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FOOD SCIENCE & TECHNOLOGY | REVIEW ARTICLE

Detection of foodborne pathogens by qPCR: A practical approach for food industry applications

María-José Chapela^{1*}, Alejandro Garrido-Maestu^{2,3} and Ana G. Cabado³

Abstract: Microbiological analysis of food is an integrated part of microbial safety management in the food chain. Monitoring and controlling foodborne pathogens are traditionally carried out by conventional microbiological methods based on culture-dependent approaches in control laboratories and private companies. However, polymerase chain reaction (PCR) has revolutionized microbiological analysis allowing detection of pathogenic microorganisms in food, without the necessity of classical isolation and identification. However, at present, PCR and quantitative polymerase chain reaction (qPCR) are essential analytical tools for researchers working in the field of foodborne pathogens. This manuscript reviews recently described qPCR methods applied for foodborne bacteria detection, serving as economical, safe, and reliable alternatives for application in the food industry and control laboratories. Multiplex qPCR, which allows the simultaneous detection of more than one pathogen in one single reaction, saving considerable effort, time, and money, is emphasized in the article.

Subjects: Science; Microbiology; Food Science & Technology; Food Analysis; Food Microbiology

Keywords: PCR; qPCR; pathogen; detection; industry

1. Introduction

In order to ensure food safety, it is necessary to consider all aspects of the food production chain. The free movement of safe and wholesome food is an essential aspect of the internal market and contributes significantly to the health and well-being of citizens, and to their social and economic interests. A high level of protection of human life and health should be assured in the pursuit of community policies (EC, 2002).

ABOUT THE AUTHORS

The authors perform their research activity in the Food Safety Division and Microbiology and Bioassays Laboratory of ANFACO-CECOPECA. Here, they have developed an extensive work on development of new methods and implementations, in order to provide fast and reliable tools which are intended for food industry, with the final goal of enhancing food safety, by determining the cause specific problems such as marine biotoxins and foodborne pathogens detection, control, reduction, and/or elimination from their food products.

PUBLIC INTEREST STATEMENT

This manuscript is intended to provide readers, especially for those who develop their professional activity related in the food industry, a general idea about how molecular methods, particularly qPCR, may be applied for controlling foodborne pathogens. Two main fields are covered, on the one hand, a more scientifically oriented overview of PCR/qPCR is included, summarizing from the technique fundamentals to primer/probe design and advanced multiplex applications. On the other hand, a detailed description of available methodologies is provided for the different bacterial pathogens covered, intended to be helpful for people looking for direct industry applications.

During the year 2012, the number of food alerts and notifications received through the European alert information system or Rapid Alert system for Food and Feed (<http://www.efsa.europa.eu>) has risen to a total of 2,801 for food products. This number includes different contaminants, of which 462 (16%) have been alerts, 869 (31.5%) have been information notifications, and 1476 (52.5%) were rejections in border. Microbiological contamination corresponds to 21% of notifications, that *Salmonella* spp. has the highest risk and causes 49% of the microbiological contamination (Agroalimentaria, 2012). All these notifications and risks have led to extensive analyses of foodstuffs before their release and distribution to the market.

Networking of laboratories of excellence, at regional and/or interregional level, with the aim of ensuring continuous monitoring of food safety, could play an important role in the prevention of potential health risks for consumers as well as of unnecessary economic costs. In this sense, the availability of quick and quality assays allows giving precise results in a few hours and results as a great economic and safe advantage. Among microbiological risks that can lead to foodborne diseases, *Salmonella*, *Listeria monocytogenes*, and *Escherichia coli* are pathogenic micro-organisms that keep rising in numbers through recent years (Commission, 2011). According to several ISOs: ISO 6579, ISO 11290-1 and -2, and ISO 16654 for *Salmonella*, *L. monocytogenes*, and *E. coli*, respectively (ISO, 1996, 1998, 2001, 2003) complete analyses for these pathogens can take from two to seven days since the sample arrives to the laboratory. This period can be longer if the pathogenic serotype have to be determined, what means that foodstuffs have to be immobilized and waiting for the release until obtaining the final results. It is worth to mention that if the food is fresh, it can be completely spoiled and even if is a ready-to-eat product, it can loss the quality and not be allowed for consumption. In the case of products with a short shelf life, if the immobilization is very long, the foodstuffs can reach the expiry date or be close to that with all the consequent commercial problems that can prevent to place these products in the market and all derived economic losses included those that have to be paid due to the destruction of food.

All these facts highlight the necessity of the development of fast, feasible, and accurate analytical methods in order to allow a product to be released immediately or, at least, in a very short period of time, to prevent or decrease no necessary costs and food spoilage. Figure 1 shows a schematic comparison of traditional culture methods and quantitative polymerase chain reaction (qPCR) detection methods.

2. Impact of multiplex real-time polymerase chain reaction in routine food control

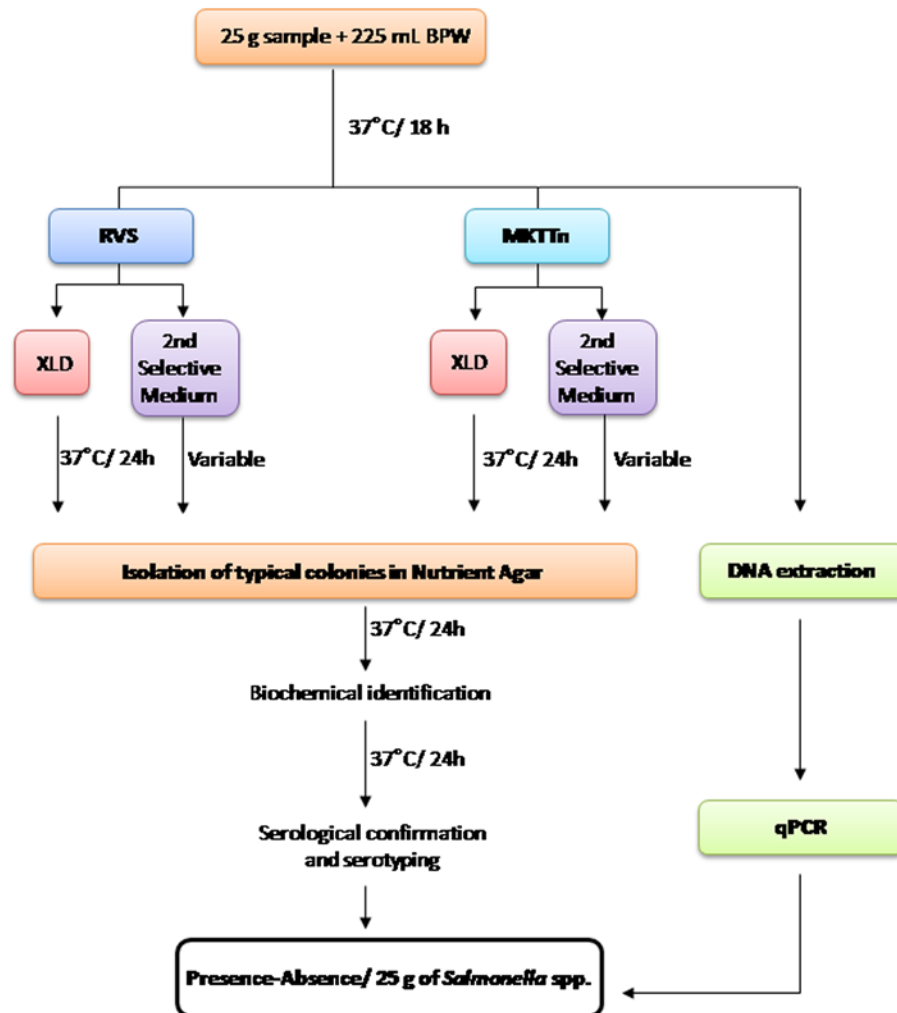
The food microbiological testing is experiencing an exciting momentum due to the development in the analytical field of new and accurate approaches. Polymerase chain reaction (PCR) has revolutionized traditional microbiological analysis by allowing detection of pathogenic micro-organisms directly in the food, without the necessity of classical isolation and identification. Since its discovery it has become an essential analytical tool for researchers working in the foodborne pathogens field (Wernars, Delfgou, Soentoro, & Notermans, 1991; Wernars, Heuvelman, Chakraborty, & Notermans, 1991). This technique was used in more than 300,000 scientific publications, and was applied in different areas of research, including food safety, due to its versatility, specificity, and sensitivity. In recent years, PCR has been successfully applied in food science for microorganisms identification (Rodríguez-Lazaro et al., 2007), for detection of ingredients in food products (Lopez-Calleja et al., 2013) and different meats and fish species identification (Chapela et al., 2002; Mackie et al., 1999; Quinteiro et al., 1998; Rodríguez-Lázaro, Cook, & Hernández, 2013; Soares, Amaral, Oliveira, & Mafra, 2013; Sotelo et al., 2001; Zhang, 2013).

2.1. PCR and multiplex PCR

Conventional PCR can be described as an exponential amplification of a DNA fragment. Its principle is based on the mechanism of DNA replication *in vivo*, i.e. double-stranded DNA (dsDNA) is denatured to single-stranded DNA (ssDNA) and duplicated by a DNA polymerase (Higuchi, Fockler, Dollinger, & Watson, 1993). Usually, PCR is performed using one pair of primers to amplify a specific sequence; however, multiplex PCR allows the simultaneous amplification of more than one target sequence in

Figure 1. Schematic comparison of traditional culture methods and qPCR detection methods.

Notes: BPW: Buffered Peptone Water; RVS: Rappaport-Vassiliadis Soya; XLD: Xylose Lysine Deoxycholate agar; MKTTn: Muller-Kauffmann Tetrathionate-Novobiocin Broth.



a single reaction by using different sets of primers (Elnifro, Ashshi, Cooper, & Klapper, 2000). This option saves considerable time and effort, and decreases the number of reactions that need to be performed to detect the desired targets in the sample. However, the presence of many PCR primers in a single tube can cause some problems, such as the increased formation of misprimed PCR products, known as “primer dimers,” and the amplification of unspecific DNA fragments. Primers should be chosen with similar annealing temperatures for multiplex PCR assays. Also, the lengths of amplified products should be similar, since large differences in the lengths of the target DNAs may favor the amplification of shorter targets over the longer ones, which results in different yields of amplified products. In standard PCR protocols, amplification products are detected by gel electrophoresis using UV luminescence and the ethidium bromide double-stranded DNA intercalating dye or similar non-toxic compounds. These procedures have the big disadvantage of relying on end-point analysis; therefore, amplification products cannot give quantitative information regarding the initial amount of target molecules (Rodríguez-Lázaro & Hernández, 2013).

2.2. qPCR and multiplex qPCR

Higuchi and collaborators (Higuchi, Dollinger, Walsh, & Griffith, 1992; Higuchi et al., 1993) constructed a system that detects PCR products as soon as they accumulate. This was the beginning of the real time or qPCR, also known as the second generation of PCR. qPCR allows the monitoring of the amplification process in real time by using fluorescence, and not only at the end of the reaction as occurs in conventional PCR. Its major advantages are that it is a fast and easy-to-perform analysis,

the extremely wide dynamic range of quantification (more than eight orders of magnitude), and the significantly higher reliability and sensitivity of the results compared to conventional PCR. Since the first scientific work published in 1996 (Heid, Stevens, Livak, & Williams, 1996), the number of publications where qPCR is used has increased nearly exponentially. Specific detection and quantification of pathogens in food by qPCR has been evaluated for a wide variety of micro-organisms, with emphasis on the main foodborne pathogens responsible for important medical and economic outbreaks: *Salmonella* spp., *L. monocytogenes*, *E. coli* O157:H7, and *Staphylococcus aureus*, among others (Rodríguez-Lázaro et al., 2013).

The major advantage of this molecular method with respect to standard methods is the shorter time required to obtain reliable results. Typical culture methods as ISO or FDA Bacteriological Analytical Manual—for *Shigella* spp., *E. coli* O157, and different human pathogenic *Vibrio* spp. (*V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*) detection—need two days to guarantee a negative result. Regarding *Salmonella* spp. and *L. monocytogenes*, three and five days are needed for a definitive negative result. In contrast, when a positive result is found, the duration of the analyses is greatly extended as additional isolation and identification steps must be performed. However, recent studies have demonstrated that times can be significantly shortened to only 24 h by optimizing qPCR methods for most of these bacteria (Chapela et al., 2010; Garrido, Chapela, Ferreira, et al., 2012; Garrido, Chapela, Román, et al., 2012; Garrido, Chapela, Román, Fajardo, Lago, et al., 2013).

2.3. Digital PCR

Digital PCR (dPCR) allows the precise quantification of nucleic acids, facilitating the measurement of small percentage differences and quantification of rare variants. It was reported to be more reproducible and less susceptible to inhibition than qPCR. Its main applications are: rare variant measurement, molecular counting, and applications that need higher precision. Due to its growing interest, a “Minimum Information for Publication of Quantitative dPCR Experiments” guide has been recently published (Huggett et al., 2013).

2.4. Limit of detection and limit of quantification of qPCR

Concerning the limit of detection (LOD) of qPCR, it was evaluated in several multiplex studies targeting different types of bacteria (including Gram positive and Gram negative) from different origins. Obtained results were similar to traditional culture methods or even lower, with values in general below 10 CFU/25 g, with the added advantage that, with multiplex qPCR, several targets were detected simultaneously (Garrido, Chapela, Ferreira, et al., 2012; Garrido, Chapela, Román, et al., 2012; Garrido, Chapela, Román, Fajardo, Lago, et al., 2013). Low levels of target pathogens can also be detected in food matrices contaminated by other dominant micro-organisms. Garrido and collaborators demonstrated in two independent studies that *L. monocytogenes* could be successfully enriched and detected in the presence of high initial numbers of other interfering bacteria, generally above 10^6 CFU/25 g (Garrido, Chapela, Román, Fajardo, Lago, et al., 2013).

However, in a great number of cases, the detection and quantification limits obtained without enrichment of the food samples prior to performing qPCR were in the range of 102–103 CFU/g (or ml) (Hierro, Esteve-Zarzoso, Gonzalez, Mas, & Guillamon, 2006; Takahashi et al., 2009). Therefore, in order to meet the microbiological criteria required by international legislations for foodstuffs, it is sometimes necessary to associate qPCR with an enrichment step of a few hours.

When qPCR was used, not only for detection, but also as a quantitative tool, linear quantifications were reported over a large range of at least five logs and very good correlations with plate counts were obtained. In some cases, however, discrepancies between microbiological counts and qPCR have been reported with higher bacterial counts with the molecular method (Martínez-Blanch, Sánchez, Garay, & Aznar, 2009). Several reasons can explain the differences: (1) the presence of intact DNA from dead cells, (2) the presence of viable but non-culturable forms, which can be quantified by qPCR but not by plate counts, (3) the fact that one CFU on plate might be generated from more than one cell, and (4) the use of PCR primers targeting varying numbers of multicopy genes

(e.g. 16S rRNA). An important distinction should be made between LOD and quantification limits (LOQ). This distinction may lead to confusing comparisons between sensitivities of different methods. The LOD is the lowest population of micro-organisms that can be detected by the method. The LOQ is the minimal population that can be accurately quantified. Usually, LOD is lower than LOQ, meaning that when qPCR gives a positive signal, the amount of template is too low and provides a quantification cycle (Cq) that falls out of the linear range of quantification curves. When quantifying pathogens in food, the lower LOQ in the food matrix should be considered and not the lower LOQ obtained from pure cultures because it takes into account the efficiency of nucleic acid extraction and possible interactions of food components with PCR amplification. In agreement with EN ISO 22174:2005, a standard for PCR application in order to detect foodborne pathogens, and with experts in PCR/qPCR (Hoorfar et al., 2003; Hoorfar, Cook, et al., 2004; Hoorfar, Malorny, et al., 2004; ISO, 2005), an increasing number of studies have included an internal amplification control to qPCR protocols, to avoid negative deviations (Calvó, Martínez-Planells, Pardos-Bosch, & Garcia-Gil, 2008; Garrido, Chapela, Ferreira, et al., 2012; Garrido, Chapela, Román, et al., 2012; Garrido, Chapela, Román, Fajardo, Lago, et al., 2013; Jofré et al., 2005; Nordstrom, Vickery, Blackstone, Murray, & DePaola, 2007).

Nowadays, the current trend is moving towards identification of several pathogens in the same reaction tube, by applying multiplex amplification. Some qPCR technologies are particularly adapted to multiplexing. For instance, using TaqMan chemistry, several sequence-specific probes can be labeled with different fluorophores and many targets can be co-amplified and quantified within a single reaction. By contrast, in theory, the widely used SYBR Green chemistry does not allow multiplexing reactions because binding takes place non-specifically in the presence of DNA. However, some authors have circumvented the problem and successfully proposed multiplex qPCR protocols with SYBR Green by performing subsequent melting curve analysis. Distinct melt curves were obtained for each target micro-organism (Wehrle, Didier, Moravek, Dietrich, & Märtlbauer, 2010). Therefore, multiplex qPCR is an interesting tool to quickly detect different genera or species which are potentially present in the same food matrices.

qPCR has revolutionized the molecular approaches in food microbiology; specific micro-organisms can be quantified in food, and there is no need for post-amplification treatment of the samples, such as gel electrophoresis, saving time and efforts. Currently, a high number of qPCR chemistries are commercially available. These can be divided into those that are not sequence specific, such as DNA minor groove-binding dyes, and those that are sequence specific and that allow simultaneous detection and confirmation of the target amplicon during the PCR reaction (Rodríguez-Lázaro et al., 2013).

2.5. qPCR primers and probes design

When a qPCR assay is designed, the most important parameters are the amplicon length and the T_m of the primers and probe. The optimal amplicon length should be less than 150 bp; however, amplicons up to 300 bp amplify efficiently. Shorter amplicons amplify more efficiently than longer ones and are more tolerant to suboptimal reaction conditions. See Table 1 for details regarding TaqMan® probes assays that may be extensive to other type of qPCR methods.

Table 1. General guide for qPCR hydrolysis probes design

Primers length	20–24 bp
Primers T _m	60 ± 2°C
Amplicon size	140–300 bp
Probe length	20–24 bp
Probe T _m	Primer T _m + 10°C
Additional considerations	Avoid “G” in 5’ end, secondary and repetitive structures
	Probe placed no more than 10 bp from the forward primer and not less than 2 bp

Notes: T_m is the melting temperature; “G” is the guanine; and “bp” is the base pairs.

3. Importance of sample preparation methods

3.1. Enrichment broths

The combination of microbiological enrichment and molecular pathogen detection is a useful link between traditional and molecular microbiology. The advantages of both methods add up, and the disadvantages are partly eliminated. Then, the LOD of qPCR is far exceeded after a successful enrichment. Short enrichments, if performed in an appropriate way, are mostly sufficient to provide enough DNA for detection (Rossmanith & Wagner, 2010). In general, pathogenic bacteria are present in foods in very low numbers; thus, an enrichment step to increase their number is crucial in order to guarantee the achievement of legal limits (absence in 25 g for most bacterial pathogens). Extensive evaluation of optimal enrichment conditions, as well as incubation times and DNA extraction protocols has been approached for different pathogens (Gattuso et al., 2014; Rodríguez-Lázaro, Gonzalez-García, Gattuso, Gianfranceschi, & Hernandez, 2014).

When facing multipathogen detection, a popular approach is to select different broths for the enrichment of every pathogen; then DNA is extracted and multiplex qPCR is performed (Elizaquível, Gabaldón, & Aznar, 2011; Jofré et al., 2005; Ruiz-Rueda, Soler, & Calvo, 2010). An alternative is the use of a common broth for all the target pathogens, but the broth design and its performance must be carefully evaluated. In this sense, one of the first broths designed for several bacteria was the Universal Pre-enrichment Broth (UPB) for the simultaneous recovery of *Salmonella* spp. and *L. monocytogenes* (Bailey & Cox, 1992). This broth, which is still in use (Bhagwat, 2004; Hammack, Amaguana, Johnson, & Andrews, 2003), does not contain any inhibitory substance, allowing a better recovery of the bacteria due to its high buffer concentration. Previous studies have demonstrated that methods which incorporate a non-selective enrichment step gave better results than completely selective media, specially for the recovery of slow growing bacteria such as *L. monocytogenes* (Rijpens & Herman, 2004). Following this concept, Kawasaki et al. developed a similar broth named N17 (Kawasaki et al., 2005) which has been applied in different studies for recovery of *Salmonella* spp., *L. monocytogenes*, and *E. coli* O157 (Kamisaki-Horikoshi et al., 2011; Kawasaki et al., 2009, 2010, 2011; Mahmuda & Kawamoto, 2007). The N17 broth was modified in later studies by independent laboratories for an optimal performance by eliminating dextrose (Omiccioli, Amagliani, Brandi, & Magnani, 2009), and later on, the final pH of the broth was also modified by adjusting the buffering salts concentrations. This optimized broth was successfully applied for the simultaneous enrichment of *Salmonella* spp., *L. monocytogenes*, *Shigella* spp., and *E. coli* O157 (Garrido, Chapela, Román, et al., 2012; Garrido, Chapela, Román, Fajardo, Lago, et al., 2013). Excellent results were obtained with the modified version of N17 broth with all the bacteria tested, obtaining colony counts higher than 10⁶ CFU/ml for all microorganisms tested. Another non-selective enrichment broth recently developed is the simultaneous enrichment broth (SEB) developed by Kobayashi et al. SEB was applied for the simultaneous recovery of *Salmonella* spp., *L. monocytogenes*, *S. aureus*, *Bacillus cereus*, *Vibrio parahaemolyticus*, and *E. coli* O157:H7; and its performance was compared to tryptic soy broth (TSB) (Kobayashi et al., 2009). Even though satisfactory results were obtained when compared against TSB, further studies need to be performed with natural and spiked food and environmental samples.

A different approach was done by other researchers who developed selective enrichment broths for the simultaneous recovery of different bacteria, like the SEB SEL that included four antimicrobial agents—acriflavine, cycloheximide, fosfomicin, and nalidixic acid. SEL showed comparable results to UPB (Kim & Bhunia, 2008) and it was successfully applied for the recovery of *Salmonella*, *L. monocytogenes*, and *E. coli* O157 in meat products (Suo, He, Tu, & Shi, 2010).

3.2. DNA extraction protocols

Inhibition of the PCR amplification reaction is one of the most limiting factors of the PCR-based methods and it can cause complete reaction failure, leading to false negative results or reducing sensitivity of detection. Inhibition might be driven by interference with the cell lysis necessary for DNA extraction, by nucleic acid degradation or capture, and by inhibition of the polymerase activity

necessary for amplification of the target DNA. Organic and phenolic compounds and polysaccharides are also possible inhibitors (Elizaquivel & Aznar, 2008). In this sense, a critical factor when facing multipathogen detection is the selection of an appropriate DNA extraction protocol. In addition, the type of bacteria of interest must be kept in mind as it is more difficult to extract DNA from Gram positive bacteria, than from Gram negative. For Gram negative bacteria, a thermal cell lysis in the presence of a chelating resin—e.g. 6–10% (w/v) Chelex-100 suspension—is usually enough to obtain good-quality DNA (Garrido, Chapela, Román, et al., 2012; Kim & Cho, 2010; Malorny, Huehn, Dieckmann, Kramer, & Helmuth, 2009; Rodriguez-Lazaro, Jofre, Aymerich, Hugas, & Pla, 2004). Reyes-Escogido et al. developed a chelex-based method that replaced the boiling water bath by a microwave heating procedure, as the thermal lysis source, and by adding proteinase K and RNase A obtained large amounts of highly pure DNA from Gram positive and negative bacteria (Reyes-Escogido et al., 2010); but this method has been scarcely used.

In-house developed protocols have shown appropriate results regarding the quantity and purity of DNA extracted from pure and mixed cultures, as well as from natural food and environmental samples. Kawasaki et al. developed and compared a DNA extraction protocol based on the use of a strong chaotropic agent (guanidine isothiocyanate) combined with a surfactant agent (Tween 20) and an enzymatic lysis step (combination of lysozyme and achromopeptidase enhanced the enzymatic extraction) (Kawasaki et al., 2005). This protocol was successfully applied in different studies and compared against other in-house protocols, showing similar or better results, specially focused on Gram positive bacteria (Garrido, Chapela, Román, Fajardo, Lago, et al., 2013; Garrido, Chapela, Román, Fajardo, Vieites, et al., 2013; Germini, Masola, Carnevali, & Marchelli, 2009; Mahmuda & Kawamoto, 2007). Later studies reported that DNA extraction may be enhanced by the addition of a filtration step through a 11–5 µm sterile micropore filter (Kawasaki et al., 2009; Rodriguez-Lazaro, Jofre, et al., 2004), being this in agreement with other studies reporting that the presence of food particles should be avoided (Malorny et al., 2009) as it may have different effects in the qPCR reaction, like the C_q of the final fluorescence obtained (Wright et al., 2007).

On the opposite side of in-house protocols, are commercial DNA extraction kits. Different studies have been applied and several of these kits were compared, like PrepMan Ultra® (Applied Biosystems), InstaGene® (BioRad), DNeasy Tissue Kit® (Quiagen), UltraClean Microbial DNA isolation kit® (MoBio), NucleoSpin Tissue Kit® (Machery-Nagel), Nexttec kit for genomic DNA from Bacteria® (Nexttec), QIAmp DNA stool mini kit® (Quiagen), and High Pure PCR template® (Roche), among others (Elizaquivel & Aznar, 2008; Flekna, Schneeweiss, Smulders, Wagner, & Hein, 2007). Out of these, PrepMan Ultra® and InstaGene® have been extensively applied, especially with Gram negative bacteria. The DNeasy Tissue Kit is one of the most frequently selected kit as it has proven suitable for different types of samples and with different types of micro-organisms (Elizaquivel & Aznar, 2008; Elizaquivel et al., 2011; Jofré et al., 2005; Kobayashi et al., 2009; McGuinness et al., 2009).

4. Target genes and micro-organisms

The bacterial ribosomal operon (16S rRNA, 23S rRNA, and intergenic spacer (IGS) region) has been frequently used as target for PCR amplification. It is ubiquitous, bears both variable and highly conserved sequences, is easily available from public databases for many species, and often results in sensitive detection due to its multicopy nature. Detailed sequence analysis has demonstrated that the 16S rRNA gene is suitable for accurate PCR identification of many pathogens (Chakravorty, Helb, Burday, Connell, & Alland, 2007). While its discriminating power might be sufficient for some genera and species, it is not always enough to distinguish closely related species (e.g. for enterococci or *B. cereus* group members). Therefore, other housekeeping genes have been studied, as well as functional genes involved in virulence or metabolism. Greater availability of genome sequences, generalization of high-throughput molecular tools and progress in computational genomics are facilitating the set up of new qPCR protocols based on a large variety of genes, thus increasing test specificity.

Nowadays, bacterial foodborne pathogen detection by PCR and qPCR is based on the detection of one or multiple specific genes; thus, correct selection of the target along with the design/selection

of the primers and probes is a key factor for the correct identification of bacterial hazards. For every single pathogenic bacterium, there is a plethora of possible gene targets; thus lack of consensus is a general situation, even though a higher degree of agreement may be observed for the detection of certain micro-organisms.

According to the FoodNet, in 2012, 19,531 cases of infection were laboratory confirmed in the US, being the main bacterial pathogens identified *Salmonella* spp. (16.42%), *Campylobacter* spp. (14.30%), *Shigella* spp. (4.50%), Shiga-toxin-producing serotypes (STEC) non-O157 (1.16%), STEC O157 (1.12%), *Vibrio* spp. (0.41%), and *Listeria* (0.25%) (CDC, 2013). These data highlight the importance that these pathogens still represent for human health, even some have been studied for long time.

4.1. Legislated pathogens according to Commission Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs

4.1.1. *Salmonella* spp.

Nowadays, several alternatives exist for the specific detection of *Salmonella* spp. These include the *bipA* which encodes a protein of the guanosine -5'-triphosphate (GTP)-elongation binding family that displays global modulating properties by regulating a wide variety of downstream processes (Calvó et al., 2008), the *fimC*, which is involved in the synthesis of type 1 fimbria, and has been successfully applied for the detection of the species *S. enterica* (Krascsenicsová, Píknová, Kaclíková, & Kuchta, 2008; Píknová, Kaclíková, Pangallo, Polek, & Kuchta, 2005), the *ttrRSBCA* locus, which is located near the *Salmonella* pathogenicity island 2 at centisome 30.5. It is required for tetrathionate respiration in *Salmonella* (Hyeon et al., 2010; Malorny et al., 2004). Delibato et al., reported a European interlaboratory validation based on the method described by Josefsen et al., (Delibato et al., 2014; Josefsen, Krause, Hansen, & Hoorfar, 2007). Another gene used for *Salmonella* detection is the *iagA*, the invasion associated gene (Chua & Bhagwat, 2009).

One of the most frequently used genes to perform the detection of *Salmonella* spp. is the *invA*. This gene allows the entrance of the bacterium in the intestinal epithelial cells. Even though previous authors have described the existence of strains lacking this gene, like *S. Saintpaul*, while others stated, in the case of this strain, that they could successfully detect it. It was hypothesized that strains lacking this gene could not penetrate the intestinal cells, thus not being able to cause disease. A similar situation is observed with serovars Litchfield and Senftenberg, although, in general, absence of *invA* seems to be rare (Anderson et al., 2011; Calvó et al., 2008; Cheng et al., 2008; D'Urso et al., 2009; González-Escalona, Brown, & Zhang, 2012; Jofré et al., 2005; Kimura et al., 1999; Malorny, Hoorfar, Bunge, & Helmuth, 2003; Malorny et al., 2004; Rahn et al., 1992). Even though these slight discrepancies were observed by different authors, independent laboratories have selected this target for interlaboratory validation of different PCR and qPCR methods like the study of Malorny et al. (2003, 2004) in Europe, or the one carried out by Cheng et al. in the USA (Cheng et al., 2008; Cheng, Van, Lin, & Ruby, 2009).

Recently, Malorny et al. (2007) developed a qPCR method for the specific detection of serovar Enteritidis. This is of special relevance, since in the last amendment of a European Regulation 2073/2005 (EC, 2005; EU, 2011), it is specifically pointed out that the investigation of *Salmonella* spp. in chicken meat should be restricted to serovars Enteritidis and Typhimurium. The method targeted the *Prot6e* gene located on the *S. Enteritidis* specific 60-kb virulence plasmid. Even though the gene is promising, further studies should be performed, due to the fact that it fails to detect 4 out of the 79 isolates tested as they lacked the plasmid. Previous studies targeted the *sefA* gene encoding a fimbrial antigen termed SEF14. This gene is exclusively unique to *Salmonella* serogroup D serovars such as Gallinarum, Pullorum, Dublin, Rostock, Semban, and Typhi, and consequently not specific enough to identify only *S. Enteritidis*. Another conventional PCR assay used a chromosomal located fragment, called *SdfI* which has a high exclusivity but did not detect phage types 6A, 9A, 11, 16, 20, and 27 and was only tested on pure cultures (Malorny et al., 2007).

4.1.2. *Listeria monocytogenes*

Concerning *L. monocytogenes*, the most widely used targets for its specific detection are the genes *hlyA* which encodes for the listeriolysin O (Elizaquível et al., 2011; Kobayashi et al., 2009; Navas et al., 2006; Omiccioli et al., 2009; Rodriguez-Lazaro, Hernández, et al., 2004; Ruiz-Rueda et al., 2010; Suo et al., 2010), and the *prfA* which is a central virulence regulator of this bacterium (D'Urso et al., 2009; Jofré et al., 2005; Rossmanith, Krassnig, Wagner, & Hein, 2006). Additionally, the *iap* gene, coding the protein p60 essential for cell viability, has also been applied (Flekna, Stefanic, et al., 2007; Mukhopadhyay & Mukhopadhyay, 2007; Wuenscher, Köhler, Bubert, Gerike, & Goebel, 1993).

The most popular targets are *hlyA* and *prfA*. Regarding the former, the qPCR method described by Rodriguez-Lazaro, Jofre, et al. (2004) was included in a European interlaboratory assay to evaluate different methodologies' performance (Dalmaso et al., 2014). Later on, the same group of laboratories performed its validation (Gianfranceschi et al., 2014). Concerning *prfA*, it was selected for an interlaboratory validation study involving several laboratories in Europe (D'Agostino et al., 2004). In a later different report, a specific fluorescent probe was designed in order to adapt the method to qPCR (Rossmanith et al., 2006) and extensively evaluated with natural samples (Rossmanith, Mester, Wagner, & Schoder, 2010). Primers and probe targeting *prfA* were recently adopted in an in-house multiplex qPCR method for the simultaneous detection of *Salmonella* spp., *E. coli* O157, and *L. monocytogenes* returning comparable results to those of the original studies where *L. monocytogenes* was the only target (Garrido, Chapela, Román, Fajardo, Vieites, et al., 2013).

4.2. Emerging pathogens

4.2.1. *Campylobacter* spp.

Campylobacter spp. Detection has been performed in two different ways, either the main pathogenic species (*C. jejuni*, *C. coli*, and *C. lari*) or the complete genus. Different genes have been described for each species like *hipO* (gene encoding the hippuricase), *mapA* (membrane-associated protein), *rpoB* (gene encoding the β -subunit of the RNA polymerase), and the VS1 sequence of *C. jejuni*'s genome (Debretson, Habtemariam, Wilson, Nganwa, & Yehualaeshet, 2007; He et al., 2010; Mayr et al., 2010; Rantsiou, Lamberti, & Coccolin, 2010; Yang, Jiang, Huang, Zhu, & Yin, 2003).

Regarding *C. lari*, the *gyrA* (a fragment of the gyrase A subunit gene) and *pepT* (gene encoding a peptidase) have been used for the specific detection of this species.

Finally, the *ceuE* (periplasmic substrate binding protein) and the *cdtA* (gene encoding a subunit of cytolethal toxin, an important virulence factor) have been applied (He et al., 2010; Mayr et al., 2010). As it can be observed, there is not much agreement on the PCR targets for the detection of these species. Typical differentiation of *C. jejuni*, from *C. coli*, is based on hippurate hydrolysis, but atypical strains exist, thus Toplak et al. combined a *hipO* and *ccoN* (encoding the cytochrome c oxidase of *C. jejuni*), from *C. coli* *cadF* (fibronectin-binding protein) for the simultaneous detection of both species (Toplak, Kovac, Piskernik, Mozina, & Jersek, 2011).

The second alternative, detection of genus *Campylobacter* spp. without differentiating the species, may be an ideal approach from a risk assessment point of view, specially focused on thermotolerant campylobacters (*C. jejuni*, *C. coli*, and *C. lari*). Many studies have successfully applied different primers targeting 16S rRNA, specific for genus *Campylobacter* and/or thermotolerant campylobacters (Botteldoorn et al., 2008; Josefsen, Cook, et al., 2004; Josefsen, Jacobsen, & Hoorfar, 2004; Leblanc-Maridor et al., 2011; Lubeck, Cook, Wagner, Fach, & Hoorfar, 2003; Lubeck, Wolffs, et al., 2003; Wolffs, Norling, Hoorfar, Griffiths, & Radstrom, 2005).

Due to the fact that the group of thermotolerant campylobacters are the main human hazard, 16S rRNA was selected as the optimal target in an interlaboratory study performed in Europe (Josefsen, Cook, et al., 2004; Josefsen, Jacobsen, et al., 2004; Lubeck, Cook, et al., 2003; Lubeck, Wolffs, et al., 2003). A later study developed an appropriate fluorescent probe suitable for its application along with the European-validated primers (Josefsen, Jacobsen, et al., 2004).

4.2.2. *Escherichia coli* O157 and other STEC

In 2004, Perelle et al. designed a method for the identification of the most frequent clinical serotypes of Shiga-toxin producing *E. coli*, which included O26, O55, O91, O103, O111, O113, O145, and O157:H7. For this purpose, genes *stx1*, *stx2*, *rfbE* (lipopolysaccharide O-antigen synthesis gene for O157), *wbdI* (O111), *wzx* (flippase gene for O26), *wzy* (O-antigen polymerase specific for O113), *wzy* (O91), *wbgN* (glycosyl-transferase gene specific for O55), *ihpI* (based on the polymorphism between the genes encoding the putative adhesin from “O-island 29” of STEC O157 and STEC O145 O145), *eae* (adherence intimin protein for O103), and *fliC* H7 (flagellar antigen H7) were successfully applied (DebRoy et al., 2004; Gonzales et al., 2011; Kagkli et al., 2011; Perelle, Dilasser, Grout, & Fach, 2003, 2004; Samuel, Hogbin, Wang, & Reeves, 2004). Out of these, genes *stx1* and *stx2* (Shiga-toxin genes 1 and 2) are considered two major virulence factors for *E. coli* O157 and other STEC (Bonetta et al., 2011; Prendergast et al., 2011) and the *eae* gene has also been applied for serotype O157 detection by other authors (Adiguzel et al., 2012; Franz, Klerks, De Vos, Termorshuizen, & van Bruggen, 2007; Fratamico & DebRoy, 2010; Kawasaki et al., 2010; Zhang et al., 2009).

Different combinations of several genes have been published and alternative targets have been selected, like *hlyA* (hemolysin) in combination with *fliC*, *stx1*, *stx2*, *eae*, and *rfbE* (Bai, Shi, & Nagaraja, 2010). Other options included the *uidA* (β -glucuronidase gene) which was only found in O157:H7 and H- but not other O157 (Al-Ajmi et al., 2006; Elizaquível et al., 2011).

A large outbreak caused by Shiga-toxin 2 (*stx2*)-producing enterohemorrhagic *E. coli* (EHEC) O104:H4 that involved 4,000 cases, including 855 patients with hemolytic-uremic syndrome (HUS) and 53 fatalities, occurred in Germany from May to July 2011. A combination of the previously mentioned genes was specifically optimized and combined for the detection of this specific serotype of STEC including *wzy*_{O104}, *stx2*, and *fliC*_{H4} (Zhang et al., 2012).

The wide variety of possible targets is mainly associated with high number of possible serotypes included in the group STEC. Due to the fact that the most prevalent serotype associated with human infection was O157, a European validation study, gene *rfbE* was selected for the specific detection of this serotype independently of the “H” antigen. In the validation study, special attention was focused on the specificity of the primers due to the fact that this gene is also present in the genus *Vibrio* spp. As expected, after careful design and optimization of the method, optimal results were obtained (Abdulmawjood et al., 2003; Abdulmawjood et al., 2004).

4.2.3. *Yersinia enterocolitica*

Regarding *Yersinia enterocolitica*, it was observed that few studies performed the detection at the species level 16S rRNA (Lantz et al., 1998; Wolffs, Knutsson, Norling, & Radstrom, 2004). Most studies are focused on the detection of pathogenic strains of this species. There is a high degree of consensus on the application of genes *ail* (attachment invasion locus, which mediates cell invasion) as well as plasmid genes such as *yadA* (whose product is involved in autoagglutination, serum resistance, and adhesion) (Cheyne, Van Dyke, Anderson, & Huck, 2010; Jourdan, Johnson, & Wesley, 2000; Lambertz, Nilsson, Hallanvuori, & Lindblad, 2008; Lantz et al., 1998; Najdenski, Heyndrickx, Herman, Werbrouck, & Van Coillie, 2012; Wiemer et al., 2011).

Additionally, other genes like *inv* (invasive gene, which mediates cell invasion), *ystA* (which is responsible for the production of a heat-stable enterotoxin in virulent *Y. enterocolitica*), *ystB* and *ystC* (which have been observed to encode an enterotoxin present mainly in avirulent *Y. enterocolitica*), *rfbC* (which can be used to identify pathogenic *Y. enterocolitica* O:3 strains), and *virF* or *IcrF* (for *Y. enterocolitica* and *Y. pseudotuberculosis*, respectively, which encodes transcriptional activators of the *yop* regulon) have also been used (Zheng, Sun, Mao, & Jiang, 2008).

4.2.4. *Shigella* spp.

A high degree of homogeneity was observed for the detection of *Shigella* spp., both by PCR and by qPCR. The most commonly used target is the *ipaH* gene, the invasion plasmid antigen H. This gene

was also observed in some strains of entero invasive *E. coli* (EIEC) (Gómez-Duarte, Bai, & Newell, 2009; Hsu et al., 2010; Li, Zhuang, & Mustapha, 2005; Thiem et al., 2004; Wang, Li, & Mustapha, 2007). It was included in an in-house multiplex qPCR validation method which simultaneously detected *Salmonella* spp., *Shigella* spp., and *L. monocytogenes*. Excellent results were obtained with natural and spiked samples regarding relative sensitivity, relative specificity, and relative accuracy for the detection of *Shigella* spp. as well as for the other two bacteria (Garrido, Chapela, Román, et al., 2012).

4.2.5. *Vibrio cholerae*

The most widely used genes for the detection of this species are *toxR* (the transcriptional regulator for the genes encoding the major outer membrane porins OmpU and OmpT) and *ompW* (outermembrane protein) (Baron, Chevalier, & Lesne, 2007; Goel, Tamrakar, Nema, Kamboj, & Singh, 2005; Gubala & Proll, 2006; Nandi et al., 2000; Neogi et al., 2010; Sheikh, Goodarzi, & Aslani, 2012; Shuan Ju Teh et al., 2009), even though recently the *lolB* (outermembrane lipoprotein) gene has also been described as an optimal target for *V. cholerae* detection (Chua et al., 2011; Lalitha et al., 2008). Out of these, the most extensively applied and evaluated gene was the *ompW*. Further studies need to be performed with the other targets.

The most important pathogenic gene associated with *V. cholerae* is *ctxA* (gene coding for the A subunit of the cholera toxin) (Blackstone et al., 2007; Chapela et al., 2010; Fedio et al., 2007), and also *hly* (coding for a hemolysin) (Huang et al., 2009; Lyon, 2001; Saravanan, Sanath Kumar, Karunasagar, & Karunasagar, 2007; Shuan Ju Teh et al., 2009), *tcpA* (toxin-coregulated pilus A) (Sharma & Chaturvedi, 2006; Sheikh et al., 2012), and the *zot* (zonula occludens toxin) (Ang et al., 2010).

4.2.6. *Vibrio parahaemolyticus*

Detection of *V. parahaemolyticus* has been accomplished by the detection of the *toxR* gene and the *tlh* (thermolabile hemolysin) (Crocì et al., 2007; Neogi et al., 2010; Nordstrom et al., 2007; Parveen et al., 2008; Rizvi & Bej, 2010; Rizvi, Panicker, Myers, & Bej, 2006). Virulence of *V. parahaemolyticus* has historically been associated with the presence of two hemolysins encoded in the *tdh* and *trh* genes (thermostable direct hemolysin and hemolysin related with *tdh*) (Blackstone et al., 2003; Garrido, Chapela, Ferreira, et al., 2012; Nordstrom et al., 2007). Recently, the type three secretion system has been observed to be related with the pathogenic potential of the species (Hiyoshi, Kodama, Iida, & Honda, 2010; Pineyro et al., 2010).

Multiplex qPCR methods have been developed for the simultaneous detection of *tlh*, *tdh*, and *trh* (Nordstrom et al., 2007; Ward & Bej, 2006). These methods successfully detected *V. parahemolyticus* in food and environmental samples.

4.2.7. *Vibrio vulnificus*

Uniformity has been observed for the detection of *V. vulnificus* at the species level, where *vwA* (exotoxin hemolysin/cytolysin) (Campbell & Wright, 2003; Neogi et al., 2010; Panicker & Bej, 2005; Wright et al., 2007) is one of the most frequently applied targets. This homogeneity is not observed when trying to assess the pathogenic potential of the bacterium, where several targets have been proposed, such as the *rtxA*, *vcgC/E* (virulence-correlated gene clinical/environmental), and 16S rRNA type A (environmental) and type B (clinical) (Drake, Whitney, Levine, DePaola, & Jaykus, 2010; Rosche, Binder, & Oliver, 2010; Rosche, Yano, & Oliver, 2005). Recently, a polymorphism in the *pilF* gene has been described as a good marker of the pathogenic potential of *V. vulnificus* (Baker-Austin et al., 2012; Fajardo et al., 2014; Garrido-Maestu, Chapela, Román, Vieites, & Cabado, 2014; Roig, Sanjuan, Llorens, & Amaro, 2010).

Multiplex qPCR methods for the simultaneous detection of total and pathogenic *V. vulnificus* are scarce (Garrido-Maestu et al., 2014). Most methods combined specific gene targets for the detection at the species level *V. vulnificus*, *V. cholerae*, and *V. parahemolyticus* (Bauer & Rørvik, 2007; Garrido-Maestu, Chapela, Peñaranda, Vieites, & Cabado, 2014; Izumiya et al., 2011; Neogi et al., 2010; Panicker, Call, Krug, & Bej, 2004).

5. Commercial kits available for multiplex qPCR

At present, a wide variety of commercial qPCR kits are available for detection of single micro-organisms from different companies as iQ-Check from Bio-Rad, DuPont Qualicon™ BAX®, foodproof® Salmonella from Merck-Millipore, AnDiaTec® from Roche or MicroSEQ® from Applied Biosystems. All these brands have validated solutions for *Salmonella* spp., *L. monocytogenes*, *Campylobacter* spp., and *Legionella*, among others. The problem arises when a multiplex qPCR approach is desired, as the commercial kits are scarce. A possible solution may be the selection of open-formula studies that have gone through an in-house validation where parameters like specificity of the primers and probes, LOD, diagnostic specificity, diagnostic sensitivity, and diagnostic accuracy are evaluated against a reference method. In this context, a very common combination of bacterial pathogens found in the literature include simultaneous detection of *Salmonella* spp. and *L. monocytogenes* (Badosa, Chico, Pla, Pares, & Montesinos, 2009; Chua & Bhagwat, 2009; Garrido, Chapela, Román, Fajardo, Lago, et al., 2013; Jofré et al., 2005; Singh, Batish, & Grover, 2011). This combination should be more useful when the method also includes of *E. coli* O157 (Elizaquível et al., 2011; Garrido, Chapela, Román, Fajardo, Vieites, et al., 2013; Kawasaki et al., 2010; Omiccioli et al., 2009) or others food-borne pathogens like *Shigella* spp. (Garrido, Chapela, Román, et al., 2012; Wang, Li, & Mustapha, 2007).

6. Future challenges

An international standard guideline (ISO 16140:2003) was prepared by CEN/TC275 in collaboration with the Technical Committee ISO/TC34 to establish the general principle and technical procedure for the validation of alternative methods in the field of microbiological analysis of food, animal feeding stuffs, and environmental and veterinary samples. Many of the performance criteria are well recognized and accepted by numerous internationally recognized organizations for standardization (ISO, EMEA, Codex Alimentarius, etc.). There is a series of concepts that define an analytical method and that are applied to diagnostic qPCR. The main concepts are: linearity, accuracy, precision, sensitivity, and specificity. Progressive introduction of automated equipment for qPCR has enabled it to become a high-throughput method. The numerous examples cited in this review and their recentness illustrate the current interest for qPCR methods in food microbiology. However, not all fields of application are equally developed yet, in particular, the multiplex strategies as described above. There is an obvious lack of consensus considering the appropriate approach to perform the experiments and interpret the data (Bustin et al., 2009, 2010; Huggett et al., 2013).

7. Conclusions

Molecular techniques applied in food microbiology are very promising alternatives to classical cultures, biochemical and serological identification and typification methods. Among them, PCR and real-time PCR have been successfully used, not only in control laboratories, but also in research to gain deeper knowledge in the biology and pathogenesis of bacterial species.

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