Introduction

Yersinia enterocolitica, a food borne pathogen, has gained widespread recognition in recent years as a source of human infection. It is the most prevalent Yersinia species associated to disease in human. It has also been frequently isolated from animals, food and environment (Singh et al., 2003; Fredriksson-Ahomaa et al., 2006; Subha et al., 2009; Laukkonen et al., 2010; Virtanen et al., 2012; Paixao et al., 2013 and Pugazhenthi et al., 2013). The frequent clinical form of yersiniosis is acute gastroenteritis, most often resulting in self limiting diarrhea. Although most yersiniosis cases are mild infection but there can be a variety of complications depending on host factors such as age and immune status. In children, the symptoms are mainly of enterocolitis. In adults, Y. enterocolitica infection presents as pseudoappendicitis syndrome showing symptoms such as fever, abdominal pain and tenderness of right lower quadrant. In animals, it is associated with enteritis, septicaemia, placentitis and pseudotuberculosis like disease. In countries where Y. enterocolitica is a significant foodborne pathogen, the estimated carrier rate ranges from approximately 35% to 70% in swine herds and 4.5% to 100% in individual swine (Robinson-Browne, 2007). Ground pork, tongue and tonsils act as important sources of infection for human. In India, isolations of Y. enterocolitica have been reported from different foods of animal origin including pork, chicken meat, milk (Singh et al., 2003; Kushal and Anand, 2006; Subha et al., 2009; Saikia and Joshi, 2010; Arora, 2010; Hanifian and Khani, 2012 and Pugazhenthi et al., 2013). It is well established that pigs are the main reservoir for Y. enterocolitica and pork is therefore likely to be the most-important vehicle for its transmission to humans. However, the pathogen has been less frequently isolated from pork. The problem has been identified as methodological, referring to the fact that no traditional culture method works satisfactorily. There are many difficulties in recovery of Y. enterocolitica by culture methods, which includes (i) Low number of pathogens in the food. (ii) Competition between pathogen and other microorganisms during growth in liquid medium or (iii) Lack of selectivity of solid medium or may be the combination of these factors. The detection of Y. enterocolitica in pork can be significantly improved by PCR.

In recent years, researchers have developed number of PCR-based assays for the detection of pathogenic Y. enterocolitica in pork. Most of these PCR assay developed target the chromosomally located virulence-associated

Detection of Yersinia enterocolitica in Raw Pork by PCR

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ABSTRACT

In the present study, 100 pork samples including 50 each of ground pork and pig tonsils collected from the local market were examined by both PCR and culture method for presence of Yersinia enterocolitica. PCR assay based on the specific amplification of 16S rRNA gene and ail gene of Yersinia enterocolitica was carried out to amplify a chosen target DNA sequence. The detection limits of the PCR for Yersinia enterocolitica in experimentally inoculated ground pork was 10⁴ CFU per g. without enrichment, 10³ CFU per g. with enrichment for 12 h and 10² CFU per g. with enrichment for 24 h. On application of PCR for detection of Yersinia enterocolitica in pork, out of 100 samples, Yersinia enterocolitica was detected in 26 (26%). The overall percent positivity by PCR for detection of Yersinia enterocolitica from ground pork was 11 (22%) and pig tonsil 15 (30%) as compared to ground pork 8 (16%) and pig tonsil 10 (20%) by culture method, respectively.

Keywords: Y. enterocolitica, pork, isolation, PCR, tonsils.

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gene, *ail* and *ystA, yadA* and *virF* on virulence plasmid *(pYV)* and species specific gene *16S rRNA* (Nilsson *et al.*, 1998; Wannet *et al.*, 2001; Bhaduri, 2003; Nowak *et al.*, 2006; Simonova *et al.*, 2007; Hudson *et al.*, 2008). Keeping in view the great public health significance and lack of rapid and sensitive method for detection of *Y. enterocolitica*, the present study was undertaken to standardize and evaluate PCR assay for detection of *Y. enterocolitica* in pork and compare its performance with a conventional culture method for detection of *Y. enterocolitica* in pork.

**Materials and Methods**

**Sampling**

A total of 100 pork samples comprising 50 each of ground pork and pig tonsils were collected from the pigs slaughtered in the local retail market in sterile sample container with all aseptic precautions and transported to the laboratory under cold condition at the earliest.

**Bacterial strains**

The standard bacterial strains (Table 1) were revived and employed for study.

**Determination of CFU per ml of standard strain**

Standard strain of *Y. enterocolitica* (MTCC 4858) was grown for 18 h in tryptone soya broth (TSB, Hi-Media) at 28°C. 10 fold serial dilutions were made by transferring 1 ml culture into 9 ml TSB and 0.1 ml of each dilution was pipetted on to two tryptone soya agar (TSA, Hi-media) plates. The inoculum on the plates was spread with the help of a sterile spreader and the plates were incubated at 28°C for 24 h. The colonies on each plate in the range of 30-300 were counted after 24 h of incubation period. The average number of colony count of two plates indicated the bacterial load in 0.1 ml of a particular dilution of culture and thus Colony Forming Units (CFU) of *Y. enterocolitica* per ml was determined.

**DNA isolation**

DNA was isolated from 2 g ground pork and 2 g pig tonsils using Genomic DNA Purification Kit (Thermo Scientific) according to the manufacturer’s instructions. Purified genomic DNA was diluted to a concentration of 10 ng/µl in TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) and stored at -20°C.

**Standardization of PCR**

PCR was performed with two sets of primer pairs species specific *16S rRNA* gene and pathogenic *ail* gene as shown in Table 2. PCR was standardized in 25 µl of reaction volume with different concentrations of reactants under different annealing temperatures and cycling conditions using standard strain of *Y. enterocolitica* (MTCC 4858). Upon standardization, reaction mixture contained 12.5 µl master mix (Hi-Media), 8.5 µl nuclease free water, 0.5 µl of each forward and reverse primers (20 pmol) and 3 µl DNA template. PCR tubes were placed in thermocycler (Master Cycler Gradient, Eppendorf) with preheated lid (105°C) and were subjected to following PCR cycling conditions - initial denaturation at 95°C for 5 min followed by 35 cycles each of denaturation at 94°C for 1 min, annealing at 55°C for 50 sec, extension at 72°C for 45 sec and final extension at 72°C for 10 min. On completion of PCR, amplified products were analysed by agarose gel electrophoresis (1.5% agarose).

**Determination of specificity of the PCR assay**

Specificity studies were performed using different species of *Yersinia* and other bacteria (Table 1). Pure cultures of *Yersinia* spp. and other bacteria were grown in tryptone soya broth (TSB, Hi-Media) at 28°C and other bacteria at 37°C for 18 h. Bacterial DNA was isolated from 5 ml of overnight enrichment and 3 µl DNA was used as template in PCR. To amplify specifically the *Y. enterocolitica* *16S rRNA* gene, a set of primers Y1 and Y2 was used (Table 1).

**Determination of sensitivity of PCR assay**

Sensitivity studies using *Y. enterocolitica* strain (MTCC 4858) were performed to know the detection limit of the PCR with a set of primers Y1 and Y2 targeting *16S rRNA* gene. The culture of *Y. enterocolitica* was inoculated in 5 ml of tryptone soya broth (TSB, Hi-Media), incubated at 28°C for 18 h and then serially diluted (10-fold). The DNA was isolated from 5 ml of each dilution and 3 µl DNA was used as template in PCR.

To determine the detection limit of *Y. enterocolitica*, 2 g of pork samples, which have been tested to be culturally negative for *Y. enterocolitica*, were mixed with 5 ml of Peptone Sorbitol Bile broth (PSB, Hi-Media) and in these pork sample known concentration of *Y. enterocolitica* MTCC 4858 (10² to 10⁶ CFU per ml) were inoculated. Artificially inoculated pork samples were subjected to PCR assay at 0 h (no incubation), 12 h and 24 h of incubation.

**Detection of *Y. enterocolitica* in pork samples by PCR**

A total of 100 pork samples comprising 50 each of ground pork and pig tonsils were subjected to PCR for detection of *Y. enterocolitica*. 2 g of each sample was homogenized with 5 ml of Peptone Sorbitol Bile Broth. The homogenates were incubated at 28°C for 18-24 h. Bacterial DNA was isolated from 5 ml of overnight enrichment and 3 µl DNA was used as template in PCR as described above.
Isolation and identification of *Y. enterocolitica* from pork samples

*Yersinia enterocolitica* organisms were isolated from pork samples as per the method described by ISO (International Organization of Standardization) (ISO 10273, 2003). Identification of the isolates was carried out by biochemical characterization as described by Cowan *et al.* (1974) and Mac-Faddin (1976).

Comparative efficacy of PCR and culture method for detection of *Y. enterocolitica*

Comparative efficacy of PCR and culture method for detection of *Y. enterocolitica* in pork sample was evaluated using diagnostic test characteristics *viz.* sensitivity and specificity. The observational data on positivity for *Y. enterocolitica* in field pork samples by PCR and cultural method were grouped into four: Positive by both PCR and culture method, Positive by PCR and negative by culture method, Negative by PCR and positive by culture method, Negative by both PCR and culture method (Table 6).

Results

The PCR targeting 16S rRNA gene and *ail* gene were standardized which yielded a product of 330 bp and 425 bp, respectively (Table 1).

Specificity of PCR

Among the six *Yersinia* strains, only DNA from *Y. enterocolitica* strains yielded PCR products of 330 bp specific for 16S rRNA. No PCR products were observed when *Y. intermedia* or non-*Yersinia* strains were used in this study. Further, only DNA from pathogenic *Y. enterocolitica* strains (IP134, IP383 and W22703) yielded PCR products of 425 bp specific for virulence associated *ail* gene. No PCR products were observed when non-pathogenic *Y. enterocolitica* strains (MTCC 4858 and 4869), *Y. intermedia* and non-*yersinia* DNA isolates were used (Table 1 and Fig. 1).

<table>
<thead>
<tr>
<th>Bacterium tested</th>
<th>Source of strain</th>
<th>Strain</th>
<th>No. of strains</th>
<th>16S rRNA gene</th>
<th>ail gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Yersinia enterocolitica</em></td>
<td>IMTECH, Chandigarh</td>
<td>MTCC 4858</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MTCC 4869</td>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Microbial Pathogenicity</td>
<td>Lab., University of Delhi</td>
<td>W 22703</td>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Yersinia intermedia</em></td>
<td>IMTECH, Chandigarh</td>
<td>MTCC 3101</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td>MTCC 40</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td></td>
<td>MTCC 9844</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td></td>
<td>MTCC 1457</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. Primers pairs used for amplification of 16S r RNA and *ail* genes

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>F: AATACCGCGATAACGTTCTCG</td>
<td>330 bp</td>
<td>Wannet <em>et al.</em>, 2001</td>
</tr>
<tr>
<td></td>
<td>R: CTCTCTCGGAGGAAGCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ail</em></td>
<td>F: TTAATGTGTCAGGCTGAGTG</td>
<td>425 bp</td>
<td>Neubauer <em>et al.</em>, 2000</td>
</tr>
<tr>
<td></td>
<td>R: GGAGTTATCATATGAAGCGTC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. PCR detection level of *Yersinia enterocolitica* in spiked ground pork

<table>
<thead>
<tr>
<th>CFU per gm</th>
<th>10^7</th>
<th>10^6</th>
<th>10^5</th>
<th>10^4</th>
<th>10^3</th>
<th>10^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spiked Pork without enrichment (no incubation)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spiked Pork with enrichment in PSB (12 h incubation)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spiked Pork with enrichment in PSB (24 h incubation)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 4. Detection of *Y. enterocolitica* in pork samples by PCR

<table>
<thead>
<tr>
<th>Nature of sample</th>
<th>No. of samples examined</th>
<th>No. of samples positive by PCR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig tonsil</td>
<td>50</td>
<td>15 (30)</td>
</tr>
<tr>
<td>Ground pork</td>
<td>50</td>
<td>11 (22)</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>26 (26)</td>
</tr>
</tbody>
</table>

Table 5. Comparison of PCR and culture method for detection of *Y. enterocolitica* from pork samples

<table>
<thead>
<tr>
<th>Nature of sample examined (No.)</th>
<th>No. positive by PCR (%)</th>
<th>No. positive by culture method (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig tonsil (50)</td>
<td>15 (30)</td>
<td>10 (20)</td>
</tr>
<tr>
<td>Ground pork (50)</td>
<td>11 (22)</td>
<td>08 (16)</td>
</tr>
<tr>
<td>Total (100)</td>
<td>26 (26)</td>
<td>18 (18)</td>
</tr>
</tbody>
</table>

Table 6. Comparison of PCR with culture method for detection of *Y. enterocolitica* from pork samples

<table>
<thead>
<tr>
<th>Type of sample examined (No.)</th>
<th>No. positive by both PCR and culture method (%)</th>
<th>No. positive by PCR and negative by culture method (%)</th>
<th>No. negative by both PCR and culture method (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig tonsil (50)</td>
<td>10</td>
<td>5</td>
<td>35</td>
</tr>
<tr>
<td>Ground pork (50)</td>
<td>08</td>
<td>3</td>
<td>39</td>
</tr>
<tr>
<td>Total (100)</td>
<td>18</td>
<td>8</td>
<td>74</td>
</tr>
</tbody>
</table>

Sensitivity of PCR

When PCR (for 16S rRNA gene) was applied for detection of *Y. enterocolitica* in experimentally inoculated ground pork with varying concentrations (10^2 - 10^7) of organisms per g. of pork, it was found that as minimum as 100 CFU per g. of pork could be detected. The detection limit was 10^4 CFU per g. without enrichment (no incubation), 10^3 CFU per g. with enrichment for 12 h and 10^2 CFU per g. with enrichment for 24 h (Table 3, Fig. 2 to 4).
samples was attempted with standardized PCR and culture based method. The number of pork samples positive by PCR were 26 (26%) with 15 (30%) from tonsils and 11 (22%) from ground pork. In culture based method, 18 (18%) were found positive which includes 10 (20%) from tonsil and 8 (16%) of ground pork. The overall detection rate of Y. enterocolitica in pork samples by PCR was higher (26%) as compared with culture method (18%) (Table 5).

Further analysis of results revealed that number of samples positive only by PCR assay were five and three for pig tonsil and ground pork, respectively. The overall positivity for Y. enterocolitica in the pork samples by both the techniques was 26% (Table 6).

In the present study, sensitivity and specificity were calculated for detection of Y. enterocolitica in pork samples. On the basis of these characteristics there was very high sensitivity (100%) and specificity (90.24%) with PCR. The detection rate with PCR was superior as compared with culture method because it could detect Y. enterocolitica in pork even when organisms could not be cultivated.

Discussion

PCR is a well accepted method for detecting microbial DNA in a variety of samples. Using PCR, pathogenic Y. enterocolitica can be detected in samples rapidly and with high specificity. There are several reports of using PCR assay for specific amplification of Y. enterocolitica 16S rRNA gene and ail gene for detection of Yersinia in pork samples (Lantz et al., 1998; Bhaduri, 2003; Nowak et al.,...
The specificity of the PCR was verified by five Y. enterocolitica spp., one Y. intermedia and three non-Yersinia bacteria. Among the six Yersinia strains, only DNA from Y. enterocolitica strains yielded 16S rRNA specific PCR products of 330 bp. No amplification was observed with Y. intermedia and non-Yersinia strains. Further of these tested species, only DNA from pathogenic Y. enterocolitica strains (IP134, IP383 and W22703) yielded PCR products of 425 bp specific for ail gene and no amplification was observed with non-pathogenic Y. enterocolitica strains (MTCC 4858 and 4869), Y. intermedia and non-Yersinia strains (Table 2). Similar type of specificity using PCR assay based on the specific amplification of Y. enterocolitica 16S rRNA gene and ail gene has been reported earlier by other researchers as well (Nilsson et al., 1998; Wannet et al., 2001; Bhaduri, 2003; Nowak et al., 2006; Simonova et al., 2007; Hudson et al., 2008 and Renzi, 2012).

The detection limit of the 16S rRNA PCR assay for Y. enterocolitica organisms in experimentally inoculated ground pork was 10^3 CFU per gm without enrichment (no incubation), 10^4 CFU per gm with enrichment for 12 hours and 10^5 CFU per gm with enrichment for 24 hours (Table 3). Similar to our findings, Nilsson et al. (1998) also reported that as few as 10^2 Y. enterocolitica could be detected in ground pork by PCR. While - Harnett et al. (1996) detected between 5 to 10 CFU per ml Y. enterocolitica. In another study on detection, by Hudson et al., (2008) the detection limit of PCR was found to 10^4 to 10^5 CFU per ml from raw pork. Sara et al. (2009) in a study on detection of Y. enterocolitica in raw pork by conventional culture and PCR based methods reported the detection limit of 10^5 CFU per gm using CIN (Cefsulodin-irgasan-novobiocin agar) culture medium and 10^5 CFU per gm using pretreatment step with KOH, where as detection limit using PCR method was 10^3 CFU per gm for the 16S rRNA gene and 10^3 CFU per gm for the yst gene for a pre enrichment step of 24 h.

During present study, a total of 100 pork samples were subjected to PCR and Y. enterocolitica was detected in 26 (26%). Maximum number of samples from tonsils, 15 (30%) was found positive by PCR as compared to 11 (22%) from ground pork. The results of present study are strengthen by the findings of Fredriksson-Ahomaa et al., (2009) who reported the higher prevalence of Y. enterocolitica in pig tonsils (44%) as compared to other sample on application of real-time PCR. However, Fredriksson-Ahomaa et al. (2008) reported prevalence of Y. enterocolitica in tonsils of Swiss wild boars upto 24,18 and 5% by real-time PCR, PCR and culture techniques, respectively. The percentage of positive results in pig tonsils (30%) of our study are some what similar to 28.80% reported by Nowak et al., 2006 and lower than the 38.40% reported by Gurtler et al., 2005. Further DNA from 26 pork samples found positive by PCR, were subjected to a PCR assay amplifying ail gene resulting in 425 bp product specific to pathogenic strains of Y. enterocolitica. Out of these 26 samples two (one pig tonsil and one ground pork) were found positive thereby establishing them as pathogenic strains of Y. enterocolitica. There are several reports on isolation of pathogenic Y. enterocolitica from raw pork in which the incidence reported varies from 0-78% (Bhaduri et al., 1997; Fredriksson-Ahomaa et al., 1999; Vishnubhatla et al., 2000; Boyapalle et al., 2001 and Simonova et al., 2007).

The efficacy of PCR was assessed on the basis of diagnostic characteristics. The PCR was found more sensitive and specific as it detected Y. enterocolitica in pork samples even when organisms could not be cultivated with culture method. The agreement between PCR and culture method was 90.24%.

Overall PCR assay based on the specific amplification of Y. enterocolitica 16S rRNA gene and ail gene has been found not only as sensitive but also a rapid method and it can be considered as standard procedure for rapid screening of Y. enterocolitica in pork samples.

References


