

## A simplified sample preparation method from various foods for PCR detection of pathogenic *Yersinia enterocolitica*: a possible model for other food pathogens

Saumya Bhaduri\* and Bryan Cottrell

A simplified method for the direct application of multiplex polymerase chain reaction (PCR) was developed to detect plasmid-bearing virulent serotypes of *Yersinia enterocolitica* (YEP<sup>+</sup>) in a variety of foods. Strains of YEP<sup>+</sup> representing five serotypes were detected in enriched swab samples of artificially contaminated pork chops, ground pork, cheese and zucchini using multiplex PCR analysis. The method was also effective for identifying YEP<sup>+</sup> strains in naturally contaminated porcine tongues. The use of swabs eliminated time-consuming extraction of DNA from food, inhibition of PCR by food-derived DNA, interference by background flora and reduced the time needed for processing samples. The detection of other food pathogens should be feasible by this technique.

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**KEYWORDS:** detection, polymerase chain reaction, *Yersinia enterocolitica*, plasmid, virulence, food.

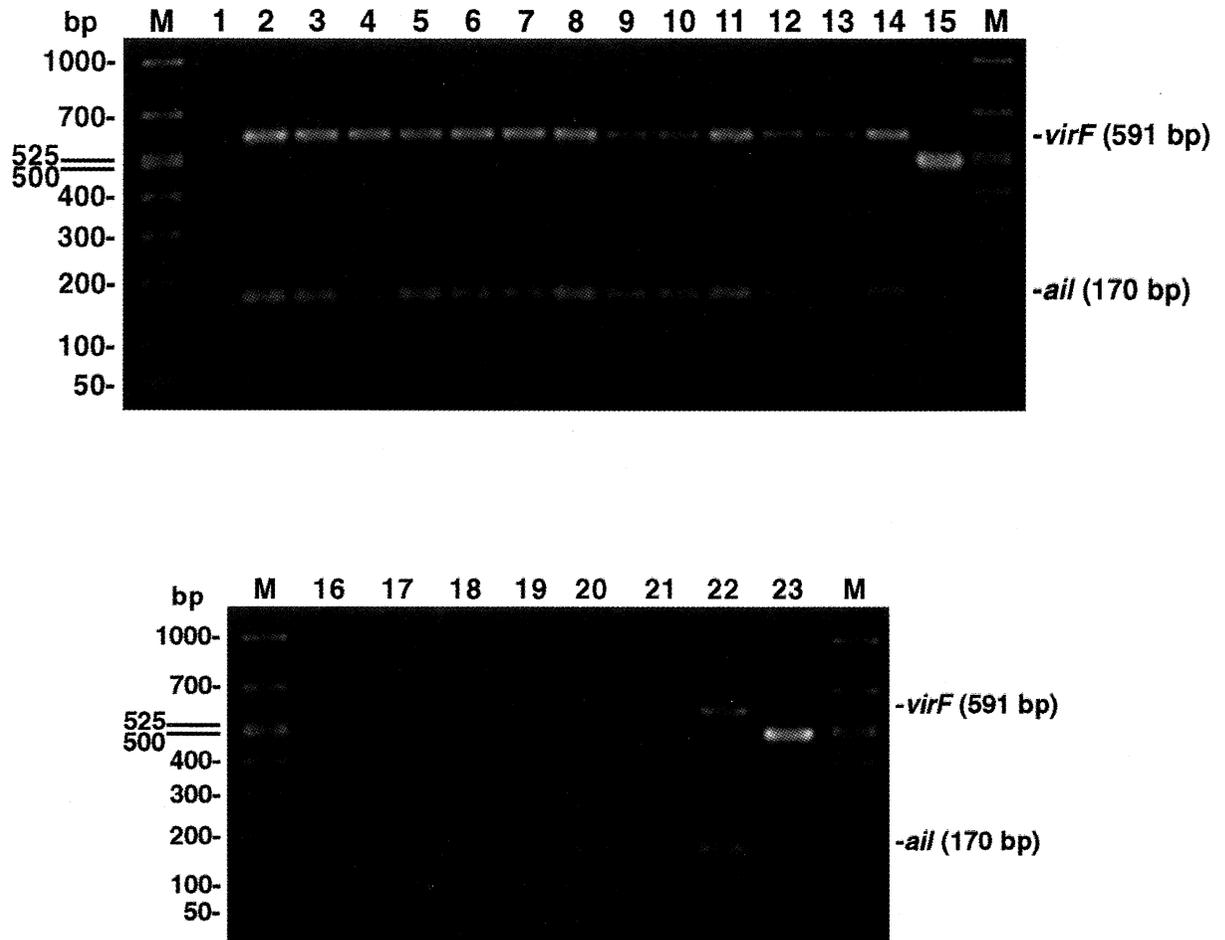
### INTRODUCTION

The polymerase chain reaction (PCR) is finding increasing application as a specific and sensitive diagnostic method for the detection of food-borne pathogens,<sup>1-6</sup> but it has met difficulties in this application due to the complex nature of food matrices. The effectiveness of PCR for detection of microorganisms in foods is hampered by the presence of substances that inhibit the PCR reaction.<sup>7</sup> The use of purified DNA samples is a requirement for reliable testing by PCR.

Several methods have been employed for the concentration and separation of bacterial DNA from food. These include enrichment followed by phenol-chloroform extraction, immunomagnetic separation,

dilution, centrifugation, filtration and adsorption.<sup>7</sup> Among the methods described in the literature, the purification of DNA from food homogenates by phenol-chloroform extraction or commercial DNA extraction kits are the most commonly used.<sup>2-4</sup> However, these techniques and others described in the literature<sup>7</sup> are relatively complicated, time consuming, require significant expertise and utilize hazardous chemicals. These factors can make the routine testing of many samples impractical.

In order to overcome these limitations, the sample preparation has been simplified by removing the food matrix from the enrichment medium through the use of swabbing. Since food surfaces are the primary site



**Fig. 1.** Detection of YEP<sup>+</sup> strains by multiplex polymerase chain reaction (PCR) amplifying the chromosomal *ail* gene (170 bp) and the plasmid-encoded *virF* gene (591 bp). Lanes; M, 50–1000 bp ladder marker; 1 and 16, negative controls. Plasmid-bearing virulent *Yersinia enterocolitica* (YEP<sup>+</sup>) strains showing the presence of the 591 and 170 bp products: lanes 2–4, zucchini; 5–7, cheese; 8–10, pork chops; and 11–13, ground pork. The panel shown is representative of the amplification results after each of the various food samples were spiked with 10, 1 and 0.5 colony forming units (CFU) cm<sup>-2</sup> concentrations of overnight culture of YEP<sup>+</sup> strains from left to right, respectively. Lanes 17–21, samples from naturally contaminated porcine tongues where lane 20 shows the presence of the 591 and 170 bp products. Lanes 14 and 22, positive controls with purified DNA from a YEP<sup>+</sup> strain showing the presence of the 591 and 170 bp products. Lanes 15 and 23, positive controls for PCR assay with  $\lambda$  as DNA template.

of bacterial contamination, non-destructive swabbing has been used widely as a sampling procedure for the enrichment of a wide variety of food-borne pathogens from meat.<sup>8</sup> To the authors' knowledge, the direct PCR testing of food samples using a combination of enrichment by swabbing and concentration by centrifugation has not been reported previously. The objective of this study was to test this method using swabs of pork chops, ground pork, cheese and zucchini, artificially contaminated with pathogenic *Yersinia enterocolitica* and enriched in a single medium with subsequent application of multiplex PCR.

## MATERIALS AND METHODS

*Yersinia enterocolitica* was selected because it is a food-borne pathogen of significance to humans. Common food vehicles in outbreaks of yersiniosis are meat (particularly pork), dairy products, tofu and raw vegetables.<sup>9,10</sup> Strains of all serotypes implicated in human disease harbour a virulence-associated plasmid of 70–75 kbp.<sup>9,10</sup>

Plasmid-bearing virulent *Y. enterocolitica* (YEP<sup>+</sup>) strains of serotypes O:3; O:8; O:TACOMA; O:5; O:27; and O:13 were used in this study for detection

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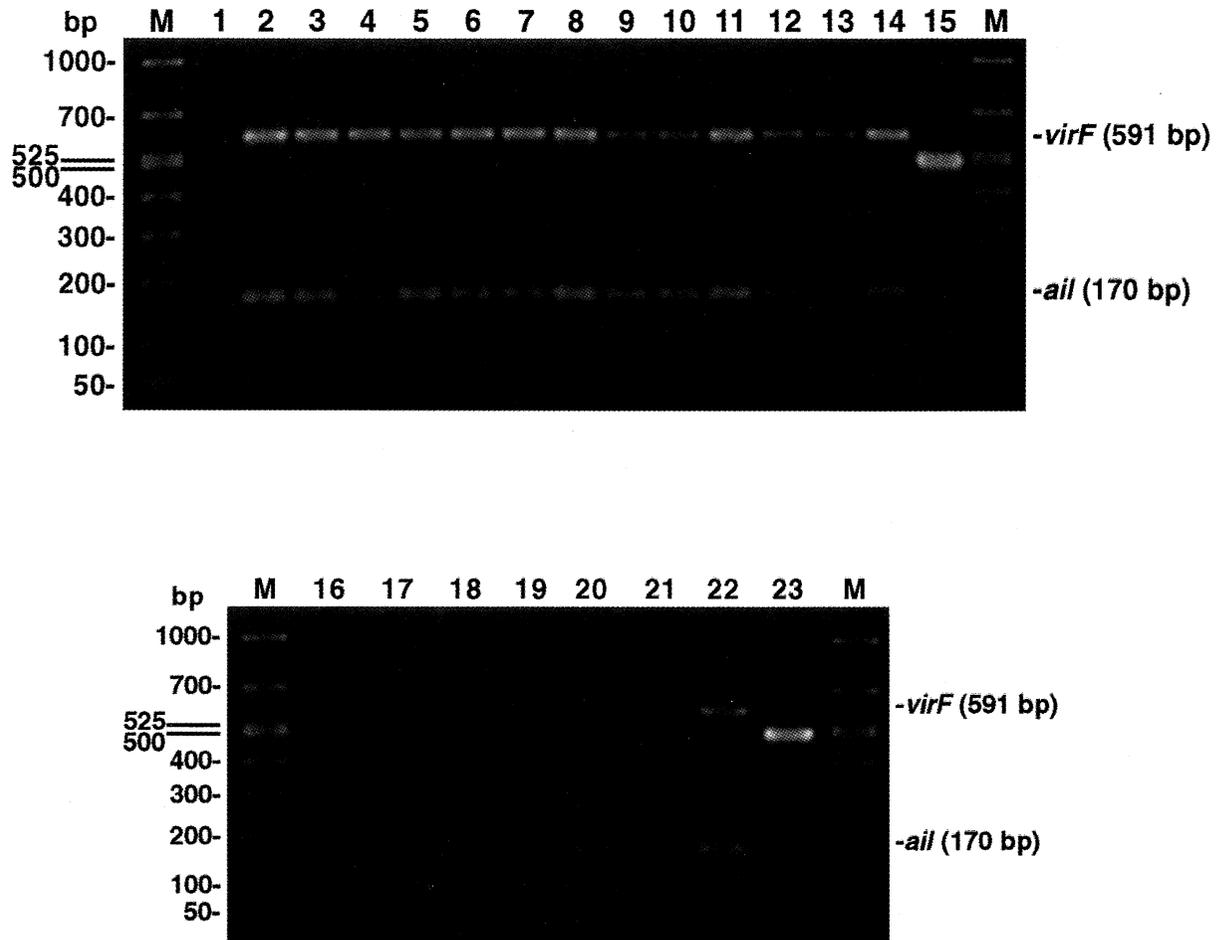
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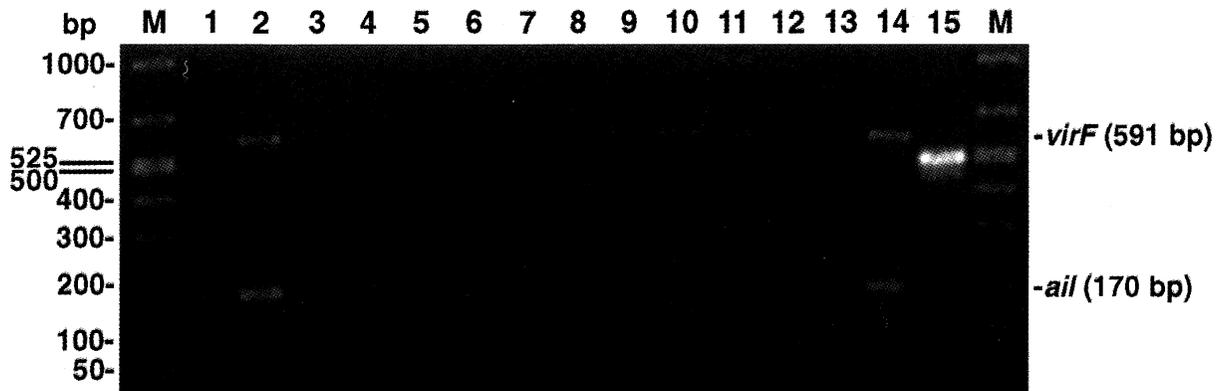
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**Fig. 2.** Comparison of the relative sensitivity of swabbing-cell pellet-derived lysate technique for DNA preparation to the conventional extraction of DNA from ground pork for direct application of polymerase chain reaction (PCR) to detect plasmid-bearing virulent *Yersinia enterocolitica* (YEP<sup>+</sup>) strains using *ail* gene (170 bp) and plasmid-encoded *virF* gene (591 bp). Lanes: M, 50–1000 bp ladder marker; 1 negative control with no template; 2, DNA from swab-pellet technique showing the presence of the 170 and 591 bp products; 3, undiluted DNA extracted from ground pork; 4–13, increasing dilutions of DNA extracted from ground pork from left to right: 8 (1:5 dilution) shows the presence of the 170 bp product; 9–11 (1:10, 1:20 and 1:100 dilutions, respectively) shows the presence of the 170 bp and 591 bp products; 14, positive control with purified DNA from a YEP<sup>+</sup> strain showing the presence of the 170 and 591 bp products; 15, positive control for PCR assay with  $\lambda$  as DNA template.

of PCR. Detailed descriptions of the strains, sources, preparation of inocula, incubation conditions and characterization of strains are given elsewhere.<sup>11,12</sup> Modified trypticase soy broth (MTSB) (Difco Laboratories, Detroit, USA) containing 0.2% bile salts #3 (Difco) and brain heart infusion (BHI) (Difco) broth were prepared as described by Bhaduri *et al.*<sup>12</sup> Pork chops, ground pork, sliced cheese and zucchini were purchased from a local market and stored at 4°C. Medallions of each food type were prepared using a 10 cm<sup>2</sup> coring tool. Porcine tongues were collected from Hatfield Quality Meats, Hatfield, USA and processed as described previously.<sup>12</sup> Cultures of YEP<sup>+</sup> were grown in BHI broth for 18 h at 25°C with shaking to a population density of approximately 10<sup>9</sup> CFU ml<sup>-1</sup>.<sup>12</sup> Dilutions containing 1000, 100 and 50 colony forming units (CFU) ml<sup>-1</sup> of YEP<sup>+</sup> strains were pipetted (100  $\mu$ l) over the 10 cm<sup>2</sup> food surfaces giving 10, 1 and 0.5 CFU cm<sup>-2</sup>. To simulate natural surface contamination, the samples were allowed to stand for 15 min. The medallions were sampled by swabbing the 10 cm<sup>2</sup> surface with a sterile 5  $\times$  5  $\times$  1.25 cm cellulose sponge moistened in 10 ml of MTSB. Each swab was placed in a sterile Whirl Pak bag containing 90 ml of MTSB and allowed to stand at room temperature for 5 min. Whole porcine tongues were swabbed and treated as described above. The enrichment bags were placed in a shaking incubator (100 rpm) at 12°C for 24 h. Irgasan (Ciba-Geigy Corp., Greensboro, USA) was then added to yield a final

concentration of 4  $\mu$ g ml<sup>-1</sup> and the samples incubated at 12°C for another 24 h.<sup>12</sup> The sampling of enriched cultures was performed after 48 h total enrichment time. Each enrichment bag was mixed immediately prior to sampling and cells from each culture were harvested by centrifugation for 10 min (7000  $\times$  g), washed twice with phosphate-buffered saline without Mg<sup>2+</sup> and Ca<sup>2+</sup>, and centrifuged.<sup>13</sup> The cells were then suspended in 45  $\mu$ l of sterile distilled water and lysed by heating for 10 min in a boiling water bath. The lysates were cooled in an ice-bath for 5 min and treated with proteinase K (2 mg ml<sup>-1</sup>) for 1 h at 37°C. The proteinase K treatment was found necessary to inactivate DNase present in YEP<sup>+</sup> strains (data not shown). The proteinase K was destroyed by heating for 5 min in a boiling water bath and the lysates were cooled. The proteinase K treated lysates were stored at -70°C and evaluated as templates for PCR analysis. The pathogenic YEP<sup>+</sup> strains were identified by multiplex PCR using the *virF* gene (transcriptional activator for the expression of plasmid-encoded outer membrane protein Yop51) from the virulence plasmid and the chromosomal *ail* gene (attachment-invasion locus).<sup>10,14</sup> The primer pairs (5'-TCATGGCAG-AACAGCAGTCAG-3' and 5'-ACTCATCTTACC-ATTAAGAAG-3') for the detection of the *virF* gene (430- to 1020-nucleotide region) amplified a 591 base pair (bp) product from the virulence plasmid.<sup>14</sup> The primer pairs (5'-ACTCGATGATACTGGGGAG-3' and 5'-CCCCCAGTAATCCATAAAGG-3') for de-

tection of the *ail* gene (664- to 833-nucleotide region) amplified a 170 bp DNA fragment from the chromosome.<sup>14</sup> The primers for both the *ail* and *virF* genes were used in a multiplex format.<sup>14</sup> The oligonucleotide primers utilized in this study were synthesized by the Appligene Company (Pleasanton, CA, USA). Polymerase chain reaction was performed using the conditions published by Bhaduri and Pickard.<sup>14</sup> Purified chromosomal and plasmid DNA from YEP<sup>+</sup> cells was used as a positive control. A negative control with all of the reaction components except template DNA was included with each test run. As an internal positive control for the PCR reaction,  $\lambda$  DNA was used. The PCR products were separated electrophoretically on a 2.0% agarose gel and detected in UV light after ethidium bromide staining.<sup>14,15</sup>

## RESULTS AND DISCUSSION

Enrichment of swabs was optimized using pork chops, ground pork, cheese and zucchini artificially contaminated with various concentrations of YEP<sup>+</sup> strain GER (serotype O:3). It was determined that Irgasan should be added after 24 h of initial enrichment at 12°C to reduce the inhibitory effect of the antibiotic.<sup>12</sup> The addition of Irgasan after 24 h and incubation for an additional 24 h at 12°C allowed the growth of YEP<sup>+</sup> strains while effectively inhibiting growth of competing microflora. Thus, YEP<sup>+</sup> strains were able to grow to a detectable level even in the presence of competing microflora. It was also determined that samples should be taken after 48 h of total incubation to allow YEP<sup>+</sup> strains time to reach detectable levels and to avoid sampling after competing microflora have begun to predominate. This technique enhances detection of YEP<sup>+</sup> strains in the presence of competing microflora through the selection of optimal incubation temperature, sampling schedule and timing for the addition of Irgasan. Thus, YEP<sup>+</sup> strains could be detected by PCR in pellets concentrated from a 48 h enrichment culture. This allowed detection of YEP<sup>+</sup> strains from zucchini, cheese, pork chops and ground pork samples spiked with 10, 1 and 0.5 CFU cm<sup>-2</sup> in a minimum of 3 days beginning with sample enrichment and concluding with PCR testing (Fig. 1, zucchini lanes 2–4, cheese lanes 5–7, pork chop lanes 8–10 and ground pork lanes 11–13). This technique has been successfully applied in the identification of different YEP<sup>+</sup> strains of five serotypes including O:3 (five strains), O:8 (five strains), O:TACOMA (four strains), O:5, O:27 (four strains) and O:13 (three strains) from the artificially contaminated zucchini, cheese, pork chops and ground pork (data not shown).

The relative sensitivity of this technique for PCR was also compared using DNA extracted from food. For comparison, DNA was prepared from an artificially contaminated ground pork medallion (10 CFU cm<sup>-2</sup>) by the above mentioned technique. Similarly ground pork was artificially contaminated with 10 CFU g<sup>-1</sup> of YEP<sup>+</sup> strain GER (serotype O:3) and enriched in 90 ml of MTSB as described previously.<sup>12</sup> DNA was extracted from a 5.0 ml portion of this enriched culture as outlined by Bhaduri and Pickard.<sup>14</sup> Since the concentration of total DNA extracted from food plays a critical role in PCR amplification of the desired sequence,<sup>2,5</sup> a series of dilutions of this DNA were made in sterile distilled water and used in the PCR reaction. Both DNAs were evaluated for PCR analysis as described above for detection of pathogenic YEP<sup>+</sup> strains. Strains of YEP<sup>+</sup> could be detected by PCR in DNA obtained using the swabbing technique (Fig. 2, lane 2). Dilutions of the DNA extracted from ground pork were used to determine the optimal concentration of this sample for use in PCR assay (Fig. 2). Both *ail* and *virF* genes were amplified at dilutions of 1:10, 1:20 and 1:100 (Fig. 2, lanes 9–11). A non-specific amplification product was noted with 1:100 dilution which was easily differentiated from the 591 and 170 bp products. A 1:5 dilution amplified only the *ail* gene (Fig. 2, lane 8). The concentration of plasmid DNA in this case may not be optimal for amplification in the multiplex PCR assay used. No amplification signal was detected in undiluted DNA or at lower (1:2.5, 1:2, 1:1.6 and 1:1.25) (Fig. 2, lanes 3–7) or higher (1:1000 and 1:10 000) (Fig. 2, lanes 13–14) dilutions. This may be due to inhibitors present in the ground pork that were not removed by DNA extraction<sup>7</sup> or to the presence of significant food DNA present in the sample. It has been determined that the optimum amount of DNA isolated from ground pork to be used for PCR amplification varies with each preparation of enriched culture (unpubl.). Similar observations were made by other investigators (pers. comm.). The isolation of DNA from homogenized enriched cultures is impractical for detection where many samples are to be analysed. The technique used provides greater sensitivity in detecting YEP<sup>+</sup> strains than conventional techniques while eliminating the necessity of using a complex extraction procedure with titration of the resulting DNA.

Since porcine tongue is an important and natural reservoir for YEP<sup>+</sup> strains<sup>9,10</sup> and naturally contaminated food samples are not easily found,<sup>12</sup> only porcine tongues were used to validate the effectiveness of this method. The successful screening of YEP<sup>+</sup> strains from naturally contaminated porcine tongue verified the effectiveness of this method. Lane

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20 in Fig. 1 shows the presence of a YEP<sup>+</sup> strain in one of the five tongues tested (Fig. 1, lanes 17–21). The data presented here is the result of one of several groups of porcine tongues tested. A total of 17 tongues were analysed and seven (~41%) were positive for YEP<sup>+</sup> strains. These results are in agreement with the detection of YEP<sup>+</sup> strains by culture technique in both artificially and naturally contaminated food samples as reported previously.<sup>12</sup>

### CONCLUSION

Swabbing offers several advantages over other food enrichment techniques for the preparation of samples to be tested by PCR. By removing the food matrix from the enrichment medium, non-specific food-derived DNA and competing background flora are reduced. Interference by background flora is also reduced by using a selective medium. This allows the use of total recovered target cells present in the enriched culture for use in a lysate DNA preparation without additional purification steps. Sample handling is substantially simplified since the method eliminates the need for homogenization. This work indicates that swabbing of food samples, when used in combination with PCR amplification of a cell pellet-derived lysate, is an effective and time-saving method for the detection of YEP<sup>+</sup> strains in various foods. The detection of other food pathogens should be feasible by this technique using suitable enrichment media specific for the target pathogen. Further studies are in progress to determine the applicability of this technique for the detection of pathogens in food samples using an appropriate enrichment medium.

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