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Development of a multiplex PCR detection of *Salmonella* spp. and *Shigella* spp. in mussels

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A. VANTARAKIS, G. KOMNINOU, D. VENIERI AND M. PAPAPETROPOULOU. 2000. Multiplex PCR amplification of *invA* and *virA* genes was developed enabling simultaneous detection in mussels of *Salmonella* spp. and *Shigella* spp., respectively. Simultaneous amplification of products of 215 and 275 bp was obtained either by using mixtures of individual strains of *Sh. dysenteriae and Salm. typhimurium* or spiked contaminated mussels with both bacteria. In the case of the mussels, 10–100 cells of *Salmonella* spp. and *Shigella* per millilitre of homogenate were detected by the multiplex PCR following a pre-enrichment step to increase sensitivity and to ensure that detection was based on the presence of cultivable bacteria. Also, the sensitivity and specificity of this method was evaluated. Multiplex PCR amplification was shown to be an effective, sensitive and rapid method for the simultaneous detection of pathogens in mussels.

INTRODUCTION

Salmonella spp. species is one of the most important groups of food-borne pathogens worldwide. They are often the aetiological agents of gastroenteritis associated with the consumption of contaminated shellfish, particularly raw oysters and mussels (Archer and Young 1988; Miescier *et al*. 1992; Wilson and Moore 1996). Conventional methods for the detection of *Salmonella* spp. in shellfish are generally cumbersome and time consuming.

The genus *Shigella* consists of four species of Gram-negative, non-motile, non-spore-forming, rod-shaped bacteria, namely *Sh. boydii*, *Sh. dysenteriae*, *Sh. flexneri and Sh. sonnei* (Holt *et al*. 1994). By means of human transmission, *Shigella* spp. may contaminate several kinds of foods. Therefore, as with other pathogens, it is important to detect the presence of *Shigella* spp. in foods. Virulent *Shigella* spp. organisms cause human bacillary dysentery which causes mild diarrhoea, fever, abdominal cramps and severe fluid loss (Ward and Hart 1996). All virulent strains harbour a 120–230-kbp virulence plasmid (Hale *et al*. 1983), which was first described for *Sh. flexneri* (Kopecko *et al*. 1980). Loss of this plasmid results in avirulence (Sansonetti *et al*. 1982), however, it has been shown that implication in virulence can be on both the virulence plasmid and the chromosome. One such gene, *virA*, has been identified on the virulence plasmid of *Sh. flexneri* 2a, and it has been implicated in invasion and intercellular dissemination (Uchiya *et al*. 1995).

The currently recommended method of monitoring shellfish for the presence of microbial pathogens requires culturing followed by a series of presumptive and confirmatory tests that can often take more than 5 days to complete (APHA 1985; D'Aoust 1989; Miescier *et al*. 1992). Traditionally, food-borne micro-organisms are detected by plating homogenates onto highly selective media (hazard test), although in some cases a pre-enrichment step is required. After several days of incubation, the presence and number of specific micro-organisms is determined (Hayes 1985). This plating technique, based on the phenotype of the bacteria, is labour-intensive and can take several weeks to obtain a finite result (Hill *et al*. 1985). On the other hand, rapid, highly sensitive and specific techniques based on genetic characteristics have been developed. DNA probe hybridization and PCR are the best known of these techniques and have been used as hazard tests for the detection and identification of food-borne micro-organisms (Hill *et al*. 1985; Olsen *et al*. 1995; Batt 1997). Although these less time-consuming methods have been developed in recent years, currently no rapid and reliable detection method is available.

Since its introduction in the mid 1980s, polymerase chain reaction (PCR) technology has proved to be an invaluable method for the detection of pathogens in food. The PCR represents a rapid procedure with both high sensitivity and specificity for the immediate detection and

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identification of specific pathogenic bacteria from various food matrices (Lantz *et al*. 1994; Hill 1996). In a few cases, multiplex PCR has been used to detect several pathogens in a single reaction (Way *et al*. 1993; Brasher *et al*. 1998; Soumet *et al*. 1999).

Legislation in Greece requires the absence of *Salmonella* and *Shigella* in foods and in shellfish particularly. In Greece, mussels comprise 90% of the production of shellfish. Apart from these two pathogens, no other pathogens have ever been reported in mussels, which is why we only selected *Salmonella* and *Shigella* in this study. Because the presence of *Salmonella* and *Shigella* should be evaluated before the commercial use of these mussels, a rapid and simultaneous method for the detection of these two pathogens in mussels could help to solve this matter.

In this study, multiplex PCR amplification was used for the simultaneous detection of viable *Salmonella* and *Shigella* species in mussels.

MATERIALS AND METHODS

Determination of cfu. *Salmonella* spp. and *Shigella* spp. were grown on plate count agar (Oxoid) to achieve isolated colonies. One colony of *Salmonella* or *Shigella* was suspended in 1 ml of buffered peptone water and 10-fold serial dilutions (10^{-1}–10^{-7}) were made. Aliquots (10 μl) of selected dilutions were made up to 1 ml with buffered peptone water and spread on plate count agar. After incubation at 37 °C, the colonies were counted.

Culture method. The detection of *Salmonella* and *Shigella* in mussels was performed according to standard methods of the American Public Health Association (APHA 1985).

PCR primers. The primers used for the detection of the *invA* and *virA* genes in *Salmonella* and *Shigella*, were developed by Villalobo and Torres (1998) and Rahn *et al*. (1992), respectively.

Preparation of samples. The mussels were shucked and the whole meat was homogenized in a Waring blender (Waring Products Division, New Hartford, CT, USA). To 25 g of mussel meat, 90 ml of buffered peptone water (Oxoid) was added. For the evaluation of sensitivity of the method, known concentrations (10^1–10^7 cells) of *Salmonella* spp. and *Shigella* spp. were added together in 1 ml of the homogenate. Also separate seeded samples were prepared for detection of *Salmonella* and *Shigella* separately. All the samples were analysed both by plating for viable count determinations and by extracting DNA for multiplex PCR detection.

Also, in a few samples, prior to the PCR and plate count analysis, the mussel meat was incubated in sterile buffered peptone water for 22 h at 37 °C for pre-enrichment.

DNA extraction from mussel samples. To recover DNA for PCR amplification, 1 ml of the homogenized mussel meat samples was treated with guanidine isothiocyanate (Sigma-Aldrich) at a final concentration of 5·9 mol l^{-1}, mixed by vortexing and incubated at 65 °C for 90 min. The suspension was diluted with sterile distilled water to a final concentration of guanidine isothiocyanate 0·3 mol l^{-1}, mixed by vortexing and transferred to a boiling water bath for 5 min. The samples were cooled to room temperature and sodium acetate was added to a final concentration of 0·3 mol l^{-1}. The samples were then centrifuged at 14 000 $\times g$ rev min^{-1} in a microcentrifuge for 10 min and the supernatants were transferred to new tubes and extracted twice with an equal volume of chloroform. The DNA was precipitated with 95% ethanol at −20 °C and sedimented by centrifugation. The DNA was resuspended in sterile distilled water and its concentration was estimated by photometry. Between 5 and 10 μl of the final dilution was used for PCR DNA amplification.

Multiplex DNA amplification (PCR). Amplifications for both species were made in the same 50-μl reaction mixture, which contained 10 mmol l^{-1} Tris-HCl (pH 8·3), 50 mmol l^{-1} KCl, 1·5 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} deoxynucleoside triphosphate (Amersham-Pharmacia), 0·25 μmol l^{-1} of each specific primer and 2·5 U of Taq Polymerase (Life Technologies Ltd, Paisley, UK) with either isolated DNA, a bacterial dilution or a diluted mussel sample. Temperature conditions were as follows: initial denaturation for 2 min at 94 °C, denaturation for 45 s at 94 °C, hybridization for 30 s at 50 °C, and polymerization for 45 s at 74 °C. Thirty-five cycles were carried out as mentioned above. Aliquots of 12 μl of the amplification reaction solutions were run on a 3% (w/v) agarose gel Nuisive 3 : 1 (FMC, Rockland, Maine, USA) stained with 0·5 mg ethidium bromide (Sigma-Aldrich) per ml. The DNA was observed by irradiating the gel with u.v. light at 264 nm. When a negative amplification was obtained a new PCR was performed and 25 μl of the resultant solution was run on an agarose gel to confirm the result.

Sensitivity and Specificity of primers. The primers were tested for their sensitivity of simultaneous detection of *Salmonella* and *Shigella* in various concentrations (10–10^7 bacteria ml^{-1} of homogenate. They were also tested for their specificity against other bacteria (*Escherichia coli*, *Klebsiella* spp., *Aeromonas* spp. *Enterobacter cloacae*).

RESULTS AND DISCUSSION

The method developed involves homogenization of mussel samples, extraction of DNA and simultaneous amplification of *invA* gene for *Salmonella* spp. and *virA* gene for *Shigella* spp. The primers *Sal3*, *Sal4* and *invAfor* and *invArev* were found to be very successful in this study for the simultaneous detection of *Salmonella* spp. and *Shigella* spp. in mussels. The 215-bp product used for the detection of *Shigella* strain was easily differentiated from the 275-bp product used to detect *Salmonella* (Fig. 1). This method enabled us to detect both pathogens in the same sample. The specificity and sensitivity of both sets of primers separately has also been previously studied (Wang *et al*. 1997; Villalobo *et al*. 1998).

Legislation in Greece requires the absence of *Salmonella* spp. and *Shigella* spp. in 25 g of mussel meat. Since it is not possible to directly carry out PCR on 25 g of mussel meat, usually a pre-enrichment step is necessary. In our study this was not necessary for the detection of *Salmonella* spp. but it was necessary for *Shigella* spp. The sensitivity of detection of *Salmonella* spp. in multiplex PCR without pre-enrichment was very high (less than 10 cells ml^{-1} of homogenate), whereas that of *Shigella* spp. was rather low

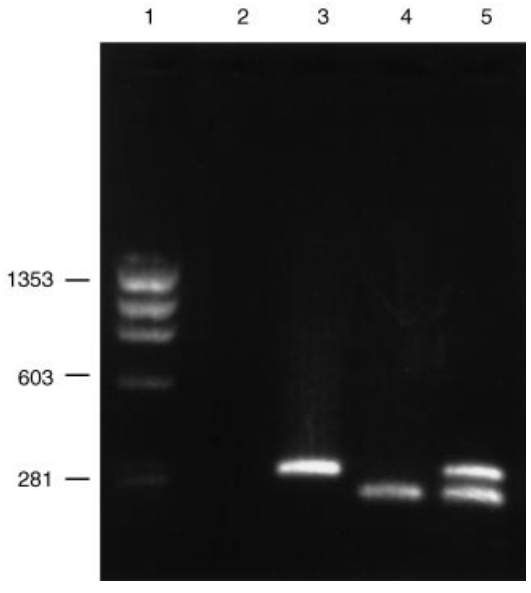

Fig. 1 Agarose gel electrophoresis of PCR products from different template DNAs. The PCR product of *Salmonella* spp. is 275 bp and the PCR product for *Shigella* is 215 bp. The sources of the DNA in the lanes are as follows: Lane 1: $\phi \times 174 \times$ HaeIII marker; lane 2: negative control; lane 3: positive sample for *Salmonella*; lane 4: positive sample for *Shigella*; lane 5: multiplex PCR product for *Salmonella* and *Shigella*

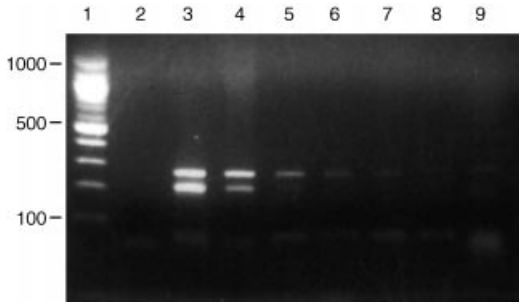

Fig. 2 Agarose gel showing sensitivity of multiplex PCR assay for detection of *Salmonella* spp. and *Shigella* spp. in mussels. Lane 1: marker 100 kbp ladder; lane 2: negative control; lanes 3–8: *Salmonella* and *Shigella* (10^7, 10^6, 10^5, 10^4, 10^4, 10^2 and 10 bacteria ml homogenate^{-1}, respectively)

(1000 cells ml^{-1} of homogenate) (Fig. 2). Therefore, we included in our method the pre-enrichment step for both in order to develop a multiplex method, which detects both *Salmonella* spp. and *Shigella* spp. in the mussels at very low numbers. Also, by including the enrichment step, we were able to detect only cultivable pathogens. Following incubation in buffered peptone water for 22 h, it was possible to detect as few as 10–100 *Shigella* spp. and *Salmonella* spp. cells per millilitre of homogenate. Epidemiological studies on *Shigella* spp. have established that 10 cells are sufficient as an infective dose (Wachsmuth and Morris 1989). This number of bacteria could easily be present in a contaminated mussel sample. The results show that multiplex PCR (including the enrichment step) with *virA* primers for *Shigella* spp. and *invA* primers for *Salmonella* spp. could be a useful hazard test because their sensitivity is similar to that reported elsewhere (Bej *et al*. 1991). Also, the ability to perform the same analysis for more than one bacterium in parallel is very important. Because of the need to enrich the samples, detection of *Salmonella* spp. and *Shigella* spp. was performed in 2 d. Shorter culture time for pre-enrichment such as 6 h should be enough for *Salmonella* and *Shigella*. However, overnight culture is convenient for the 8-h working schedule.

The multiplex PCR was tested for its specificity against other bacteria such as *E. coli*, *Klebsiella* spp., *Aeromonas* spp. and *Enterobacter cloacae* (Fig. 3). Only *Salmonella* and *Shigella* species gave the characteristic bands (275 and 215 bp). The choice of these bacteria was made because these are common in Greece.

This method was also evaluated for the detection of both pathogens in mussel samples taken from various parts of Greece. Only *Salmonella* spp. was detected in these samples (data not shown).

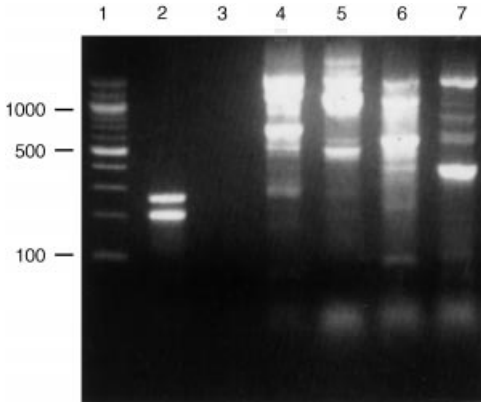

Fig. 3 Agarose gel showing specificity of multiplex PCR assay for detection of *Salmonella* spp. and *Shigella* spp. in mussels. Lane 1: marker 100 kbp ladder; lane 2: multiplex PCR product for *Salmonella* and *Shigella*; lane 3: negative control; lanes 4–7: *Klebsiella* spp., *Aeromonas* spp., *Enterobacter cloacae* and *Escherichia coli*, respectively)

Cost factors are likely to be considered when selecting the method for detection of pathogens in foods and PCR seems to be more expensive than conventional method (if confirmation of positive results is not taken under consideration). However, it should kept in mind that the multiplex PCR saves a considerable amount of money allowing a simultaneous detection of several pathogens.

The major contribution of this study is the development of a protocol using the same conditions for PCR detection of more than one important bacterial species (which concern public health) in food. We believe that this protocol could be used for simultaneous detection of other microorganisms as well, using appropriate primers. The protocol could be developed as a routine procedure for foodborne pathogen detection.

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