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RESEARCH ARTICLE

MULTIPLEX PCR FOR THE DETECTION OF YERSINIA ENTEROCOLITICA AND SALMONELLA ENTERICA SEROVAR ENTERITIDIS FROM PORK

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ABSTRACT

In the present investigation, a multiplex PCR was standardised for the simultaneous detection of *Y. enterocolitica*, *S.* Typhimurium and *S.* Enteritidis. For that, a common enrichment protocol was developed for the simultaneous recovery of the organisms. Modified Tryptic Soy Yeast Extract Broth (TSBYE) at 37^0C for 16 h. was selected as an uniform enrichment protocol for the simultaneous enrichment of *Y. enterocolitica*, *S.* Typhimurium and *S.* Enteritidis. Multiplex PCR was standardised using primers *ail*, *stm* and *sen* for *Y. enterocolitica*, *S.* Typhimurium and *S.* Enteritidis respectively. The PCR technique could detect a concentration of up to 1 cfu/ g of *Y. enterocolitica*, *S.* Typhimurium and *S.* Enteritidis in mixed culture. The multiplex PCR standardised did not show any non-specific reaction with other competing organisms. A total of 210 samples from pigs were collected including 128 pork samples, 51 tongue samples and 31 tonsil samples from different retail outlets and slaughter houses at Thrissur and Eranakulam, Kerala, India. One sample of pork was positive for *Y. enterocolitica* and seven samples were positive for *S. Enteritidis.*

INTRODUCTION

Over the past decade, there has been a marked increase in the reported incidence of food borne illnesses due to the bacterial contamination of food, which is a major challenge to food safety. There is a need for the development of improved and rapid pathogen detection methodologies in order to assure safe food for the consumers. Yersinia enterocolitica and Salmonella enteric a are well-recognized human food borne pathogens, which are implicated in food borne disease outbreaks. Healthy pigs are the carriers of S. enteric a and Yesinia strains that are pathogenic to man (Bonardi *et al*, 2013). Consumption of pork is the main source for Yersiniosis in human and healthy pigs are known to be the primary reservoir of Y. enterocolitica (Fredriksson-Ahomaa *et al*, 2007). According to Wang *et al*. (2013) consumption of contaminated chicken, pork and other meat and meat products acts as the common sources of salmonellosis. These availability of reliable, rapid and multiplexed detection of food borne pathogens becomes increasingly important for food industry.

Cloak *et al*. (1999) investigated the possibility of using buffered peptone water, as a single enrichment broth for the simultaneous recovery of Y. enterocolitica, S. Enteritidis and L. monocytogenes. Sharma and Carlson (2000) used a single enrichment broth, which was prepared by mixing equal volumes of gram-negative broth and Trypticase soy broth for the simultaneous detection of Salmonella strains and E. coli O157:H7.

According to Nilsson *et al*. (1998), virulence gene, ail is a chromosomally located marker mostly limited to pathogenic strains of Y. enterocolitica which encodes an outer membrane protein that contribute to adhesion, invasion, and resistance to complement-mediated lysis. Nowak *et al*. (2006) and Kot *et al*. (2011) also employed ail gene for the detection of pathogenic Y. enterocolitica.

Liu *et al*. (2012) used specific primer sets stm 4495 and sen 1392 for the detection of S. Typhimurium and S. Enteritidis respectively. Whereas Ramya *et al*. (2012) used primers for sefA gene for S. Enteritidis and Moussa *et al*. (2012) used primers for sefA gene for S. Enteritidis and fliC gene for S. Typhimurium.

Wang *et al*. (1997) standardised a PCR protocol for the simultaneous detection of 13 species of food borne pathogens including Y. enterocolitica and Salmonella spp. using primers for enterotoxin gene and invA gene respectively.
High occurrence of Y. enterocolitica was reported by Khere *et al*. (1999) who obtained an overall prevalence of eight per cent for Y. enterocolitica and 17.3 per cent for Y. intermedia from pork. Bonardi *et al*. (2003) reported a lower prevalence of 0.67 per cent of Y. intermedia in pork, which is in agreement with the present study. When Bonardi *et al*. (2013) screened 726 samples from pig including rectal, throat, floor, wall and carcass swabs, Y. enterocolitica was isolated only from 0.55 per cent of samples.

Higher prevalence of Salmonella in pork was a reported by Van *et al*. (2007) and Yang *et al*. (2010) who obtained a prevalence of 64 and 31 per cent respectively. Vanantwerpen *et al*. (2013) attempted confirmation of Y. enterocolitica isolates obtained from tonsils of 1397 fattening pigs by observing the presence of ail gene in 26.8 per cent tonsils.

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Bolton *et al*. (2013) made a study to investigate the presence of Y. enterocolitica at different stages of production from birth to chilled carcasses. A total of 726 samples consisting of rectal, throat, floor, wall and carcass swabs and water samples were tested using bacteriological analytical method. Yersinia enterocolitica could be detected from two floor swabs, one wall swab and one rectal swab with an overall prevalence rate of 0.55 per cent.

A study was conducted by Cortez *et al*. (2006) for the identification of Salmonella spp. isolated from chicken abattoirs by multiplex-PCR using three sets of primers targeting the invA, pefA, and sefA gene sequences from Salmonella spp., S. Typhimurium and S. Enteritidis respectively. Out of 288 samples tested, Salmonella spp. was detected in 10 per cent of the samples, whereas serovars Enteritidis and Typhimurium were identified in 5.6 per cent and 2.4 per cent of samples respective

MATERIALS AND METHODS

Procurement of reagents

Standard cultures of *Y. enterocolitica* (MTCC 859), *S.* Typhimurium (MTCC 1144), were procured from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Reference culture of *S.* Enteritidis was procured from repository of Indian Veterinary Research Institute (IVRI), Izatnagar. Maintenance of pure cultures was carried out by regular sub culturing onto Nutrient Agar slants at 25 days interval. Molecular reagents and chemicals used in the study were procured from Sigma, Bangalore, Fermentas, Bangalore, Thermo Scientific (EU) and Sigma- Aldrich (USA).

Development of common enrichment protocol for the recovery of Y. Enterocolitica, S. Typhimurium and S. Enteritidis.

Six different broths including Peptone Water (PW), Nutrient broth (NB), Luria Bertani broth (LB), Selenite Cystein broth (SC), Brain Heart Infusion broth (BHI) and Tryptic Soy Yeast extract broth (TSBYE) were compared for the growth of *Y. enterocolitica*, *S.* Typhimurium and *S.* Enteritidis. Growth of the organisms in different incubation temperatures (32^0 C and 37^0 C) and different incubation periods (12h., 16h., and 18h.) were also evaluated using different broths under comparison.
A loopful of individual bacterial culture was inoculated into five different broths and incubated at six different incubation conditions i.e., 32^0 C for 12 h., 16 h., and 18 h. and at 37^0 C for 12 h., 16 h. and 18 h. Duplicate tubes were used for each enrichment condition. After the period of incubation, optical density (OD) was measured using spectro- photometer at 600nm (Perkin Elmer, Lambda 25). The entire protocol was repeated for six times.

***Standardisation of Multiplex PCR Protocol For Y. Enterocolitica, S.* Typhimurium *and S.* Enteritidis**

Three virulence markers were assayed by PCR for the detection of *ail* gene for *Y. enterocolitica*, *stm* gene for *S.* Typhimurium and *sen* gene for *S.* Enteritidis. Gradient PCR was carried out at different annealing temperatures and 60^0 C was selected. PCR was carried out at different concentrations of MgCl2 and 2 mM was found to be optimum. The PCR reagents used were as follows: PCR reaction buffer (10X), Taq DNA polymerase (1U/µl), dNTP mix (10 m*M*), $MgCl_2$ (25m*M*), forward and reverse primer set (100n*M*/ml) and sterilized milliQ water. The amplification conditions were Initial denaturation of 7 min followed by 35 cycles of 1 min for final denaturation at 950 C, 40 sec at 60^0 C for annealing and 2 min at 72^0 C for extension and one cycle of 10 min at 720C for final denaturation. The PCR products were separated in a 1.5 per cent agarose gel and stained with Ethidium bromide(10mg/ml).

In order to determine the sensitivity of standardised PCR protocol, five different concentrations of *Yersinia enterocolitica, S.* Enteritidis and *S.* Typhimurium were studied *i.e.*, 10^3, 10^2, 10, 10^0, 10^{-1} cfu/ml.

The specificity of the standardized PCR protocol was tested by screening the reference strains as well as other commonly prevalent and cross-reacting bacterial species such as *E. coli, Enterohemorhagic E. coli, L. monocytogenes, S. aureus* and *V. parahaemolyticus*.

Prevalence of Yersinia Enterocolitica, Salmonella Typhimurium And Salmonella Enteritidis in Pork

A total of 210 samples from pig, including 128 pork samples, 51 tongue samples and 31 tonsil samples were collected from different retail outlets and slaughter houses at Thrissur and Eranakulam, Kerala, India. The samples were taken, 50 g each from retail portions of boneless pork, tongue and tonsil samples.

A 25 gram portion of each sample was aseptically transferred to 225 ml of modified TSBYE in a stomacher bag. Then it was homogenized in a stomacher (Smasher, AES, France) for 120 sec. and incubated at 37^0 C for 16 h. Two milliliter aliquot from the homogenised sample was used for DNA extraction by boiling and snap cooling method and multiplex PCR protocol was carried out. One milliliter of aliquot from pre-enriched samples were taken into an eppendorf tube and centrifuged at 1000 X g for 10 min. at 4^0 C. The supernatant was discarded and the pellet obtained at the bottom was washed twice in one millilitre of sterile milliQ water by re-centrifugation at 1000 X g for 10 min. at 4^0 C. The pelleted cells obtained finally were resuspended in 100µl of Molecular grade water, kept in a boiling water bath for 10 min. and then immediately chilled on crushed ice for 30 min. Then the samples were centrifuged at 1000 X g for five minutes and supernatants were stored at -20 °C for further use as template for PCR. From this 2µl of template DNA was used directly for the multiplex PCR.

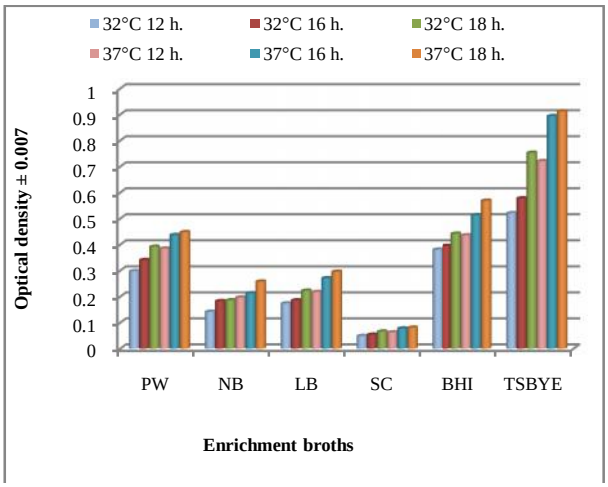
Statistical analysis

All data were analysed by ANOVA and Duncan's multiple range test (DMRT) using SPSS,
Version 22.0.

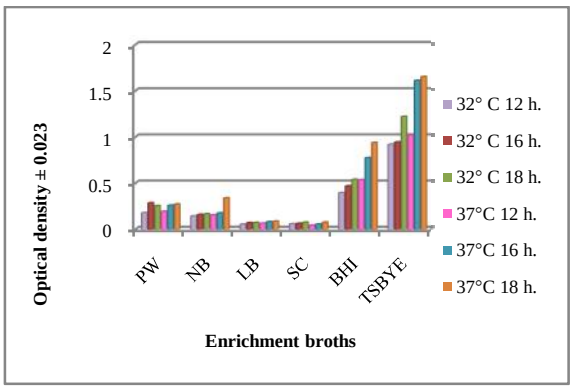
RESULTS

The growth of *Y. enterocolitica*, *S.* Typhimurium and *S.* Enteritidis in different broths at different incubation conditions were analysed with respect to optical density after the period of incubation. The optical density was measured using Spectrophotometer at 600nm. The mean OD values of the organisms at different incubation conditions are represented in graph 1, 2, 3 and 4. Three way ANOVA test was carried out for comparing OD values in different broths at different conditions. The analysis revealed that, there was significant

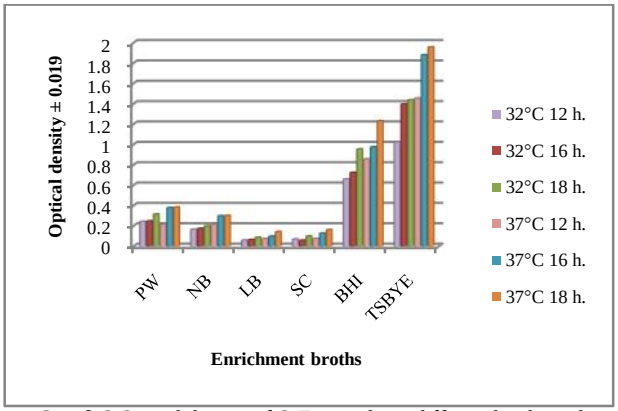
difference in the growth of *Y. enterocolitica*, *S. Enteritidis and S.Typhimurium* individually and as mixed culture in different broths. The organisms showed significantly higher growth in TSBYE with respect to optical density and bacterial count when inoculated individually and together. TSBYE showed significantly higher ($p<0.01$) optical density and when compared to all other media. Optimum growth of all the organisms was observed at 37^0 C than 32^0 C. However, better growth of all the organisms was observed at 18h. and 16. of incubation, 16 h. was selected for the common enrichment for the rapid recovery of the pathogens individually and in mixed culture. So TSBYE at 37^0 C for 16 h. was selected as the common enrichment protocol for the isolation of *Y. enterocolitica*, *S.* Typhimurium and *S.* Enteritidis.



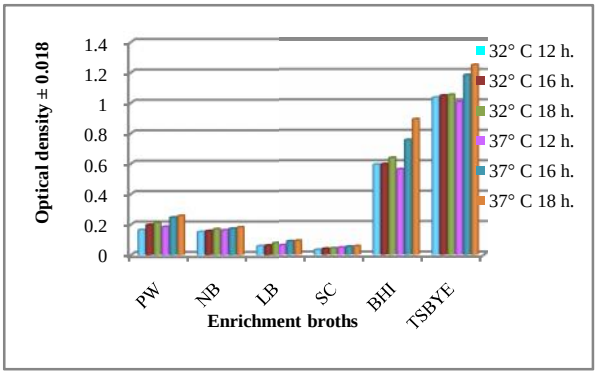
Graph 1 Optical density of *Y. enterocolitica* in different broths and different incubation conditions



Graph 2 Optical density of *S.* Typhimurium in different broths and different incubation conditions



Graph 3 Optical density of *S.* Enteritidis in different broths and different incubation conditions



Graph 4 Optical density of mixed culture in different broths and different incubation conditions

Standardisation of multiplex PCR

Standardised multiplex PCR protocol allowed the simultaneous amplification of virulence associated genes of *Y. enterocolitica*, *S.* Enteritidis and *S.* Typhimurium namely *ail*, *sen* and *stm* to their respective 351, 656 and 915 bp. This PCR could detect a concentration of up to 1 cfu/ g of all the organisms. The multiplex PCR did not show any non-specific reaction with other competing organisms (Figure 1)

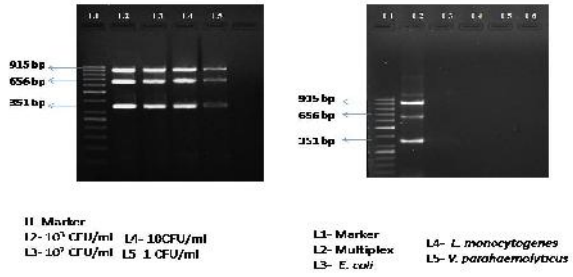


Figure 4 sensitivity and specificity of multiplex PCR

Prevalence of Yersinia Enterocolitica, Salmonella Typhimurium And Salmonella Enteritidis in Pork

Two hundred and ten samples of pork collected from different sources were subjected to multiplex PCR for the detection of *Y. enterocolitica*, *S.* Typhimurium and *S.* Enteritidis One sample of pork from Angamali showed the presence of virulence gene, *ail* of *Y. enterocolitica* in the multiplex PCR (Figure 2).

Salmonella Enteritidis was detected in six samples of pork and one sample of tongue by multiplex PCR (Figure 3). None of the sample was positive for *S.* Typhimurium.

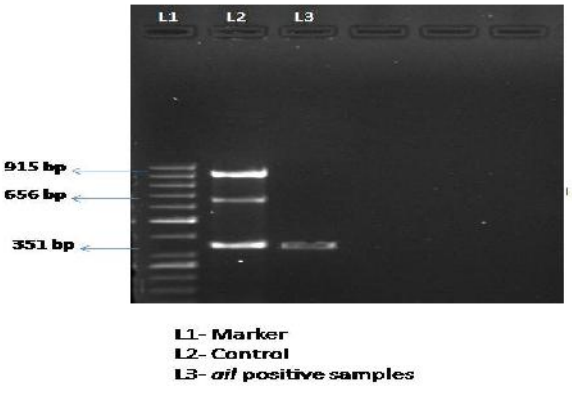


Figure 5 Yersinia enterocolitica positive sample by multiplex PCR

The statistical analysis revealed that there was no significant difference between the occurrences of Salmonella in pork and tongue samples. The overall prevalence of *Y. enterocolitica* and *S.* Enteritidis was found to be 0.48 per cent and 3.33 per cent in the samples from pig.

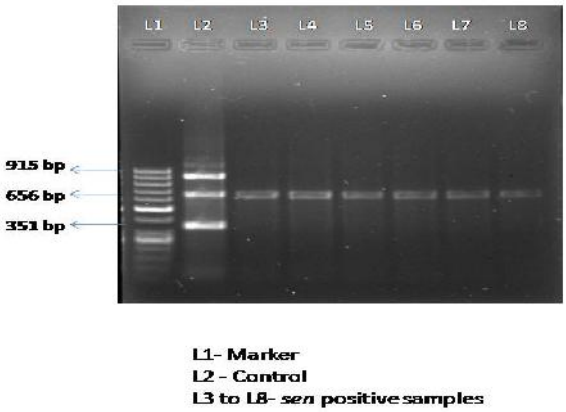


Figure 6 *S.* Enteritidis positive samples by multiplex PCR

DISCUSSION

Multiplex PCR assay standardized in the present study can detect more than one pathogen simultaneously by amplifying more than one target gene in a single reaction, which can save time and lab our cost. Detection of *Y. enterocolitica* in meat and other products is of particular concern in relation to consumer safety as these organisms are capable of growing both in raw and cooked meat on refrigeration temperature, which could lead to the development of significant number of the organisms. Detection of Salmonella on meat indicate the need for greater awareness of the risk associated with the production and handling of meat. The primary reservoir of Salmonella is the intestinal tract of animals and birds.

During slaughter, the intestinal contents can spill and contaminate the muscles and organs of the animals and birds which is the important source of presence of enteric pathogens in meat. With raw meat, contamination may be less important as cooking to an internal temperature of 60^0 or more prior to consumption eliminate Salmonella. However, inadequately cooked meat and cross contamination cause greater risk of Salmonella poisoning. Personnel involved in the production, distribution and retail handling and sail should be aware of potential risk from cross contamination and steps to be taken to limit the transfer of the pathogen between raw and cooked products.

CONCLUSION

In general, food borne illnesses is a major public health problem and a variety of food products especially chicken and pork meat acts as the important source of food borne illnesses like Salmonellosis and Yersiniosis. The changing food habits of the consumers pose a risk to such food borne diseases. In order to reduce the risk of contamination and infection due to these organisms, it is important to adopt good hygienic practices during slaughter. The presence of virulence genes from the isolates shows the pathogenic potential of the organisms. Implementation of preventive measures and consumer food safety education efforts are needed to reduce the risk of food borne diseases. Proper cooking of the meat before consumption and improving personnel and meat hygiene in the line of meat production from farm to fork should be adopted to ensure safety of meat for human consumption.

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