to detect a food-borne pathogen in food and to discriminate bacteria and Archaea in an environmental sample.

2.1. Detection of *Salmonella enterica*

Salmonella enterica is widely known as notorious food-borne pathogen and responsible for human salmonellosis [32]. *S. enterica* is often transmitted to humans through chicken meat or

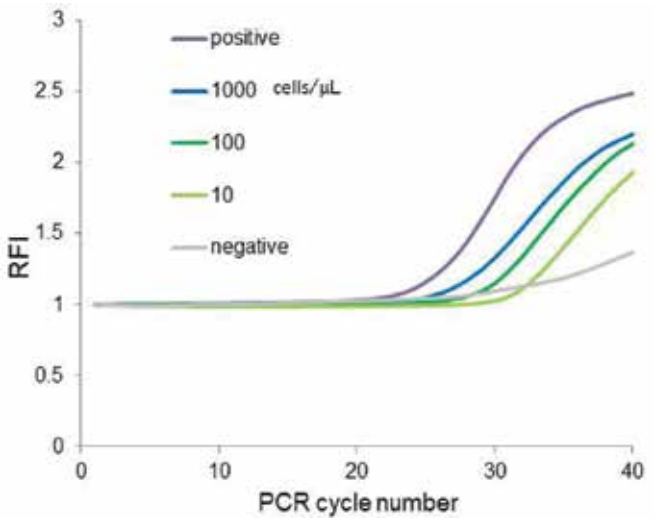

Figure 2. Hot cell-direct PCR of *S. enterica* cells in real-time PCR system.

eggs, because chickens sometimes carry *S. enterica* in their intestines. The incubation period after infection may last from 72 h to several days and infection can cause abdominal pain, diarrhea, vomiting or fever in a patient. This pathogen is currently detected by a culture method and suspicious foods are tested by cultures that take several days to grow and test. However, in order to avoid the spread of infection, a rapid method to detect the pathogen is required. Since *S. enterica* is known to have a specific gene *invA*, which is the gene coding for the invading factor into a host cell, a detection method based on amplification of *invA* by PCR exists [33]. To amplify *invA*, a commercialized PCR detection kit (Cycleavage salmonella detection kit, TAKARA, Osaka, Japan) was used. The kit contains a fluorescent probe (cycling probe) to detect the PCR product of *invA*, and fluorescence caused by the probe increases as the PCR product increases, similar to the TaqMan probe. This kit is promising tool for hot cell-direct PCR.

This kit contains a primer/probe mix, Taq polymerase, RNaseH, buffer and a dNTP mixture. The primer/probe mix is a mixture of primers and probes to detect the *invA* gene. The probe for the *invA* gene was labeled with 6-carboxyfluorescein (FAM) and a quencher. The increase in fluorescence from FAM occurred after an amplification of the *invA* gene. After the probe was hybridized to the PCR product, it was cut by RNaseH. Then, the fluorescence intensity is increased by uncoupling the quencher from the probe.

We examined hot cell-direct PCR of *S. enterica* using this kit, as follows [29]. First, a suspension of bacterial (*S. enterica*) cells was mixed with all the kit's reagents in a microtube and the mixture was heated at 95°C for 2 min to lyse cells. In the same tube, PCR (DNA denaturation 95°C × 5 s, annealing of primer 55°C × 10 s and elongation of DNA 72°C × 30 s) cycles were performed without the addition of any reagent. These processes only involved change in temperature of the reaction mixture. Through PCR, fluorescence of the FAM probe increased and was measured using a real-time PCR system (ABI 7500).

Hot cell-direct PCR was using a range of concentrations (10–1000 cells/μL) of *S. enterica* suspension (**Figure 2**). At each concentration, fluorescent intensity increased with each PCR cycle. In real-time PCR, relative fluorescent intensity (RFI) is unity (value 1) at the start of PCR and RFI increases as the number of PCR cycle proceeds. The number of PCR cycles with a recognizable increase in RFI depends on the concentration of amplicon of the target gene with higher concentrations showing a smaller number of cycles while lower concentrations have a larger number of cycles. As shown in **Figure 2**, a lower concentration of *S. enterica* showed a larger number of cycles with a recognizable increase in RFI. In addition to this experiment, selectivity of the reaction was confirmed under the coexistence of *Escherichia coli* cells. A cell suspension of *S. enterica* (1000 cells/μL) and various concentrations of *E. coli* (1000–1,000,000 cells/μL) were mixed and hot cell-direct PCR of *invA* was examined. At any concentration of *E. coli*, and the same number of cycles resulted in a recognizable increase in RFI curves. The lysate of both *S. enterica* and *E. coli* did not have an effect on the PCR of *invA* and hot cell-direct PCR of this specific gene is a useful and easy way to detect *S. enterica*.

2.2. Detection of *Bacillus cereus*

Bacillus cereus is a food-borne bacterium that infects humans mainly from cocked carbohydrates such as rice and pasta, with symptoms being vomiting and diarrhea. Conventionally *B. cereus* in food has been tested by a culture- based method that takes 7 days to identify the bacterium. Such a long period required for its identification has hampered efforts to avoid the spreading of *B. cereus* infection. Thus, a rapid and reasonable method to detect and identify *B. cereus* is required. The diarrheal syndrome is caused by enterotoxins produced by *B. cereus* with known enterotoxins being HBL, NHE and CytK1. HBL is the gene product of *hblA*, *hblC* and *hblD* and NHE is that of *nheA*, *nheB* and *nheC* [34]. Among these enterotoxins, the toxicity of *B. cereus* is correlated with the amount of secreted NHE. Thus, NHE is regarded as an important enterotoxin related to food-borne disease. Ester et al. reported that NHE is produced by most of strains of *B. cereus* and that the carrying rate of the NHE gene in *B. cereus* is in 99.69% of strains. Thus, detection of the NHE gene will demonstrate its existence in most *B. cereus* strains.

Yang et al. reported the detection of *B. cereus* targeting one of the NHE gene, *nheB* [35]. They reported a PCR primer for the amplification of *nheB*. Since we have succeeded in hot cell-direct PCR of *invA* in *S. enterica*, we tried to perform hot cell-direct PCR of this specific *B. cereus* gene [36]. There is no commercialized kit for the amplification of *nheB*. We performed PCR of *B. cereus* utilizing the primer set reported by Yang et al., forward primer (SGF3: GCACTTATGGCAGTATTTGCAGC) and reverse primer (SFR3: GCATCTTTTAAGCCTTCTGGTC), to amplify *nheB* of two *B. cereus* strains. After PCR using these primers, the *nheB* amplicon was observed by electrophoresis. To develop the hot cell-direct PCR procedure of *nheB*, the TaqMan probe to detect the PCR amplicon is necessary. Since there is no report on the TaqMan probe of *nheB*, we designed probes. The *nheB* amplicon is 152 bp long. From the amplicon sequence, we designed probes using the "Primer Express Software Version 3.0 (Applied Biosystems) software", in which the sequences had a 30–80% of GC contents, and where the length was 15–30 bp and contained no GGGG nor AAAAAA. The selected sequence was termed probe 1 (5′-ATTATGCCGGCTCATACGTATGCAGCTG-3′). To construct the TaqMan probe, the 5' end

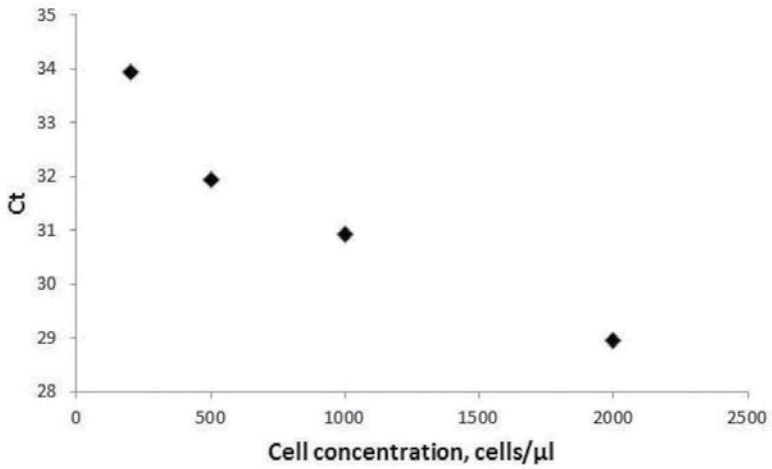

Figure 3. Ct in hot cell-direct PCR of *B. cereus*.

was labeled with FAM and the 3' end was labeled with Dark Quencher. Probe 1 was examined to detect the *nheB* amplicon by the real-time PCR system. With the use of probe 1, as PCR cycles proceeded, florescence of the sample solution increased. Using the primers (SGF3 and SGR3) and probe 1, hot cell-direct PCR of *B. cereus* was performed. The *B. cereus* suspension was mixed with *Ex Taq HS* (TAKARA, Japan), dNTP mixture, probe 1, primers and *Ex Taq*™ buffer (TAKARA). The sample mixture was heat-treated at 95°C × 5 min (lysis), and PCR cycle of 95°C × 30 s, 55°C × 30 s and 72°C × 30 s was repeated. By measuring fluorescence using real-time PCR, the sample containing *B. cereus* cells showed an increase in fluorescent as the number of PCR cycles increased. In real-time PCR, cycle number increase, there is a recognizable increase in RFI, defined as Ct. The Ct with an RFI value of 1.2 was examined with various concentrations of *B. cereus* cells (100, 500, 1000 and 2000 cells/µL). At a lower concentration of cells, a larger Ct was observed (**Figure 3**). These results demonstrated that *B. cereus* can be detected by hot cell-direct PCR as *S. enterica* and that hot cell-direct PCR can be performed using TaqMan probe as well as a cycling probe.

2.3. Discrimination of Archaea and Bacteria

All living organisms on earth are classified into prokaryotes (including Archaea and bacteria) and eukaryotes. In an environmental community, less than 1% of the total numbers of prokaryotes have species that are able to be cultured [37]. Most Archaea species grow in habitats with extremely limited conditions, including temperature, altitude, salinity, pH, and anaerobic conditions and thus are difficult to cultivate in the laboratories. Not only archaeal species but also some bacterial and eukaryotic species coexist in the same environment [38].

Molecular analysis of environmental samples has clear advantages for discovering and characterizing microbial diversity and understanding interactions between microbes and with abiotic environmental factors. The culture-independent analysis of the small subunit ribosomal RNA (ssu rRNA) gene, 16S rRNA for Archaea and bacteria and 18S rRNA for

Eukarya in environmental samples or by metagenomic analysis provide valuable information about microbial diversity. The advantages of utilizing the ssu rRNA gene are: (1) ssu rRNA genes exist and the stability of their sequences are conserved in all living organisms; (2) nucleotide length (about 1600 bp for 16S rRNA) is sufficient for phylogenetic analysis; (3) conserved regions and variable regions are distributed throughout the gene, and are comparable to relatively closely-related species; (4) it is easy to obtain universal or specific primers for sequence analysis; (5) a huge number of sequence data are available. Fundamental techniques for molecular analysis of Microsphaera are based on PCR, electrophoresis and sequencing.

There are nine variable regions in 16S rRNA: V1 at nucleotide position 69–99 (*E. coli*), V2 (137–242), V3 (433–497), V4 (576–682), V5 (822–879), V6 (986–1041), V7 (1117–1173), V8 (1243–1294) and V9 (1435–1465). On the other hand, consensus sequences such as positions 515–533, 1390–1407 and 1492–1507 of *E. coli* are suitable for universal PCR primers of all living organisms [38, 39].

Differentiation of archaeal and bacterial species in an environmental sample has been possible using hot cell-direct PCR with two sets of PCR primers and two different fluorescence-labeled TaqMan probes. The primers and probes were designed with specific sequences in conserved regions of the 16S rRNA gene of Archaea and bacteria, respectively [40]. For example, the following primers and probes were used to analyze geothermal microflora in our study: A21F: 5'-TTCCGGTTGATCCYGCCGGA as the forward primer for Archaea, B27F: 5'-AGAGTTTGATCCTGGCTCAG as the forward primer for bacteria, and AB547R: 5'-TTACC GCGGCKGCTGGCAC as the reverse primer for Archaea and bacteria. TaqMan probes were designed with FAM and BHQ1 for Archaea: FAM-AP113 (5'-ACGGCTCAGTAACACGTGGC TAA)-BHQ1, and 5'-carboxytetramethyl rhodamine (TAMRA) and BHQ2 for bacteria: (TAMRA)-BP368 (5'-ACTCCTACGGGAGGCA GCAGTAGG)-BHQ2 (**Figure 4**).

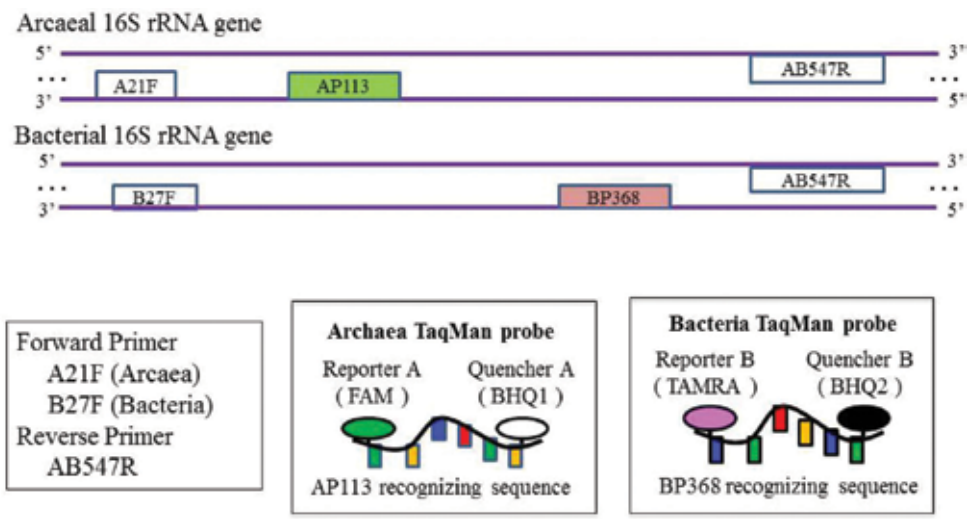

Figure 4. Primers and probes for the discrimination of Archaea and bacteria.

In this method, the cells in a sample were lysed, and then DNA was amplified by PCR with a TaqMan probe and analyzed sequentially by real-time PCR in one tube. The reaction mixture consisted of the cell-containing sample, primer sets and TaqMan probes for Archaea and bacteria, Premix Taq DNA polymerase, and buffer. PCR was performed with a Real-time PCR system using the following program: an initial lysis step at 95°C for 5 min; 30 cycles at 95°C for 30 s, 58°C for 30 s and 72°C for 30 s. The fluorescence intensity of FAM and TAMRA were analyzed sequentially. As model Archaea and bacteria, *Metallosphaera sedula* TH-2 and *E. coli* B were examined. The sample containing *M. sedula* with A21F primer and AP113 probe only showed an increase in RFI of FAM and no increase with other primers or probes. In contrast, the *E. coli* sample with B27F primer and BP368 probe showed an increase in RFI.

The RFI of FAM and TAMRA increased significantly in the reaction mixture that contained Archaea and bacteria, respectively [40]. In the Microsphaera analysis, a combination of unicellular fractionation and this method should be useful for differentiating Archaea and bacteria at the single cell level.

The most important aspect for the sensitive and robust amplification of a PCR product is the selection of a suitable primer set. A number of primers were designed and investigated for ssu rDNA, but there was no perfect match yet, especially for Archaea. Much effort is still required to evaluate primers [41].

3. Hot cell-direct RT-PCR of gene expression

Hot cell-direct PCR is a promising method to amplify the genes of microbes and to detect microbial species. Furthermore, we aimed to apply this method to detect expressed genes as well as specific genes in cells. Usually the analysis of an expressed gene is carried out after m-RNA extraction from lysed cells. Extracted m-RNA is reverse-transcribed into complementary DNA (c-DNA). The obtained c-DNA is amplified by PCR and the amplicon is analyzed by electrophoresis or real-time PCR (**Figure 5 (B)**). This method is called reverse transcription PCR (RT-PCR) because PCR is carried out after the reverse transcription of m-RNA. RT-PCR needs the extraction of the expressed gene (m-RNA) from lysed cells, and this process is similar to gene extraction of PCR. However, extraction of m-RNA is more difficult than DNA extraction, because RNA molecules are easily degraded by RNase in saliva and sweat which is secreted from humans around the working area. Such degradation of RNA molecules through the extraction process should be avoided when performing RT-PCR. Thus, RT-PCR has to be performed by a highly trained worker under sterile conditions. If the extraction process is omitted, such problems can be eliminated.

We investigated RT-PCR without the extraction of m-RNA from lysed cells as in hot cell-direct PCR and aimed to establish hot cell-direct RT-PCR, which was performed only by heat treatment after preparation of the RT-PCR reagent mixture with target cells [30]. Reverse transcription in RT-PCR is catalyzed by reverse transcriptase (RTase). Generally speaking, lysis buffer containing detergent is used to lyse cells and in the buffer cells are lysed by heat treatment. RTase used in general RT-PCR is inactivated by detergents and heat treatment, like most proteins. Therefore, RNA molecules were extracted from lysed cells and washed after lysis.

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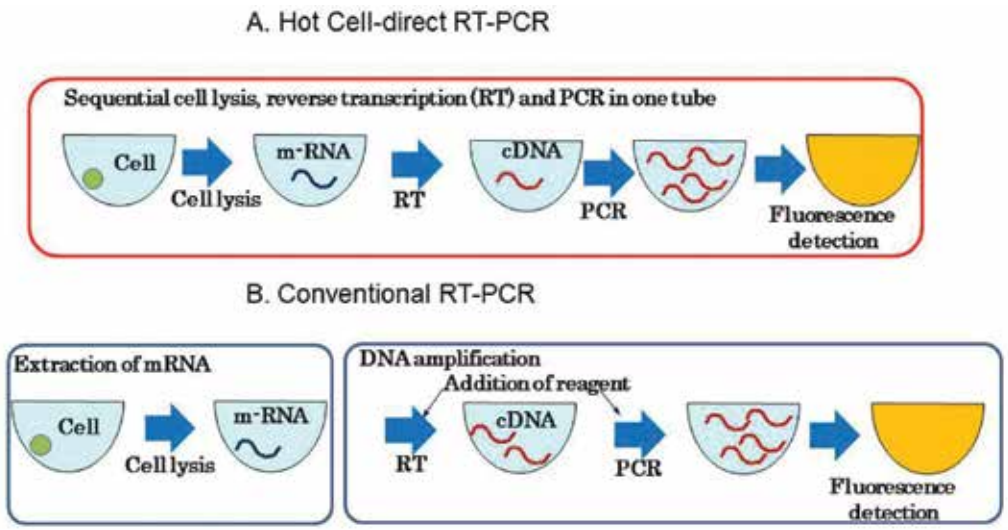

Figure 5. (A) Hot cell-direct RT-PCR and (B) Conventional RT-PCR.

Reagent for reverse transcription containing RTase was added to the washed RNA and reverse transcription was performed. In order to achieve our purpose, we considered the use of heat-stable RTase in solution. Heat-stable RTase can be added before cell lysis and lysis and reverse transcription can be accomplished in the same tube without extracting m-RNA (**Figure 5 (A)**).

As a candidate of heat-stable RTase, we discovered heat-stable DNA polymerase with reverse transcriptase activity, which can act as RTase and DNA polymerase, namely Tth DNA polymerase, which has good reverse transcription activity in the presence of Mn^{2+} [42]. As a suitable reagent for Tth DNA polymerase, we examined the Tth polymerase kit (Roche Applied Science). The kit contains Tth DNA polymerase (5 U/mL), 0.1% TritonX-100 and 25 mM $Mn(OAc)_2$. To avoid the degradation of RNA molecules by RT-PCR, we used RNase inhibitor. We examined several genes expressed in eukaryotic cells. In this review, mainly describe our investigation using Jurkat cells. Jurkat cells are known human T-cell lymphocytes. Among the expressed genes, we examined the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene, because it is a housekeeping gene; that is, expression of the gene is constantly observed. We examined RT-PCR of *GAPDH* in Jurkat cells using a double dye probe designed for the detection of *GAPDH* (Nippon EGT, Japan) [30].

At first, cell lysis was confirmed with various concentrations of a detergent (TritonX-100). To the Tth DNA polymerase kit, TritonX-100 was added at a concentration of 0. 0.001, 0.01, 0.1 and 1% and mixed with proliferated Jurkat cells (1000 cells/μL). The mixture was heat treated at 94°C for 10 min to lyse cells. The lysate was reverse transcribed (42°C for 15 min) and PCR was performed as follows: 95°C for 30 s to denature the DNA, and 60°C for 60 s of annealing and elongation. After the reaction, PCR products were confirmed by electrophoresis. The band of the *GAPDH* amplicon was observed at all concentrations of TritonX-100. It was predicted that a higher concentration of detergent would degrade the cell membrane more strongly than a lower concentration. However, the lowest concentration of sample indicated the thickest

band and a higher concentration of detergent resulted in a thinner band. The detergent in Tth DNA polymerase (0.1%) was enough to lyse cells and no additional detergent was necessary to obtain the PCR product of the *GAPDH* gene.

At first, reverse transcription was carried out at 42°C, in which most of the reaction was performed using non heat-stable reverse transcriptase. In our study, the reaction temperatures examined were 42, 50, 60 and 70°C to optimize the reaction using Tth DNA polymerase. Relative to initial fluorescence intensity was measured by PCR cycles with a real-time PCR system. PCR cycle number (Ct) with an RFI of 1.3 at various temperatures was compared (**Figure 6**). At the same concentration of *GAPDH* m-RNA, more efficient reverse transcription will produce a higher concentration of its cDNA and reduce the number of PCR cycle numbers at the same RFI. At 42°C, Ct was 22 and it was 18, 17 and 19 at 50, 60 and 70°C, respectively. The smallest Ct was observed at 60°C. Then 60°C appeared to be the most efficient temperature for reverse transcription catalyzed by Tth DNA polymerase [43].

Conventionally RT-PCR is performed using the extracted m-RNA from lysed cells. To confirm the efficiency of hot cell-direct RT-PCR, we compared hot cell-direct RT-PCR and conventional PCR, at the same concentration of cell suspension. In conventional RT-PCR, total RNA was extracted from a given concentration cell suspension after cell lysis. In hot cell-direct RT-PCR, the same number of cells was used as that used in conventional RT-PCR. In this study, a cell concentration of 1000, 500, 250 and 125 cells/μL was compared in both RT-PCR methods. The number of genes doubled in one cycle of PCR amplification.

When the sample with a certain concentration of gene needs the N cycle to reach the desired Ct, another sample with half the concentration of that gene needs N + 1 cycles to reach that Ct. When RT-PCR is performed for a sample of total RNA, the same phenomenon will be observed as in hot cell-direct RT-PCR. In the case of total RNA, the Ct of 1000, 500, 250 and 125 cells/μL was 16, 17, 18 and 19, respectively. The observed result agreed with the expected one. As in hot cell-direct RT-PCR, Ct was almost the same as in RT-PCR using total RNA. These results demonstrate that in hot cell-direct RT-PCR, the cell lysate did not affect RT-PCR and that the

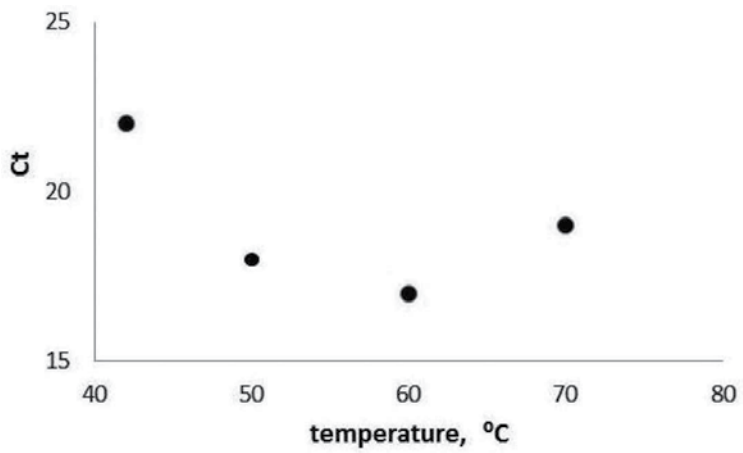

Figure 6. Ct in hot cell-direct RT-PCR of Jurkat cells at various RT temperature.

same amount of PCR amplicon was produced as RT-PCR with total RNA, without needing to extract m-RNA from the cell lysate. We examined hot cell-direct RT-PCR of other genes (β-actin and IL-2) expressed in Jurkat cells by real-time PCR and amplification of expressed genes was also observed, as for the *GAPDH* gene. Thus, hot cell-direct RT-PCR is useful and very easy to detect expressed genes.

4. Application of hot cell-direct PCR to single-cell analysis

Recently, single-cell analysis has drawn attention to the analysis of characteristics of each cell in a single-cell population, because it has been suggested that each cell has different characteristics, even in the same population and with the same genes. The most traditional single-cell isolation technique is micromanipulation. Patient training and experience of the operator are necessary conditions to perform micromanipulation. As the operator takes only one cell at a time, it is a low throughput method. On the other hand, a fluorescence-activated cell sorter (FACS) is a high throughput method to analyze cells. It can separate cells one by one rapidly and classify them depending on their shape and size with the assistance of fluorescent staining. However, this is not appropriate to identify a gene or expressed gene in a single cell. To characterize a single cell, single-cell isolation has been investigated with the use of microfluidic devices. The microfluidic method is a promising method to isolate cells from a large number of cells easily and rapidly. We have developed an original compact disk (CD)-shaped microfluidic device with microchannels and microchambers for single-cell isolation and a rapid and easy single-cell isolation method on the device. On this CD-shaped device, after applying the cell suspension in the microchannel, cells are easily isolated in microchambers on the channel by the rotation of the device, without the need for any micropump. By applying the cell suspension to many microchannels, cells can be isolated in a large number of microchambers simultaneously. On one device, 24 microchannels are arranged and on one microchannel 300 microchambers are arranged. In total, 7200 microchambers on one device are available for single-cell isolation (**Figure 7**) [28, 44]. This device was fabricated using heat-resistant material, silicone and glass, and can be used in heating reactions such as PCR. On the device, single-cell isolation and hot-cell direct PCR or RT-PCR can be achieved very easily. After PCR, fluorescence of each microchamber should be measured and the existence of the target gene is reflected by the increase in fluorescence. By hot cell-direct RT-PCR on the device, gene analysis can be performed easily. In our study, detection of *S. enterica* cells was based on hot cell-direct PCR of the *invA* gene. As an application of the detection of expressed gene analysis, detection of *GAPDH* in isolated Jurkat cells is shown.

4.1. Detection of *Salmonella enterica*

As a way to detect food-borne bacteria, application of our device to *S. enterica* was investigated. *S. enterica* was detected after PCR of *invA* as described above. When a cell trapped in a microchamber is *S. enterica*, fluorescence increases, caused by amplification of *invA* after hot cell-direct PCR. When the entrapped cell is not *S. enterica*, fluorescence of the chamber does not

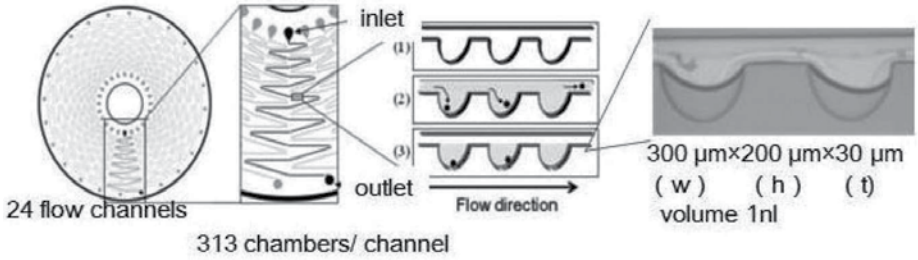

Figure 7. CD-shaped microfluidic device.

increase. Therefore, only the microchamber entrapping *S. enterica* cell shows an increase in fluorescence.

The isolation of microparticles such as bacterial cells into microchambers on the device depends on a Poisson distribution [29]. The isolation of *S. enterica* cells was confirmed by applying a cell suspension (50–400 cells/μL) to a microchannel. As the cell concentration increased, the number of microchambers entrapping a cell increased, and at a concentration range of 50–200 cells/μL, the number of cells observed in a microchamber was one or zero. However, at a concentration of 400 cells/μL, the number of cells was occasionally two or more. At the same concentration range, *S. enterica* cells were applied to the device and hot cell-direct PCR was performed according to the same temperature change protocol in a microtube. The fluorescence of each microchamber was measured using an epifluorescent microscope customized to observe the microchambers on the device [28].

S. enterica cells mixed with PCR reagent were applied to the inlet of the microchannel and isolated in microchambers by rotation of the device. Before hot cell-direct PCR, fluorescence of each microchamber was measured under a fluorescent microscope. Thereafter, the device was set to a thermal cycler, which has a customized stage for the device to perform temperature changes. In the thermal cycler, cells were lysed by heat at 95°C for 2 min and followed by 40 PCR cycles. After completing the PCR cycles, the fluorescence of each microchamber was measured again. Then the fluorescence ratio of after to before hot cell-direct PCR was evaluated.

At the range of 50–400 cells/μL, the microchambers showed increased fluorescence and the number of microchambers with increased fluorescence depended on the applied concentration of *S. enterica* cells (**Figure 8**). A single *S. enterica* cell was confirmed by hot cell-direct PCR on the device. When *E. coli* cells (1000 cells/mL) were mixed with *S. enterica* cells, almost the same increase in fluorescence was observed. This method can be applied to single-cell detection of a specific cell type.

Since *S. enterica* is known to infect chicken meat, we investigated the detection of *S. enterica* from ground chicken on the CD-shaped microfluidic device by hot cell-direct PCR. After the sample of ground chicken was suspended in buffered peptone water (BPW), the sample solution from meat was filtered through a filter bag. Using the filtrate, sample detection limit was examined and 40,000 cells/mL (40 cells/μL) could be determined but not a lower concentration. After sampling through the filter bag, the filtrate was cultured in medium for 4–8 h at

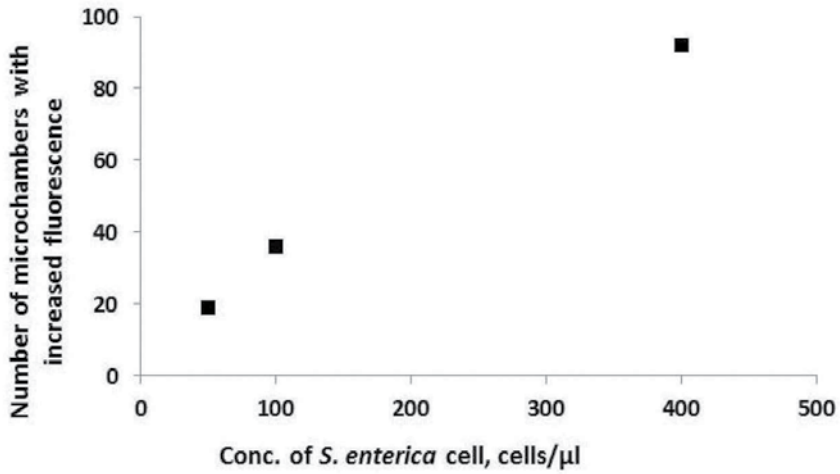

Figure 8. Dependence of number of microchambers with increased fluorescence on concentration of *S. enterica* cells.

30°C and from the cultured medium *S. enterica* cells were collected by centrifugation and the detected on the device. After culture, 30 cells/g were detected in the sample [28].

Conventionally, *Salmonella* sp. is detected with a culture-based method that takes several days to identify the bacterial species. However, the hot cell-direct PCR method using the microfluidic device takes a very short amount of time (8–12 h including enrichment culture) and a small amount of reagent. This method can be applied to detect various bacteria when primers and TaqMan probe for the specific gene are available. We therefore consider this method to be promising for microbial research.

4.2. Analysis of expressed gene in single Jurkat cells

Analysis of expressed genes in single cells has garnered much interest. For example, differences in gene expression of cells at an early stage of development are very interesting, but it is not easy to assess because the number of cells is limited. In general, biological phenomena are analyzed based on cell populations. However, phenotypic variation has been observed in one population of cloned cells by FACS. This suggests that individual cloned cells have different phenotypes and gene expression is different from cell to cell. To investigate actual differences in gene expression of individual cells, evaluation of gene expression in an isolated single cell is necessary. The m-RNA extracted from a single cell is so little that it cannot be amplified and detected by RT-PCR in a tube-based analysis. On the other hand, a microfluidic method can concentrate cells in a small volume of reagent after isolating the target cell, allowing for the detection of an expressed gene after RT-PCR of the gene in the cell. If the evaluation and analysis of gene expression of an isolated single cell is enabled by hot cell-direct RT-PCR on the microfluidic device above, it may provide a solution. We investigated the possibility of analyzing an expressed gene in single cells by using Jurkat cells by hot cell-direct RT-PCR on the CD-shaped microfluidic device for single-cell isolation.

Initially, *GAPDH* gene expression was examined. Proliferated Jurkat cells were harvested and suspended in the RT-PCR reagent mentioned above and the mixture was applied to the microfluidic device. The concentration of cells in the suspension was 200–400 cells/μL and the cells were isolated in microchambers. On the device, cell lysis (95°C for 10 min), reverse transcription (60°C for 15 min) and PCR cycles were executed according to the hot cell-direct RT-PCR temperature program. The number of microchambers showing higher fluorescence after RT-PCR corresponded to the value evaluated using a Poisson distribution. After 40 PCR cycles, the before-to-after PCR fluorescence ratio of microchambers was evaluated and compared. The chamber entrapping no cells did not show any increase in fluorescence and the fluorescence ratio was almost 1.0, little variation was observed. The chamber entrapping a cell showed a remarkable increase in fluorescence while the fluorescence ratio showed a wide range (2.30 ± 0.41) [45]. This variation in the fluorescence ratio of the chamber entrapping a single cell was larger than that of the chamber with no cell. This result indicates the possibility that the gene expression level of a single cell was different in each cell. This heterogeneity in gene expression was considered to be caused by different cell cycle phases, although the *GAPDH* gene is known as a housekeeping gene and is constitutively expressed. Furthermore, we are now investigating the expression of other genes besides the *GAPDH* gene in cultured human cells.

5. Conclusion

Hot cell-direct PCR and RT-PCR are effective methods to detect genes and expressed genes very easily, as described above. Specific cell detection is possible by using the CD-shaped device, allowing for rapid and easy biological assays.

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Chapter 4

Regulatory Concern of Polymerase Chain Reaction (PCR) Carryover Contamination

Yuan Hu

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66294

Abstract

Currently, DNA amplification techniques have become important detection tools. However, the extreme sensitivity of such techniques can easily result in contamination. This is a major problem in using these techniques routinely in a regulatory agency such as the Food and Drug Administration (FDA) because false-positive polymerase chain reaction (PCR) results will fail our mission. Preventing PCR carryover contamination and a capacity to rapidly determine false PCR positives are crucial. In the past, several methods have been used to prevent amplicon carryover contamination. In this chapter, we provide practical suggestions for PCR carryover contamination detection and prevention that work well with most PCR applications in our laboratory.

Keywords: polymerase chain reaction (PCR), carryover contamination, nested PCR, real-time PCR, single-tube nested real-time PCR

1. Introduction

Polymerase chain reaction (PCR) amplification techniques have provided means for the rapid and sensitive detection of pathogens [1]. The number of applications of PCR is still growing, and more and more amplification-based techniques are now used in FDA field laboratories to detect pathogens, such as *Salmonella*, *Escherichia coli* 0157:H7, *Shigella*, *Vibrio*, hepatitis A virus (HAV) and noroviruses (NoVs) [2]. A significant challenge facing us is that the sensitivity of PCR can easily result in contamination and consequently in false-positive PCR. A small amount of previously amplified PCR product or potential target sequences that infiltrate laboratory supplies and equipment or that are present in an aerosol can easily contaminate the sample and PCR reagents in the tests. Therefore, prevention of carryover

contamination from previous PCR amplifications has become a high priority. As a first line of defense to prevent contamination of PCR with a previously generated amplicon, mechanical separation of the PCR laboratory into different rooms or laboratory benches is needed. Secondly, chemical, UV, and enzymatic methods can be applied to inactivate any prior amplicon generated in the laboratory. Additionally, rapid identification of contaminants and their sources is needed to prevent false-positive PCR results. For this purpose, we developed a rapid method to detect PCR carryover contamination by DNA sequencing. The combination of the above methods plus good laboratory technique should be able to totally eliminate PCR carryover contamination and allow us to perform accurate and sensitive PCR routinely in regulatory setting.

1.1. Polymerase chain reaction

In 1983, Dr. Kary Mullis at Cetus Corporation conceived of polymerase chain reaction. There is not any technique that has had a greater impact on the practice of molecular biology than PCR. PCR-based methods are powerful techniques [3]. This technique is centered around the ability of sense and anti-sense DNA primers to hybridize to a DNA of interest. When put into use, agents of infectious diseases can be detected at extremely low levels. After extension from the primers on the DNA template by DNA polymerase, the reaction is heat-denatured and allowed once again, to anneal with the primers. After another round of extension, a multiplicative increase in DNA products is observed. When critical controls are set, this technique becomes a quantitative process. Therefore, a minute amount of DNA can be efficiently amplified in an exponential fashion to result in an easily manipulable amount of DNA. The current sensitivity and detection limit is at a level as low as 10–50 copies per ml. Although the PCR is extremely easy and fast, PCR product carryover contamination impedes the routine use of these techniques routinely in regulatory laboratories.

1.2. PCR-based technology

1.2.1. Reverse transcription PCR (RT-PCR)

PCR uses DNA as a starting material. When RT-PCR is carried out, the starting material is RNA. In this method, RNA is first transcribed into complementary DNA (cDNA) by reverse transcriptase from total RNA or messenger RNA (mRNA). The cDNA is then used as a template for the quantitative PCR (qPCR). Real-time quantitative PCR (RT-qPCR) is used in a variety of applications such as food-borne RNA virus and avian flu virus detection. With this technique, we can detect the target RNA at an extremely low level in samples. RT-PCR is an increasingly popular method for RNA virus detection, but DNA contamination in RNA preparations is also a concern. In order to minimize the possibility of carryover contamination in RT-PCR, it is critical to minimize the number of handling and pipetting steps.

1.2.2. Real-time quantitative PCR (qPCR)

Traditional detection of amplified PCR product relies upon gel electrophoresis. qPCR is an advanced form of the traditional PCR. It is a major development in PCR technology that enables

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the reliable detection and measurement of products generated during every cycle of the PCR process. This technique became possible after the introduction of an oligonucleotide probe that was designed to hybridize within the target sequence. Due to the 5′ nuclease activity of Taq polymerase, amplification of the target-specific product can be detected through cleavage of the probe during PCR. These assays are very sensitive and can detect as few as 10–100 viral copies per reaction. qPCR techniques have evolved into a variety of other branches including real-time PCR by Taqman (Roche), LightCycler by (Roche), SmartCycler by (Cepheid), etc. Some of them are now widely in use for virus and bacteria detection in regulatory laboratories. Unlike other PCR methods, qPCR does not require post-PCR product handling, preventing potential PCR product carryover contamination.

1.3. PCR contamination

All the PCR methods are powerful techniques. Unfortunately, the exquisite sensitivity of these techniques makes them vulnerable to contamination [4, 5]. One of the most important rules when performing PCR is to avoid contamination. This chapter will outline necessary precautions to prevent contamination as well as procedures for detecting and cleaning suspected contamination.

2. Potential sources of contamination

2.1. Cross contamination between samples

A large number of target organisms in sample handling may lead to pre-amplification sample cross contamination [6, 7]. The sources of contaminants between samples are diverse and can all contribute to the contamination of the finished PCR product. These sources may include reagents, disposable supplies, sample carryover, improper handling procedures, etc.

2.2. Cross contamination between nucleic acids

Cross contamination between nucleic acids is a major problem in all PCR laboratories. Nucleic acids from organisms or plasmid clones derived from organisms that have been previously analyzed and that may be present in large numbers in the laboratory environment could be a source of contamination. Contaminants can also be introduced by unrelated activities in neighboring laboratories. These sources of contamination are problematic as they may lead to pre-amplification cross contamination [8–10].

2.3. PCR product carryover contamination

The most important source of contamination is from the repeated amplification of the same target sequence, which leads to accumulation of amplification products in the laboratory environment. Even minute amounts of carryover can lead to false-positive results. A typical PCR generates theoretically as many as 10^8 copies of target sequence [11]. If uncontrolled, amplification products will contaminate laboratory reagents, equipment, and ventilation

systems. Carryover of previously accumulated amplified DNA is considered the major source of contamination.

3. Methods to control contamination

Contamination between samples and from previous PCR amplicon generation is a significant potential source of invalid PCR results [12]. The first two forms of contamination described above can be easily avoided by using careful technique and good quality control practices. Generally, most PCR-based assays consist of three steps: DNA sample processing, PCR amplification, and amplification product detection (excluding real-time PCR). It is in the latter step that carryover contamination often occurs through methods that include gel electrophoresis, solid phase hybridization, solution hybridization, and capillary electrophoresis [7]. Methods to prevent amplification product carryover contamination have been developed in the past ten years [13–17]. Basically, there are mechanical, chemical, UV light irradiation, and enzymatic methods and closed-tube PCR detection formats, all of which can help to prevent amplification product carryover contamination [7]. The following section will focus on more recent practices and methods that have been used in our laboratory to eliminate carryover contamination.

3.1. Mechanical method

Our laboratory was designed and operated in a way that prevents contamination of reactions with PCR products from previous assays and cross contamination between samples. It includes the separation of areas of the laboratory where samples and reagents are prepared from the areas where amplification is performed and amplification products are analyzed. This unidirectional workflow can reduce the opportunity for contamination to occur. A typical PCR laboratory should be divided into at least three to four different areas—(1) sample preparation, (2) PCR mix preparation, (3) PCR product detection, and (4) RNase free area—if the PCR method involves RNA sample.

3.2. Chemical method

General cleaning practices are important for controlling PCR carryover contamination. All surfaces in the PCR area should be routinely decontaminated to prevent cross contamination. The PCR work bench is required to be cleaned with 10–15 % sodium hypochlorite solution (bleach), followed by removal of the bleach with 70 % ethanol.

3.3. UV irradiation method

UV irradiation is an easy method to inactivate amplification product involved in carryover contamination. The method is based on the ability of UV light to induce thymidine dimer formation in the DNA that makes the contaminating nucleic acid inactive as a template for further amplification (**Figure 1**). A good practice is to expose all of the PCR supplies to UV light for 5–20 min as the nucleic acid will be damaged by absorbing the UV light energy at

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254 nm wavelength [19]. UV irradiation is an integral feature of our PCR laboratory, and the Spectrolinker XL-1500 (Spectronics Corporation, Westbury, NY) is used to eliminate contamination that may occur during PCR tests. All of our PCR tools are stored in a UV light box (C.B.S Scientific, Co. Del Mar, Ca). PCR master mix preparation and specimen setup are also carried out in this UV light box.

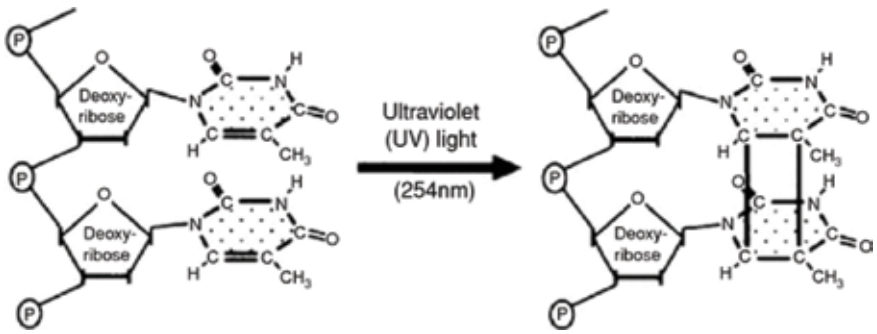

Figure 1. Action of UV light on the nucleic acids [18].

3.4. Enzymatic method

Uracil-DNA glycosylase (UNG) is a DNA repair enzyme [20] that can recognize and remove uracil residues from DNA (**Figure 2**). In 1990, the use of UNG to inactivate PCR products was first reported [21]. This method employs uracil (dUTP) instead of thymine (dTTP) during PCR to generate amplification products with distinguishing characteristics relative to the native DNA template. Because the newly synthesized amplicons contain dUTP, they are susceptible to hydrolysis by UNG. This method is the most widely used contamination control technique in our laboratory.

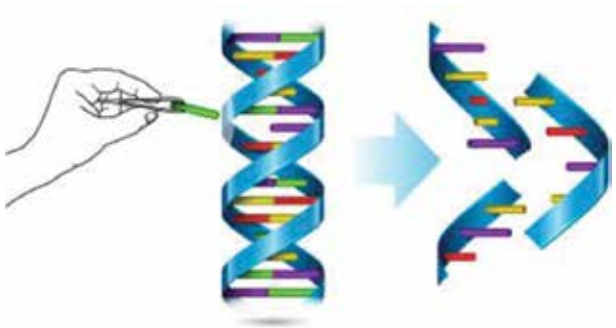

Figure 2. Replace dTTP with dUTP during PCR amplification and the PCR product will contain uracil. Prior to PCR, the PCR mixture is treated with uracil-DNA glycosylase (UNG). During the denaturation step, temperature is elevated to 95°C, resulting in cleavage of apyrimidinic sites and fragmentation of carryover DNA. As the template contains thymidine, it will not be affected by the UNG treatment (source: Sopachem Life Sciences).

Briefly, this carryover prevention technique consists of three steps:

1. The dUTP is incorporated into all PCR products, substituting dUTP for dTTP or incorporating dUTP during synthesis of the primers [15, 22].
2. Before PCR, mixtures are treated with UNG (Applied Biosystems, Foster City, CA) at room temperature for 10 min to hydrolyze and remove any contaminating amplification products that may be present in the PCR mixtures. This technique also has a hot start function [15].
3. UNG is thermally inactivated at 95°C for 5 min prior to the actual PCR.

3.5. Another amplification format without the risk of carryover contamination

3.5.1. Real-time PCR-based technology to avoid contamination

In traditional PCR, amplification and detection of the target DNA sequence occur separately. To determine if a sample contains the target sequence, post-amplification handling of the amplicon is required. A more recent technological development, real-time PCR [23], allows for the simultaneous amplification and detection of a target sequence through the use of fluorescent-labeled probes (**Figure 3**). In comparison to conventional PCR, real-time PCR can reduce the chance of carryover contamination. The new generation of amplification technology simultaneously amplifies and detects target DNA without exposing the amplification products to the laboratory environment. Currently, we developed several real-time PCR methods, such as single-tube real-time PCR and single-tube nested real-time PCR (**Figure 4**) to simultaneously detect multiple pathogens in a closed system which has substantially reduced the possibility of false-positive results due to amplification product carryover contamination [24, 25].

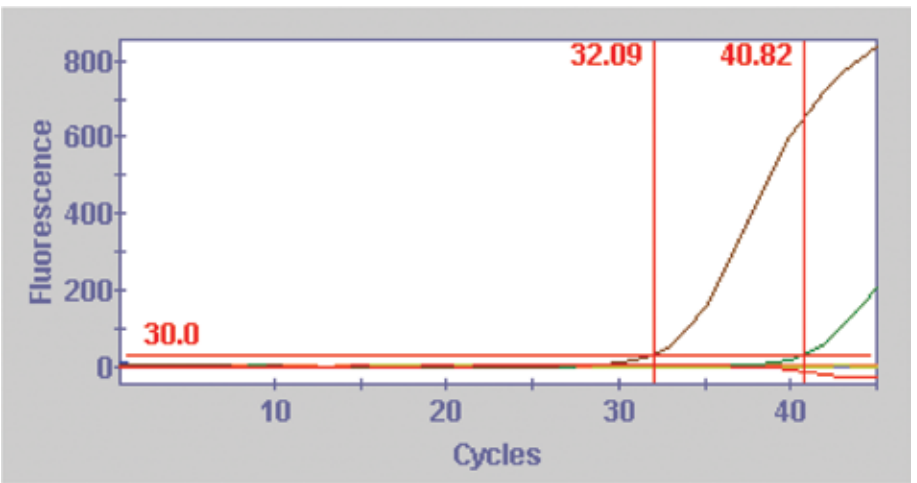

Figure 3. A typical result of graphical view from our laboratory.

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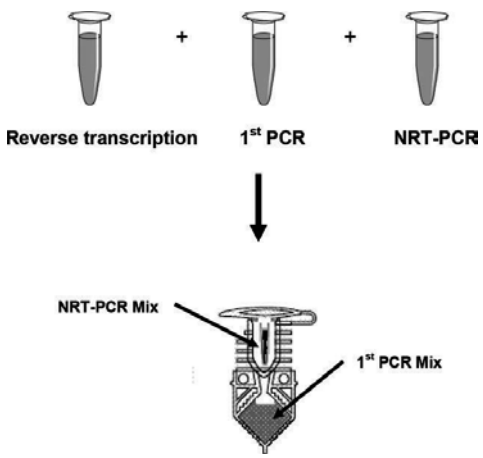

Figure 4. Single closed-tube nested real-time-PCR system: in order to reduce the chance of carryover contamination, all reactions including reverse transcription, conventional PCR, first PCR, nested PCR, and real-time TaqMan detection are performed in a single closed tube [24].

4. Method to detect contamination

In the context of this discussion, contamination is defined as the unwanted presence of a PCR amplicon. At times, PCR contamination is present but difficult to ascertain. If a significant contamination problem appears in a PCR laboratory, we need to walk through the procedure of testing for contamination and, if necessary, replace all reagents. The PCR parameters considered for potential sources of contamination include amplification setup, amplification product handling, and DNA aerosol and storage. Carryover contamination is determined by the following methods in our laboratory.

4.1. Internal controls

Appropriate control reactions are helpful in determining whether DNA contamination has occurred. It is important to use a special PCR-positive control which is different from the sample DNA, such as a DNA fragment with a deletion or base alteration in the region of amplification [26]. PCR products can be assessed on a gel to distinguish the control from the native PCR products. Negative controls are also very important and must be included with each run because the first sign of contamination trouble is usually the appearance of an amplification product in the negative or blank controls [27].

4.2. DNA sequencing

Techniques to sequence PCR products were developed in our laboratory in the past few years [15, 28]. This confirmatory sequencing ensures that the PCR product has the expected sequence. The direct comparison of PCR product sequences from a sample and a control is the

best way to determine whether two PCR products are similar or different. After comparison of the DNA sequence variation between the PCR products and the control, the cross contamination of samples can be detected. In some suspected cases, we directly sequenced PCR products by using the ABI BigDye Terminator Cycle Sequencing kit with a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Briefly, each cycle consists of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min. After 25 cycles, the fluorescent extension products are purified by a simple isopropanol precipitation step. Software is available on websites [29] to perform a wide range of different types of sequence alignment. DNA sequence data were analyzed by the Geneious, the GenBank sequence database, and the BLAST program from the National Center for Biotechnology Information (NCBI). Accurate identification of any contamination is required for the proper function of FDA field laboratory. The DNA sequencing method should be an ideal technology for this purpose.

5. Discussion

PCR is a very powerful and extremely sensitive amplification technique, but there is always the peril that a tiny amount of contamination of the DNA target may lead to false-positive results. It has become necessary to systematically address the issue of PCR contamination, especially in the FDA, a regulatory agency. To overcome this issue, effective methods have been successfully developed and used to avoid carryover contamination in our regulatory laboratories in the past few years [15, 24, 25].

- We have effectively established and maintained a unidirectional workflow from a PCR clean to a PCR dirty area, thereby reducing the opportunity of contamination to occur.
- Samples were set up on a bench that was isolated from PCR product testing areas.
- All PCR master mixes were prepared in a separate room or at least on a separate bench. Also, we always used a separate laboratory coat, gloves, tubes, and filter pipette tips in the different PCR working areas.
- A separate aliquot of water stock for each round of PCR was addressed.
- All PCR work benches were decontaminated with 10–15 % bleach and 70 % alcohol. All the pipettes, pipette tips, tubes, racks, and gloves were UV-irradiated.
- A different pipette tip was used when pipetting each of the PCR reagents, even the same master mix to each tube.
- The PCR tubes were kept closed during the procedure. The tubes were opened only when necessary because of potential aerosols that are dangerous with respect to contamination. Minimizing the number of pipetting and mixing steps in PCR master mix preparation is also very important from the perspective of aerosol contamination.
- It is very important to schedule PCR when not handling plasmids to prevent cross contamination.

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- dUTP was incorporated into all PCR products which can subsequently be selectively destroyed by UNG.
- Optimization of PCRs is also important. G + C-rich products may be more difficult to inactivate by UNG because of the lower concentration of uridine triphosphate(UTP).
- Positive controls consisted of a low copy number of the desired nucleic acid target and should never be prepared or stored with the samples.
- Other amplification methods, such as real-time PCR [23] or closed-tube PCR [25] which can reduce the chances of carryover contamination, are now being used more routinely in our laboratory.
- A rapid DNA sequencing method to precisely detect contamination was established.

6. Conclusion

Standard precautions should always be employed during all PCR-based testing, whether it is real-time PCR or conventional PCR. All the regulatory laboratories should have their own appropriate controls and systematic measures to prevent and detect contamination. When contamination does occur, we need to accurately determine which reagent is contaminated. All of us should also understand that our individual working habits directly affect our work quality. We believe all the above methods can reduce the risk of contamination and ensure the efficacy of all PCR results.

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Chapter 5

Multiplex Polymerase Chain Reaction Assay for Early Diagnosis of Viral Infection

Hiroko Tsunemine, Yuriko Yoshioka, Miho Nagao, Yasuhiro Tomaru, Toshiharu Saitoh, Souichi Adachi, Norio Shimizu and Takayuki Takahashi

Additional information is available at the end of the chapter

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Abstract

Viral reactivation is one of the most serious complications for immunocompromised patients. Under immunosuppressive conditions, some viruses can be reactivated solely or simultaneously and may thus cause life-threatening infection. Therefore, the prompt and proper diagnosis of viral reactivation is important for the initiation of preemptive therapy. For this purpose, we recently developed a multiplex-virus polymerase chain reaction (PCR) assay. The multiplex PCR assay is designed to qualitatively measure the genomic DNA of 12 viruses at once: cytomegalovirus (CMV), human herpesvirus type 6 (HHV-6), HHV-7, HHV-8, Epstein-Barr virus (EBV), varicella-zoster virus (VZV), BK virus (BKV), JC virus (JCV), parvovirus B19 (ParvoB19), herpes simplex virus type 1 (HSV-1), HSV-2, and hepatitis B virus (HBV). When a specific PCR signal is obtained, the viral load is determined by a quantitative real-time PCR. The qualitative multiplex and quantitative real-time PCR procedures take only 3 hours to complete. With this assay system, we can identify viremia at the early stage and thereby prevent it from progressing to overt and symptomatic viral infection in immunocompromised patients, such as those receiving hematopoietic stem cell transplantation.

Keywords: multiplex PCR, viral infection, viral reactivation, immunocompromised host, hematopoietic stem cell transplantation, immunosuppressive therapy

1. Introduction

Viral reactivation is a major cause of morbidity and mortality in patients receiving chemotherapy, immunosuppressive therapy, and hematopoietic stem cell transplantation (HSCT). This occurs in a host via an internal or an external trigger, such as immunosuppression, to activate from the latent state of viral infection, which lasts for a long time following primary infection. In immunocompromised hosts, some viruses can be reactivated solely or simultaneously and may cause life-threatening infection, such as pneumonia, meningitis, encephalitis, and lymphoproliferative disorders [1, 2]. Therefore, the prompt and appropriate detection of viral reactivation is important for the initiation of preemptive therapy. Among viruses that cause these infections, human herpes and polyoma family viruses are important in patients in an immunocompromised state.

Polymerase chain reaction (PCR) is a useful tool for detecting or monitoring viral genomes. However, a conventional viral PCR assay detects only a single virus. Therefore, we recently developed a qualitative multiplex PCR assay to quickly and simultaneously detect 12 kinds of viral DNA genomes, including eight herpes family viruses, from various samples such as blood [3–5], cerebrospinal fluid (CSF), ocular fluid [6, 7], bronchoalveolar lavage fluid [8], urine, and gastrointestinal mucosa. This qualitative multiplex PCR assay can be combined with quantitative real-time PCR to determine the viral load when a specific PCR signal is detected by the multiplex PCR.

In this chapter, we describe the frequency of viral reactivation and its clinical significance in immunocompromised patients, such as those with hematologic malignancies, inflammatory bowel diseases, and collagen diseases, with special relevance in patients receiving allogeneic hematopoietic stem cell transplantation (HSCT).

2. Materials and methods

2.1. Samples

From April 2011 to May 2016, specimens were obtained for monitoring viral infections from blood, urine, cerebrospinal fluid, and intestinal mucosa from patients in Shinko Hospital and the Pediatric Department of Kyoto University Hospital, when patients developed symptoms such as fever, cough, headache, consciousness disorder, liver dysfunction, nausea, abdominal pain, and diarrhea. Blood samples were obtained as negative controls from 12 healthy volunteers with informed consent. The present study was a single institutional clinical study designated "Multiple Virus-Analytic Study by Multiplex PCR" and was approved by the review board of Shinko Hospital. Individual patients provided their written informed consent.

The plasma was separated from ethylenediaminetetraacetic acid (EDTA)-2Na-chelated whole blood, and the supernatants were obtained from the urine and cerebrospinal fluid (CSF). DNA was extracted from 400 μl of these samples using a QIAamp MinElute Virus Spin Kit (Qiagen,

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Valencia, CA, USA). DNA was extracted from gastrointestinal mucosa using a QIAamp DNA mini kit (Qiagen) and eluted with 100-μl elution buffer.

2.2. Multiplex-virus PCR assay

The multiplex-virus PCR was designed to identify the following 12 species of virus at once: cytomegalovirus (CMV), human herpesvirus type 6 (HHV-6), HHV-7, HHV-8, Epstein-Barr virus (EBV), varicella-zoster virus (VZV), BK virus (BKV), JC virus (JCV), parvovirus B19 (ParvoB19), herpes simplex virus type 1 (HSV-1), HSV-2, and hepatitis B virus (HBV). The sequences of the primers and probes for these 12 viruses have been described [3]. Multiplex PCR amplifications were set up in two capillaries: capillary A was for HSV-1, HSV-2, VZV, HHV-6, CMV, Parvo B19, BKV, and JCV, and capillary B was for EBV, HHV-7, and HHV-8. Each capillary contained 5-μl DNA extract, specific primers, and AccuprimeTaq (Invitrogen, Carlsbad, CA, USA). The PCRs were performed using a LightCycler (Roche, Basel, Switzerland) with the following protocol: an initial denaturation step for 2 min at 95 °C, followed by 40 PCR cycles at 95 °C, then 2 s at 58 °C, and then 15 s at 72 °C. Hybridization probes were then mixed with the PCR products by 3000-rpm centrifugation for 3 s, and the melting curves were analyzed using a LightCycler (Roche) with the following protocol: an initial denaturation step for two cycles of 0 s at 40 °C, then 10 s at 95 °C, followed by hybridization at 40 °C for 20 s and melting at 40–80 °C (ramp rate, 0.2 °C/s). Specific hybridization with probes and individual PCR products were dissociated at the specific temperature for each virus and, as a result, the fluorescence signal disappeared (**Figure 1**).

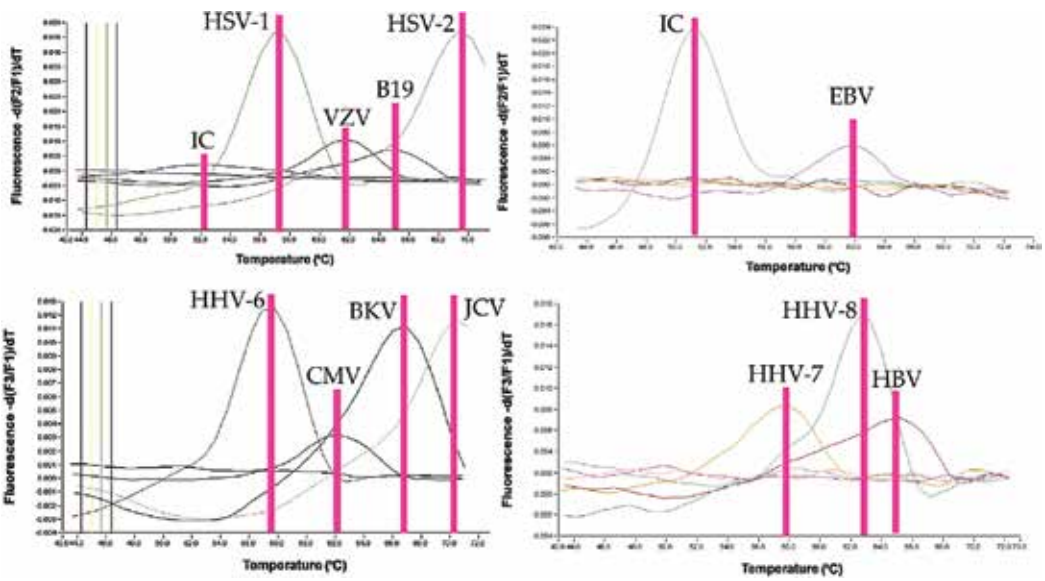

Figure 1. The melting curve analysis of multiplex-virus PCR.

Virus	Primer sequence	Probe sequence
HSV1/ HSV2	F: CGCATCAAGACCACCTCCTC	P: Cy5-TGGCAACGCGGCCCAAC-iowaBK
	R: GTCAG CTCGTG RTTCTG	P: 3FAM-CGGCGAIGCGCCCAG-iowaBK
VZV	F: TCACTACCAQTCATTTCTATCCATCTG	P: HEX-TGTCTTTCACGGAGGCAAACACGT-iowaBK
	R: GAAAACCCAAACCGTTCTCGAG	
EBV	F: CTGGGCAAG G AG CTGTTG	P: 6FAM-CTCGGCTGTGGAGCAGGCTT-iowaBK
	R: GGrCGrTTGTAAAATTGCA	
CMV	F: TCG CGCCCGAAGAGG	P: Cy5-CACCGACGAGGATTCCGACAACG-iowaBK
	R: CGGCCGGATTGTGGATT	P: Cy5-CACCGACGGATTCCGACAACG-iowaBK
HHV6	F: GAAGCAGCAATCGCAACACA	P: Cy5-AACCCGTGCGCCGCTCCC-iowaBK
	R: ACAACATGTAACTCGGTGTACGGT	P: Cy5-AACCCGTGCGCCGCTCCC-iowaBK
HHV7	F: CGGAAGTCACTGGAGTAATGACAA	P: HEX-CTCGCAGATTGCTTGCTTGCTTGGCCATG-iowaBK
	R: ATCGTTGCCTATTTCTTTTTGCC	
BKV/JCV	F: GGAAAGTCTTTAGGGTCTTCTACCTTT	P: 6FAM-ATCACTGGCAAACAT-MGB
	R: GATGAAGATTTATTYTGCCATGARG	
Parvo B19	F: GGGTTTCAAGCACAAGYAGTAAAAGA	P: 6FAM-CAGCTGCCCCTGTGG-MGB
	R: CGGYAAACTTCCTTG AAAATG	
ADV	F: GACATGACTTTTGAGGTGGA	P: 6FAM-CCCATGGAYGAGCCCACCCT-BHQ
	R: TCGATGACGCCGCGGTG	
HBV	F: GTGGTGGACTTCTCTCAATTFTCTAG	P: 6FAM-TGTCTGCGGCGTTTT-MGB
	R: GGACAMACGGGCAACATACTT	P: 6FAM-TGTCTGCGGCGTTTT-MGB

Table 1. The sequences of primers and probes used for the multiplex PCR assay.

More recently, a more convenient multiplex-virus PCR method adding adenovirus (ADV) detection was developed using a solid-phase plate. The primers and probes are fixed in advance as a solid phase on seven-well plates (Nihon Techno Service, Ibaraki, Japan), as follows: well A is for TBP and GADPH; well B for HSV-1 and HBV; well C for BKV and HHV-7; well D for EBV and VZV; well E for HHV-6, HSV-2, and HHV-8; well F for ADV and JCV; and well G for CMV and ParvoB19. The probes in the corresponding wells were labeled with four kinds of fluorochromes: 6FAM™, HEX™, Cy5™, and ROX™. The sequences of primers and probes employed in this assay system are listed in **Table 1**. The reaction mixture at a final volume of 20 μl consisted of 2-μl DNA extract (100–300 ng/well), 10-μl buffer, 0.2-μl Taq enzyme (Nihon Techno Service, Ibaraki, Japan), and 7.8 μl dH_2O. The reaction mixture was placed in each well described above, dissolving the solid-phase primers and probes, to create

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the final reaction solution. The wells were then capped and centrifuged for 3000 rpm for 3 s. Amplification and real-time fluorescence detection were performed with a model 7500 Real-Time PCR System (Applied Biosystems, CA, USA) with the following protocol: an initial denaturation step for 10 s at 95 °C, followed by 45 PCR cycles at 95 °C for 5 s and 60 °C for 30 s. Of note, a plate already fixed with a Taq enzyme in the solid phase in addition to the advanced fixation of primers and probes will be available in the near future from Shimadzu Corporation (Kyoto, Japan).

A sensitivity test for multiplex PCR was performed using known plasmid DNA representatives for the 12 individual DNA viruses. The plasmid DNAs were synthesized by Nihon Techno Service Company (Ibaraki, Japan). We confirmed that a minimum of 50 copies of each virus (5×10^3 copies/ml) could be detected by both the capillary and solid-phase plate systems with the plasmid DNAs at various dilutions [3, 5].

As described previously, when a specific PCR signal was obtained with the multiplex-virus PCR, the sample was subjected to subsequent quantitative real-time PCR assay to measure the viral load [3].

2.3. Statistical analysis

The cutoff value from the receiver operator characteristic (ROC) curves was evaluated for the sensitivity and specificity. A statistical analysis was performed using the EZR software program (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a modified version of the R software package that contains a graphical user interface and is designed for statistical functions that are frequently used in biostatistics (The R Foundation for Statistical Computing, Vienna, Austria) [9].

3. Results and discussion

We analyzed 2450 blood, 173 CSF, 129 intestinal mucosa, 483 urine samples, 18 ocular fluids, and 123 bronchoalveolar lavage fluids from 858 patients between February 2011 and May 2016 (**Table 2**). The patients included 117 who underwent allogeneic HSCT; 741 treated with chemotherapy or immunosuppressive therapy for hematologic malignancies, autoimmune diseases, and inflammatory bowel diseases, and a small number of immunocompetent hosts. The HSCT patients included 30 adults and 87 children.

3.1. Detection of viral DNAs in plasma samples

3.1.1. Viremia in immunocompromised patients

Multiplex PCR detected HSV-1 in 21 (0.9%), VZV in 24 (1.0%), EBV in 224 (9.1%), CMV in 430 (17.6%), HHV-7 in 11 (0.4%), HHV-6 in 209 (8.5%), Parvo B19 in 9 (0.4%), BKV in 163 (6.6%), JCV in 13 (0.5%), and HBV in 16 (0.7%) samples of 2450 blood samples from all patients (**Table 2**). Neither HSV-2 nor HHV-8 was detected in any of the samples. None of

the 12 DNA viruses were detected in any of the 12 healthy volunteers. The frequency of viral detection in patients who received allogeneic HSCT was higher than in patients treated with chemotherapy or immunosuppressive therapy. The most frequently detected virus in allogeneic HSCT patients was CMV (48.7%; 52/117), followed by HHV-6 (41.9%; 49/117), and EBV (29.9%; 35/117). Multiple detection of ≧2 viruses was observed in 8.1% patients (198/2450 samples), and coinfection with 4 kinds of viruses was observed in 3 cases. The most frequent coinfection in all samples was a combination of CMV and EBV (1.9%; 47/2450), followed by CMV and HHV-6 (1.3%; 33/2450). Coinfection of CMV and HHV-6 was observed more frequently in allogeneic HSCT patients (11.1%; 13/117) than in the other patients (0.4%; 3/741).

Virus	Plasma		Plasma(allo-HSCT)		CSF		Urine		Ocular fluid		BALF	
	Sample number	Positive (%)	Sample number	Positive (%)	Sample number	Positive (%)	Sample number	Positive (%)	Sample number	Positive (%)	Sample number	Positive (%)
HSV-1	21/2450	0.9	15/1395	0.9	6/173	34.7	0	0	1/18	5.6	1/123	0.8
HSV-2	0	0	0	0	4/173	23.1	0	0	0	0	0	0
VZV	24/2450	1	13/1395	0.9	6/173	3.5	0	0	2/18	11.1	1/123	0.8
CMV	430/2450	17.7	284/1395	20.4	1/173	0.6	14/380	3.7	3/18	16.7	13/123	10.6
HHV-6	209/2450	8.5	164/1395	11.8	8/173	4.6	7/380	1.8	1/18	5.6	3/123	2.4
HHV-7	11/2450	0.4	4/1395	0.3	0	0	0	0	0	0	7/123	5.7
HHV-8	0	0	0	0	0	0	0	0	0	0	0	0
EBV	224/2450	9.1	116/1395	8.3	2/173	1.2	4/380	1.1	2/18	11.1	39/123	31.7
BKV	163/2450	6.6	119/1395	8.5	3/173	1.7	194/380	51.1	0	0	1/123	0.8
JCV	13/2450	0.5	2/1395	0.1	2/173	1.2	54/380	14.2	0	0	0	0
HBV	16/2450	0.7	6/1395	0.4	1/173	0.6	0	0	0	0	0	0
ParvoB12	9/2450	0.4	0	0	1/173	0.6	0	0	0	0	0	0
ADV	N.D.	0	N.D.	–	N.D.	–	65/350	18.6	N.D.	–	N.D.	–

allo-HSCT; allogenic hematopoietic stem cell transplantation, BALF;bronchoalveolar fluid, N.D.; not determined.

Table 2. Absolute numbers and percentages of PCR-positive specimens separated by material.

3.1.2. Multiplex-virus PCR and CMV antigenemia assay

The correlation of the results by multiplex-virus PCR and those by CMV antigenemia assay (LSI Medience Corp., Tokyo, Japan), which has been widely used to monitor CMV reactivation, was examined using 144 plasma samples. In Japan, preemptive therapy is usually conducted when the antigenemia test gives three positive cells/two slides, as a criterion for preventing CMV disease. In our study, the virus multiplex PCR negativity corresponded to a median 0 positive cells/2 slides (range: 0–19) by the CMV antigenemia method, and the PCR positivity corresponded to a median 5 positive cells/2 slides (range: 0–419, $P < 0.001$). The median CMV

DNA copy number determined by quantitative real-time PCR was 280 copies/ml (0–11,000) and 13,000 copies/ml (70–500,000) in negative and positive samples by multiplex PCR, respectively ($P < 0.0001$). An ROC curve analysis showed that a CMV viral load of 2400 copies/ml in the plasma corresponded to 3 positive cells/2 slides by CMV antigenemia (sensitivity: 88.9%, specificity: 80.2%) and that a CMV viral load of 120 copies/ml in plasma was the threshold value of positivity by multiplex PCR (sensitivity: 95.6%, specificity: 81.2%). These results indicated that multiplex-virus PCR was able to detect the level of the CMV viral load to determine whether or not antiviral therapy should be started, and quantitative PCR was indispensable for determining the need for preemptive therapy.

3.1.3. Viremia in allogeneic HSCT patients

Using this multiplex PCR assay, Inazawa et al. prospectively examined 105 patients who underwent allogeneic HSCT once a week for viral reactivation, from pretransplantation to 42-day posttransplantation. The detection of viremia peaked at 21 days posttransplantation, and the most frequently detected virus was HHV-6 (60.0%), followed by EBV (10.5%), CMV (10.5%), and HHV-7 (8.6%). Cord blood transplantation, steroid treatment, and anti-thymoglobulin use were significant risk factors for viral reactivation after allogeneic HSCT [5].

3.1.4. Viremia in patients with liver dysfunction

Using a multiplex-virus PCR assay and subsequent quantitative real-time PCR, Ito et al. examined 37 patients with unexplained liver dysfunction not due to HBV or hepatitis C virus (HCV) infection for 12 species of DNA viruses and 6 RNA hepatitis virus (hepatitis A virus, HCV, hepatitis D virus, hepatitis E virus, hepatitis G virus, and transfusion-transmitted virus; TTV). The patients included 19 with hematologic disease and 18 with immunocompetence or other diseases. The detected viruses were TTV (38% of patients), HHV-6 (35%), EBV (14%), CMV (8%), hepatitis G virus (3%), and HHV-7 (3%). The relationship between liver dysfunction and HHV-6, EBV, and CMV infections was confirmed based on the time course of liver dysfunction and the detection of these viruses [3].

3.1.4.1. Case presentation 1: early diagnosis of visceral disseminated VZV infection by multiplex-virus PCR

A 48-year-old female underwent unrelated allogeneic HSCT for acute myeloid leukemia and received immunosuppressive therapy with tacrolimus. Four months after the transplantation, she developed severe abdominal pain. Computed tomography and upper gastrointestinal endoscopy were unremarkable; however, VZV-DNA was detected in the plasma by multiplex-virus PCR as a screening test, and a high copy number of VZV (65,000 copies/ml) was confirmed by the quantitative PCR. A diagnosis of visceral-disseminated VZV infection was made. Three days after starting intravenous acyclovir, she developed vesicular eruptions and hypoesthesia on the right leg. A high level of VZV (14,000 copies/ml) was also detected in the cerebrospinal fluid (CSF), indicating she also had meningitis/myelitis. Her symptoms were improved, and VZV-DNA became undetectable in both plasma and CSF after the dosage of acyclovir was increased. Visceral-disseminated VZV infection is a life-threatening disease associated with a

high mortality rate in patients following up HSCT and organ transplantation [10, 11]. Screening by multiplex-virus PCR was very useful in terms of the early diagnosis of VZV disease and consequent treatment.

3.1.4.2. Case presentation 2: the role of multiplex-virus PCR in intestinal complications after allogeneic HSCT

A 58-year-old female underwent allogeneic HSCT for high-risk myelodysplastic syndrome. She developed abdominal pain and severe diarrhea 1 month after the transplantation. Colonofiberscopy showed diffuse mucosal erosion and edema. The multiplex-virus PCR did not detect any virus DNA in the blood or intestinal mucosa. The histopathological diagnosis was intestinal graft-versus-host disease (GVHD). Parenteral methylprednisolone was started in addition to tacrolimus, but her symptoms were not improved. CMV was detected in the plasma 2 weeks after the initiation of methylprednisolone, and the virus copy number was 180 copies/ml. Another colonofiberscopy revealed exacerbation of mucosal erosion and edema, and CMV became detectable in both the intestinal mucosa and plasma by multiplex-virus PCR. At this time, the CMV copy number in the intestinal mucosa and plasma were 33,000 and 47,000 copies/ml, respectively. Treatment with ganciclovir was started. On an immunohistochemical examination, CMV-infected cells were observed in the intestinal mucosa in addition to GVHD histologic features. Her abdominal symptoms lasted for 3 months but were gradually improved by treatment of both GVHD and CMV infection. In this patient, intestinal GVHD was complicated by CMV colitis, and it was difficult to distinguish the CMV colitis from intestinal GVHD by endoscopy because a histopathological examination takes some time. In this setting, the multiplex-virus PCR examination was useful because the PCR assay enabled us to perform early intervention for CMV infection and prevent exacerbation of the viral disease.

3.2. Virus detection in CSF samples from patients with central nervous system (CNS) symptoms

A total of 173 CSF samples were obtained from 142 patients with central nervous system (CNS) symptoms, such as a fever with headache and consciousness disturbance. The most frequently detected viruses by the multiplex-virus PCR were HHV-6 (N = 9; 5.2%), followed by HSV-1(N = 6; 3.5%), VZV (N = 6; 3.5%), HSV-2 (N = 4; 2.3%), BKV (N = 3; 1.7%), EBV (N = 2; 1.2%), JCV (N = 2; 1.2%), CMV (N = 1; 0.6%), ParvoB12 (N = 1; 0.6%), and HBV (N = 1; 0.6%) (**Table 2**). HHV-6 was detected in the CSF in 17.1% of patients who underwent allogeneic HSCT, a significantly higher prevalence than that in the remaining patients (1.4%) who received chemotherapy and immunosuppressive therapy. In one patient who received allogeneic HSCT, HSV-1 and BKV were simultaneously detected when the patient was complicated by consciousness disturbance. HHV-7 and HHV-8 were not detected in all patients. The median HHV-6 copy numbers in the CSF and plasma samples at the same time were 3800 (range: 290–110,000) and 1250 (range: 10–1900) copies/ml, respectively, indicating a higher HHV-6 load in the CSF than in the blood.

HHV-6 encephalitis after allogeneic HSCT is a serious and potentially fatal complication. The reported incidence of this encephalitis after allogeneic HSCT varies from 0 to 21.4%, and it

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typically occurs around 2–6 weeks after transplantation [12, 13]. The incidence of HHV-6 detection in CSF in our institution tended to be higher than that reported in previous studies, possibly due to frequent monitoring of HHV-6 in the plasma and CSF during longer follow-up after transplantation. A recent prospective study reported that a high-plasma HHV-6 load is associated with an increased risk of developing HHV-6 encephalitis [14]. In particular, a plasma HHV-6 load exceeding 10,000 copies/ml significantly increases the risk of HHV-6 encephalitis following allogeneic HSCT [14]. In comparison with previous reports, our patients had relatively low copy numbers of HHV-6 in both the plasma and CSF. Although the reason for this difference in results is unclear, it may be due to different quantitative PCR methodologies or differences in the sampling time.

Human herpesviruses other than HHV-6 (HSV, VZV, HHV-7, CMV, and EBV) and two human polyomaviruses (BKV and JCV) also cause serious meningitis or encephalitis [15, 16]. These viruses are latent in many sites, including the CNS and hematopoietic cells after primary infection during childhood or adulthood [15, 17–20]. Encephalitis caused by reactivation of these viruses can develop even in healthy individuals, as well as in immunocompromised hosts. However, immunocompromised hosts are at a higher risk of severe disease and mortality [21], and even in those that survive, a majority are left with serious neurologic impairments, such as memory disturbance and seizure [22]. Therefore, the early diagnosis by multiplex-virus PCR and preemptive therapy is of great importance, although the standardization of the PCR method is needed.

3.2.1. Case presentation 3: convulsion attack during the treatment with foscavir after allogeneic HSCT

A 25-year-old man underwent allogeneic HSCT for acute lymphoblastic leukemia, and monitoring for viral reactivation was performed weekly using multiplex-virus PCR. Four weeks after transplantation, HHV-6 was detected in the plasma at a viral load of 120,000 copies/ml. The HHV-6 viral load was decreased to 1200 copies/ml 1 week after the initiation of foscavir therapy, but the patient developed convulsions with consciousness disturbance. Head magnetic resonance imaging (MRI) revealed no abnormal findings. A CSF examination revealed a white blood cell count of 3/mm^3 with 100% mononuclear cells, and the concentrations of protein and glucose were within normal ranges. Multiplex-virus PCR was positive for HHV-6 at 110,000 copies/ml but negative for HSV-1, HSV-2, VZV, CMV, BKV, and JCV. The cause of the convulsions was considered to be HHV-6 encephalitis, and the dosage of foscavir was doubled. The HHV-6 level in CSF became undetectable, and his consciousness was normalized 2 weeks after starting the increased dose treatment. No neuropsychological problems persisted. In this patient, it was difficult to make a diagnosis of HHV-6 encephalitis because he had already received preemptive therapy for HHV-6 reactivation, and encephalitis due to other unknown viruses needed to be excluded first. Although multiplex-virus PCR is useful for a prompt differential diagnosis, the risk of developing HHV-6 encephalitis should be taken into consideration when a high-blood viral load is observed, even just once.

3.3. Detection of viral DNA in urine samples

A total of 380 urine samples were obtained from 58 allogeneic HSCT patients who developed hematuria, micturition pain, and pollakisuria. The most frequently detected virus was BKV in 194 of 380 samples (51.1%), followed by ADV in 65 (17.3%), JCV in 54 (14.2%), CMV in 14 (3.7%), HHV-6 in 7 (1.8%), and EBV in 4 (1.1%) (**Table 2**). Herpes family viruses other than CMV were not detected. The median copy number in BKV viruria was 1.7×10^7 copies/ml, that of ADV was 2.7×10^5copies/ml, that of JCV was 8.75×10^5 copies/ml, that of CMV was 2.5×10^3 copies/ml, that of HHV-6 was 2.4×10^3 copies/ml, and that of EBV was 3.4×10^3 copies/ml. In 30 of 194 BKV viruria, coinfection with ADV was observed. Most EBV and HHV-6 viruria were coinfected with BKV or JCV. The median copy number of CMV in the plasma (6.6×10^3 copies/ml) was higher than that in the urine. CMV viruria, therefore, might be caused by viral transposition from the blood stream to the urinary tract. By contrast, in 184 urine samples with detectable BKV, plasma samples were simultaneously examined for BKV, and BKV was detected in 61 plasma samples (33.2%). The median copy number of BKV in the plasma was 5.9×10^3 copies/ml, which was lower than that in the urine, suggesting that BKV had a greater affinity for the urinary tract. Similarly, in 47 ADV viruria, ADV was detected in 30 plasma samples (63.8%), and the median copy number of ADV in the plasma was 4.4×10^4 copies/ml.

Viral infections with polyomaviruses and ADV, mainly type 11, have been documented in allogeneic HSCT patients complicated by hemorrhagic cystitis (HC) [23, 24]. Some data have also identified CMV and HHV-6 as putative causes of HC [25, 26].

Polyomaviruses, namely BKV and JCV, are thought to cause primary infection in childhood or early adulthood and subclinically remain in the kidney and peripheral blood in 35–85% of the global population [27]. Although BKV has been reported to be detectable by PCR in the urine in 5–44% of asymptomatic and immunocompetent adults [28], BK viremia has been described in only immunocompromised patients, such as those who have undergone renal transplantation or HSCT. BKV is considered to be the main pathogen for HC in allogeneic HSCT patients [29]. Clinical manifestations of BKV reactivation include prolonged hematuria, painful dysuria, HC, and renal dysfunction. HC caused by polyomaviruses, however, spontaneously resolves in most patients [30]. A few studies have reported that BK viremia may predict the development of HC. The incidence of HC was reported to be 26–43% in allogeneic HSCT patients, and all of them had BK viremia [31]. Erard et al. observed that adult allogeneic HSCT patients with BKV viremia exceeding 10^4 copies/ml had a higher risk of developing HC than those with viremia less than 10^4 copies/ml [32].

Primary ADV infection also occurs during childhood and remains latent in the genitourinary tracts. In contrast to BKV, ADV is usually undetectable in the urine of healthy adults, indicating that ADV does not replicate itself under normal immune conditions and that severe immunosuppression allows for ADV replication [33]. The clinical manifestations of ADV are similar to those of BKV; however, the symptoms are more severe in ADV-caused HC [34] and sometimes lead to a fatal course [35]. Echavarria et al. reported that ADV viremia was a risk factor for the development of severe or fatal disease [36]. Thus, the early diagnosis of ADV HC using a PCR method such as our multiplex-virus PCR assay is important, and therapeutic interven-

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tion for ADV with antiviral agents such as cidofovir should be considered if ADV is detected in the plasma [30, 34].

4. Conclusion

Viral reactivation is a major problem and must be monitored and treated at the early stage in immunocompromised patients to avoid a fatal outcome. The qualitative multiplex-virus PCR assay that we recently developed is useful for screening and monitoring viral infection, especially multiple-virus infection, which occurs under immunosuppressive conditions, such as in patients following allogeneic HSCT. Under these conditions, the early diagnosis and preemptive therapy for viral infection is important to prevent immunocompromised patients from developing severe organ damage. Furthermore, quantitative real-time PCR may be useful for evaluating the clinical relevance of virus infections. The establishment of a cutoff value to distinguish viral reactivation from viral disease is currently difficult, as a standardized assay system is lacking. Therefore, the standardization of the multiplex-virus PCR system, as well as validation of its sensitivity and specificity, should be established in the future.

Conflicts of interest

The authors declare no conflicts of interest in association with this study.

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Chapter 6

The Advantages of Using Multiplex PCR for the Simultaneous Detection of Six Sexually Transmitted Diseases

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Additional information is available at the end of the chapter

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Abstract

Sexually transmitted diseases (STDs) are among the most common infections. Their clinical identification is difficult because STDs are often asymptomatic. Untreated infections with these pathogens can in time lead to serious consequences. It is documented that isolation of some of these bacteria from cultures is very difficult. Because there is a large number of STD pathogens which can generate coinfections, their simultaneous detection in a unique sample is very important. Multiplex polymerase chain reaction (PCR) is an advanced method of molecular biology which allows for simultaneous detection of multiple pathogens in the same sample. The advantages of the multiplex PCR method were assessed by various researchers by comparing the diagnosis results obtained with different other conventional methods. The sensitivity and specificity of these methods were analyzed on different specimens in comparison to traditional methods, such as culture media or direct microscopic examination. These studies demonstrated beyond any doubt that the multiplex PCR system is highly effective in the detection of each of multiple STD pathogens depicted from a single specimen and argued for multiplex PCR superiority in terms of sensitivity and rapidity.

Keywords: STD pathogens, DNA, simultaneous detection, multiplex PCR

1. Introduction

Sexually transmitted diseases (STDs) are an issue of great interest as they are among the most common of all infections. It is documented that STDs are more prevalent in economically underdeveloped populations. Only a few countries outside Western Europe and

North America have implemented monitoring systems for these infections. In the rest of the world, epidemiological studies are based on the results of samples provided by certain population segments (mainly symptomatic patients or prenatal controls) not necessarily representative for the majority of the population. Usually, persons presenting obvious signs (secretions, lesions or pain) interrupt their sexual activity and seek medical help. Ironically, most often those who actually transmit these infections are asymptomatic or present mild symptoms [1].

It is well-known that vaginal bacterial infections can induce human infertility, yet they are underestimated in infertility testing. Population screening and treatment of individuals presenting unexplained infertility that are detected as STD-infected persons seem appropriate in this context [2].

No STD can be regarded as an isolated problem since multiple infections are rather common and STD infections generally denote a high-risk sexual behavior that often can be associated with more serious infections. It is therefore important to acknowledge that STDs are often asymptomatic or cause nonspecific symptoms and that periodical STD testing is crucial in limiting the risk of human immunodeficiency virus (HIV) infection. STD control could significantly reduce HIV incidence worldwide, although the impact of interventions may vary depending on local epidemiological contexts. Analysis of data from several studies has suggested that a better management of STD cases is more likely to reduce HIV incidence in the early stages of an epidemic, when HIV infections are concentrated in population groups with a high prevalence of other curable STDs [3].

A correct STD diagnosis is needed to prevent further spreading of such infections in the healthy population. Several techniques and laboratory methods for highlighting these diseases were developed in the past decades. For instance, because of their high sensitivity, specificity and suitability for different types of samples, nucleic acid amplification tests (NAATs) are suitable for the diagnosis of urogenital infections. Lately, however, polymerase chain reaction (PCR) techniques are increasingly employed in such cases as they allow direct, sensitive, automated and usable detection of STD-causing pathogens on all sample types and even the simultaneous detection of several STDs.

2. The most frequent causative agent of STDs

Chlamydia trachomatis is an intracellular human pathogen. *C. trachomatis* infection is the most common STD reported in Western Europe developed countries [4]. About 75% of all cases involve young people aged 15–24 years, being more common in women than in men. It infects the epithelial cells of the endocervix in women and the urethra in men. Infection with *C. trachomatis* is often asymptomatic, especially in women, but presents a high risk for complications. The treatment is rather simple if early detected. Of note, the bacteria can be passed from mother to newborn during delivery [5].

Urogenital infection with *C. trachomatis* shows a broad spectrum of clinical manifestations, including urethritis, cervicitis and pelvic inflammatory disease (PID). Intense mucosal

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inflammation is characterized by erythema, swelling and mucous secretions caused by mucopurulent cervicitis in women and nongonococcal urehtritis (NGU) in men. It is very important to diagnose these bacteria in early stages of infection and beginning treatment as soon as possible to prevent long-term complications. It can persist in the genital tract for a long time, in a form that is resistant to immunodestruction; such symptoms are unnoticed in approximately 75–80% of women [6]. *C. trachomatis* genital infection significantly increases the risk of sequelae in the reproductive tract in women, including tubal deterioration, ectopic pregnancy and miscarriage [7]. The role of the *C. trachomatis* infection in decreasing male fertility is also well-known. Given the severity of the complications, several countries (e.g., France) made recommendations for screening these bacteria in asymptomatic young subjects [8].

Neisseria gonorrhoeae is the etiologic agent of gonorrhea, one of the most common sexually transmitted bacterial infections, producing more than 82 million new infections worldwide each year [9]. *N. gonorrhoeae* or gonorrhea is an aerobic Gram-negative bacterium presenting itself in the form of diplococci. Along with *C. trachomatis,* it is the most prevalent sexually transmitted bacterial infection. It causes urogenital mucosa infections in men and women, being a serious cause of morbidity. The disease is characterized by purulent inflammation of the urogenital system mucosa. It is also presumed that gonorrhea infection may increase susceptibility to HIV and its transmission [10].

Neisseria gonorrhoeae has developed mechanisms to alter the epithelial barriers in order to reach subepithelial tissues and colonize in the host organism. Emergence and spread of multiresistant *N. gonorrhoeae* strains and the absence of an effective vaccine are major problems worldwide. Gonococcal endocarditis is a rare (1–2%) but serious disease that occurs in patients with disseminated gonococcal infections, which are also rare (0.3–5%) [11].

Several data indicate the involvement of the gonococci in miscarriages. Screening and medical management of the *N. gonorrhoeae* infection, including screening of women visiting abortion centers, have been proposed in order to identify associated risk factors and to assess the benefits of systematic screening in avoiding complications [12].

Trichomonas vaginalis is the causative agent of trichomonosis, one of the nonviral STDs. This protozoan was first described in 1836 by Donné. Infection with this parasite can give birth to serious complications, especially in women. The spectrum of clinical presentation range from asymptomatic or slightly symptomatic, particularly in men, to severe vaginitis with abundant vaginal secretions in women [13]. Infections with this protozoan can lead to serious health problems such as infertility, preterm delivery, low birth weight [14, 15], susceptibility to cervical cancer [15] or increased prevalence of high risk human papillomavirus (HPV) infection [16]. Moreover, there is a strong association between *T. vaginalis* infection and acquisition of HIV [17]. Because it is sexually transmitted, trichomoniasis is common in populations at higher risk for other sexually transmitted infections. Its presence in an individual is a marker for high-risk behaviors and coincident STDs should be sought after. This infection is detected in approximately three quarters of the infected women's male sex partners [18]. For better cure rates, sexual partners should be treated simultaneously even if the infection is asymptomatic.

Trichomonas vaginalis infects squamous epithelial cells through direct contact, producing micro-ulcerations and microscopic bleedings in the vaginal walls and endocervix. In most cases, men are asymptomatic, but they transmit the infection to women. As the women's columnar epithelium is not affected, trichomonosis is manifest as vaginitis, but not endocervicitis. The simultaneous presence of an endocervicitis should alert the physician to check possible coinfections with *C. trachomatis* or *N. gonorrhoeae*. Severe infections are usually treated with nitroimidazole derivatives, but the number of resistant strains is constantly growing [19, 20].

The term mycoplasma is used to designate organisms in the Mollicutes class, the smallest free-living cell-wall-deficient microorganisms, the most simple life forms capable of replication outside a host cell. *Ureaplasma urealyticum*, *Ureaplasma parvum*, *Mycoplasma genitalium* and *Mycoplasma hominis* are the most common species of mycoplasmas in both men and women. Mycoplasma species (*U. urealiticum* and *M. hominis* in particular) are frequently detected in sexually active asymptomatic youngsters. However, they can induce a broad spectrum of pathological conditions in both women and men, including unexplained chronic symptoms of lower urinary tract, NGU, PID, pyelonephritis, chronic prostatitis, preterm labor and idiopathic abortion due to infection of the urogenital bodies [21].

Ureaplasma species are sometimes detected in the commensal bacteria of the lower genital tract, so their role among STD is still questioned. Some studies demonstrated that the association of *Ureaplasma* species with NGU depends on the detected species and that *U. urealiticum* is an etiologic agent of NGU, unlike *U. parvum*. In addition, it is reported that *U. urealyticum* can cause infections in the lower genital tract and is a pathogen agent of urethritis in males [22]. In assessing the role of these microorganisms in producing human diseases, their high prevalence among asymptomatic individuals should be taken into consideration.

M. genitalium is a microorganism associated with acute and chronic sexually transmitted nongonococcal urethritis in men. Data regarding infections in women suggest that *M. genitalium* is associated with urethritis, cervicitis and PID. According to some authors, individuals with clinically-significant urethritis, persistent PID or cervicitis should be tested for *M. genitalium* [23]. As in the case of *C. trachomatis*, *M. genitalium* infection is often asymptomatic.

Several authors have reported that these organisms are engaged in women infertility, preterm delivery, premature rupture of membranes and chorioamnionitis [24, 25]. In addition, some studies present mycoplasma as causative agents of male infertility. It has been reported that these infections change various sperm characteristics, such as motility, density or morphology, and that antibiotic treatment improves the quality of the sperm [26]. A recent study found out that detection rates of *M. hominis* and *U. urealiticum* in infertile couples were about two times higher than in fertile couples [27].

3. STD detection methods

Chlamydia trachomatis was first isolated from the female genital tract in 1959 [28]. Culture techniques for the isolation of these bacteria were developed since 1965. Microimmunofluorescence

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tests for seroepidemiological studies and serotyping emerged later. Currently, detection of *C. trachomatis* is accomplished via bacteriological examination or enzyme-linked immunosorbent assay (ELISA) [29].

Due to technical difficulties resulting from the need to inoculate specimens immediately after sampling, culture assays present low sensitivity (50%) and are seldom used today in the microbiological detection of *C. trachomatis*.

It is thought that *Chlamydia* immunoglobulin (IgG) antibodies persist in the body for years and therefore are used as markers for *C. trachomatis* infections [30]. These markers can be useful if a previous infection could have produced changes in the reproductive tract, but the presence of antibodies is not indicative of an infection present in the body at the time of detection, so these markers cannot be used in diagnosis.

Today, there are numerous methods available for *C. trachomatis* diagnosis, but it should be underlined that the PCR direct approach is given great interest lately. Also, the quick and easy to use loop-mediated isothermal amplification (LAMP) is often employed in detecting *C. trachomatis* [31]. Molecular biology tests based on gene amplification improved diagnostic quality in terms of sensitivity and specificity and are gradually replacing all other techniques (cell cultures, antigen tests, molecular hybridization without amplification, etc.). These techniques confirmed the high frequency of asymptomatic infections, their prevalence in women and men and evaluation of the frequency of recurrent and/or persistent infections.

In detecting *N. gonorrhoeae* infections, bacteriological cultures are yet the gold standard for diagnosis as they allow further testing of antibiotic susceptibility. Culture assays using Thayer-Martin agar have long been seen as the standard method [32]. Due to difficulties in collecting, transferring and storing specimens, these tests exhibit low sensitivity and are unsuitable for screening. Although enriched agar cultivation is still widely used for the diagnosis of *N. gonorrhoeae* infections, recent years have seen extensive usage of NAATs, especially in detecting carriers. These genetic tests enable establishing a diagnosis in 2–3 days for gonococcal urethritis or cervicitis with a sensitivity of up to 99%. In contrast, cell cultures provide a sensitivity of 85–95% in acute urethral infections and below 50% in chronic forms in women. These figures largely depend on sampling conditions, as *N. gonorrhoeae* is a rather fragile organism [33].

Precise diagnosis of gonorrhea is needed to prevent severe complications and to control transmission, especially in the case of asymptomatic infections. Molecular approaches such as hybridization assays or nucleic acid amplification tests have revolutionized the diagnosis of gonococcal infection due to their increased accuracy compared to the culture media and their ability to simultaneously test multiple species. NAAT should be the technique of choice in the diagnosis of coinfections and screening. In addition to diagnostic, molecular approaches have been successfully applied for testing *N. gonorrhoeae* in tracking genetic diversity.

Suspicion of *T. vaginalis* is often considered by clinicians in symptomatic women presenting a combination of vaginal discharge, vulvar irritation and unpleasant smell. Unpleasant smell and low irritation is more likely to be correlated with bacterial vaginosis than with trichomonosis, the irritation being more prominent in the latter case. After completing the physical

examination, it is useful to determine the pH of the vaginal secretion. Vaginal pH is higher than 4.7 in most women presenting trichomonosis, but high pH values are also seen in most women with bacterial vaginosis, therefore further investigations are required for an accurate diagnosis. A final diagnosis is based on the positive detection of this parasite. This can be determined microscopically in saline serum, using a magnification of 400×, but the sensitivity of this method is about 60%. A crucial step in the infection process involves a dramatic morphological change in the parasite. Free ovoid cells that resemble the familiar image of a flagellated protozoan take an amoeboid shape in contact with the urogenital tract [34], making microscopic detection more difficult. Pap smears can detect *Trichomonas* infections, but the Gram staining is useless here. Microscopic evaluation is time consuming and standardization in the interpretation of positive results is rather ambiguous [18]. Another disadvantage is that the microscopic examination should be performed within the first 10 min after sample collection [35].

Microscopic examination of the smear and/or culture is presently the most commonly used method in the detection of *T. vaginalis*. Because of its very slow growth rate, strict requirements for nutrients and specific culture embodiments that are not widely available, *T. vaginalis* is difficult to be grown in culture media [36]. Not only are such media very expensive, they also present a low sensitivity compared to the PCR methods recently introduced and require microscopic evaluation. Another disadvantage is that in women *T. vaginalis* is undetectable several months after metronidazole therapy [37]. Therefore, a precise diagnosis in women and men based on cell cultures is rather difficult to be made and the newer molecular techniques are more and more brought into action [38]. In the last decade, several PCR assays were developed and studies comparing the two methods in the detection of *T. vaginalis* demonstrated a higher sensitivity and specificity on behalf of the PCR.

The contribution of flow cytometry technology in investigating adhesion of the extracellular parasite to human host cells has been clearly demonstrated. This methodology can be optimized and the test can be used in a format in which several different strains may be analyzed simultaneously [39]. It is, however, a difficult method for the diagnosis of trichomooasis. A recent study [40] indicated the advantages of using DNA in the detection of *T. vaginalis.*

As mentioned above, *U. urealyticum, U. parvum, M. hominis* and *M. genitalium* are the most common mycoplasma species in both men and women. While the first three can be grown in culture media, the latter cannot. However, growing on culture embodiments is difficult and lasts longer, up to 4 days for *U. urealyticum* [36]. Also, the technique requires biochemical identification to determine the species of mycoplasma involved. Mycoplasmas are hard to grow in normal laboratory conditions, their simplistic genome demanding more complex environments. Difficulties in their cultivation and identification in clinical samples that often contain other bacteria or fungi have further complicated causal diagnosis and their association to disease. Mycoplasma infections are often associated with the presence of asymptomatic carriers that may or may not develop the disease [41]. Thus, identification of the virulence factors in mycoplasma has been complicated by the difficulties encountered in isolating the strains.

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M. genitalium detection using culture cells is no longer employed in routine clinical practice because its growth takes several weeks and presents technical difficulties [42]. According to some authors, *M. genitalium* requires a special medium and incubating for up to 8 weeks to be successfully detected [43]. Although many researchers would rather turn to serological methods when documenting an infection caused by these bacteria [44], such diagnosis approach is not appropriate due to cross-reactivity with *M. pneumoniae*. For a precise diagnosis, a direct identification is therefore recommended.

The safe detection of *M. genitalium* and other bacteria was for a long time hampered by the absence of a commercially available diagnostic test. In recent years, however, NAATs revolutionized the detection and study of mycoplasma. These techniques contributed greatly to the DNA-based improvement of the detection, identification and serotyping methods [45]. In addition, the DNA-based method enabled the identification as separate species of *U. urealyticum* and *U. parvum*, previously considered as biovariants (biovar 1 and biovar 2) of *U. urealyticum* [46].

Over 50 mycoplasma genome sequences are now available in public databases, revealing a genetic diversity more complex than first predicted. This growing set of data is extremely valuable in the study of organisms otherwise difficult to cultivate, offering new means for testing and molecular diagnostics [47].

4. Multiplex PCR methods as diagnostic tools for STDs

Multiplex PCR methods for the simultaneous diagnosis of several STDs are more and more often employed in recent years. These methods place a number of primer pairs corresponding to specific DNA sequences of various STD pathogens all in one reaction tube, such commercial ready to use kits being available. While some kits can simultaneously detect two commonly associated STDs, such as *C. trachomatis* and *N. gonorrhoeae* infections, others can manage three pathogen detections. However, kits containing the primer pairs needed for the simultaneous detection of 6–12 STD pathogens are gaining momentum on the market. The most effective and widely used kits are presently those simultaneously detecting the top six most frequent STD agents, namely *C. trachomatis*, *N. gonorrhoeae, T. vaginalis, U. urealyticum, M. hominis* and *M. genitalium*, agents which play hard to get for other detection methods and often exhibit multiple associations.

Another asset is that easily collectable urine samples are adequate DNA sources for these PCR techniques along with the urethral/vaginal discharge swabs. About 30–50 mL of first void urine (FVU) samples collected in sterile polypropylene containers early in the morning, at least 4 h after the previous urination, are more than enough for a successful analysis. FVU is definitely less invasive compared to the harvesting of vaginal or urethral secretions, an aspect reported to have influenced a significant number of subjects to avoid STD identification tests previously [48]. As urine specimens can be self-collected in intimacy, an increased number of patients tend to favor such option that also enhances the applicability of the screening programs [49]. The fact that urethral secretions were found to present increased sensitivity

and specificity compared to FVU in immunological analyzes for male subjects seems to hold lesser relevance, anyway differences have diminished since NAAT were first performed [46]. Concerning the female subjects, the use of urine specimens in STD identification produced similar results to vaginal or endocervical secretions [50]. It is known that assay sensitivity may decrease if samples are repeatedly frozen/thawed or stored for longer periods of time because nucleic acids can easily degrade. Urine samples hold another advantage as well: they can be stored at 4–8°C up to 7 days prior to the processing.

Thanks to this easy and noninvasive sampling, the multiplex PCR can be used to detect STDs in both symptomatic and asymptomatic individuals and could prove to be a useful screening tool for the general population.

A 15 min centrifugation at 15,000g and subsequent supernatant removal and pellet resuspension are needed as a successful DNA extraction requires concentrated pathogen suspensions. Both urethral/vaginal discharge swab specimens and urine samples should be brought to room temperature prior to the centrifugation. One can use up to 10 mL amounts of sample to increase the DNA extraction yield. Several commercially available kits for DNA extraction can be used according to the manufacturer's instructions, e.g., QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany), AccuPrep Genomic DNA Extraction Kit (Bioneer, Seoul, Korea) or MasterPure™ Complete DNA and RNA Purification Kits (Epicentre Biotechnologies, USA).

In our marketing era, such new techniques and gadgets (kits) need to be accompanied by studies assessing their performance compared to other traditional methods.

One such study conducted in 2010 [36] compared the multiplex PCR assay for the simultaneous detection of *T. vaginalis*, *U. urealyticum* and *M. hominis* with data obtained using microscopic and culture techniques. Three cotton swabs were obtained from each of the 240 women enrolled in the study based on claims of lower genital tract infections. For the detection of *T. vaginalis,* swabs were inoculated in a modified Diamond's Trypticase-yeast medium including fetal bovine serum, streptomycin, penicillin G and amphotericin B and a wet mount was prepared for its detection by direct microscopic examination. The samples were incubated at 37°C in carbon dioxide atmosphere and observed for a week under a light microscope. The presence of *M. hominis* and *U. urealyticum* was assessed by culture detection using the Mycoplasma Duo kit (Sanofi Diagnostic). A number of different primer sets were synthesized, PCR amplifications and ethidium bromide detections in 1% agarose gels were performed for a multiplex PCR to be designed for the simultaneous detection of *T. vaginalis, M. hominis* and *U. urealyticum*. Results were then favorably compared to more conventional methods for the detection of *T. vaginalis, M. hominis* and *U. urealyticum*. When tested on vaginal swabs, the multiplex PCR correctly detected 14 out of 14 *T. vaginalis*-positives, 95 of 98 *U. urealyticum*-positives and 22 out of 22 *M. hominis*-positive samples. In contrast, the microscopic detection of *T. vaginalis* exhibited a 28.6% sensitivity, whereas culture methods identified 71.4% of the trichomonad infections documented with multiplex PCR. While the kit's sensitivity in the detection of *U. urealyticum* was 96.9%, a value comparable to culture test sensitivity (91.8%), identification of *M. hominis* was greatly improved (100 vs. 36.3% sensitivity). The study argued for multiplex PCR superiority in terms of sensitivity and rapidity (hours vs. days/weeks).

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Another study [43] in 2011 compared the results of 113 STD patients tested for six sexually transmitted microorganisms (*C. trachomatis, M. hominis, M. genitalium, N. gonorrhoeae, T. vaginalis* and genital *Ureoplasma*) with an automated Seeplex® (Seegene, Korea) multiplex PCR-based STD6B autocapillary electrophoresis (ACE) system vs. culture and conventional PCR-based tests on genital and urinary specimens. *C. trachomatis* was cultured on cycloheximide-treated McCoy cells, while A7, arginine broth and agar media were used for *M. hominis* and *Ureaplasma*. Conventional culture media were employed for *N. gonorrhoeae*. *N. gonorrhoeae* and *C. trachomatis* was also tested with the PCR-based COBAS Amplicor. On the other hand, in-house validated PCR assays were performed for *T. vaginalis, N. gonorrhoeae* and *C. trachomatis*, while a Hy-Mycoplasma PCR Detection Kit (Hy-Labs, Israel) enabled the detection of *M. hominis*, *M. genitalium* and *Ureaplasma*. All six pathogens (*T. vaginalis, M. hominis, M. genitalium, C. trachomatis, N. gonorrhoeae* and *Ureaplasma*) were subjected to a multiplex PCR amplification and the products were separated and detected via automated capillary gel electrophoresis. The sensitivity of the STD6B ACE kit was 100% in case of *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, *T. vaginalis* and *M. hominis*, and 98% for the *Ureaplasma*. Specificity was also 100% for *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium* and *T. vaginalis*, while for *M. hominis* and *Ureaplasma* figures were 99 and 97%, respectively. These results prove beyond any doubt that the integrated STD6B ACE system is highly effective in the detection of each of the six STD pathogens depicted from a single specimen.

Seeplex® STD6 ACE Detection kit (Seegene, Korea) was evaluated in yet another Korean study, completed in 2012, which was conducted on 739 subjects [51]. Cervical swabs were collected from the women enrolled, while men were requested to provide 30–40 mL of FVU. The six pairs of dual priming oligonucleotide primers specifically targeting the *C. trachomatis, M. hominis, M. genitalium, N. gonorrhoeae, T. vaginalis* and *U. urealyticum* genes were tested against a combined monoplex PCR. All specimens were tested with the multiplex PCR for the six pathogens and amplification products were separated with a LabChip® DX Seeplex® assay system (Caliper, USA). All specimens were retested with six monoplex PCRs (one for each of the STD agents) and a duplex strand displacement amplification (SDA) for *C. trachomatis* and *N. gonorrhoeae* was accomplished with a BD ProbeTec™ (Becton–Dickinson Microbiology System, USA). An absolute correlation between the multiplex and monoplex PCRs was found in terms of both sensitivity and specificity. The results of the multiplex PCR and duplex SDA were 99.7% concordant in the case of *C. trachomatis* and 100% for *N. gonorrhoeae*. The results confirmed that the simultaneous detection of several species in one sample was feasible and that the clinical sensitivity provided by the multiplex PCR was on an equal footing with the monoplex PCR and duplex SDA, proving to be fully adequate for routine detection of several STDs while holding an upper hand in terms of cost-effectiveness and rapidity.

Anyplex™ II STI-7 Detection Kit (Seegene, Korea) was tested to the limit in a 2013 Korean study [46] aiming to investigate the accuracy and reliability of a real-time multiplex PCR assay employed for the detection of seven STD agents (*C. trachomatis, N. gonorrhoeae, T. vaginalis, M. genitalium, M. hominis, U. urealyticum* and *U. parvum*) in clinical samples. A total of 897 specimens (696 FVU and 201 endocervical swabs) were collected from 365 symptomatic patients and 532 asymptomatic volunteers and five diagnostic methods were employed as the speci-

mens were subjected to parallel testing using four NAATs and one *Mycoplasma* detection kit. The RT-PCR amplification was performed for all seven microorganisms with Anyplex™ II STI-7 Detection Kit. PCR amplification was performed using the Seeplex® STD6 ACE Detection Kit, in accordance with the manufacturer's protocol. A battery of kits were deployed for detecting the pathogens: *C. trachomatis* and *N. gonorrhoeae*—SDA technology using BD ProbeTec™; *T. vaginalis* and *M. genitalium*—AmpliSens® (EPH PCR Kit-InterLabService Ltd, Russia); *M. hominis* and *U. urealyticum*—Mycoplasma IST 2 Kit (bioMérieux, France). Test sensitivity and specificity of each method for every STD agent were calculated in parallel and conclusions were that the multiplex real-time PCR (Anyplex™ II) demonstrated outstanding results compared with other diagnostic tools as it yielded 100% sensitivity and very high specificity in the detection of *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, *M. genitalium* and *M. hominis* and was also useful in discriminating between *U. urealyticum* and *U. parvum*. Despite the increased workload and higher costs due to the larger number of tests conducted, multiplex real-time PCR was found to be equivalent or superior to other assessment methods and in the future is expected to become a standard diagnostic tool.

We ourselves have used a Seeplex® STD6 ACE Detection kit in a study on 224 Romanian subjects including persons with STD symptoms and asymptomatic individuals involved in unprotected sex with multiple partners, couples experiencing unexplained infertility or others requesting STD screening for various reasons. Each individual contributed with 30–50 mL FVU samples and DNA extraction was accomplished with MasterPure™ Complete DNA and RNA Purification Kits (Epicentre Biotechnologies, USA). A 2% agarose gel containing ethidium bromide was used with 0.5× Tris-borate 0.1 mM EDTA (TBE) as running buffer. The PCR products were visualized using a UV transilluminator. An example of gel electrophoresis results can be visualized in **Figure 1**.

The accuracy of the method was demonstrated by the lack of contamination (no amplicons in the negative control band) and the presence of positive control bands corresponding to all six DNA fragments of known molecular weight included in the marker used to determine the size of the amplification products. Also, internal controls indicated proper PCR amplification in each microtube (see **Figure 1**).

About 74 (33.03%) of the 224 subjects, both symptomatic and asymptomatic, were positive for one or more STDs. All six pathogen agents were detected with the multiplex PCR kit, several double or triple infections demonstrating associative patterns. The following double associations were detected in the study: *C. trachomatis* and *N. gonorrhoeae*, *C. trachomatis* and *U. urealyticum*, *N. gonorrhoeae* and *U. urealyticum*, *C. trachomatis* and *M. hominis*. Several triple infections grouped *C. trachomatis*, *N. gonorrhoeae* and *U. urealyticum*.

The bacteria most frequently identified in the analyzed urine samples was *C. trachomatis*, found in 20.9% of the cases. Consequently, a number of associations between *C. trachomatis* and other STD agents (*N. gonorrhoeae*, *U. urealyticum* and *M. hominis*, respectively) were found. These results are consistent with studies conducted in other countries or international statistics indicating *C. trachomatis* as the most common STD agent [4, 52, 53]. Detection of *C. trachomatis* in several asymptomatic patients demonstrated again how easily this infection can pass unnoticed.

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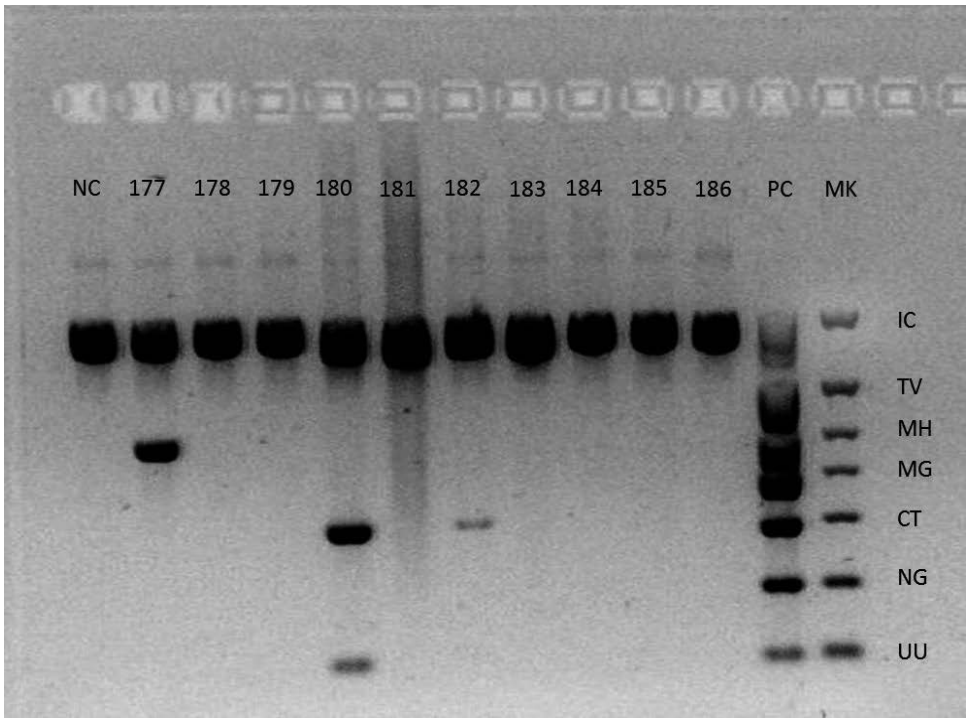

Figure 1. STD pathogens—agarose gel electrophoresis. NC: negative control, 177–186: samples, PC: positive control, MK: marker, TV: *T. vaginalis*, MH: *M. hominis*, MG: *M. genitalium*, CT: *C. trachomatis*, NG: *N. gonorrhoeae*, UU: *U. Urealyticum*.

Some studies recommended that all sexually active women should be tested, pregnant ones or those that have experienced miscarriages in particular, given the high prevalence of *C. trachomatis*/*N. gonorrhoeae* infections [54, 55]. In the US, concomitant empiric treatment of *C. trachomatis* and *N. gonorrhoeae* is recommended for all gonorrhea positives considering the frequent association between the two pathogens [56]. However, results indicate that several other associations (such as those between *C. trachomatis* and *U. urealyticum* or *M. hominis*) are often encountered, suggesting that a population screening for *C. trachomatis*/*N. gonorrhoeae* is not good enough and a multiplex method for the simultaneous detection of the most frequent six or more STD agents would be more appropriate, considering the different associations that may arise.

N. gonorrhoeae was detected in 7.1% of the cases, as was the case of *U. urealyticum*. These results are consistent with other studies indicating a high prevalence of both bacteria, the most frequent STD agents except for *C. trachomatis* [9, 57]. A notable finding is that we detected patients positive to *N. gonorrhoeae* that presented no or very atypical symptoms of gonococcal infection, which could have resulted in inadequate treatment without this DNA-based analysis that allowed us to identify the presence of the gonococcus. The *N. gonorrhoeae*/*U. urealyticum* coinfection we found was another confirmation that both *C. trachomatis* and *N. gonorrhoeae* can associate with other STD pathogens, an additional argument for the use of multiplex kits able to detect a large number of STDs in the same sample.

A recent study in Korea [58] found a 3.3% infection rate for *T. vaginalis* in the general population and argued that the rate was higher in women over 50 years of age. This followed a previous study documenting a 2.4% infection rate for *T. vaginalis* in the same area 15 years before and the significant increase lead researchers to recommend PCR-based testing of *T. vaginalis* in all women over 50 years old, irrespective of their symptomatology. Our study detected but one (0.44%) case of *T. vaginalis* infection, suggesting that in our area prevalence is much lower than the literature data based on other than NAAT determinations (e.g., physical and microscopy examinations or cell cultures) indicated. NAAT seems to be for now the only accurate method to diagnose this parasite. Our results are consistent with those conducted in other countries where *T. vaginalis* was detected in similarly low percentages compared to other STD [43, 51]. However, the incidence of trichomonosis has gradually decreased over the past 40 years. This may be due to the large amount of metronidazole and other imidazoles generally used in the treatment of bacterial vaginosis. Although the number of people infected with *T. vaginalis* is low, the analysis is justified considering its asymptomatic manifestation and the serious complications it produces.

In a study conducted in the United States, *M. hominis* and various *Ureaplasma* species were detected in 21–53% of the cases involving asymptomatic women and 40–80% of the sexually active women, respectively, their prevalence being slightly lower in men [59]. In our study, mycoplasma infections were detected in 25 individuals (a rate of 11.1%), the most commonly encountered being *U. urealyticum* (in 7.1% of the patients). Although *M. hominis* and *M. genitalium* were less represented (1.7 and 2.2% of the patients, respectively), these infections cannot be ignored. More so considering that *M. genitalium* was detected in an asymptomatic patient previously found negative for different STDs investigated on specific culture media (including ones for *M. genitalium*) in a number of different specimens (urine, urethral discharge). This last example reaffirmed the sensitivity of the multiplex PCR method over traditional alternatives.

5. Advantages of the multiplex PCR method

The advantages of the multiplex PCR method were assessed by various researchers comparing the diagnosis results obtained with different other conventional methods. The sensitivity and specificity of these methods were analyzed on different specimens in comparison to traditional methods, some of these considered to be the gold standard in diagnosis [36, 43, 46, 51]. Although this method can sometimes increase the workload, implying more protocol steps (DNA extraction, purification—if necessary, PCR amplification, electrophoresis), it still holds a huge advantage in terms of the exact diagnosis of STD. Except for special circumstances, such as when testing susceptibility to antibiotics of the various bacterial agents, NATTs have become the most sought after tests in STD detection. Regarding bacterial resistance to antibiotics, the DNA extracted and identified as belonging to such bacteria may be genetically tested to determine the genes that induce resistance to antibiotics or the different mutations that cause such resistance.

Although the symptoms are often similar, the treatment may differ depending on the STD agent detected. Without an accurate diagnosis, treatment is often ineffective. Clinicians often employ point-of-care (POC) tests to diagnose vaginal infections and STDs. For example, most

clinicians rely on Amsel's clinical criteria in defining bacterial vaginosis: increased vaginal pH, the presence of amines, clue cells observed on wet mounts and homogeneous vaginal secretions [60]. Although bacterial cultures and other methods are still widely used in STD detection, PCR tests are relevant, reproducible, sensitive and specific enough tools implying low costs and simplicity. In addition, the availability of commercial NAATs that include several STD pathogens allows more detailed studies regarding the relationship between such organisms in the etiology of these diseases. As well documented, all STDs occur in populations at high risk for other sexually transmitted infections. Their presence in a given individual is a marker for high-risk behaviors and coincidental infections should be sought after, as well as other more serious sexually transmitted diseases such as HIV.

An advantage of this method is its great sensitivity consisting in the detection of STD pathogens in individuals who have previously been tested with conventional methods and came out with negative results.

The greatest advantage of this method is the detection of double or multiple coinfections. This is an important argument for promoting multiplex testing in the same sample. These results could have important implications in epidemiology and treatment by improvements in the accuracy of determining the possible synergies and interactions between such microorganisms.

STD screening for certain categories of population (e.g., patients attending sexual health clinics, infertile persons or women who had miscarriages) is necessary not only to identify symptomatic persons in order to diagnose and treat their infection but also to identify asymptomatic individuals who serve as possible infection carriers in order to reduce morbidity and help controlling these STDs. The multiplex PCR method provides a good opportunity to argue for STD screening.

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Chapter 7

A Real-Time PCR-Based Diagnostic Test for Organisms in Respiratory Tract Infection

Takashi Hirama

Additional information is available at the end of the chapter

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Abstract

Respiratory tract infection, especially pneumonia, is a significant cause of morbidity and mortality worldwide. Although rapid and accurate identification of the pathogens and the corresponding treatment, which is based on the microbiological results, is required in the healthcare setting, the current clinical tests lack high sensitivity and flexibility. As of yet, a comprehensive approach has not been able to work these issues out. Meanwhile, the development of molecular techniques enables the detection of organisms from respiratory specimens speedily as well as precisely and aids the settlement of such issues. With our novel approach that employs relative quantification, we successfully set the cutoff value to discriminate the causative pathogen from colonizing commensal organisms by real-time PCR. In this way, a diagnostic system for respiratory pathogens was devised and validated through clinical sample testing. In this chapter, a real-time PCR-based test capable of differentiating causative pathogens in respiratory specimens is described, and also its principle and the utility of this approach are illustrated.

Keywords: PCR, real-time PCR, pneumonia, respiratory tract infection, sputum, HIRA-TAN, commensal organism, foreign organism, non-commensal organism, nucleic acid amplification test, NAAT

1. Introduction

Pneumonia is a common disease in healthcare settings and is a significant cause of morbidity and mortality worldwide. Despite there being a mere two-dozen species of pathogens responsible for most cases of pneumonia, the causative pathogen cannot be identified by clinical tests involving smear and culture of sputum, antigen tests, and serological assays in up to 50% of the cases [1–4]. Therefore, the identification of the causative pathogen(s) with

high detection power will allow for the selection of targeted antibiotics. Accordingly, timely identification has been shown to reduce the mortality rate and, on a long-term basis, the emergence of drug-resistant pathogens [5]. To identify such pathogens and thus to obtain the desired benefits, a clinical test is required that is sensitive, rapid, accurate, easily performed, and cost-effective.

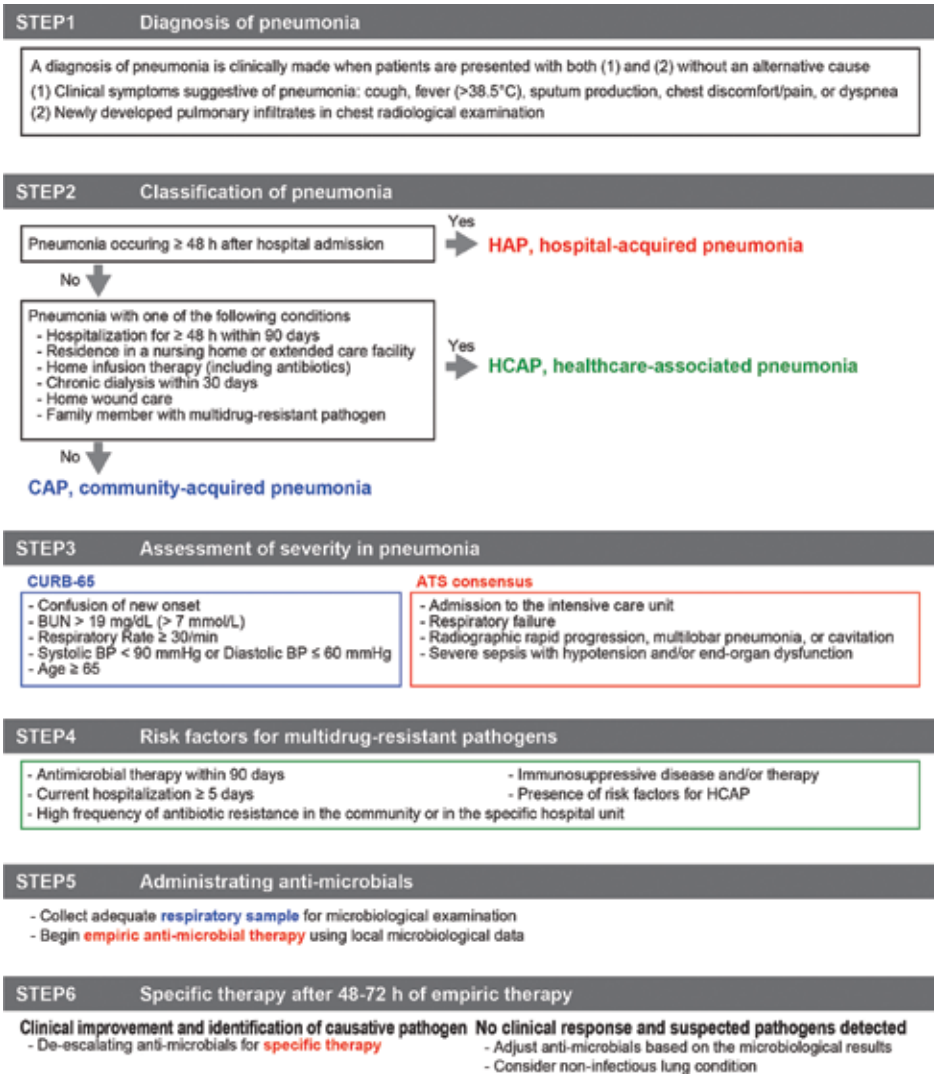

Figure 1. The overall strategy and assessment of pneumonia. The current consensus for diagnosis through treatment of pneumonia was summarized by STEP1 through STEP6 [3, 4]. Although examples of assessment, such as CURB-65 for CAP and ATS consensus for HAP, were illustrated in STEP3, an appropriate index ought to be chosen in each situation.

The development and wide usage of molecular techniques, such as polymerase chain reaction (PCR), brought about remarkable advances in clinical medicine. Detection of causative

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pathogens in pneumonia has been optimized in the last few decades, and from that point, PCR has played a principal role in laboratory medicine [6–8]. Currently PCR-based approaches, however, are mainly used to identify foreign organisms (such as *Mycoplasma pneumoniae* or *Legionella pneumophila*) in respiratory specimens [2, 9–11]. Even if such clinical tests have a multiplex detection system, identification of foreign organisms alone shows limited clinical value since the majority of pneumonia is caused by commensal organisms (such as *Streptococcus pneumoniae* or *Haemophilus influenzae*). Thus, distinguishing causative pathogens from among detected commensal organisms in respiratory samples, which contain colonizing organisms, has been challenging due to the highly sensitive detection power of PCR.

In this chapter, a real-time PCR-based test which is capable of differentiating therapeutic targets from detected colonizing commensal organisms in respiratory samples is described, and also its principle and the utility of this approach are illustrated.

2. Clinical approach and current strategy for pneumonia

Pneumonias are as of now classified as either the community-acquired pneumonia (CAP) or the hospital-acquired pneumonia (HAP), depending on the place pneumonia is acquired (**Figure 1**). Each has a specific spectrum of causative pathogens and allows medical professionals to speculate on the causative pathogen and initiate empirical antimicrobial therapy covering most of the speculated pathogens (**Figure 2**). Following confirmation of the causative pathogen, the antimicrobial should be switched/de-escalated to more specific and appropriate medication.

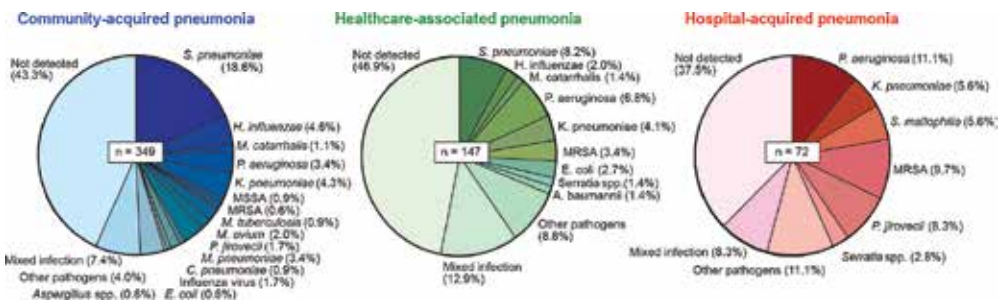

Figure 2. Etiology of three types of pneumonia diagnosed by conventional methods in clinical study. Five-hundred and sixty eight patients were enrolled in the prospective study where seven institutions participated [12]. Three-hundred and forty nine, 147, and 72 patients who met the criteria of pneumonia shown in **Figure 1** were categorized as community-acquired pneumonia (CAP), healthcare-associated pneumonia (HCAP), and hospital-acquired pneumonia (HAP), respectively, in which causative pathogen(s) were summarized in pie charts. In this study, all patients tested Gram stain and culture of expectorated sputum, urine antigen for *Streptococcus pneumoniae*, and real-time PCR for *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Chlamydophila psittaci*, *Coxiella burnetii*, *Legionella pneumophila*, *Bordetella pertussis*, *Mycobacterium tuberculosis*, *Mycobacterium intracellulare*, *Mycobacterium avium*, *Mycobacterium kansasii*, and *Pneumocystis jiroveci*. Urine antigen was tested for in the case of *L. pneumophila* for admitted cases only and influenza antigen for flu season only. Mixed infection indicates the cases in which two or more causative pathogens were identified at one time.

3. Organisms in respiratory tract infection

3.1. Conventional diagnostic tools for causative pathogens

A wide variety of laboratory tests including culture-based methods, antigen tests, and serology have been available for diagnosis and treatment of pneumonia (**Table 1**). Nevertheless, despite comprehensive evaluations with a range of different tests, as many as 40% of the causative pathogens causing CAP, HCAP, and HAP remain undiagnosed (**Figure 2**). While defining the pathogenic role of the respiratory organisms during pneumonia is still difficult, a commensal organism and foreign organism are often considered to be a causative pathogen when the below criteria are met. These criteria have provided legitimate results and thus have been used in clinical practice.

3.2. Commensal organism

Commensal organisms form part of human normal flora in the respiratory system (e.g., *Streptococcus pneumoniae*, *Haemophilus influenzae*, or *Pseudomonas aeruginosa*). However, in specific situations as when the host defense is weakened, the number of organisms grows beyond their typical ranges and then causes pneumonia. Since the majority of them reside in the respiratory tract, when they are detected from respiratory sample, there is no unequivocal criterion to discriminate causative pathogen from colonizing one by the sputum examination.

3.3. Foreign organism (non-commensal organism)

Foreign organisms account for a small portion of pneumonias (e.g., *Mycoplasma pneumoniae*, *Legionella pneumophila*, or *Mycobacterium tuberculosis*) (**Figure 2**). They do not reside in the respiratory tract or are rarely identified from healthy individuals, and thus their detection indicates that they are the causative pathogens.

3.4. Criteria for commensal organisms to be the causative pathogen

A commensal organism that fulfills at least one of the following three criteria is considered to be a causative pathogen when (1) an organism is identified from the normally sterile site (blood or pleural effusion); (2) a morphologically compatible organism, coexisting with abundant neutrophils, is observed through Gram staining and later confirmed by sputum culture; or (3) for *Streptococcus pneumoniae* only, the urine antigen test is positive.

3.5. Criteria for foreign organisms to be the causative pathogen

Foreign organisms that fulfill at least one of the following two criteria are considered to be a causative pathogen when (1) an organism is identified by culture, antigen test (involving serum, urine or nasopharyngeal specimen), or PCR test or (2) a paired serological test reveals a significant increase in antibody titer (more than four times).

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	Rapid test	Confirmed diagnosis
Commensal organisms		
Gram-positive cocci		
Streptococcus pneumoniae	Gram stain, urine antigen	Culture
Streptococcus milleri group		Culture
Staphylococcus aureus	Gram stain	Culture
Gram-negative cocci		
Moraxella catarrhalis	Gram stain	Culture
Gram-negative rods		
Haemophilus influenzae	Gram stain	Chocolate agar culture
(Non-fermenter)		
Pseudomonas aeruginosa	Gram stain	Culture
Acinetobacter baumannii		Culture
Stenotrophomonas maltophilia		Culture
Burkholderia cepacia		Selective agar culture
(Enterobacteria)		
Klebsiella pneumoniae	Gram stain	Culture
Escherichia coli		Culture
Serratia marcescens		Culture
Anaerobes		
Anaerobes	Gram stain from sterile sample	Anaerobic culture
Foreign organisms		
Atypical organisms		
Mycoplasma pneumoniae	NAAT	Serology CF, PPLO culture
Legionella pneumophila	NAAT, urine antigen	BCYE culture
Chlamydophila pneumoniae	NAAT	Serology MIF
Chlamydophila psittaci	NAAT	Serology MIF
Coxiella burnetii	NAAT	Serology IIF
Mycobacterium Tuberculosis	NAAT, AFB smear	Lowenstein-Jensen culture
Opportunistic organisms		
Actinomyces israelii		Anaerobic culture, microscopy for sulfur granules
Nocardia asteroides	Gram stain, AFB smear	Culture
Pneumocystis jiroveci	NAAT, Giemsa stain	
Fungi		
Aspergillus fumigatus	GMS stain, Galactomannan test	Sabouraud agar culture
Cryptococcus neoformans	Antigen test	Sabouraud agar culture
Histoplasma capsulatum	Antigen test	Serology CF, Sabouraud agar culture
Coccidioides immitis		Serology ID, Sabouraud agar culture
Viruses		
Influenza virus	NAAT, rapid antigen	
Parainfluenza virus	NAAT	Serology EIA
RS virus	NAAT, rapid antigen	
Human metapneumovirus	NAAT	Serology EIA
Adenovirus	NAAT, antigen test	
Cytomegalovirus	pp65 antigen	
Parasites		
Paragonimus westermani	Microscopy for ova	Microscopy for ova or worms

Representative laboratory tools to diagnose clinically significant pathogens which cause pneumonia are listed [13]. NAAT, nucleic acid amplification test; CF, complement fixation; PPLO, pleuropneumonia-like organism; BCYE, buffered charcoal yeast extract; MIF, micro-immunofluorescence; IIF, indirect immunofluorescence; AFB, acid-fast *Bacillus*; GMS, Gomori methenamine silver; ID, immunodiffusion; EIA, enzyme immunoassay.

Table 1. Current laboratory diagnosis of pathogens causing pneumonia.

3.6. Issues with applying conventional diagnostic tools for identifying causative pathogens in respiratory samples

Although an organism requires specific agar or a unique detection assay, applying such different kinds of diagnostic tools for all patients with pneumonia is not practical and feasible from the standpoint of labor required. Moreover, the most significant issue is the culture-based method, which is a standard test for respiratory samples containing colonizing organisms or normal flora. It is not capable of discriminating a causative pathogen from isolated commensal organisms.

4. A real-time PCR for respiratory samples

4.1. Applying molecular techniques to identifying the causative pathogens in respiratory tract infection

Nucleic acid amplification test (NAAT), such as a PCR-based test or reverse transcription-PCR (RT-PCR), for purulent sputum is a logical and beneficial strategy. Firstly, if PCR cannot detect a suspected pathogen, the pathogen is less likely to be the causative pathogen due to the highly sensitive nature of PCR which can amplify even small numbers of pathogens. Secondly, PCR is capable of identifying foreign organisms that cannot grow on the standard culture agar. Thus, their detection conclusively yields the causative pathogens. Thirdly, PCR is a speedy test, and the result can be delivered to medical professionals in an early phase of the treatment. Finally, from the standpoint of NAAT, organisms causing respiratory tract infections can be simply divided into two categories; commensal organism and foreign organism (see Sections 3.2 and 3.3). Therefore, PCR does not require the pathogen-specific agar or growth conditions since all organisms are dealt with at the nucleic acid (DNA or RNA) level in the laboratory.

4.2. Issues with applying molecular diagnostic tools for identifying causative pathogens in respiratory samples

A major problem associated with a PCR-based test, similar to conventional methods, is its inability to discriminate a commensal organism causing pneumonia from the same organism colonizing in the airway.

Given the unique aspect of sputum, we assumed that, although setting a cutoff value by the direct quantification of bacterial cell number in respiratory samples would fluctuate and thus provide indistinct discrimination between causative pathogen and colonizing organism, using the relative quantification would be more stable even using the sputum which lacks homogeneity and reproductivity. To overcome this challenge, we proposed the "battlefield hypothesis," in which the ratio of pathogen to human cells in the respiratory samples would be an indicator for the dominant pathogen in the "pneumonia battlefield." The principal of this hypothesis is that the relative number of combatants (i.e., pathogens) causing the current state of pneumonia is considered a major determinant.

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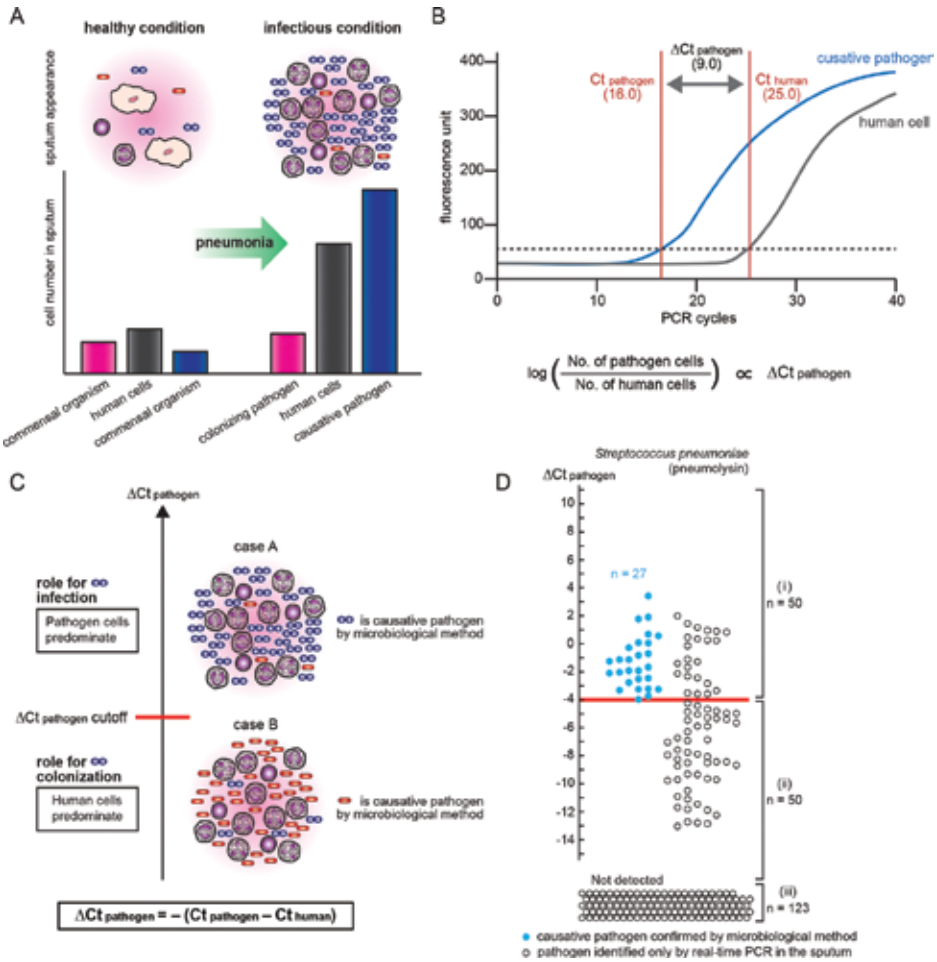

Figure 3. Battlefield hypothesis. (A) When pneumonia occurs, the number of human inflammatory cells (gray) increases at the inflammation site where that of the causative pathogen (blue) outnumbers the human cells. Meanwhile, the colonizing pathogens (red) lag behind. Thus, the ratio of causative pathogen cells to human cells was considered as a practical indicator for the discrimination of the causative pathogen from the colonizing organisms. (B) The ratio of cell numbers between two cell types in sputum is measureable by quantitative PCR. The Ct (threshold cycle) is the number of PCR cycles at which the fluorescent signal passes the threshold. Ct_{human} is the Ct for the human-specific gene, $Ct_{pathogen}$ is the Ct for the pathogen-specific gene, and both are log proportional to the number of the cells during PCR cycles, which is accordingly formulated to $\Delta Ct_{pathogen} = -(Ct_{pathogen} - Ct_{human})$. (C) Since $\Delta Ct_{pathogen}$ indicates the ratio of pathogen cells to human cells, the $\Delta Ct_{pathogen}$ cutoff can be determined. Two examples of pneumonia cases were displayed; case A is *Streptococcus pneumoniae* (*S. pneumoniae*) pneumonia (Gram-positive cocci in purple), and case B is *Haemophilus influenzae* (*H. influenzae*) pneumonia (Gram-negative bacilli in red). In case A, the $\Delta Ct_{S.\ pneumoniae}$ value above which a pathogenic role of *S. pneumoniae* in pneumonia was confirmed should show a higher value, while a $\Delta Ct_{S.\ pneumoniae}$ value would demonstrate lower value in case B in which *H. influenzae* dominated human cells and *S. pneumoniae* played a colonizing role. (D) Determination of the $\Delta Ct_{pathogen}$ cutoff in a case of *S. pneumoniae* pneumonia. $\Delta Ct_{pathogen}$ was measured for commensal organisms in a pilot study (n = 223). Respiratory samples from patients with pneumonia in which *S. pneumoniae* was identified using commensal organism criteria (1)–(3) (see Section 3.4) were illustrated as blue circles, and samples in which none of criteria (1)–(3) was met were depicted as white circles. The $\Delta Ct_{S.\ pneumoniae}$ cutoff (a red line) was determined as the smallest $\Delta Ct_{S.\ pneumoniae}$ for the blue circles. Sputum in which *S. pneumoniae* was not detected and $\Delta Ct_{S.\ pneumoniae}$ was not applied is shown at the bottom (shown as "not detected").

4.3. Battlefield hypothesis

With the battlefield hypothesis, the ratio of the cell number of a commensal organism to human cell numbers is assumed to be an index of the organism's pathogenic role. When pneumonia occurs, the number of human cells, mostly inflammatory cells, drastically climbs at the site of infection where that of causative pathogen exceeds the human cells (**Figure 3A**). On the other hand, pathogens that merely colonize the affected area do not proliferate. In a real-time PCR-based system specializing in quantification, the specific primers and probe can amplify the target sequence log proportionally, in which the ratio of pathogen to human cells is formulated as $\Delta Ct_{pathogen} = -(Ct_{pathogen} - Ct_{human})$ (**Figure 3B**). As indicated by the battlefield hypothesis, a threshold value that discriminates commensal organisms from organisms colonizing the airway would be set up as $\Delta Ct_{pathogen}$ cutoff (**Figure 3C**).

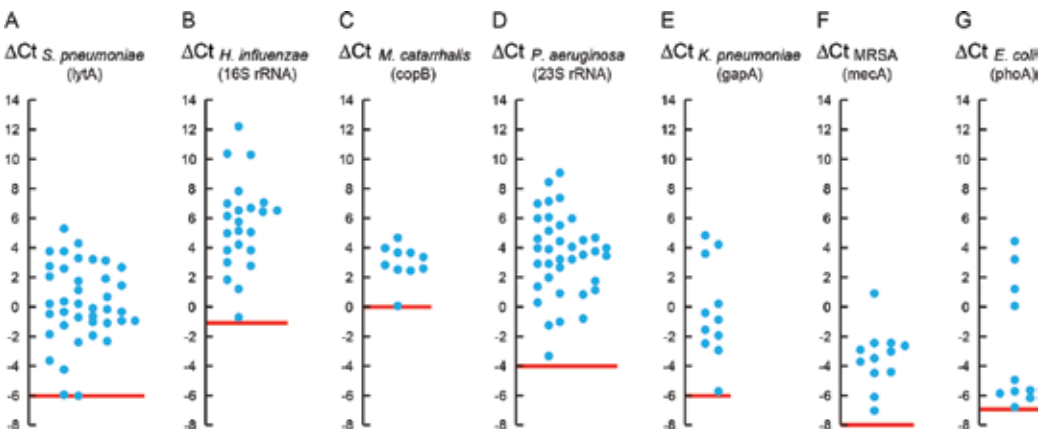

Figure 4. Determination of $\Delta Ct_{pathogen}$ cutoff. The $\Delta Ct_{pathogen}$ was measured for seven representative commensal organisms from the past clinical study (n = 533, from May 2007 to January 2009 at the Saitama Medical University Hospital and the participating institutes). Samples from patients with pneumonia in which a causative pathogen was identified using criteria (1)–(3) (see Section 3.4) are shown as blue circles. The $\Delta Ct_{pathogen}$ cutoff (a red line) was defined as the smallest $\Delta Ct_{pathogen}$ for the blue circles. The $\Delta Ct_{pathogen}$ cutoff was defined below: (A) −6 for *S. pneumoniae*, (B) −1 for *H. influenzae*, (C) 0 for *M. catarrhalis*, (D) −4 for *P. aeruginosa*, (E) −6 for *K. pneumoniae*; (F) −8 for MRSA, and (G) −7 for *E. coli*. Brackets indicate the target gene specific for each pathogen.

In order to verify the hypothesis, we first screened *S. pneumoniae* by specific real-time PCR (targeted to the pneumolysin gene) in 223 pneumonia patients (**Figure 3D**). By setting the cutoff as the smallest value of $\Delta Ct_{pathogen}$, the samples were classified into three groups: (i) samples with $\Delta Ct_{pathogen}$ > cutoff (−4), in which case the organism was considered to have a high chance of being a causative pathogen which was called a "therapeutic target"; (ii) samples with $\Delta Ct_{pathogen}$ < cutoff, in which case the detected organism had little chance of being a causative pathogen since the pathogen-to-human ratio has never been shown in a causative pathogen in pneumonia; and (iii) samples lacking organism detection, in which case the pathogen was unlikely to be a causative pathogen.

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	Gene (accession #) PCR product		Sequence (5'–3')		Gene (accession #) PCR product		Sequence (5'–3')
Control				**Foreign organisms**			
1	*Homo sapiens*			11	*Mycoplasma pneumoniae*		
	SFTPC	Fw	GCAGTGCCTACGTCTAAGCTG		16S rRNA	Fw	AGTAATACTTTAGAGGCGAACGGGTGA
	(U02948.1)	Rv	TAGATGTAGTAGAGCGGCACCTC		(NC_000912.1)	Rv	TCTACTTCTCAGCATAGCTACACGTCA
	130 bp	Dp	CGAGATGCAGGCTCAGCACCCTC		227 bp	Dp	ACCAACTAGCTGATATGGCGCA
				12	*Chlamydophila pneumoniae*		
					53KD-antigen	Fw	GCAACCACGGTAGCAACACAAATTA
					(E12535)	Rv	AATTGAGCGACGTTTTGTTGCATCT
Commensal organisms					364 bp	Dp	AGCGGCTGTCAAATCTGGAATAAAAG
2	*Streptococcus pneumoniae*			13	*Chlamydophila psittaci*		
	lytA	Fw	ACGCAATCTAGCAGATGAAGCA		ompA	Fw	GTATGTTCATGCTTAAGGCTGTTTTCAC
	(AE005672)	Rv	TCGTGCGTTTTAATTCCAGCT		(X56980.1)	Rv	TCCCACATAGTGCCATCGATTAATAAAC
	75 bp	Dp	TGCCGAAAACGCTTGATACAGGGAG		291 bp	Dp	CCAGAAGAGCAAATTAGAATAGCGAGCA
3	*Haemophilus influenzae*			14	*Coxiella burnetii*		
	16S rRNA	Fw	TTGACATCCTAAGAAGAGCTCAGAGA		Transposase	Fw	GTCTTAAGGTGGGCTGCGTG
	(Z22806.1)	Rv	CTTCCCTCTGTATACGCCATTGTAGC		(M80806)	Rv	CCCCGAATCTCATTGATCAGC
	267 bp	Dp	ATGGCTGTCGTCAGCTCGTGTT		295 bp	Dp	AGCGAACCATTGGTATCGGACGTTTATGG
4	*Moraxella catarrhalis*			15	*Legionella* spp.		
	copB	Fw	GACGGGTGAGTAATGCCTAGGA		16S rRNA	Fw	AGGCTAATCTTAAAGCGCCAGGCC
	(U69982.1)	Rv	CCACTGGTGTTCCTTCCTATATCT		(FR799709)	Rv	GCATGCTTAACACATGCAAGTCGAAC
	298 bp	Dp	AGTGGGGGATCTTCGGACCTCA		198 bp	Dp	CATATTCCTACGCGTTACTCACCCGT
5	*Pseudomonas aeruginosa*			16	*Legionella pneumophila*		
	23S rRNA	Fw	TCCAAGTTTAAGGTGGTAGGCTG		mip	Fw	TAACCGAACAGCAAATGAAAGACG
	(AJ549386)	Rv	ACCACTTCGTCATCTAAAAGACGAC		(S72442.1)	Rv	AAAACGGTACCATCAATCAGACGA
	94 bp	Dp	AGGTAAATCCGGGGTTTCAAGGCC		264 bp	Dp	TGATGGCAAAGCGTACTGCTGAA
6	*Klebsiella pneumonia*			17	*Bordetella pertussis*		
	gapA	Fw	TGAAGTATGACTCCACTCACGGT		BP485	Fw	CGAGCCACTGTTTCTATTGATTGA
	(M66869)	Rv	CTTCAGAAGCGGCTTTGATGGCTT		(BX640412)	Rv	CGGGCCTCATCTTCGTTCAG
	670 bp	Dp	CCGGTATCTTCCTGACCGACGA		118 bp	Dp	TGTGCGTGTTTTCCCCAGAGCCCC

	Gene (accession #) PCR product		Sequence (5'–3')		Gene (accession #) PCR product		Sequence (5'–3')
7	*Staphylococcus aureus*			18	*Mycobacterium tuberculosis*		
	femB	Fw	TGGCCACTATGAGTTAAAGCTTGC		MPB64	Fw	ATCCGCTGCCAGTCGTCTTCC
	(DQ352467)	Rv	TCATAATCAATCACTGGACCGCGA		(NC_000962)	Rv	CTCGCGAGTCTAGGCCAGCAT
	162 bp	Dp	CGAGGTCATTGCAGCTTGCTTACTTA		238 bp	Dp	CCGGACAACAGGTATCGATAGCGCC
8	*Escherichia coli*			19	*Mycobacterium intracellulare*		
	phoA	Fw	CGAAGAGGATTCACAAGAACATACC		ITS 16-23S rRNA	Fw	AGCACCACGAAAAGCACTCCAATT
	(M29670)	Rv	GGTCTGGTCGGTCAGTCCAA		(AM709724)	Rv	CGAACGCATCAGCCCTAAGGACTA
	94 bp	Dp	CGGGCCATACGCCGCAATACGCA		243 bp	Dp	CCTGAGACAACACTCGGTCGATCC
				20	*Mycobacterium avium*		
					16S rRNA	Fw	CAAGTCGAACGGAAAGGCCTCT
					(M29572)	Rv	GCCGTATCTCAGTCCCAGTGTG
Drug resistance-related genes					257 bp	Dp	TACCGGATAGGACCTCAAGACGC
9	Metallo-beta-lactamase			21	*Mycobacterium kansasii*		
	IMP	Fw	GGCAGYATTTCCTCTCATTTTCATAGC		dnaJ	Fw	ACCCGTGTGATGAGTGCAAAGGC
	(AY625689)	Rv	AATTTGTRGCTTGAACCTTACCGTCTT		(AB292544.1)	Rv	GTAAAGCTGACCGGAACTGTGACG
	134 bp	Dp	ATTCTCGATCTATCCCCACGTATGCA		231 bp	Dp	AGGACGGACAGCGGATCAGACT
10	Methicillin-resistant *S. aureus*			22	*Pneumocystis jiroveci*		
	mecA	Fw	AACTACGGTAACATTGATCGCAAC		5S rRNA	Fw	GTGTACGTTGCAAAGTACTCAGAAGA
	(AY786579)	Rv	CTTTGGTCTTTCTGCATTCCTGGA		(AF461782)	Rv	GATGGCTGTTTCCAAGCCCA
	112 bp	Dp	AGATGGTATGTGGAAGTTAGATTGGGA		346 bp	Dp	CTAGGATATAGCTGGTTTTCTGCGAA
				23	*Nocardia* spp.		
					16S rRNA	Fw	CCTTCGGGTTGTAAACCTCTTTCGAC
					(DQ659898)	Rv	TTGGGGTTGAGCCCCAAGTTTTCA
					191 bp	Dp	AAGAAGCACCGGCCAACTACGTGC

A database search was used to select potential DNA sequences to identify microorganisms of interest. The primers and probes were validated using genomic DNA from the microorganism of interest as well as screened against 74 other microorganisms [12]. Fw, forward primer; Rv, reverse primer; Dp, detection probe (TaqMan).

Table 2. Primers and probes for the real-time PCR.

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4.4. Determination of the $\Delta Ct_{pathogen}$ cutoff

We then confirmed that the primers and probes were specific to seven representative commensal organisms (*S. pneumoniae*, *H. influenzae*, *Moraxella catarrhalis* (*M. catarrhalis*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Klebsiella pneumoniae* (*K. pneumoniae*), Methicillin-resistant *Staphylococcus aureus* (MRSA), and *Escherichia coli* (*E. coli*)) and the human genome and were able to quantify their cell numbers by counting the copy numbers (Ct value) for each in sputum. The pathogen-to-human cell number ratio was measured using 533 sputum samples collected in the past clinical study between May 2007 and January 2009 (**Figure 4**). Based on the microbiological examination and its criteria (see Section 3.4), the $\Delta Ct_{pathogen}$ cutoff to discriminate the causative pathogens was determined by the smallest $\Delta Ct_{pathogen}$ value in seven organisms (**Figure 4**).

4.5. HIRA-TAN system

Accordingly, we devised a PCR-based test for sputum samples that can distinguish causative pathogens from detected commensal organisms. Moreover, combining the described PCR system for "commensal organisms" with a PCR detection system for "foreign organisms" constitutes the HIRA-TAN (human cell-controlled identification of the respiratory agent from "TAN," which means sputum in Japanese), which involved 23 PCR with organism-specific target genes for quantifying 7 commensal organisms, 13 foreign organisms, 2 drug resistance-related genes (DRRG), and the human specific gene (as the internal control) (**Table 2**). HIRA-TAN was capable of screening 23 target genes simultaneously and diagnosing the therapeutic targets among commensal and foreign organisms in a single assay, which was able to be completed within 4 h. The technical details in the real-time PCR and the HIRA-TAN system were discussed in more detail in Refs. [12, 14].

4.6. Criteria for detected organisms to be the therapeutic target by HIRA-TAN system

In the HIRA-TAN system, the cutoff value was determined for each commensal organism, which enabled us to discriminate the *bona fide* therapeutic target from other commensal organisms detected by real-time PCR,. with the assumption being that the high relative levels of any of the given candidate pathogens would increase its likelihood of being the causative pathogen. Thus, commensal organisms were diagnosed as the "therapeutic target" when $Ct_{pathogen}$ exceeded the given cutoff value. Foreign organisms were diagnosed as the "therapeutic target" when detected by real-time PCR.

Technically, since opportunistic organisms, such as *Pneumocystis jiroveci* and *Nocardia* spp., were not normally identified from healthy individuals, they were classified as foreign organisms. Methicillin-resistant *S. aureus* (MRSA) was judged as therapeutic target in HIRA-TAN both when *S. aureus* (femB gene) was detected above −7 in the $\Delta Ct_{S.\ aureus}$ cutoff and when DRRG (mecA gene) was also detected above −8 in the ΔCt_{MRSA} cutoff altogether. IMP-producing *P. aeruginosa* (multidrug resistance *P. aeruginosa*, MDRP) was judged as therapeutic target in HIRA-TAN both when *P. aeruginosa* was detected above −4 in the $\Delta Ct_{P.\ aeruginosa}$ cutoff and IMP gene was detected altogether.

5. Materials and methods

5.1. Respiratory specimen

In Section 5, samples and their treatment will be described. Sputum, induced sputum, or sputum obtained by intratracheal aspiration (sputum hereafter) was collected from patients with pneumonia. The sample was homogenized by pipetting and dispensed into two tubes; one was submitted for a standard microbiological test (microscopic examination and culture) and the other for nucleic acid extraction and real-time PCR analysis. To assess the pathogenic role with an appropriate sample, sputum with M2–P3 macroscopic appearance and a Ct_{human} <27 (the human-specific gene with Ct (threshold cycle) value by the real-time PCR) were studied [15]. Classification of the gross appearance of the sputum (M1, M2, P1, P2, and P3) was according to Miller and Jones [16].

5.2. DNA preparation from sputum

There are several kit options available for DNA extraction depending on the sample type; however, only column-based extraction has been used for most respiratory specimens due to their viscosity [14]. The sample was diluted with an equal volume of phosphate-buffered saline (PBS) and homogenized by vortexing. 200 μL of the homogenate was mixed with 200 μL AL buffer (Qiagen, Tokyo, Japan) containing 20 μL proteinase K (Takara Bio Inc., Shiga, Japan), and the resultant mixture was incubated at 56°C for 1 h. The DNA was extracted with 100 μL TE buffer using QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan). The DNA concentration, based on the absorbance, was determined in a spectrophotometer GeneQuant Pro (GE Healthcare, Tokyo, Japan). The ratio of nucleic acid to protein absorbance (260 nm/280 nm) was calculated as an index of the purity of DNA samples [14].

5.3. Real-time PCR

Although a variety of PCR methodologies and devices are available, an illustration of the details of our approach is given. The final solution of the PCR contained 12.5 μL of the Takara Premix Ex Taq (Takara Bio Inc., Shiga, Japan), 300 nM of each primer, 100–300 nM of the fluorescence-labeled TaqMan probe, 1.0 μL of purified DNA, and deionized distilled water up to 25.0 μL. The PCR for 23 target genes was multiplexed in 16 reactions and amplified using in a single assay. The PCR was performed by starting at 95°C for 30 s followed by 40 cycles at 95°C for 8 s, 61°C for 25 s, and 72°C for 20 s using the SmartCycler II (Cepheid, Sunnyvale, CA). The sequences of primer and probe were described in **Table 2**.

6. Practical application of HIRA-TAN system

6.1. Prospective study

We designed a prospective study to investigate the validity of the cutoff values we set up for the commensal organisms in the HIRA-TAN system. The aim of the study was the proportion

of samples in which $\Delta Ct_{pathogen}$ was greater than the cutoff value (diagnosed as the therapeutic target by HIRA-TAN), compared to the proportion of the samples in which each commensal organism was shown to be the causative pathogen (diagnosed as the causative pathogen by microbiological methods (**Table 1**)). The study was performed between February 06, 2009, and October 14, 2010, at the Saitama Medical University Hospital and other six participating institutes. Five-hundred and sixty eight patients with pneumonia were enrolled, and the results of the microbiological examinations were summarized in Ref. [12] and **Figure 2**. The identification rates for *S. pneumonia*, *H. influenzae*, *M. catarrhalis*, *P. aeruginosa*, *K. pneumoniae*, MRSA, and *E. coli* by the HIRA-TAN were 91.6% (87/95; 95% CI 84.1–95.6%), 96.7% (29/30; 95% CI 82.8–99.2%), 90.9% (10/11; 95% CI 58.7–97.7%), 93.2% (41/44; 95% CI 81.3–97.5%), 80.6% (29/36; 95% CI 64.0–89.9%), 78.1% (25/32; 95% CI 60.0–88.5%) and 87.5% (14/16; 95% CI 61.6–96.0%), respectively (**Figure 5A–G**). The response of the antibiotics and the relief of pneumonia for the cases in which such commensal organisms were the causative pathogens showed a consistent clinical course as predicted from the results of the HIRA-TAN.

6.2. Overall identification capacity of therapeutic targets by real-time PCR-based test

Overall performance of the HIRA-TAN system to identify both the therapeutic targets (commensal organisms judged by the ΔCt cutoff and foreign organisms detected by the real-time PCR) was altogether 60–70% en masse, which was comparable to what was attained by an extensive search using multiple detection methods [10, 18, 19]. However, it is supposed to reach its limit to identify the causative pathogens using primers and probe of only bacteria, and for a thorough investigation, incorporating PCR systems for viruses, anaerobes, and fungus will be required.

6.3. Beneficial aspects of PCR-based system for identifying pathogen

The most prominent feature of the HIRA-TAN is its ability to identify the causative pathogen for the pneumonias from among the commensal organisms detected in the sputum. Clinically, without this ability, this system would have been only partially useful, since more than half of pneumonias are caused by commensal organisms (**Figure 2**). And this system does not require the use of pathogen-by-pathogen identification methods (unique agar or a specific antibody for an organism). The easily performed comprehensive test covers a wide variety of pathogens in a single assay, which will reduce the time and labor spent on cumbersome procedures. The HIRA-TAN procedure now provides a comprehensive detection system for causative pathogens of pneumonia.

The HIRA-TAN system can also be expanded to include more pathogens, thereby increasing its abilities. The addition of any respiratory viruses or particular fungi to the screening protocol is straightforward [6, 9–11, 20]. Likewise, the inclusion of other commensal organisms, such as *Acinetobacter baumannii*, *Burkholderia cepacia*, or *Stenotrophomonas maltophilia*, will also be beneficial in nosocomial pneumonia. In addition, the most challenging category is anaerobes. Since investigating the anaerobe's contribution to respiratory tract infection by molecular techniques has not been attempted, indicating that setting a cutoff value for anaerobes with HIRA-TAN system will be beneficial. Thus, the commensal organisms that have conformed to

the scheme shown in **Figure 3A–D** and all non-commensal organisms become candidates, which will make the greater detection rate by the HIRA-TAN than the current 60–70% performance.

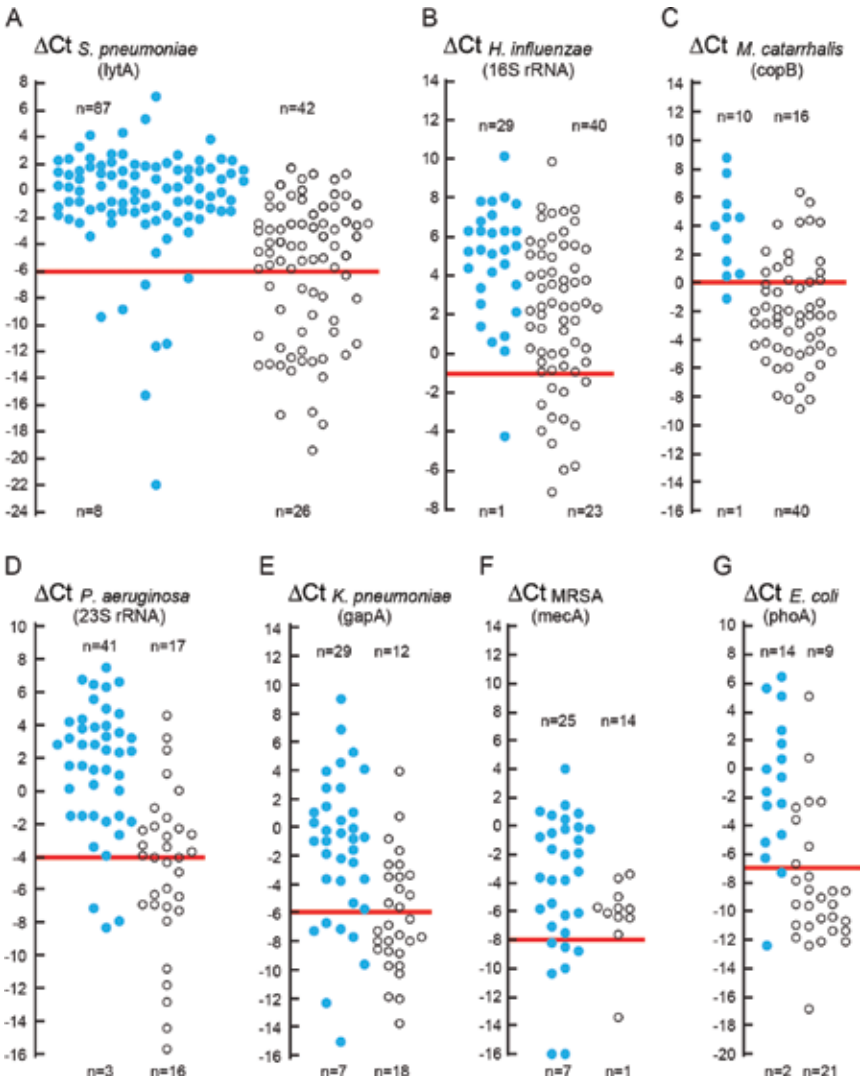

Figure 5. Evaluating $\Delta Ct_{pathogen}$ for each commensal organism in the prospective study (n = 568). Samples from patients with pneumonia in which a causative pathogen was identified using criteria (1)–(3) (see Section 3.4) are shown as blue circles, and samples from patients with pneumonia in which none of criteria (1)–(3) was met were shown as white circles. The HIRA-TAN identified the causative pathogens: (A) 91.6% (87/95; 95% CI 84.1–95.6%) for *S. pneumoniae*, (B) 96.7% (29/30; 95% CI 82.8–99.2%) for *H. influenzae*, (C) 90.9% (10/11; 95% CI 58.7–97.7%) for *M. catarrhalis*, (D) 93.2% (41/44; 95% CI 81.3–97.5%) for *P. aeruginosa*, (E) 80.6% (29/36; 95% CI 64.0–89.9%) for *K. pneumoniae*, (F) 78.1% (25/32; 95% CI 60.0–88.5%) for MRSA, and (G) 87.5% (14/16; 95% CI 61.6–96.0%) for *E. coli*. Brackets indicate the target gene specific for each pathogen. The 95% confidence interval (CI) was calculated using the formula for the binomial probabilities by the software Mathematica Ver.8TM (Wolfram Research, IL, USA) [17].

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6.4. Drawback of newly emerged PCR system

Currently the ability of the HIRA-TAN system to determine if MRSA is the causative pathogen is lacking. This is largely due to the fact that the determination and establishment of the cutoff value for a given microorganism in the HIRA-TAN system still require the conventional sputum examination. To date we have not been able to determine the ΔCt_{MRSA} cutoff, and this will take more time and the availability of properly analyzed clinical samples to be established. Likewise, the diagnosis of *Pneumocystis jiroveci* pneumonia is hardly confirmed since the definitive diagnosis for this organism by conventional method has not been established.

7. Conclusion

In this chapter, the principle and utility of a real-time PCR-based diagnostic test for the causative pathogen in respiratory samples was described. Although rapid and accurate identification of pathogens and corresponding treatment based on the microbiological results are required in the healthcare setting, the current clinical tests lacking high sensitivity and a comprehensive approach have not been able to work these issues out. Development of molecular techniques and their usefulness enables the detection of organisms from the clinical specimens speedily as well as precisely and aids the settlement of such issues. With our novel approach that employs the relative quantification, we successfully set up the cutoff value to differentiate the causative pathogen from colonizing commensal organisms by PCR, with which a real-time PCR-based diagnostic system was devised and validated through clinical sample testing. Although this may be only one instance among many comprehensive systems, innovating such systems will help patients struggling with these disorders in the future.

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Chapter 8

PCR Technique for the Microbial Analysis of Inanimate Hospital Environment

Urška Rozman and Sonja Šostar Turk

Additional information is available at the end of the chapter

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Abstract

Discipline of molecular ecology and molecular techniques such as polymerase chain reaction (PCR) offers a possibility to study and reveal the microbial diversity in environmental settings with complicated mixed communities, non-culturable organisms, interfering contaminants and low levels of target DNA. Hospital environment represents a new ecological niche for clinically important nosocomial pathogens and antibiotic-resistant microorganisms, which have been commonly found on various hospital surfaces. Accurate characterization of microbial communities depends on several factors, starting with sample collection and conditional enrichment step. In the step of nucleic acid isolation and purification, the DNA, as a dominant signature molecule, is extracted followed by removing co-extracted impurities. PCR target sequences are often 16S rDNA gene, functional gene probes or species-specific probes, depending on the objective of the study. Furthermore, properly prepared PCR amplicons can serve as a basis for characterizing microbial community. The PCR technique is a powerful tool for the analysis of microbial diversity of environmental ecosystems. In a hospital environment, advantages of detecting pathogens and antibiotic-resistant bacteria need to be pointed out.

Keywords: microorganisms, hospital environment, inanimate surfaces, DNA extraction, PCR

1. Introduction

Characteristics of the hospital environments are very specific where inanimate environment can be colonized with a wide range of microorganisms [1, 2]. The cultured microorganisms represent only a small fraction of natural microbial communities, hence the microbial diversity

in terms of species richness and species abundance is grossly underestimated [3]. Therefore, the discipline of molecular ecology and molecular techniques such as polymerase chain reaction (PCR) offers a possibility to study and reveal the real-microbial population complexity and a possibility to overcome limitations of culture-based approaches. Due to the power of the PCR to amplify small amounts of DNA, organisms occurring in small numbers in an environment are now detectable [3]. Special challenges in environmental settings are complicated mixed communities, interfering contaminants and low levels of target DNA [4]. The specifics of environmental samples are low to medium concentration of target cells, low sample homogeneity and high degree of PCR inhibition [5].

Many types of pathogenic microorganisms have been found on various common hospital surfaces. Most common nosocomial bacteria present and detected on inanimate hospital surfaces, using specific marker genes, are presented in **Table 1**.

Microorganism	Locality	Marker gene
Clostridium difficile	Bed, sink, toilet, wall, rails, call button, stretcher [6–9]	tcd_D, tcd_E, tcd_C, cdu_2, cdd_3 [10]
Klebsiella pneumoniae	Bed frame, over-bed table, bedcovers, drains, sinks, shower, drug trolley, blood pressure cuff, laryngoscope, stethoscope [11–13]	bla_{TEM}, $bla_{CTX\text{-}M}$ [14] bla_{SHV} [15] bla_{KPC} [16]
Staphylococcus aureus	Air, bed, mattress cover, bathroom floor, bed linen, chairs, table, floor, shower, drug trolley, blood pressure cuff, laryngoscope, stethoscope [13, 17–20]	*spa* [21] fem_A [22, 23]
Acinetobacter baumannii	Bed rails, sinks, tables, curtains, door handles [24]	$bla_{OXA\text{-}23}$, $bla_{NDM\text{-}1}$ [25] omp_A [26] bla_{OXA}, bla_{VIM}, bla_{MBL} [27]
Pseudomonas aeruginosa	Bed, tables, ward sinks and surgical equipment, shower, drug trolley, blood pressure cuff, laryngoscope, stethoscope, water [13, 28]	las_A [29]
Escherichia coli	Bed, shower, drug trolley, blood pressure cuff, laryngoscope, stethoscope, water [13]	bla_{TEM}, $bla_{CTX\text{-}M}$ [14] bla_{SHV} [15] bla_{KPC} [16]
Legionella pneumophila	Drinking water [30]	*mip* [30]
Vancomycin resistant enterococci [VRE]	General areas in patients' rooms and toilets, light switch [31]	van_A [31]
ESBL Enterobacteriaceae	Mechanical ventilator, showers, beds, wall, sinks, toilet, hospital room [32, 33]	bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{CMY}, bla_{IMP}, bla_{VIM} [32, 33]

Table 1. Most common nosocomial bacteria present and detected on inanimate hospital surfaces.

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Hospital environment also represents a new ecological niche for clinically important antibiotic-resistant microorganisms. Along with identification through the amplification of conserved genomic sequences, PCR can also be used to detect antimicrobial resistance or virulence genes [34].

In molecular approaches, for studying microbial population complexity, DNA is the dominant signature molecule that phylogenetically dissects microbial communities and substantially increases our insight into microbial diversity. This method does not provide a clear distinction between viable and non-viable organisms, so may not be an accurate reflection of the microbial load present on a surface [34]. On the other hand, accurate determination of the total bacterial load is important in many microbiological applications but cannot be obtained with traditional bacterial cultivation methods. These classical incubation methods based on phenotypic detection of microorganisms are also time-consuming and can work poorly with slow growing or viable but non-culturable (VBNC) organisms [35]. The PCR method clearly has potential in environmental studies where there may be low numbers of viable but non-culturable microorganisms [34] that can still be active cells and can maintain their infectivity in the case of pathogenic bacteria [36–38].

2. Sample collection

Accurate characterization of microbial communities depends upon several factors, starting with the sample collection step that is often ignored as a source of problem [3] in afterwards analysis. Three major obstacles are the sample volume, sample site accessibility and sample transport.

Sampling sites in hospitals can be divided in two large groups:

- Inanimate surfaces (stethoscope, hospital textiles, beds, sinks…)
- Hospital water (drinking water, waste water)

For each of the mentioned group, the appropriate sampling method is required.

2.1. Swabbing and elution

The swab-rinse sampling technique was first described in 1916 by Manheimer and Ybanez [39]. Current recommendations for surface sampling suggest the use of a pre-moistened flocked nylon swab [40], followed by a dry flocked nylon swab for soaking up any remaining liquids. Ekrami et al. [13] used cotton tipped sterile swabs moistened in sterile brain-heart infusion broth. Swabs for environmental surface sampling have been used in numerous studies, and they allow the recovery of microorganisms from hard-to-reach surfaces such as behind taps [41], drains [6] and bed rails [34, 42]. The swab can be transferred into eluting solution (e.g. 0.9% NaCl with 0.2% Tween 80) [43, 44] and vortexed to obtain the substrate for DNA isolation.

2.2. Contact plate sampling

For direct surface sampling, RODAC (replicate organism detection and counting) plate is a common choice [45]. Small Petri dishes are filled in order to provide a convex surface, optionally with a nutrient or selective growth medium. The plate is then pressed onto any flat surface and incubated. The resulting colony count can be expressed as cfu/area [46]. The efficiency of this method depends on the evenness of the surface tested [47]; therefore, Rabuza et al. [48] concluded that the RODAC plate method used for sampling microorganism on textiles has certain limitations due to the rough, uneven three-dimensional (3D) fabric surface. Regarding the use of molecular techniques, each selected colony of interest can be used for DNA isolation and subsequent PCR analysis.

2.3. Elution

When sampling hospital textiles, as a part of inanimate environment that can contribute to the transmission of healthcare associated infections, the elution method is the most effective. This method is based on the principle of eluting microorganisms from textiles; therefore, microorganisms that have penetrated into the deeper structure of the 3D structure of the textile material are also collected. Microorganisms trapped in the 3D structure of the material will not be detected by the RODAC plate method, neither by swabbing, but they can be captured by elution, either destructive or non-destructive [48].

2.4. Water samples filtration and concentration

Since a fundamental limiting factor in the assessment of microbial quality of waters, and especially drinking water, is often the very low number of each organism present, most samples needs to be concentrated, usually by sterile filtration. Bacteria are generally recovered on membrane filters with porosities of 0.22–0.45 μm. Subsequently, membrane filters may be incubated on the solid media or soaked in the liquid media [49].

2.5. Enrichment in growth medium

Generally, the purpose of environmental monitoring is to establish an aerobic colony count of bacteria from a surface, which can be processed without enrichment. This provides a direct enumeration of the level of microbial contamination of a surface [34]. On the other hand, enrichment steps can be used to increase detection in the case of identifying specific multi-resistant or virulent pathogens, which may be the cause of an outbreak. In this case, the number of organisms is not strictly required, but rather their presence or absence [34]. Typically, the number of pathogenic microorganisms is low [50] and their recovery is low because they are under stressed conditions. Therefore, the chances of detecting pathogenic bacteria will be greater by using an enrichment step. Usually enrichment step is also used prior to detection by PCR. Special attention must be paid to false positive results, since PCR does not discriminate between viable and non-viable organisms [49]. Again, disadvantages of DNA-based methods may be partly overcome by including a pre-enrichment step that allows organisms to multiply before gene probe tests are applied [51].

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3. Nucleic acid isolation and purification

When collecting hospital environmental samples by methods described above, protocols for DNA isolation from water samples or pure cultures are usually successfully applied [52], but to date none have been accepted as a standard procedure [4]. The extracted DNA is a mixture of DNA referred as a community DNA, which is ideally a representative of all populations within the sample community; however, in reality, the extraction efficiency of different types of microorganisms can vary widely [52].

DNA/RNA isolation and purification must be achieved through methods efficient enough for releasing DNA from the cells; too rigorous conditions could lead to highly fragmented nucleic acids [3]. DNA or RNA, which is not released from the cells, will not contribute to the final analysis of diversity. RNA extraction is a special case because of the possible RNases effect in the procedures [3] and also short half-life of mRNA [52]. Since DNA is mostly found in low concentrations and DNA solution also contains high concentration of saline, this is of concern in later disturbance of PCR. The most popular application for both cases is simple alcohol precipitation, where many varieties and also commercially available kits exist [53].

To remove co-extracted impurities one or more purification steps are often necessary. However, the number of purifications performed should be minimized as much as possible since large portions of the extracted DNA may be lost during the purification process [52].

4. PCR target sequence

Depending upon the objective of the study, gene probes to target various genes can be designed. Generally, the gene probes can be divided into the phylogenetic probes, to obtain information about taxonomy and phylogeny of microorganism; functional gene probes, to search for the unique activity of the microbial community; species-specific probes.

4.1. Phylogenetic probes

PCR target sequence is often 16S rDNA gene due to the presence of variable and conserved regions used as a phylogenetic marker [54]. Full length 16S rDNA gene can be amplified either directly or after reverse transcription of rRNA with a set of primers binding to conserved regions of the 16S rRNA/rDNA [3]. On the other side, 16S rDNA gene often fails to discriminate between species and strains level; therefore, the 23S rRNA gene and the ITS regions are also well employed [4].

Clifford et al. [55] described a set of 16S rRNA real-time PCR primers, designed to have the same optimal annealing temperature, and displaying high specificity for four clinically important pathogens (e.g. *Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*).

The amplification of 16S rDNA gene can also be useful for initial estimation or pre-screening of the microbial diversity by denaturing gel electrophoresis of amplified 16S rDNA products [3].

4.2. Functional gene probes

The target gene may code the production of a unique enzyme, where the positive gene probe indicates that the environmental sample contains the genetic potential for that particular activity [52]. In hospital environmental samples, this special activity usually refers to the antibiotic resistance. Szczepanowski et al. [56] designed and synthesized 192 resistance-gene-specific PCR primer pairs to detect plasmid-borne antibiotic-resistance genes in wastewater treatment plant bacteria. Perreten et al. [57] described rapid and efficient screening of Gram-positive bacteria for the presence of up to 90 of the most prevalent and transferable antibiotic resistance genes using microarray technology.

4.3. Species-specific probes

Primers can also be species specific, as for detecting environmental pathogens. The target sequences that are unique to a particular microbial species allow screening of an environmental sample for the presence of that specific microorganism [52]. There is a vast majority of literature describing species-specific primers for pathogen microorganisms usually find in the hospital environment and on top of that, primers described for identification of microorganisms in clinical samples are working equally well.

4.4. mRNA

Messenger mRNA is turned over rapidly in living bacterial cells, with very short half-lives inside the cell [58, 59] and has therefore been proposed as marker for cell viability [51]. mRNA is also desirable to target when detecting those microbial cells that are in a viable but non-culturable (VBNC), i.e. a dormant state in the environment, since live cells are considered those capable of cell division, metabolism (respiration) or gene transcription (mRNA production). There are several reports on the existence of many microorganisms, including human pathogens, in the environment in a VBNC state that are shown to be potentially infectious when suitable conditions prevail [36, 60–62].

5. Inhibition of PCR amplification

Materials co-extracted with nucleic acids strongly inhibit DNA modifying. Sometimes dilution of the DNA template could be useful, but very low DNA concentrations may influence the PCR efficiency. Therefore, it is desirable to avoid the effects of PCR inhibitors in the amplification reactions. Although the methods described in chapter 'Nucleic acid isolation and purification' can remove the majority of the environmental contaminants and are useful for various molecular biological studies; there is no standard protocol for removing all possible

inhibitors that can be applied for all types of environmental samples [60]. In addition, various biotic and abiotic components of environment, such as blood, urine, feces, tissue, skin, bleach and detergent, can act as the source of PCR inhibitor [63–65], affect lysis efficiency and may interfere with subsequent DNA purification and enzymatic steps [3].

Finally, it should be noted that community DNA extracts may also contain non-microbial DNA, usually origination from humans (patients, hospital workers and visitors) [52] and co-extracted non-target DNA can also inhibit the PCR [66, 67].

6. PCR-based microbial complexity analyses

Properly prepared PCR amplicons can serve as a basis for further analysis of microbial populations.

Culture-independent methods based on amplification and sequencing of 16S rRNA genes allow identification of thousands of different bacteria in a single sample [68–70] when combined with high-throughput DNA sequencing, and hundreds of samples can be multiplexed simultaneously. Therefore, 16S rRNA gene has become a mainstay for characterizing microbial community structure [70, 71]. Hewitt et al. [72] used culture-independent next-generation sequencing to survey bacterial diversity in neonatal intensive care unit surfaces with amplification of the bacterial small subunit [16S] ribosomal RNA gene sequence using 'universal' barcoded primers. They found averaging approximately 100 bacterial genera per surface containing many known opportunistic pathogens, as well as abundant groups whose pathogenic potential and ability to resist antibiotic treatment are poorly understood [72].

Oberauner et al. [73] used the 16S rRNA pyrosequencing approach to study the intensive care units (ICU) environmental microbiome. The phylogenetic spectrum combined species associated with the outside environment, taxa closely related to potential human pathogens and beneficials as well as included 7 phyla and 76 genera [73]. A similar methodology was obtained by Poza et al. [74], who amplified a hypervariable region of the bacterial 16S rRNA gene to explore the bacterial diversity at inanimate surfaces of the ICU wards. Detected microbiota contained a total of 3000 operational taxonomic units. The identified representatives were 16 canonical bacterial phyla, members of the phyla Firmicutes (mainly *Staphylococcus* and *Streptococcus*) and Actinobacteria (mainly *Micrococcaceae, Corynebacteriaceae* and *Brevibacteriaceae*), the phylum Proteobacteria (mainly by members of the families Enterobacteriaceae, Methylobacteriaceae and Sphingomonadaceae), the phyla Proteobacteria, Bacteroidetes, Deinococcus-Thermus and Cyanobacteria, Proteobacteria (mainly due to the high abundance of Enterobacteriaceae members) [74]. 16 S rDNA PCR and sequencing was also employed in study of Xu et al. [75], where 53 isolates from environmental water-associated sites in a haematology unit, and the outer surfaces of cleaning lotion containers sited throughout a tertiary referral hospital were investigated. Sequence analysis was able to identify 51 isolates, mostly Gram-positive bacteria. Nine different genera were identified from the haematology unit and 13 from the cleaning lotion containers [75].

For the estimation of the total microbial population, purified PCR 16S rRNA amplicons can be separated by denaturing high-performance liquid chromatography (DHPLC), with afterward sequencing of chosen fractions (outstanding peaks on DHPLC chromatograms). Described methodology was used by Rozman for estimation of the total microbial population complexity on hospital textiles, where 63 bacterial genera/species were identified, *Acinetobacter* spp., *Corynebacterium* spp., *Staphylococcus* spp., *Sphingomonas mucosissima* and *Stenotrophomonas maltophilia* being mostly abundant [76].

7. Conclusion

DNA molecules can survive in the environment for long periods of time [49] therefore the PCR technique is a powerful tool for the analysis of microbial diversity of environmental ecosystems that can solve many questions in the area of microbial ecology and microbial community structure. Offered advantages of detecting pathogens and antibiotic-resistant bacteria or resistant genes in environmental samples need to be pointed out. PCR has been available for three decades and has become a gold standard to use in microbiology; moreover, several modified PCR assays are the essential tool for use in environmental microbiology.

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Chapter 9

PCR: A Powerful Method in Food Safety Field

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Additional information is available at the end of the chapter

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Abstract

In this chapter, application of the polymerase chain reaction (PCR) technique in food safety, considering all the branches of this concept, is presented. The area of interest contains important analysis for both human health and the identification of food adulteration. PCR techniques used for detection of genetically modified organisms (GMO) in different matrices, identification of different animal species in meat and dairy products, as well as the detection of food infection with food-borne pathogens and toxicogenic fungi are described. The working methods and result analysis are exemplified, starting with DNA isolation adjusted to different matrices, detection of target genes, and validation for all of these methods. Techniques of simplex PCR, primer multiplexing, primer design, validation of the laboratory methods, optimization of the PCR results, and result interpretation through the analysis of the electrophoresis gels and sequencing data are studied. At the same time, the obtained results, the obstacles encountered, and how they were overcome could be an example for specific analysis developed with less resources and also for adapting the existent validated methods to the new laboratory conditions. The practical applicability and the consumer's demands are of great importance and always must be considered in developing and validating those methods.

Keywords: PCR application, food safety, method validation, species identification, GMO detection

1. Introduction

Food safety concept has been emerged in the last decades as a scientific discipline concerning the handling, processing, storage, and packaging of food in order to prevent food-borne illness. This concept has been developed and extended arriving to include practice considerations of food hygiene, labeling, additives, and other exogenous chemical residues but also

the biotechnology products. The primary categories of food-borne pathogenic organisms are fungi, bacteria, viruses, and parasites. Intrinsic food components include nutritional factors and thousands of contaminant compounds naturally present in foods. Hazardous chemicals in foods include naturally occurring toxicants, agro-industrial contaminants, and food additives. Naturally occurring toxicants are chemicals from the natural environment that occur in foods and animal feeds, such as mycotoxins, aquatic biotoxins and phytoalexins, intrinsic components of plants, bacterial toxins, cyanobacterial toxins, and food decomposition components. Worldwide, if the system of safety risk management is not fully functional, food contaminated with pathogens or their toxins could be sent to the consumers. Besides, because of a low economic efficiency, the producers could appeal to food adulteration. Whatever problem occurred, the specialists in food control have to be prepared to apply the most appropriate analysis methods. The huge progress registered in the last period in the area of nucleic acid research determined the development of analysis methods with direct applicability for different practically aims. The multitude of resources used in the DNA analysis is available all over the world leading to the development and improvement of laboratory techniques [1]. The common food markets, the regulations in this area, and the increased demanding of the consumers determine the manufacturers to send to consume products appreciated due to their organoleptic qualities, origin and composition, and last but not least their safety. Illnesses associated with foods are not very abundant. Nevertheless, when they occur, the adverse effect on human society and the food supply availability has been proved to be significant. Therefore, studies developed in research laboratories play an important role in identifying the sources of food-borne health risks, in developing procedures and products that reduce the effects of health and economic hazards. These studies must provide the assurance of a safe, wholesome food supply. The way of choosing a food may reflect aspects of lifestyle (vegetarianism and a diet based on organic food), religion (the absence of pork in some diets), diet, and health issues (e.g., the presence of the allergens). In addition, accurate labeling is important to support fair trade. Supplementary information can be added on a descriptive label as a consequence of branding and of regulations for the marketing of products. While regulations enshrined in national and international law are the underlying of mandatory label information, unfortunately, those regulations are not sufficient to prevent food fraud. Most often manufacturers choose to mislead the consumer by adding or substituting ingredients in food with cheaper ones for a higher economic gain [2] which leads to an impaired overall quality of the food. To prevent these frauds, there are a multitude of options available to identify species whose products constitute ingredients in food such as meat or dairy products. First, there are physical identification performed by monitoring the labeling and the microscopic analysis and the identification of lipid, volatile organic compounds and proteins, and nucleic acid analysis [3]. Initial methods for identifying food composition were based on morphological characteristics such as flavor, color, shape, or taste [4]. European countries are still using microscopic methods for detecting animal or plant material in food and feed which in most cases is limited [3]. The authenticity of products of animal origin and traceability issues are increasingly important in modern society, as can be inferred, for example, from the relative recent events regarding adulteration of meat products, with species that are not declared, such as horsemeat [5], adulteration of products of sea food

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type, the deliberate introduction of animal proteins in feed ingredients, or fraudulent mislabeled or label unlisted foods are a serious problem affecting the end consumer in many ways . Today, many consumers are concerned about animal products they consume, and accurate labeling is important for consumer information in the choices they make.

2. Design of a qualitative PCR investigation

A qualitative polymerase chain reaction (PCR) investigation has as starting points the materials/matrices which will be analyzed and the specific DNA sequence which will be amplified. Considering the types of matrices and the primers used for amplification, the factors influencing to analyze uncertainty in laboratory should be considered. The sample homogeneity is an important factor, in a direct relation with the matrix type.

2.1. DNA extraction and reference sample preparation

Seeds (wheat, soybean, and maize), forages, and food products as substitutes for meat based on soy protein (granules, textured, and schnitzel) have to be grinded at the same speed of the mill, and particles with the same size are selected. The minimum quantity is 1000 grains (approximate 200 g for soybean and 300 g for maize) when seeds are analyzed. For the mentioned food products, all the package content is grinding. Meat, usually sampled from fresh material, could be used directly, but we recommend its dehydration. Thus it will be easier to manipulate, the long-term storage of reference sample will be facilitated in terms of time and space, and the samples used for DNA extraction will be homogeneous. The samples can be freeze-dried (the best option), but air drying in special condition can be also used. Considering that air drying takes a long time (several weeks for best results), a free contaminant environment, a constant air flow, and a temperature below 24 °C have to be ensured to avoid DNA degradation. Before drying the samples, they need to be chopped in small pieces, and when the process is finalized, the material is ground to obtain homogeneous samples for DNA extraction. The same preparation method could be used when different meet products are analyzed (salami, sausages, etc.) [6, 7].

Food products as milk, soybean milk, yogurt, cheese, tofu, and pate need to be shaken/mixed. From the homogenized material, the samples are collected. From grinded/mixed material, a 1 g test sample is collected. From the test sample, two analytical samples (100 mg) are taken according to the quartering method for DNA extraction. Besides, a quantity of 50 g is stored as a reference, if the analysis fails or if it needs to be repeated. Other factors that must be followed are DNA quantity and quality. To meet these requirements, all the DNA samples which will be compared are extracted based on the same DNA extraction method, selected according to the matrix specificity. Each DNA extraction procedure is based on the elimination of all the materials present besides DNA in a specific matrix. Therefore different matrix compositions require different physical and/or chemical treatments to extract DNA.

Nowadays a large variety of DNA extraction kits was developed, but using conventional, standardized methods gives a much greater flexibility.

In our work the method described by SR EN ISO 21571 was suitable for a very wide range of matrices, from seeds, flour, or food to meat, dairy, or meat products. The method comprises a step of thermal lysis, in the presence of cetyltrimethylammonium bromide (CTAB), followed by several extraction steps for the removal of protein or polysaccharide compounds. For some matrices different enzymatic steps are necessary. For example, the samples containing high quantity of starch have to be treated with α-amylase; for the meat-derived products, a longer treatment (up to 3 h) with proteinase-K will remove proteins and will facilitate the tissue dissociation, and the RNA can be removed by RNase treatment. One of the most important concerns during the DNA extraction procedure is cross contamination, which can be monitored through the following control samples: environment control (EC), a nuclease-free water (100 μl) sample, present in an open tube during the DNA extraction process; extraction blank (EB), obtained by performing all stages of standard DNA extraction procedure, except the introduction of biological sample; positive control for target DNA (PDT), extracted from positive samples, namely, certified reference or validated in-house materials; and negative control for target DNA (NDT), extracted from negative samples, namely, certified reference or validated in-house materials.

To evaluate the DNA yield and purity, the absorbance method was applied. Therefore a spectrophotometer is used to measure the absorbance at 260 nm for DNA concentration and also the ratios $A_{260/280}$ and $A_{260/230}$ for DNA purity. It is known that the aromatic amino acids from proteins absorb at 280 nm and other contaminants as organic compounds or chaotropic salts absorb at 230 nm. $A_{260}/A_{280} = 1.7–2$ and $A_{260/230} > 1.5$ for a good-quality DNA [8]. If the quality was not appropriate, the DNA samples were subjected to an advanced purification through sodium acetate ($C_2H_3O_2Na$) precipitation. All the DNA samples used in an experiment are diluted at the same concentration (25–100 ng/μl) and stored at −20 °C, until use. The concentration of the DNA samples originated from unprocessed products as seeds or meats are usually higher and their quality meets the purity requirements. But the DNA quality is not optimal if highly processed materials are analyzed, mainly due to the DNA degradation during the mechanical and thermal treatments. But, considering that PCR technique amplifies short DNA fragments (few hundreds base pairs), it is assumed that at least few copies of that fragments are intact in the DNA samples.

Considering that the DNA samples are further on evaluated by PCR, it is necessary to determine if they are amplifiable, namely, inhibitors do not interfere with the polymerase activity, and the DNA fragments have suitable length. Therefore, the first step in each PCR experiment is an amplification reaction with species-specific primers: lectin for soybean, zein for maize, etc.

Another fundamental factor is the calibration with certified materials or validated in-house materials.

For genetically modified organism (GMO) analysis, the certified reference materials (CRM) provided by the Institute for Reference Materials and Measurements (IRMM) were used. For soybean analysis the materials with certified GM content have the following concentrations: <0.3, 1.0, 5.0, 10.0, 20.0, and 50.0 g/kg.

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In most cases the animal reference material can be purchased from entities that are developing [9], but it may be found that some of them are missing from the market and also, in some cases, are expensive. For research purposes we found that it is better to prepare own reference material followed by in-house validation, respecting all the good laboratory practice (GPL) regulations. The best reference material is prepared from the muscular tissue, but in its absence, the organ tissue can be used as well. Samples of the blood can be also collected, but this is not the best option because in this case, the blood-free circulating DNA (even remaining from other species tissue intake) is extracted together the species DNA.

In a preliminary phase of a method validation, it is indicated to isolate and purify the DNA from a fresh raw material, and thus the obtained DNA is of best amplifiable quality. In our experiments the reference materials for animal species identification were validated in-house. The target animal species were cattle, swine, poultry, and fish. Two different types of materials were obtained, according to the analyzed samples origin—meat or milk. To obtain reference materials from meat, fresh muscle was collected from the already mentioned animal species. They were prepared as previous described, starting from 200 g of fresh tissue. The DNA samples were amplified with the species-specific primers to validate the DNA identity. These DNA samples were further on used as reference materials, with a content of 100 % validated animal species.

Milk samples from different animal species were collected for reference materials used for cheese analysis, namely, cow, sheep, and goat. Further on, cheeses were manufactured in our laboratory from fresh milk by a traditional recipe for the preparation of fermented cheese: milk was filtered, stirred, and heated at 30 °C for 1 h and allowed to clot for 2 days (free of added rennet). After 2 days it was heated again, followed by the whey separation for 12 h. Therefore, the reference materials were cow's milk cheese (100 %), goat milk cheese (100 %), and sheep milk cheese (100 %) which were further validated with species-specific primers.

The reference materials used for *Fusarium* species identification strains were validated in-house. For the species with the highest frequency in our country, the fungal strains were isolated and identified according to their morphological description and microscopic analysis (shape of the macroconidia, the presence or absence of chlamydospores). Their identity was confirmed by DNA sequencing of the gene encoding the elongation factor 1 alpha (TEF) and comparison with the available databases.

Other factor involved in analysis uncertainty is the amplification reaction. All the DNA samples used in an analysis series are diluted at the same concentration, to provide similar number copies of the target sequences. All the reactions follow the same amplification conditions programed in a calibrated thermal cycler. The NTC- non-template control has to be used besides the already mentioned controls. This consists of an amplification mixture where the DNA sample is replaced with sterile distilled water.

Considering that the qualitative PCR needs the amplification product analysis, the electrophoresis and the gel evaluation are the last step.

The regular amplification products are few hundred base pair length; therefore, they can be separated by agarose gel. The gel concentration is correlated with the DNA fragment size,

being ranged between 1.5 and 2.5 %. To monitor the precision, accuracy, and the lack of cross contamination, the following samples are analyzed on each gel: analyzed samples (usually two repetitions for each), **EC**, environment; **EB**, extraction blank; **NDT**, negative DNA template; **PDT**, positive DNA template; and **NTC**, non-template controls.

The specificity of the amplified fragments is determined through similarity with the positive DNA template controls and comparing with a molecular weight marker. To evaluate the amplified fragment size, visual observation or the use of specific software could be done. Therefore, to validate a result obtained by qualitative PCR, which is a positive/negative result, few conditions need to be met: same length for the amplified fragments of unknown sample and positive DNA control; environment, extraction blank, negative DNA template, and non-template controls need to be negatives. If all controls are not showing the expected results, the qualitative PCR test is not validated [10, 11].

To obtain reliable results in PCR experiments, it is necessary to meet the good laboratory practice requirements. Therefore, to avoid the cross contamination risks, all the working steps have to be developed in different rooms. A DNA-free area has to be organized where the pre-PCR is performed, namely, the preparation of the PCR mixtures to avoid the original reagent contamination. After amplification the copy number of the interest sequences is very high; therefore, the post-PCR step, namely, electrophoresis, needs to be done in a special area, which is not connected with the rest of laboratory.

3. Detection of genetically modified organisms (GMO) in agriculture products, forage, and food products based on PCR

Nowadays, according to the European Union regulations, all the food or forage products containing GMO (genetically modified plants) in a concentration higher than 0.9 % need to be labeled. It is known that the GM product with the higher prevalence on the European market is GM soybean [12]. Even if it is not approved for cultivation in EU, it is imported in very large quantities and represents the most important ingredient of forages used for livestock [13]. Therefore, the implementation of the detection methods for this product is of great importance. They were developed starting from the molecular sequence specific for the construct used for genetic transformation [14]. This sequence includes the promoter responsible for the transcription of the whole genome of a *Cauliflower mosaic virus* named CaMV 35S promoter or 35S promoter; the region CTP4, encoding a transit polypeptide involved in protein transport to chloroplast originating from *Petunia* hybrid; and the gene CP4 EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), a mutant gene originated from *Agrobacterium* sp., which is insensitive to glifosat action and the terminal sequence nopaline synthase (nos) [15].

For the primary screening, the detection of the 35S promoter was used as a target sequence, using the primers p35S-cf3 and p35S-cr, or the terminal sequence nos, using the primers HA-nos118-f şi HA-nos118-r. The expected amplicon was a fragment of 123 bp length when the primers are placed in the promoter sequence or 118 bp if the primers were specific for the nos terminal sequence [6, 16, 17]. Considering that the analyzed DNA is extracted from different

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processed matrices, the controlling of its quality is necessary. First the absorbance measurements are done by spectrophotometer to evaluate the DNA concentration and quality followed by PCR amplification with primers specific for the analyzed species (lectin) to determine if the DNA samples are amplifiable (**Table 1**).

Organism/target gene	Primer sequences 5'…3'	Annealing temperature (°C)	Amplicon size (bp)	Reference
GMO3/GMO4 *Lectin* gene	GCCCTCTACTCCACCCCCATCC GCCCATCTGCAAGCCTTTTTGG	63	118	[18]
p-35S-cf3/p-35S -cr4, 35S promoter	CCACGTCTTCAAAGCAAGTGG TCCTCTCCAAATGAAATGAACT TCC	62	123	[18]
zein3-zein4 Zein gene	AGTGCGACCCATATTCCA GACATTGTGGCATCATCATT	60	277	[18]
mg1–mg2	TATCTCCACTGACGTAAGGGATGAC TGCCCTATAACACCAACATGTGCTT	61	430	[18]
HA-nos 118-f/HA-nos118-r, nos gene	GCATGACGTTATTTATGAGATGGG GACACCGCGCGCGATAATTTATCC	62	118	[18]
CW/CX Rubisco gene	CGTAGCTTCCGGTGGTATCCACGT GGGGCAGGTAAGAAAGGGTTTCGTA	63	150	[26]
Fusarium ssp./ region TEF	ATGGGTAAGGAGGACAAGAC GGAAGTACCAGTGATCATGTT	54	760	[30]
Fusarium graminearum	GTTGATGGGTAAAAGTGTG CTCTCATATACCCTCCG	53	500	[46]
Fusarium proliferatum	CGGCCACCAGAGGATGTG CAACACGAATCGCTTCCTGAC	65	230	[46]
Fusarium verticillioides	CGCACGTATAGATGGACAAG CACCCGCAGAATCCATCCATCAG5	65	700	[46]
ef1/ef2	ATGGGTAAGGAGGACAAGAC GGAAGTACCAGTGATCATGTT	53	700	[47]
ef1/ef22	ATGGGTAAGGAGGACAAGAC AGGAACCCTTACCGAGCTC	53	450	[47]
Cattle/16S rRNA	TAA GAG GGC CGG TAA AAC TC GTG GGG TAT CTA ATC CCA G	60	104	[42]
Swine/12S rRNA-tRNA	CTA CAT AAG AAT ATC CAC CAC A ACA TTG TGG GAT CTT CTA GGT	60	290	[42]
Poultry/12S rRNA	TGA GAA CTA CGA GCA CAA AC GGG CTA TTG AGC TCA CTG TT	60	183	[42]
Fish/12S rRNA	TAA GAG GGC CGG TAA AAC TC GTG GGG TAT CTA ATC CCA G	60	224	[42]
Horse/mtDNA	CCATCCCTACGCTCCATTCCC TGTTTTCGATGGTGCTTGCG	60	280	Unpublished own research
Salmonella typhimurium/alfa gene	CCT TTC TCC ATC GTC CTG AA TGG TGT TAT CTG CCT GAC CA	56	120	[36]

Organism/target gene	Primer sequences 5'…3'	Annealing temperature (°C)	Amplicon size (bp)	Reference
Escherichia coli/ fimA	GCT GGG CAG CAA ACT GAT AAC TCT C CAT CAA GCT GTT TGT TCG TCC GCC G	56	750	[36]
Bos taurus 12S rRNA-16S rRNA	GTACTACTAGCAACAGCTTA GCTTGATTCTCTTGGTGTAGAG	55	256	[45]
Capra hircus/12S rRNA	CGCCCTCCAAATCAATAAG AGTGTATCAGCTGCCAGTAGGGTT	55	326	[45]
Ovis aries/12S rRNA	ATATCAACCACACGAGAGGAGAC TAAACTGGAGAGTGGGAGAT	55	172	[45]

Table 1. The sequences of the primers used in described experiments.

Our aim was to implement in our laboratory the standardized methods used for DNA isolation from different matrices containing soybean [18] and the qualitative PCR used for GM detection [19].

The first step was the method validation, considering that it was a standardized method used for the proper aim, started from the mentioned types of matrix, developed in our laboratory conditions. Therefore it was not necessary to evaluate the specificity, but the limit of detection (LOD) and the repeatability were determined. According to the standardized method, the absolute limit of detection was not determined, but it was demonstrated that it can detect 50 copies of the DNA originated from Soybean 40-3-2 GTS. In our experiments the samples extracted from the certified reference materials (CRM) were analyzed, each amplification reaction containing 50 ng of DNA (2 μl DNA, concentration 25 ng/μl). For all the reference materials, the expected amplicon (118 bp) was visible. According to the literature data, it is known that there is only one copy of the transgene in the haploid genome [20] and the soybean haploid genome is 1.13×10^9 bp, equivalent with 1.13 pg DNA (a fragment with a 1000 bp length is equivalent with 10^6 pg DNA).

Consequently a quantity of 50 ng of DNA contains approximate 40×10^3 soybean genomes. The transgene copy numbers depending of GM concentration in 50 ng of DNA are as follows: 10 % = 4000, 5 % = 2000, 1 % = 400, 0.5 % = 200, and 0.1 % = 40 copies.

Thereby the investigations performed in our laboratory allowed the emphasizing of 40 transgene copies (50 ng DNA 0.1 % MG), lower than the value mentioned in the standard.

To verify the detection limit, soybean samples with different GM concentrations were prepared in the lab, because products with similar GM concentrations were not available on the market. As started materials flour was obtained from conventional and 100 % GM soybean and also from substitute of meat by grinding. First we prepared a 1 % GM soybean flour (1 g MG and 99 g conventional soybean flour) which was used for 0.1 % MG (90 g conventional and 10 g 1 % MG soybean flour) and 0.1 % textured—substitute of meat (90 g substitute of meat and 10 g 1 % MG soybean flour). Two samples from each experimental variant were analyzed, starting from the first step. The DNA was extracted from the samples in five experimental series, one

series per week. All the DNAs were evaluated spectrophotometrically, diluted at 25 ng/μl, and amplified with the lectin and nos primers.

All the samples with a 0.1 % GM concentration (40 transgene copies) were positive each time, pointing out 100 % repeatability.

In conclusion it was demonstrated that the detection limit for the GM soybean detection is 40 transgene copies or material with 0. 1% GM content in condition of repeatability.

Experiments with lower GM concentrations were not performed because the European legislation requires product labeling only if the GM concentration is higher than 0.9 %; therefore, the detection of concentrations lower than 0.1 % is not necessary.

Analysis is performed in condition of repeatability. The repeatability conditions are considered when the results of the independent tests are obtained based on the same method, in the same laboratory, and with the same equipment in short interval of time. Therefore in our experiments, five identical series were performed in the conditions previous described. The materials used for analysis were based on soybean as follow: meat substitute granules and schnitzel, pate, pate with pepper, mayonnaise sauce, vanilla dessert, yogurt chocolate drink, tofu, forage, and laboratory-prepared flour 100 % and 1 % MG.

The DNA was extracted from each sample in two repetitions, in five experimental series, one series/week. First time the quality, integrity, and concentration of the extracted DNAs were determined. The quality of the DNA extracted from the food samples was not optimum (the values for OD280/260 and OD 230/230 were less than the recommended ones) because they were highly processed. Therefore the amplifications with species-specific primers (lectin) were performed to demonstrate that the DNA is amplifiable by PCR reaction. Previously all the samples were diluted to 25 ng/μl. All the analyzed samples were positive with lectin primers, pointing out that DNA samples can be used in amplification reactions. The positive and negative controls were as expected, and the length of the resulted amplicon was 118 bp, same size for both analyzed samples and certified reference material.

Further on, the DNA samples were amplified with *nos*-specific primers. The analyzed samples had similar results for all fifth experimental series. The size of the resulted amplicon and the positive and negative controls were, as expected, identical with the certified reference material.

The gel analysis pointed out that all of the nine types of food products (textured, pate, milk, cheese, yogurt, mayonnaise, and desert drink) were negative. The chicken forage was positive, as a result of MG soybean, but it was properly labeled. Positive results were determined for flour samples 100 % MG and 1 % MG prepared in our laboratory; the differences between band intensities for 100 and 1 % GM flour were visible for all the fifth experimental series [21, 22]. We can conclude that the results were similar for all the fifth experimental series in terms of repeatability conditions, with DNA extracted from different matrices: agricultural products, food, and forages.

When the detection method was implemented and validated in our laboratory, different samples containing soybean were analyzed. We analyzed samples of food products derived from soybean traded on Romanian market. Thus 50 unlabeled food products were analyzed

such as baby food and dietary products, soy beverages and desserts, tofu, yogurt, and substitutes for meat based on soy protein (grains, textured, and schnitzel). None of the analyzed samples was positive for the GM transgene, pointing out the correct labeling of the food products based on soybean.

Besides, some products like salami and sausages were tested for the soybean and GM content. It turned out that all the processed meat products contained soybean, because their DNA was amplified with the lectin primers. For three samples the results were positive also for the GM gene (nos gene). These results opened other questions—were the transgenic fragments emphasized in highly processed meat products originated from added soybean, or were they already present in the meat? As it was previously described, for GM detection a very short DNA fragment (118 bp) is identified; therefore, its transfer from forage to animal tissue is possible [23]. This assumption is supported by the literature data, which mentioned that transgenic DNA can be detected in different animal tissues, probably transported by body fluids in the same way as other nutrients as exogenous DNA which does not interfere with any metabolic pathway [24, 25].

However the traceability of the transgene sequences from forage to the final processed product was not studied before. In our studies we used as biological materials samples of the liver, muscle, and stomach collected from five pigs, normally fed with GM soybean in a local farm. For comparison a forage sample used for animal fed was analyzed.

The DNA was extracted from both fresh animal tissues and processed ones based on CTAB method [19]. Samples from each organ/animal were chopped, boiled under high pressure (121 °C, for 30 min), dried, ground, and homogenized. Therefore the steps usually used in food industry were simulated.

First, the DNA extracted from fresh material was subjected to absorbance evaluation to determine the concentration and its quality. Further on, the primers species-specific for pork were used for PCR, to determine if the DNA samples are amplifiable. It turned out that all the analyzed samples were positive, excepting those originated from forage.

To emphasize the presence of plant DNA in the analyzed samples, the chloroplast ribulose-bisphosphate carboxylase-1.5 (RuBisCo) gene was amplified [26]. All the analyzed samples were positive, with pointing out the transfer of the plant DNA to the animal tissue.

For soybean detection the primers designed for the lectin gene were used, and for transgene fragment, the nos sequence was amplified. Considering that the chloroplast genes are present in many copies in each cell in contrast to lectin gene which is a single-copy gene, the PCR reactions needed to be suitable adapted.

In these conditions the specific 118 bp fragment for lectin gene was identified in all the analyzed samples, with differences related to the organ of origin. Therefore the highest number of residual lectin sequences was detected in the liver tissue, followed by the muscle tissue and slightly observable in the stomach tissue. The samples originated from the same organs but from different animals had homogenous intensities, indicating a specific mechanism of transfer.

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Finding that fragments from the soybean genome could be identified in tissues collected from the animals fed with this forage, the next goal was to determine if the transgene sequence could be detected by a regular PCR screening. Our results showed that the presence of the nos target gene was not similar in the same organ originated from different animals suggesting that its transfer can be possible but randomly. It was appreciated that the lower content of GM sequences may be caused by the complex composition of forage which can contain a mixture of conventional and GM soybean. Even if the transgene sequences are in lower concentration than those originated from conventional ones, they are still detectable.

The next objective was to determine whether the plant DNA sequences could be also detected in the processed materials. The PCR results obtained through lectin and nos primer amplifications emphasized that the DNA sequences present in fresh tissues were not totally degraded during the chopping, heating, and grinding. We can conclude that DNA sequences transferred from feed to animal are still detectable, even after mechanical and thermal treatments. Considering that according to EU legislation the food products containing more than 0.9 % GMO have to be labeled but the meat of animals fed with GM forage does not require labeling, it is of great importance to follow the transgene traceability along the food chain. If the transgenes are detected in a food product, they can be originated either from meat or from added soybean. Knowing the origin of transgenic sequences absolves the manufacturer of mislabeling.

4. Detection of *Fusarium* infection in agricultural, forage, and food products based on PCR

A complex widespread disease of the small grains (wheat, maize, or barley) is *Fusarium* head blight (FHB) produced by the *Fusarium* sp. infection. Besides the considerable loss of yield, it has the ability to produce mycotoxins which are harmful to human and animal consumer. For this reason it is of great importance to identify *Fusarium* infections before the toxins are synthesized and in the same time to determine its toxicogenic potential [27].The first aim of our research was to evaluate the specificity of the primers mentioned in the literature for the detection of the *Fusarium* strains present in our country. For this, 60 *Fusarium* strains were isolated from wheat samples, collected from different Romanian locations. As a result of the microbiological evaluation and microscopic analysis of the colonies, it was pointed out that *Fusarium graminearum* had the highest incidence (70.68 %), followed by *Gibberella fujikuroi* complex (13.79 %). Within *G. fujikuroi* complex, some small differences were observed, indicating the affiliation to *Fusarium proliferatum* and *Fusarium verticillioides*, but an accurate determination at this level was impossible. Consequently all the isolates were generally classified as belonging to *G. fujikuroi* complex (complex species *G. fujikuroi*, GFSC) [28]. Besides, for three other strains, the morphological identification was impossible because of the spores' absence; therefore, they were collectively classified as *Fusarium* sp. (5.17 %). Three other strains were classified as *Fusarium equiseti* considered to be a secondary invader. It was also identified one strain of *Fusarium avenaceum* (1.72 %), one *Fusarium solani* (1.72 %), and another one *Fusarium cerealis* (1.72 %) which is considered to be pathogenic for humans. Considering that

the simple observation of the phenotypical symptoms produced by *Fusarium* sp. and the microbiological evaluations are time consuming and uncertain, with low precision the new methods based on DNA sequence for pathogen monitoring were applied, using the primers mentioned in the literature [29] (**Table 1**).

The DNA was extracted from the collected seeds, based on CTAB method (SR EN ISO 21571). It was considered that this method allows the isolation of DNA from both wheat and infected microorganisms. For all of the strains considered to be *F. graminearum*, the specific primers were used for amplification. The preparation and identification of the samples used as reference materials were previously described. From these, positive results were obtained for 39 strains, whereas three samples were not amplified. The absence of amplification indicated either the lack of primer specificity due to DNA mutations or the fact that the isolates are not belonging to *F. graminearum* species. The amplification based on *F. proliferatum* and *F. verticillioides* primers of the corresponding strains was positive, pointing out the species identity. To overcome these uncertainties, the PCR followed by DNA sequencing was performed for all of the other strains whose identity was not confirmed by regular amplification.

4.1. PCR followed by DNA sequencing

During the last years, the DNA sequencing techniques had a very important development; therefore, the availability and costs make them accessible for each laboratory. They can be applied in many domains, including the establishment of the identity for the fungi belonging to *Fusarium* genus. In this respect genes as TEF, the gene encoding the elongation factor 1 alpha, were found to be an excellent tool. TEF gene has a special phylogenetic utility because (i) it is very specific for *Fusarium* genus, (ii) non-orthologous copies of the gene were not detected, and (iii) universal primers were designed, appropriated for phylogenetic studies [30]. Besides this gene is always present as a single copy and has a very high degree of polymorphism. Since September 2003, a database containing TEF sequences for hundreds of strains of the genus *Fusarium* was created—Fusarium-ID publicly available database which contain data regarding 78 species and almost 6000 sequences.

The molecular identification based on DNA sequencing involves the following steps: initiation of a pure culture, fungal DNA isolation, PCR amplification for the TEF gene, purification for the amplification product, and fragment DNA sequencing. The primers used for TEF gene amplification were ef1, ef2, and ef22 primers (**Table 1**). These primers amplify a region of the TEF gene delimited by three introns and four exons. In some laboratories the pair of primers ef1 and ef2 is used, generating an amplicon of 700 bp which provides enough information for an accurate species identification. As an alternative the pair of primers ef1 and ef22 could be used, with an amplicon of 450 bp. This is part of the previous fragment, generating also a good identification process.

In our research the pair of primers ef1/ef2 was used for the amplification of 18 isolates with uncertain identity. Previously, fungal pure cultures were obtained starting from single colony, and the DNA was extracted from the mycelium for all the 18 strains with uncertain identity. First, the eight isolates classified as *G. fujikuroi* complex were analyzed. After sequencing, five strains were identified as *F. proliferatum*, with 98–99 % of identity in Fusarium-ID database.

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Besides, one strain from each of the following species was identified: *F. verticillioides* (99.5 % identity Fusarium-ID), *Fusarium subglutinans* (91 % identity NCBI), and *Fusarium andyiaze* (99 % identity Fusarium-ID). The three samples classified as *Fusarium* sp. because of lack of spores were confirmed to be *F. graminearum*, with percent of 99 % identity in both Fusarium-ID and NCBI databases. The same results were emphasized for the isolates characterized as *F. graminearum* by phenotypical and microbiological observations, but were not amplified by the specific primers recommended by the literature for *F. graminearum*. In other situations the morphological data were confirmed by sequencing; therefore, three isolates were confirmed to be *F. equiseti* and the other one as *F. avenaceum*. Our results pointed out that screening with specific primers recommended in the literature had a 93 % percent of success, but the PCR amplification followed by DNA sequencing can provide the most reliable and accurate method for *Fusarium* species identification. Therefore, if we are interested in a general screening of the agricultural products, the regular PCR amplification can be used. Further different samples collected both from fields and Romanian market were analyzed.

Biological material: eight samples of commercial feeds (labeled A, B, C, D, E, F, G, and H) were subjected to screening for pathogenic fungal species. First, eight samples of commercial complex forages were subjected to screening for the most common pathogenic fungal species, namely, *F. graminearum* and *F. proliferatum*. As reference materials the DNA extracted from fungal strains with identity confirmed by sequencing were used. To evaluate the amplifiable quality of DNA samples, the (RuBisCo) gene was amplified, considering that the forage samples had a complex composition based on vegetal products. All the samples had positive results pointing out the lack of inhibitors. Besides, all of them were positive for both *Fusarium* species, underlining their fungal contamination [31]. Next, samples were collected from seven locations, where GM corn (MON 810) was cultivated in 2010. From each location both GM and correspondent conventional corn samples were analyzed. After DNA extraction from all the collected samples, the identity and in the same time the amplifiable quality were evaluated using the zein primer amplifications. To confirm the GM identity, the 35S promoter was used as target gene, with mg1 and mg2 primers. All the results were as expected; thus, the amplifications with primers specific for the most common *Fusarium* species were performed. *F. graminearum* and *Fusarium culmorum* were identified in both conventional and GM corn, with a higher prevalence in the first type. *F. proliferatum* was identified only in conventional corn. The results were predictable considering that GM corn lacks the European borer attack, making more difficult for fungal infection [32].

In other experimental series, ecological products from specialized market, labeled as "bio," were analyzed: wheat, barley, rye, durum wheat pasta, biscuits, and sunflower seeds, in comparison with conventional products as corn flour, wheat flour, and integral cereals. Taking in account that all the samples were originated from plants, the RuBisCo gene was used to evaluate the amplifiable quality of DNA. Then, the fungal amplifications were performed with *F. graminearum* and *F. culmorum* primers. A specific amplification for *F. graminearum* infection was emphasized for bio wheat and barley samples. Besides, many other unspecific bands were visualized for the other bio samples, making the result interpretation more difficult. For the conventional products, the corn and wheat flour were positive for *F. graminearum*, but both

specific and unspecific bands were visualized. The other species, *F. culmorum*, was not identified in any of the analyzed samples [33]. These results showed that both ecological or "bio" products and conventional ones can be infected with fungal strains; therefore, they are not always a solution for healthy food. The obtained results enable us to conclude that the fungal screening based on PCR-specific primers can be applied when agricultural products as grains or forages collected from fields or silages are analyzed. For processed materials the identification is arbitrary and difficult, probably due to the fungal DNA degradation. Besides, when DNA is extracted from vegetal samples that are possibly infected, it is assumed that the fungal DNA is in very low quantities compared to the plant DNA. If more precise results are necessary, the inoculation is recommended, followed by morphological and molecular analysis: PCR with specific primers or PCR followed by sequencing.

5. Detection of food-borne pathogens in forage and food products based on PCR

Assessment of the food quality and safety has a great importance for human health. Some of the most common pathogens that are found in foods and can be easily transmitted from animals to humans are bacterial strains *Salmonella* and *Escherichia coli* [34]. They also produce significant damage in livestock, since infections lead to high disease and death rates. Traditional methods for identifying pathogens in food poisoning, which causes disease in humans, usually include multiple subcultures and biotype or serotype-identification steps. They are not effective in terms of time and cost, being also very laborious [35]. In most of the cases, a rapid identification of the agent that was causing food poisoning is essential for a pertinent intervention in order to overcome the pathogenic effects. Methods based on molecular techniques for identifying bacterial DNA have proved to be efficient for the detection of pathogens in feed and forage product. Considering this frame of work, the aim of our studies is to develop and validate a qualitative PCR-based method for the rapid identification of *Salmonella* and *E. coli* pathogens. The primers that were used are designed for the identification of specific *Salmonella* spp. and pathogenic *E. coli* genes: fimA and afa, respectively, being described to provide highly specific, rapid, sensitive, and reliable results [36].

Bacterial strains of *Salmonella* ssp. and pathogenic *E. coli* O157:H7 were provided on the courtesy of Microbiological Department of our institution, as free of culture media cells, in safe condition, the DNA being extracted immediately without other preparations. The working DNA solution was established at 5 ng/μl for the reference sample. In order to validate a method of detection, the usual steps were followed.

First, the primer specificity was evaluated. The *Salmonella* and *E. coli* primers were challenged in simplex PCR with nontarget species and different types of muscular tissue of fish, ruminant, pork, and poultry; maize grains, two samples of commercial forage and *Fusarium* ssp. along with the negative controls in order to detect possible cross-reactions; and unspecific amplifications products. As positive control for primer specificity, DNA extracted from bacterial cells was analyzed. In no case a cross-reaction neither unspecific amplicons was observed. For the

positive controls, the primers generated specific fragments of appreciatively 750 bp for *E. coli* strain and 120 bp for *Salmonella* strains. To determine the assay sensitivity for specific DNA quantity, DNA extracted from bacterial cells was prepared in five serial DNA dilutions: 5, 2.5, 1.5, 1, and 0.5 ng/μl, respectively. The dilutions were used as DNA template for PCR amplification with pathogen-specific primers. The assay sensitivity was evaluated according to PCR product intensity, as it can be visualized in agarose gel. The intensity of amplicons was found to decrease gradually from 2.5 ng/μl concentration continuing till the 0.5 ng/μl limit, where the amplicons are still perceptible. For *E. coli* targets, this concentration can be considered a lower threshold for the PCR assay sensitivity, but for *Salmonella* targets, it could be established even at a low concentration. To establish the limit of detection (LOD) expressed in percent, the bacterial genomic DNA was progressively diluted in vegetal genomic DNA suspension with the same concentration. Starting from 50 % bacterial DNA template, five serial DNA dilutions were obtained: 25, 10, 5, 2.5, and 1 %, respectively. For these samples the amplifications with specific primers were performed along with the reference sample. No differences were noticed among the first three dilutions in both cases. In this assay it was found that for 1 % concentration of pathogen, the amplicon can be easily detected suggesting that the method can be very sensitive and accurate.

Applicability on food and feed matrices was representing the last step of in-house validation process. In this experiment we submitted two commercial feed samples, two meat food products, two food products containing eggs, and two samples of cheese. Following the PCR detection, we had no positive results except the reference samples. Therefore, it will be necessary for the correct validation of the method to develop an experiment with the legal control agencies to provide contaminated food samples. The PCR method proposed in this study can be used as an initial screening in detecting food pathogens. In several hours accurate results can be at the disposal of control organisms, thus facilitating the further analysis, and even will indicate the test that should be applied.

6. Detection of different animal species in meat and dairy products based on PCR

Nowadays, the incidence of food fraud has a wide prevalence all over the world, but sometimes its identification is difficult. To ensure compliance with regulations and to implement punitive measures, when necessary, robust analytical tests are needed. Food adulteration industry could be systematized into four main subsections: (A) the origin of animal products and feeding regime of animals from which they originated (such as certified regional products); (B) replacement of the ingredients in the recipe with other animal species, tissue, fat, or protein of other origin; (C) changes in food processing methods thus altering the original recipe; and (D) additions of nonself components, such as water or additives and flavor enhancers [37]. Therefore, molecular analytical methods have been developed based on the analysis of lipids [38], protein [39], and, most recently, nucleic acids [40]. However, methods that rely on lipids and proteins are limited because protein biomarkers are easily denatured and the amount of protein can be significantly altered by food processing so attention turns to techniques based

on the analysis of nucleic acid. They have superior stability and universal traceability in all the organism cells; thus, DNA-based techniques are reliable, robust, accurate, and fast. Simultaneous detection of several animal species has been extensively developed by PCR techniques, the research being adapted to the producers' requirements to authenticate products [41]. For this type of analysis, both nuclear DNA and mitochondrial DNA were used, but the systems that promise to work in the case of all living animal species—the mitochondrial D-loop region, cytochrome b, cytochrome oxidase subunit I (COI), 16s rRNA, 12S rRNA, and NADH dehydrogenase subunit 5/6 genes—are used intensely nowadays for DNA barcoding. According to the literature data, a lot of DNA markers were developed to evaluate the food/feed compositions. But each experiment has specific particularities; therefore, there are important issues that have to be taken in consideration. First, the matrix of the selected product has to be considered in order to adjust the DNA extraction protocol as described above. The amount of starting material/product will be established in accordance with the degree of processing and the matrix nature and also the animal species that can be identified. The next step is the selection of the biomarkers which will be evaluated. A biomarker needs to meet some requirements to be suitable for detection of animal species in a product, as follow: to be highly specific for the species of interest and to be abundant in its genome. In this respect the most used are the mitochondrial genes 16s rRNA and 12s rRNA that has been proved to be successfully applied for some animal species [42]. There are many primer pairs developed on the basis of these biomarkers, which can be acquired and validated in any laboratory, but usually the best way is to design own primer pairs according to the experiment requirements. For this purpose, the most used DNA sequence is cytochrome C oxidase I (COI), which is specific for species from insects, fish, shellfish, birds, to farm animals and primates. Research focused on very different animal species has shown that sequence variation in the 5' region of COI allows the identification of 98 % of them [43]. The design of animal species-specific primers involves the amplification of the 700 bp fragment of the COI gene biomarker and selection of the sequences which are most suitable to be used for an amplification reaction. The specific primer pairs can be easily designed using dedicated softwares, in order to fulfill all the requirements of the experiment. That softwares can be acquired and used off- and online; however, it is necessarily to confirm the specificity of the newly designed primers by aligning them with sequences from the suitable data basis. In our experiments reliable results have been obtained using NCBI database. The most important issue for primers designed to be used in multiplex PCR reaction is their expected amplicon size. The differences between the amplicon lengths generated with different pairs of primers, each of them specific for an animal species, have to be easily discriminated in electrophoresis gel. The fragments overlapping seriously affect the result of the analysis. Besides, a special attention must be directed into selection and preparing the reference materials, as already described. In our laboratory we have experienced the validation and application for analysis of several methods used for identification of food and feed component species. Our first experiments were focused on the development and implementation of an endpoint simplex PCR for detection of fish or fish products in feedstuffs. This is of great interest for farmers because of the European legislation [44]. The factors followed up for in-house validation of the detection method were specificity, sensitivity, and limit of detection, using an appropriate reference material, prepared in laboratory from fresh muscular

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tissue of common regional fish species as previous described. The detection of possible cross-reactions and unspecific amplification products was followed to evaluate the primer specificity. For this aim the fish primers were used in simplex amplification reactions with different types of templates, namely, DNAs extracted from samples: (a) containing target sequence, muscular tissue of fish and forage containing 2 % fish material, and (b) nontarget sequence—muscular tissue of ruminant, pork and poultry, and soybean and maize. Besides, all the already mentioned positive and negative controls were analyzed. It was demonstrated that only the samples containing fish material were positives, with amplified fragments of 230 bp length as expected. No amplification was visible neither for the other samples nor for the negative controls, pointing out the reaction specificity. To evaluate the assay sensitivity, templates with different content of fish DNA were analyzed. First, the sample of DNA extracted from fish raw muscular tissue was precisely quantified by spectrophotometric method. Then, seven serial DNA dilutions—100, 50, 10, 1, 0.1, 0.01, and 0.001 ng/μl—were prepared and used as templates in amplification reactions. The assay sensitivity was evaluated according to the PCR product intensity as it can be visualized in agarose gel. The highest concentrations of 100 and 50 ng/μl generated very strong bands, but the intensity of amplicons decreased gradually at lower concentrations, starting from 10 ng/μl and continuing to 0.001 ng/μl. At the last concentration, the band was still perceptible; therefore, it was considered that the lower threshold for the PCR assay sensitivity in our laboratory was 0.001 ng/μl. The next goal for method validation was to establish the limit of detection (LOD) expressed in percent of fish material in a mixture. Thus, pure fish genomic DNA with known concentration was progressively diluted in vegetal genomic DNA suspension, resulting five serial DNA dilutions: 1, 0.5, 0.05, 0.005, and 0.001 %, respectively. All of them were used as DNA template for PCR amplification. As positive control, the 100 % fish genomic DNA, considered as reference material, was used. It was shown that it is nearly impossible to discriminate among concentrations of fish material above 0.5 %, but the intensity decreases dramatically in the case of 0.001 % fish DNA template. Considering that for the last concentration the PCR products were still detectable, the detection threshold in our laboratory was established to 0.001 % fish material in a mixture. Further on, the method repeatability was evaluated to complete the validation process. Therefore the already described steps were repeated three times, one time/week with similar results. Applicability on food and feed matrices represents the last step of the process. In our research we analyzed four commercial feed samples, three of them being labeled by producers as non-contaminated with fish material. The PCR detection of fish material was performed for all of them. Positive results were obtained for two of the labeled samples as non-contaminated. Evaluating the intensity of used positive control (0.05 % fish material), it was emphasized that the fish material contamination was below the mentioned percent, probably being due to the manufacturer's faulty procedures, and this might not exceed the limits of regulation. It was demonstrated that the PCR method proposed in this study can be considered as a further improvement of conventional assays. The test could be useful in the control of different food and feed products, to verify the origin of the raw materials, especially in products submitted to denaturing technologies, for which other methods cannot be applied. The next experiments were focused on development and implementation of Multiplex PCR for simultaneous detection of different animal species. We considered the animal groups that are commonly used in European

countries as ingredients of meat-derived and dairy products. The validation of two endpoint multiplex PCR methods for detecting the main used animal species in meat and dairy products was performed following the same pattern. Three sets of primer designed for different regions of mitochondrial DNA—12S rRNA, tRNA Val, and 16S rRNA—of cattle, swine, poultry, and fish [42] were used to evaluate the meat product adulteration. When adulteration of dairy products was studied, the detection of cow, goat, and sheep milk was aimed, using the specific primers [45]. For in-house validation, the same factors were followed up—specificity, sensitivity, and limit of detection—using appropriate reference materials, prepared in laboratory from fresh muscular tissue collected from cattle, swine, poultry, and fish and from cow, goat, and sheep milk, as previously described. From each material the DNA was extracted based on the described methods. To evaluate primer specificity, two different mixtures of DNA were prepared: one from cattle, swine, poultry, and fish DNA, named bulk A, and the other from cow, goat, and sheep, named bulk B. Bulk A was template for different amplification reactions with each pair of primers separately. Besides, the same bulk DNA was amplified with the mixture of the four pairs of primers. The primers in the simplex had the same concentration as in multiplex reaction. A single, visible fragment was shown for each simplex reaction, with the expected length, similar with the reference materials, pointing out the primer specificity. The multiplex amplification generated four bands, visible, perfectly separated with the length specific for each animal species targeted. To get these results, the electrophoretic analysis must be conducted in optimal condition to permit the correct separation of the multiplex PCR products; otherwise amplicons overlapping would seriously affect the analysis result. The same results were obtained when dairy products (bulk B) were analyzed with specific pair of primers, emphasizing the primer specificity. The next step was determination of the assay sensitivity and establishing the limit of detection (LOD); the assay sensitivity and the limit of detection were evaluated according to PCR product intensity as it can be visualized in agarose gel. The bulk DNA sample composed as a mix of the animal species DNA (equal amounts of 200 ng/μl DNA solution of each species) was diluted in maize DNA to obtain templates with known percent concentrations of animal material. Starting from the considered 100 % DNA bulk, seven serial DNA dilutions were performed—10, 1, 0.1, 0.01, 0.005, 0.002, and 0.001 %, respectively—all of them were used as DNA template for multiplex PCR amplification, with the four pairs of primers simultaneously. Discrimination between samples with DNA concentrations higher than 0.1 % was not possible, but the band intensity decreases gradually at lower concentrations, being faint but detectable in the case of 0.001 % concentration. Considering the results it was concluded that a concentration of 0.002 % represents the lowest concentration that gives reliable results. Therefore 0.002 % was considered the threshold for the sensitivity and the limit of detection (LOD) for the described multiplex PCR assays. Further on, different processed meat and dairy product matrices were analyzed, to evidence the multiplex method applicability. First, the DNA extracted from commercial product matrices were submitted to specific simplex PCR assay using the different primer systems in separate PCR amplifications. The obtained results were compared with those obtained in similar multiplex PCR amplification. A total concordance between the results can be considered successful completion of the multiplex PCR in-house validation process. For detection of animal species in dairy products, a number of 20 milk-derived products were analyzed. Pure goat and sheep milk cheeses and

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yogurt but in most cases products obtained from the mixtures of the three types of milk were purchased from the local market according to the labeled composition. The food and feed samples analyzed for detection of different types of meat could be classified in three main categories: forage samples—commercial products developed for farm animals, pet food either dried or canned, and a large variety of meat-derived products such as hamburgers, sausages, salami, and other products that are obtained from animal tissue flour, all of them being purchased from the local market stores. Using the described methods allowed, it was possible to detect all the specific ingredients from each product and to identify incorrect labeling of several processed meat products and fraudulent addition of cow milk in dairy products that were labeled as pure goat or sheep milk-containing dairy products.

New experiments were focused on horse meat detection. In the context of the big issue of fraudulent addition of horse material in alimentary products that were labeled as containing beef meat, we were endorsed to develop a time- and cost-efficient PCR-based method for horse DNA detection, following the same pattern for method validation. The main goal of this experiment was to design a pair of primers for horse DNA detection which can be successfully used in duplex PCR experiments along with cattle-specific primers that were already validated in our laboratory. For this purpose, we designed specific primers that amplify a 280 bp fragment from cytochrome b mitochondrial gene (GenBank code *JQ340166.1*). Considering that our aim was to use both pairs of primers, horse and cattle in the same amplification reaction, a high difference between the generated DNA fragments was necessary (290 bp for horse and 104 for cattle). Genomic DNA extracted from horse (*Equus caballus*) fresh muscle tissue was used as reference material for in-house validation process. First step was to confirm the species identity; therefore, the fragment generated by the newly designed primers was sequenced and compared with the specialized databases. Thus, the specificity for horse species was confirmed, allowing the primers used in future investigations. The next step was to evaluate primer specificity when different templates are amplified. For these aim different ten types of DNA extracted from raw materials—horse, bovine, sheep, goat, swine, chicken, alfalfa, soybean, and *F. graminearum*—and a DNA mixture of 5 % horse and 95 % beef were amplified with the horse-specific primers. The results were as expected, only the last samples being positive. The assay sensitivity was achieved by amplification of serial dilution from the same horse DNA matrix: 100, 50, 10, 1, 0.1, 0.05, 0.01, 0.005, and 0.001 ng/μl. Slightly visible positive results were recorded even in the case of the lowest concentration; however, the detection in this case may become hazardous; therefore, the threshold for the sensitivity was set at 0.005 ng/μl. In order to determine the detection limit, horse DNA was diluted in vegetal (alfalfa) DNA suspension reaching to the following concentrations: 100, 20, 5, 1, 0.5, 0.1, 0.05, 0.005, and 0.001 %. The band intensity decreases gradually at lower concentrations, being detectable in the case of 0.005 % concentration. This concentration was considered as detection limit for the horse DNA detection. In this experiment a limit of detection for mixtures of heat treated and minced horse and beef meat in different percentages was also established. The mixtures were prepared with a content of 50, 20, 5, 1, 0.5, 0.1, 0.05, and 0.005 % horse and beef meat mixture. In this situation a limit of detection at 0.1 % of horse material was established.

Our experiments focused on identification of the animal species in different matrices enabled us to develop and validate a series of methods as follow: detection of fish meal in complex forages, a multiplex PCR method for identifying the animal species (pork, ruminants, poultry, and fish) in food products, detection by multiplex PCR of cow milk in products made from other species (sheep, goat) milk, and detection of horse meat in food products. The in-house validation process was very laborious but still not very difficult. Even if previously described methods are applied, they have to be adapted to own laboratory conditions (equipments, reagents, manipulators). Particularly, in the case of multiplex PCR, the molar concentrations of the primers have to be adjusted so that their competition must not interfere with the final result.

7. Conclusions

The requirements for food safety are determined by the universal demand of the consumer that all the food products are in conformity with the demands concerning health, diet, lifestyle, culture, and religion. Therefore concerns in this area have exceeded the boundaries of safety for the health of the consumer, getting to heed religious prohibitions, food-borne allergies, and food fraud of animal-derived products. PCR-based applications have seen a significant development in recent decades, many techniques being developed for detection of any potential risk that could arise from food intake. These methods are powerful tools especially when they have immediate practical applications being developed in concordance with current society demands. This chapter is describing the in-house validation processes of few PCR-based methods that were successfully applied in our laboratory and that were chosen according to the present requirements of consumers and food safety authorities.

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Chapter 10

Site-Directed Mutagenesis by Polymerase Chain Reaction

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Additional information is available at the end of the chapter

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Abstract

Since genomic data are widely available, many strategies have been implemented to reveal the function of specific nucleotides or amino acids in promoter regions or proteins, respectively. One of the methods most commonly used to determine the impact of mutations is the site-directed mutagenesis using the polymerase chain reaction (PCR). There are different published protocols to develop single or multiple site-directed mutagenesis. In this chapter, we reviewed the enzymes commonly used in site-directed mutagenesis, the methods for simple and multiple site-directed mutagenesis in large constructs, mediated by insertion of restriction sites. Other methods reviewed include high-throughput site-directed mutagenesis using oligonucleotides synthesized on DNA chips, and those based on multi-site-directed mutagenesis, based on recombination. Software tools to design site-directed mutagenesis primers are also presented.

Keywords: site-directed mutagenesis, polymerase chain reaction, plasmids

1. Introduction

With the advent of new technologies during the last decade, an important amount of genomic data has been provided by next-generation sequencing. With the information provided by genomic sequences, many gene polymorphisms, insertions, or deletions have been significantly associated with disorders that include mono- and polygenetic diseases to cancer. However, to demonstrate that these mutations, located either in coding or uncoding regions, are involved in

the illness, it is necessary to evaluate them in a simpler context using other molecular strategies. Taking the advantage of direct manipulation of DNA as well the availability of sequences, site-directed mutagenesis using polymerase chain reaction (PCR) has become an essential tool for studies of key sequences in regulatory regions and/or the relationship between the structure and function of proteins. Many strategies have been developed to simplify the method and increase its efficiency. Commonly, site-directed mutagenesis is used to introduce mutations in a DNA fragment, genome or plasmid, either by PCR or restriction endonucleases digestion. In this chapter, we summarized different strategies to perform the site-directed mutagenesis using PCR.

2. Site-directed mutagenesis

Mutagenesis is usually employed to understand the regulatory regions of genes and the relationship between the protein structure and its function [1]. Depending on the number of sites to be mutated, site-directed mutagenesis can be divided into two types: simple or multiple mutations [2]. For single mutations, methods are based on the amplification of double-stranded DNA from plasmids using complementary oligonucleotides carrying the mutation of interest [3]. Due to its simplicity, the low number of hours spent, and high efficiency, this is one of the most common strategies to introduce mutations in DNA fragments. For multiple mutations, methods incorporate the desired mutations simultaneously in the same reaction or they are obtained after several rounds of mutations.

There are a number of commercial kits for simple mutagenesis. These kits are easy to use but regularly have trouble getting large deletions [4]. With the intention of overcoming the limitations of commercial kit, other methods have been developed for other applications [5].

2.1. Enzymes used in site-directed mutagenesis

To ensure an accurate amplification by PCR, versions of the high-fidelity DNA polymerases are usually available for site mutagenesis. The common trait of this kind of polymerases is their low error rate. High-fidelity DNA polymerases contain a proofreading domain consisting of polymerase activity 5′-3′ and exonuclease activity 3′-5′ to remove wrong incorporated nucleotides. **Table 1** shows the most representative enzymes to amplify DNA by PCR.

The DNA polymerases *Pfu*Turbo and KOD are very useful to amplify products with complementary primers, however, Phusion DNA polymerase and others cannot. The failure probably is due that Phusion requires high annealing temperatures, and it is able to promote the formation of a perfect matching of complementary primers more than the formation of the primer-template duplex containing mismatches [6].

Another important part of site-directed mutagenesis is eliminating the template with a methylation-recognizing-nuclease, as *Dpn*I. Although digestion of *Dpn*I can eliminate fully methylated parental DNA, around 20–30% of hemimethylated molecules (parental strand combined with PCR-generated strand) could not be removed due to hemimethylated DNA, and the PCR product would be more resistant to *Dpn*I [7].

Enzyme	Published error rate (errors/bp/duplication)	Fidelity relative to *Taq*
Taq	$1–20 \times 10^{-5}$	1×
AccuPrime- *Taq*, HF	N/A	9× better
KOD	N/A	4× better, 50× better
Pfu	$1–2 \times 10^{-6}$	6–10× better
Phusion hot start	4×10^{-7} (HF buffer),	>50× better (HF buffer),
	9.5×10^{-7} (GC buffer)	24× better (GC buffer)

Source: Taken from McInerney et al. (2014) [8].

Table 1. Published fidelity (error rate) values for DNA polymerases.

2.1.1. Phusion™ high-fidelity DNA polymerase

This enzyme is manufactured by New England Biolabs, Phusion high-fidelity DNA polymerase is recommended for DNA amplification with high fidelity and robust performance. This DNA polymerase has a unique structure obtained from a fusion of the dsDNA-binding domain to a *Pyrococcus*-like proofreading polymerase. The error rate reported by the provider webpage is 50-fold lower than that of *Taq* DNA polymerase and sixfold lower than that of *Pyrococcus furiosus* DNA polymerase [9]. This polymerase generates blunt-ended products and can amplify from GC-rich templates.

2.1.2. Q5® High-fidelity DNA polymerase

This recombinant enzyme is also produced by New England Biolabs, and described with both, fidelity and robust performance. According to providers webpage, Q5 DNA polymerase is composed of a novel polymerase fused to Sso7d DNA binding domain. Its error rate is >100-

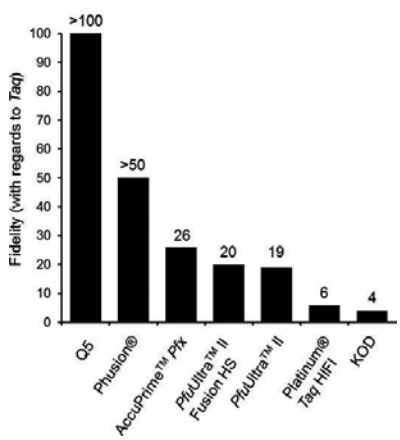

Figure 1. Comparison of fidelity rate among high fidelity DNA polymerases. Modified from: https://www.neb.com/products/pcr-polymerases-and-amplification-technologies/q5-high-fidelity-dna-polymerases/q5-high-fidelity-dna-polymerases.

fold lower than that of *Taq* DNA polymerase and 12-fold lower than that *Pyrococcus furiosus* (*Pfu*) DNA polymerase (**Figure 1**). This polymerase has been optimized with a buffer system to allow a robust amplification in GC-rich regions. Moreover, New England Biolabs webpage has included a Tm calculator to determine the appropriate annealing temperature for primers in GC-rich regions.

2.1.3. AccuPrime™ Pfx

This product is a preparation of DNA polymerase obtained from *Thermococcus* species strain KOD [10, 11]. ThermoFisher Scientific mentions that this polymerase is highly processive and possesses a fast chain extension capability. The preparation contains an antibody bound to the inactive polymerase at ambient temperatures, but it dissociates after the initial denaturation step at 94°C.

2.1.4. PfuUltra high-fidelity DNA polymerase

The *Pfu*Ultra high-fidelity DNA polymerase (Agilent Technologies, La Jolla, CA, USA) is a formulation of a genetically engineered mutant of *Pfu* DNA polymerase and the ArchaeMaxx polymerase-enhancing factor. According to the manufacturer, this enzyme exhibits an average error rate threefold lower than *Pfu*Turbo DNA polymerase and 18-fold lower than *Taq* DNA polymerase. *Pfu*Ultra high-fidelity DNA polymerase is described and it provides a robust amplification of long and complex genomic targets. The ArchaeMaxx factor eliminates dUTP, a PCR inhibitor, and promotes shorter extension times, higher yield, and greater target length capabilities. In fact, the ArchaeMaxx factor functions as a dUTPase.

2.2. Endonuclease *Dpn*I

The use of *Dpn*I endonuclease, which is able to cut methylated DNA, has enabled progress on site-directed mutagenesis. Plasmid DNA extracted from bacteria contains methylated DNA, which makes it susceptible to *Dpn*I enzyme [12], whereas the DNA amplified by PCR does not contain methylated DNA. Based on this principle, the PCR using plasmid DNA as template is useful for the *in vitro* replication and mutagenesis and *Dpn*I digestion. PCR products digested by *Dpn*I have been widely used in mutagenesis.

2.3. Site-directed mutagenesis in large constructs

The QuikChange mutagenesis kits (Agilent Technologies) have become the standard to develop site-directed mutagenesis due to the simplicity of their protocols and their high efficiency. The single-site mutagenesis approach use a pair of complementary mutagenic primers to amplify a target plasmid [2]. The main problem in mutagenesis with large inserts (up to 2100 bp) is due to the efficiency, which is lower than small and medium inserts. In this case, one limitation for site-directed mutagenesis is the size of the target plasmid. The factors that may affect the efficiency of the method are focused on the quality and efficacy of the polymerases and primers used [13].

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Wang et al. [14] have described a method to generate site-directed mutagenesis in large genes. The method consists of two PCR products with four primers (containing mutations and restriction enzyme sites). Fragments are transiently ligated into TA cloning vectors, and, after cutting with appropriate enzymes, fragments are ligated into a final vector (**Figure 2**).

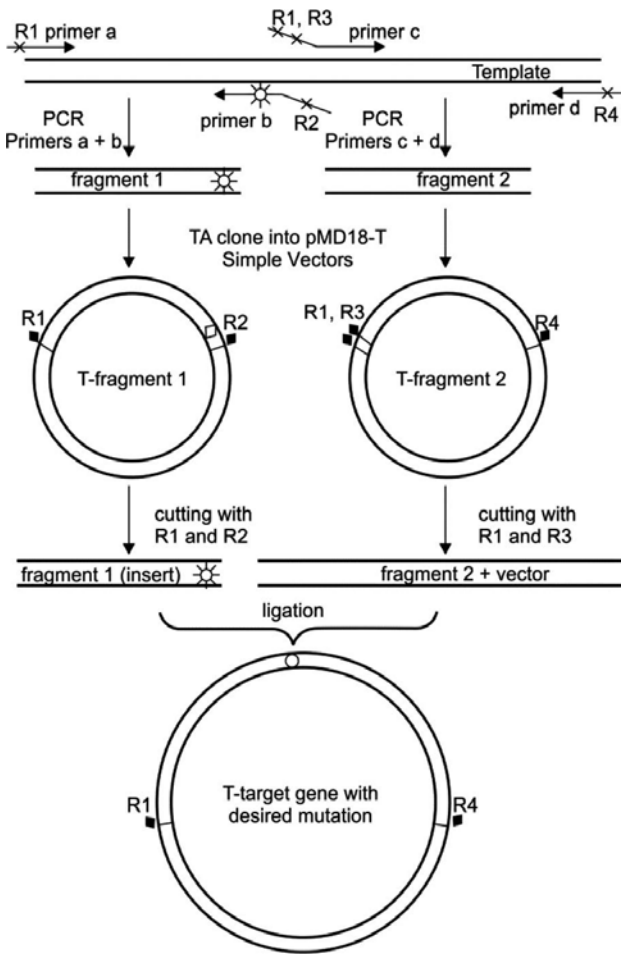

Figure 2. Schematic diagram for site-directed mutagenesis in large constructs. Two target gene segments are amplified from template DNA by PCR using four primers, including two flanking primers (a and d). Restriction enzyme sites (R1 and R4) are incorporated. The mutation of interest (indicated by a star) and/or a same-sense mutation are introduced to create restriction enzyme sites (R2 and R3). Next, PCR fragments are TA-subcloned into T-vectors, and one fragment is cut (R1 and R2) and inserted into the T-vector (predigested by R1 and R3) containing another fragment.

Munteanu et al. [13] used the KOD Hot Start polymerase in combination with high performance liquid chromatography purified primers to achieve site-directed mutagenesis in big plasmid (up to 16 kb). The procedure allowed the incorporation of single or multiple base changes using 6 cycles of PCR instead of 18.

2.4. Site-directed mutagenesis mediated by insertion of restriction sites

The method described by Rouached et al. [15] takes the advantage of the plasticity of the genetic code and the use of compatible restriction sites (**Figure 3**). Method is developed in two steps. First, target DNA is subcloned in a vector, which is the template for the next two PCRs. One reaction amplifies from the start codon to mutagenized site that contains the new introduced restriction site. The other reaction amplifies from the mutagenized site con-

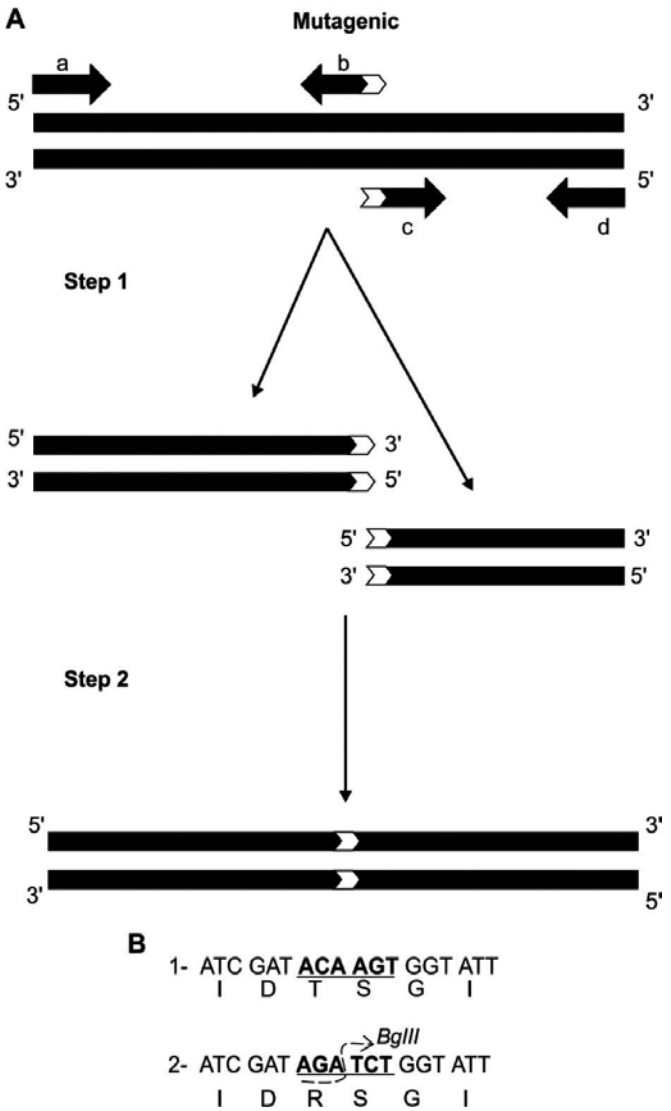

Figure 3. Mutagenesis strategy by restriction enzyme site insertion. Panel A, Two PCR products are obtained from the full length coding sequence. Mutations, including the restriction enzyme site, are contained in primers b and c. PCR product are subcloned by separate in cloning vectors. Next, two fragments are digested with the appropriate enzyme and ligated to reassemble by in-frame coding sequence. Panel B, example of amino acid substitution after introduction of *Bgl*II restriction site.

taining the restriction site to the end of the coding sequence. After amplification, PCR products are digested with the appropriate enzyme and ligated. Primers containing the restriction site are partially overlapped to allow an in-frame assembly of the whole coding sequence [15].

Zhang et al. [16] reported a method of site-directed mutagenesis where they introduced restriction enzyme sites to facilitate the mutant screening (**Figure 4**). The method uses a dsDNA plasmid as a template. In order to select the restriction enzyme sites to be introduced, authors translate the DNA sequence into amino acid sequence, and afterward the amino acid sequence is reversely translated into DNA sequence again with degenerate codons. This approach allows selection of a large number of sequences with silent mutations, which contains several restriction enzyme sites. The transformants are screened by digesting with the appropriate enzyme [16].

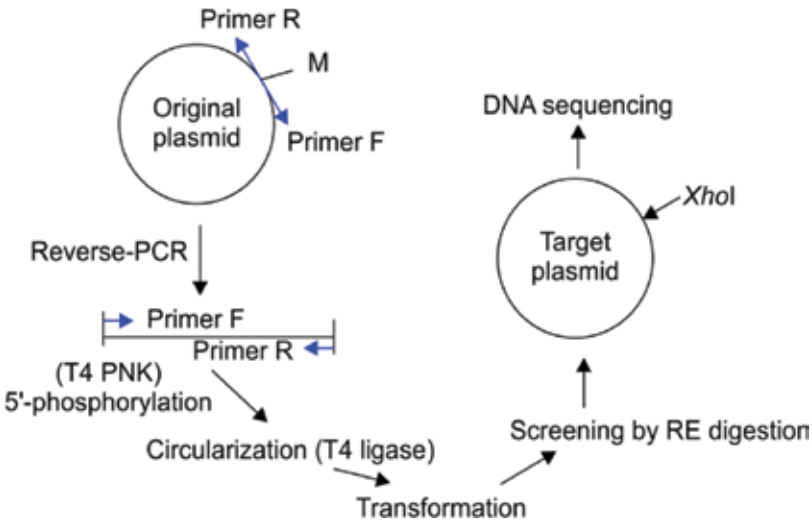

Figure 4. Schematic diagram of the site-directed mutagenesis introducing a restriction enzyme site. Primers contain a new restriction enzyme site. Next to PCR assay, product is phosphorylated and ligated to be transformated in a *E. coli* strain. After restriction enzyme digestion (*Xho*I), plasmid is selected to be sequenced. PNK, polynucleotide kinase; RE, restriction endonuclease.

2.5. High-throughput site-directed mutagenesis using oligonucleotides synthesized on DNA chips

In order to generate a series of constructs with multiple mutants unlimited by the cost of oligonucleotides, Saboulard et al. [17] described the first generation of a library of single and multiple site-directed mutants using a mixture of oligonucleotides synthesized on DNA chips (**Figure 5**). They used the human interleukin15 gene as a model. The library produced 96 different clones in 37 different codons using pools of oligonucleotides. Authors described this approach as straightforward and flexible way to address resolve the problem of massive mutagenesis after successive rounds [17].

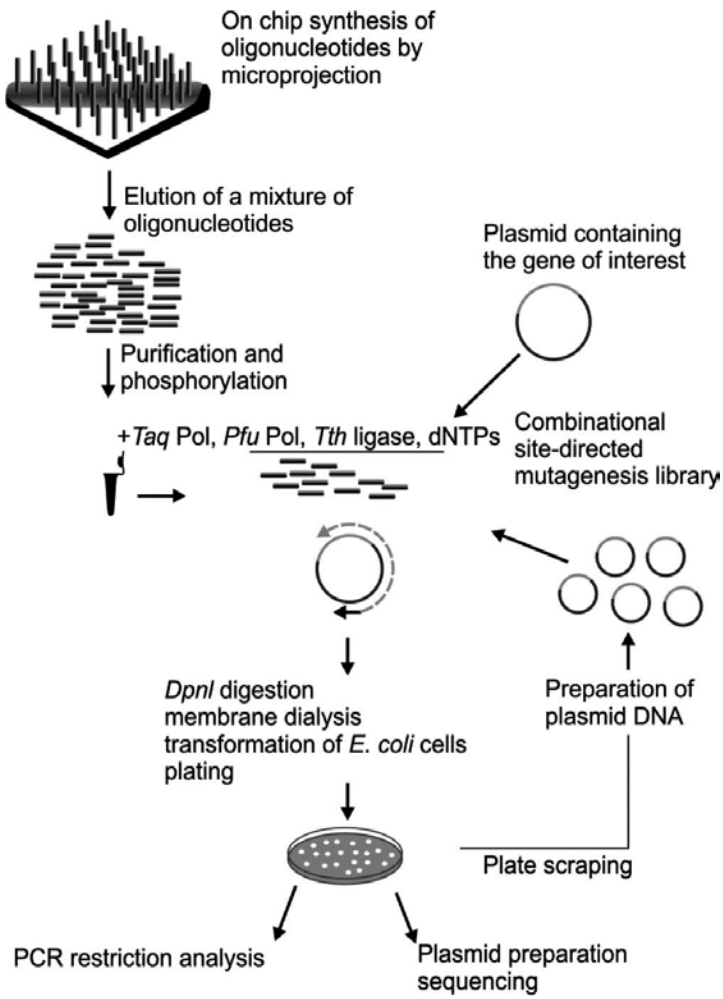

Figure 5. Strategy for massive mutagenesis using oligonucleotides synthesized on DNA chips. The oligonucleotides have been synthesized *in situ* by microprojection on chips. These oligonucleotides, eluted as a mix, are purified and phosphorylated. Next, target plasmid is amplified in a single-strand with the mutagenic oligonucleotides. The amplified product is digested using *Dpn*I, dialyzed and transformed in *E. coli*. Several successive rounds can be performed. Analysis of the clones is made by sequencing and/or by PCR and restriction analysis. Taken from Saboulard et al. [17].

2.6. Methods for multiple site-directed mutagenesis

Fushan et al. [18] developed a method to introduce multiple and complex mutations on plasmids without intermediate subcloning. The procedure is depicted in **Figure 6**. By sequential rounds, each one with PCR amplification with two nonoverlapping pair of primers, the mutation is introduced at the 5′ end of one or both internal primers. Next, PCR products are mixed and ligated to be amplified with external primers. These external primers are suppression adapters to limit the amplification only to target DNA. In order to generate other mutations, an aliquot of the previous reaction is used as a template [18].

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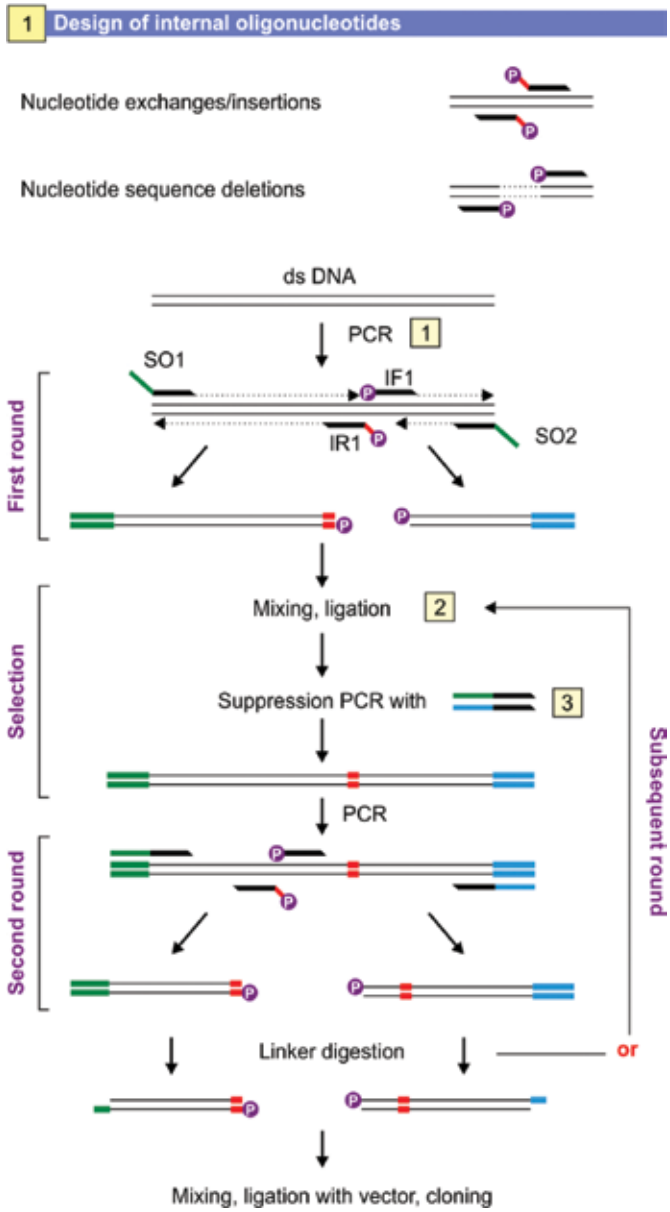

Figure 6. Schematic representation of site-directed mutagenesis by amplification, ligation and suppression PCR. Target DNA is amplified by using two pairs of primers (SO1, IR1, SO2 and IF1). Mutations are on the 5′ end of IR1. After amplification, DNA fragments are ligated generating different types of products (right panel). Then, DNA is amplified with suppression primers SO1 and SO2 where predominant products are type C molecules. After first round of mutagenesis, an aliquot of product is used to the next round to introduce a new mutation. After multiples rounds, the final product is digested with restriction enzymes to be subcloned in a vector.

Another method to generate multiple site-directed mutagenesis is that developed by Holland et al. [19]. They named their method as AXM mutagenesis. Scheme of Holland's method is

shown in **Figure 7**. By PCR, using a prone-error polymerase, a large and mutated DNA fragment is generated with a modified primer containing phosphorothioate linkage at 5′ end. After amplification, a bacteriophage T7-exonuclease treatment allows the removal of strand synthesized with the nonmodified primer. The resulting PCR product is a megaprimer, which is used in a subsequent mutagenesis reaction. The DNA base excision repair pathway in *Escherichia coli* favors the nucleotide base-change to DNA synthesized by megaprimer instead of the complementary uracilated DNA sequence [3, 19]. The method facilitates a rapid generation of multiple mutagenic sites in parallel. A recently modified method of this procedure has been published, where the Eco29k I enzyme is incorporated to selectively degrade the original DNA [3].

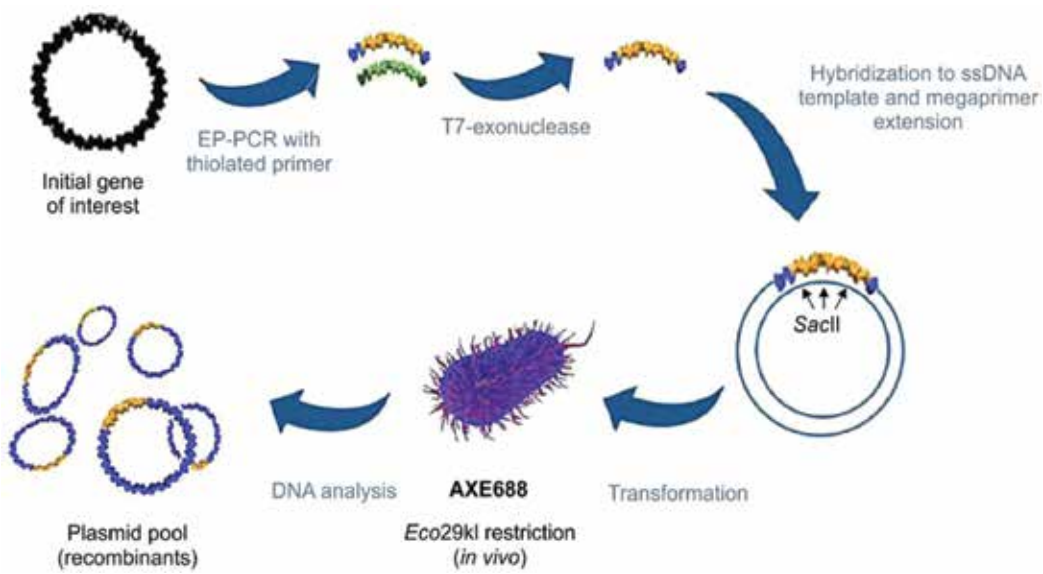

Figure 7. Schematic procedure of AXM mutagenesis. An error-prone PCR (EP-PCR) reaction using a reverse primer containing phosphorothioate linkages on its 5′ end is carried out. The double-stranded DNA is treated with T7 exonuclease to selectively degrade the unmodified strand of the dsDNA molecule. The resulting megaprimer is then annealed to the uracilated, circular, single-stranded phagemid DNA and used to prime *in vitro* synthesis by DNA polymerase. The final product is transformed into *E. coli* AXE688 cells, where the uracilated strand is removed by the uracil N-glycosylase, allowing the survival of the newly synthesized, recombinant strand generated by the megaprimer. The *Eco*29k I enzyme selectively degrades the original DNA retaining *Eco*29k I recognition sites.

2.7. Methods for multi-site-directed mutagenesis based on recombination

Trehan et al. [20] reported a method named REPLACR-mutagenesis (recombineering of Ends of linearized PLAsmids after PCR), which is able to create mutations (substitutions, deletions and insertions) in plasmids by *in vivo* recombineering (**Figure 8**). REPLACR-mutagenesis requires the transformation of PCR products in bacteria expressing Red/ET recombineering proteins. This method can used a variety of plasmids up to bacterial artificial chromosomes [20].

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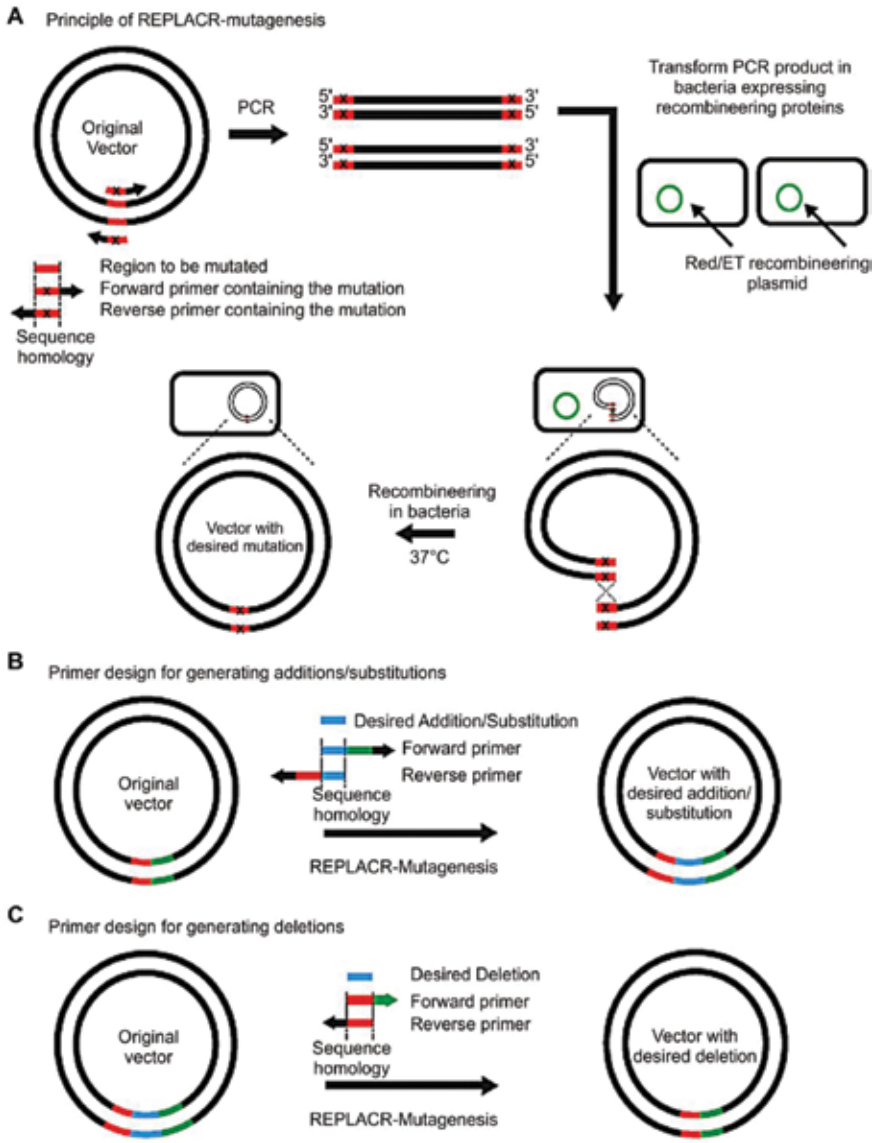

Figure 8. Principle of REPLACR-mutagenesis and primer design strategy for sequence substitution, insertion or deletion. (A) Primers containing the desired mutation overlap for recombination. Bacteria expressing the recombineering proteins (Redγ, β, α and RecA) are transformed with the PCR product. Recombination takes places inside the bacteria and after extraction of plasmid, mutations are confirmed by PCR and sequencing. (B) Example of forward and reverse primers contains the desired addition/substitution. (C) Example for generating deletion mutants, where the forward primer contains the sequence adjoining the sequence to be deleted, and the reverse primer contains a sequence homologous to the forward primer and the adjoining sequence in the vector.

Liang et al. [2] developed a method for the simultaneous introduction of up to three mutations in a plasmid DNA via homologous recombination. The strategy is depicted in **Figure 9**, and it is compatible with a variety of mutations, including degenerate codons in plasmids of different

sizes [2]. The procedure consists of a single multiplex or three independent PCR assays. Each pair of primers contains the desired mutation. Final PCR products have homology at end-terminal to be recombinated. After PCR, a 15-min pulse of recombination activity is carried out and sample is transformed in *E. coli*.

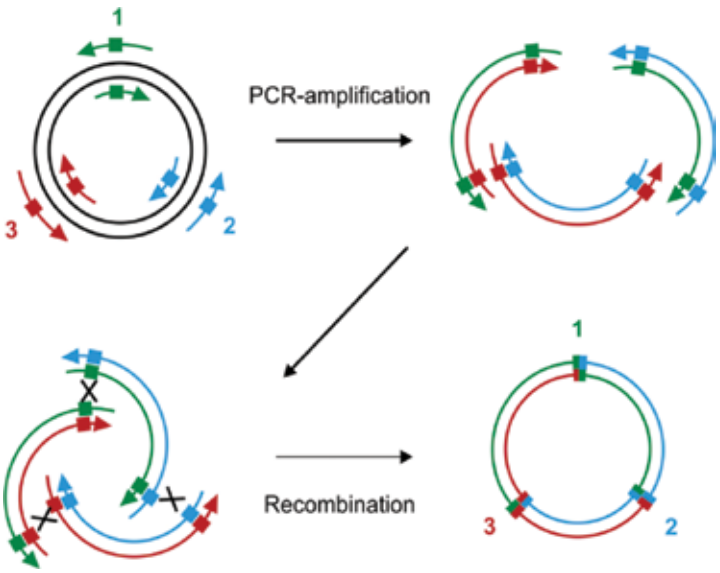

Figure 9. Strategy for multi-site-directed mutagenesis by homologous recombination. Numbers denote the mutation sites. To introduce three mutated sites, typically three pairs of overlapping forward and reverse primers are used unless a pair of long primers is used to cover the mutation sites that are in very close proximity.

2.8. Software tools to design site-directed mutagenesis primers introducing "silent" restriction sites

The critical points of site-directed mutagenesis are the primer design and the annealing temperature. Specific software programs, such as Primer Generator and SiteFind [21, 22], can be used for the design of a restriction enzyme site within the mutation primers without altering the translated amino acid sequence [12].

For example, SiteFind allows the introduction of a restriction site near to the point mutation in manner such that the restriction site has no effect on the peptide sequence. Based on the redundancy of genetic code, a peptide can be encoded by different DNA sequences. Then, the novel restriction site can be used as a marker to be easily screened [22]. The software can work with sequences up to 400 bp.

Another program developed is SDM-Assist, which creates primers to site-directed mutagenesis based on their thermodynamic characteristics. The primer contains the desired mutation and a restriction site for identification of mutant constructs. The algorithm consider factors such as Tm, GC content, and secondary structure [23].

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2.9. Conclusion—key results

The site-directed mutagenesis using PCR has been used in molecular biology to modify gene sequences. Methods described here have allowed the introduction of single or multiple mutations into the same target. Despite the wide range of commercial kits for site-directed mutagenesis, there is a constant search to improve the efficiency and simplicity, with a concomitant reducing of costs.

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