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# OPTIMIZATION OF MULTIPLEX PCR FOR DETECTION OF GRAM POSITIVE FOODBORNE PATHOGENS FROM RETAIL QUAIL MEAT

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This experimental design was developed to optimize the conventional and multiplex PCR reaction for detection of *Staphylococcus aureus* and *Bacillus cereus* from quail meat samples. Conventional PCR was optimized with annealing temperature 50°C, each primer concentrations of 10pmol, 200ng of DNA template and 10µl Qiagen master mix and 35 number of PCR cycles. In the same way multiplex PCR was optimized with annealing temperature of 50°C, 20pmol of each primer, 200ng DNA template of each bacteria and 23µl of Qiagen multiplex master mix. Quail meat samples were collected from chain stores (n=40), retail market (n=40) and UVAS quail farm (n=20). Prevalence of *S. aureus* through conventional culture method was detected to be 80 percent. While through conventional and multiplex PCR recovery was 85 percent. Prevalence of *B. cereus* through conventional culture method was 65 percent, while through conventional and multiplex PCR recovery rate was 72 percent. Statistically no significant difference ($p$>0.05) was found between conventional culture method, conventional PCR and multiplex PCR. Similarly, no significant difference ($p$>0.05) was observed in recovery rate of *S. aureus* and *B. cereus* in quail meat collected from chain stores, retail market and UVAS quail farm.
**Keywords:** Foodborne pathogens, *S. aureus, B. cereus,* Multiplex PCR, quail meat

## INTRODUCTION

Foodborne diseases are of main concern throughout the world. Gram negative bacteria cause most foodborne diseases, but some gram positive bacteria like *Staphylococcus aureus, Listeria monocytogenes* and *Bacillus* species etc. are also of great concern (Le Loir *et al*., 2003). Food is a tremendous medium by which pathogens can reach a suitable colonization site in a new host (Newell *et al*., 2010). *S. aureus* causes the most recurrent food poisoning worldwide. It produces enterotoxins due to which in lumen loss of water occurs that results in diarrhea and vomiting (Brizzio *et al*., 2013). Two types of food poisoning are caused by *Bacillus cereus* i.e. diarrheal syndrome and emetic disease (Yuan *et al*., 2012).

Food and Drug Administration's (FDA) main concern is to protect the community from the microbial infectivity of food supply. FDA has listed some bacteria as foodborne pathogens which includes, *Clostridium botulinum, Listeria monocytogenes, Clostridium perfringens, Staphylococcus aureus, Bacillus cereus, Salmonella, Shigella, Escherichia coli, Campylobacter, Vibrio* spp. and *Yersinia* spp. (Wang *et al*., 1997). Due to consumption of food contaminated with *B. cereus, C. perfringens* and *S. aureus*, there were 1229 reported outbreaks of gastroenteritis in United States from 1998 to 2008 (Bennett *et al*., 2013). In another study, major bacterial pathogens isolated from meat were *B. cereus, E. coli* and *S. aureus* (Haileselassie *et al*., 2013).

Quail meat is a rich source of different vitamins like riboflavin, niacin, vitamin B6, pantothenic acid and thiamin. This is the reason quail meat is favorite of most people as compared to the other types of the poultry meat (Hamad *et al*., 2012). By using conventional and standard methods such as culture and colony counting methods the bacterial pathogens present in foods can be identified which may take up several days and hours to yield results (Velusamy *et al*., 2010). For rapid, specific detection PCR based techniques are being increasingly used with better differentiating power (Pinto *et al*., 2005). Multiplex PCR is more rapid method as compare to conventional PCR (Chen *et al*., 2012). To optimize multiplex PCR, relative concentration of primers, amount of DNA template, balance between the Magnesium Chloride and dNTPs, concentration of *Taq* DNA polymerase, and concentration of PCR buffer are important (Markoulatos *et al*., 2002).

## MATERIALS AND METHODS

***Revival of positive control*:** ATCC culture of *S. aureus* (ATCC 25923) and *B. cereus* (ATCC 11778) was revived in University Diagnostic Laboratory (UDL), University of Veterinary and Animal Sciences, Lahore by culturing in

**Table 1. Primers sequences used in this study for detection of *Staphylococcus aureus* and *Bacillus cereus*.**

| Gene name | Sequence 5'-3' | Annealing Temperature | Amplicon Size (bp) |
|---|---|---|---|
| Nuclease gene | Forward | | 276 |
| (*Staphylococcus aureus*) | CGATTGATGGTGATACGGTT | 50°C | |
| | Reverse | | |
| | CAAGCCTTGACGAACTAAAGC | 50°C | |
| Hemolysin gene | Forward | | 185 |
| (*Bacillus cereus*) | CTGTAGCGAATCGTACGTATC | 50°C | |
| | Reverse | | |
| | TACTGCTCCAGCCACATTAC | 50°C | |

Tryptic Soy Broth (Oxoid, UK) for preliminary enrichment. After primary enrichment, *S. aureus* was cultured on Mannitol salt agar (Kateete *et al*., 2010) and *B. cereus* was cultured on Mannitol egg yolk Polymyxin agar (Tallent *et al*., 2012). Bacteria were confirmed through microscopy, gram staining and biochemical testing (Cowan *et al*., 2004; Smith *et al*., 2004). DNA was extracted by using Phenol, Chloroform and Isoamyl Alcohol (PCI) method (Cheng and Jiang, 2006).

***Optimization of conditions for conventional and Multiplex PCR*:** The sequence of PCR primers was selected from specific regions of *S. aureus* (*nuc* gene) and *B. cereus* (hemolysin gene) listed in Table 1. For conventional and multiplex PCR, the amplification reactions were done in 25µl and 50ul reaction mixtures respectively. Different PCR conditions were optimized by changing the concentrations and cycling conditions i.e. Primer concentrations 8pmol, 10pmol, 15pmol and 20pmol, DNA template concentrations 200ng, 250ng, 300ng and 400ng, concentration of master mix 8µl, 10µl, 12µl and 15µl, annealing temperature from 50 to 60°C, and number of PCR cycles 25, 30, 35 and 40. After PCR reaction, product was separated and visualized through 2 percent gel electrophoresis (Wang *et al*., 1997). After PCR reaction, product was separated and visualized through 2 percent gel electrophoresis as shown in Figure 1.

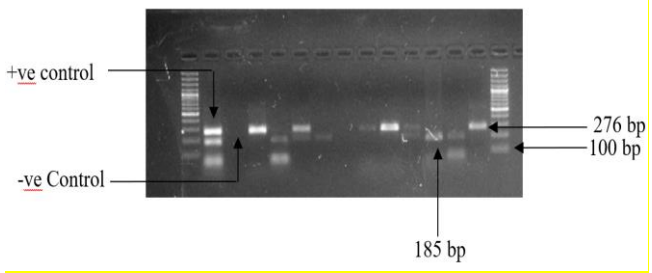


**Figure 1. Ethidium bromide stained Agarose gel (2%) showing PCR result using DNA ladder (100 bp) 276 bp: *S. aureus* (*nuc* gene), 185 bp: *B. cereus* (hemolysin gene).**

***Sample collection*:** A total 100 slaughtered quails were purchased from various chain stores, retail stores and UVAS quail farm (40 from chain stores, 40 from retail market and 20 from UVAS quail Farms). The samples were transported to University Diagnostic Lab, University of Veterinary and Animal Sciences, Lahore for isolation and identification of *S. aureus* and *B. cereus*. Pre-enrichment was done in Tryptic soy broth by incubating at 37°C for 24 hours.

***Isolation and identification of S. aureus and B. cereus through biochemical tests*:** After initial enrichment, samples were inoculated onto selective media. For *S. aureus* Mannitol salt agar and for *B. cereus* Mannitol egg yolk Polymyxin agar was used. Confirmation of *S. aureus* was done by using catalase and coagulase test (Ref). *B. cereus* was confirmed through starch agar and Voges Proskauer test (Quinn *et al*., 2011).

***Direct detection of S. aureus and B. cereus through conventional and Multiplex PCR*:** Qiagen DNA Isolation kit was used to extract DNA from quail meat. Optimized conventional PCR was performed in 25µl that include 10 pmol (1µl) of each primer, 4µl of DNA template (50ng/µl), 10µl of Qiagen master mix and 9µl PCR grade ultra-pure water. The amplification conditions were initial denaturation at 95°C for 5 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension 72°C for 1 min and final extension at 72°C for 10 min. The PCR products (10µl of each) were separated by electrophoresis in 2% Agarose gel containing ethidium bromide (1µg/ml) as staining dye. Same samples were processed through optimized multiplex. PCR amplification was done in 50µl which include 2µl (20pmol) of forward and reverse primer of each bacteria, 4µl of each DNA template (50ng/µl), 23µl of Qiagen multiplex master mix and 11µl PCR grade ultra-pure water. The amplification conditions were initial denaturation at 95°C for 15 min, then 35 cycles of denaturation at 94°C for 30sec, annealing at 50°C for 90 sec, extension 72°C for 90 sec and final extension at 72°C for 10 min. The PCR products (10µl of each) were separated by electrophoresis in 2% Agarose gel containing ethidium bromide (1µg/ml) as staining dye.

## RESULTS AND DISCUSSION

This study was aimed for rapid detection of gram-positive foodborne pathogens in retail quail meat. This aim was

achieved by optimizing the conventional and multiplex PCR for *S. aureus* and *B. cereus.* Optimization was done by changing the conc. of primers, DNA template, master-mix, annealing temperature and number of PCR cycles. In conventional PCR, four different concentrations of primers were used in present study but best results were obtained when 10pmol and above concentration was used. It is best to use 10pmol rather than high conc. of primer because bands were equally bright at 1µl and above concentrations to avoid the chemical wastage and possibility of dimers. Similarly, primer optimization was done for multiplex PCR. Different concentrations of each primer of *S. aureus* and *B. cereus* were used. Sharp bands were observed at 20pmol and above concentration. To avoid chemical wastage 20pmol was used in the study. It is better to use lesser concentration of primer because at higher concentration of primer non-specific amplification may occur. It was noticed that as primer concentration increases there are more chances of dimmers or nonspecific amplification (Malorny *et al*., 2003). DNA quantification was done by Nano Drop (Thermo Scientific USA). Concentration of DNA samples was adjusted to 50ng/µl. In conventional PCR best results were obtained when 200ng or above conc. of DNA template was used which produced bright and sharp bands. So, by using the minimum volume of DNA template best PCR results were obtained. In multiplex two sharp and bright bands were observed when 200ng or above concentrations were used (Fig.1). In conventional & multiplex PCR, 200 ng was the lowest concentration of DNA at which bright bands were observed respectively. To avoid chemical wastage lesser amounts were used in this study. Annealing temperature is one of most important parameters in optimization. Primer annealing temperature and time depends on the length, base composition and primer's concentration. Temperature ranging from 50-60°C was checked for conventional PCR as well as for multiplex PCR. Bright bands were observed at 50°C. In 1997, a protocol for multiplex PCR was established in USA for the PCR detection of 13 foodborne pathogens. In this study, sharp bands were also observed at 50°C (Wang *et al*., 1997).

By changing the number of PCR cycles, bright bands were observed at 35 cycles. At 40 cycles, same bands were observed but to save time, we used option of 35 cycles.

In the present study, detection of gram positive foodborne pathogens was done by using three different methods i.e. conventional biochemical testing, conventional PCR and multiplex PCR. One hundred quail meat samples were processed for detection of *S. aureus* and *B. cereus*. Prevalence of *S. aureus* was 80% from quail meat samples when detection was done through biochemical testing. Out of 40 samples taken from chain store 32 samples (80%) were positive. While 40 samples were taken from retail market and 31 samples (78%) were positive. Out 0f 20 samples collected from UVAS quail farm 17 samples (85%) were positive. The highest prevalence was observed in the samples that were collected from the UVAS quail farm. Prevalence of *S. aureus* was 85% from quail meat samples when detection was done through conventional and multiplex PCR. The results were same when detection was done by these two methods. Forty samples were taken from chain store and 35 samples (88%) were positive, while out of 40 samples from retail market 33 samples (83%) were positive. Out of 20 samples collected from UVAS quail farm 17 samples (85%) were positive. Highest prevalence was observed in the samples that were collected from the chain stores. In a study, PCR detection of *S. aureus* from different food samples was done to check the specificity of *nuc* targeted primers. Total 164 food samples were processed and 69 samples was positive by the conventional biochemical testing. When detection was done by conventional PCR 74 samples were positive. The results were compared and high level of result agreement (93.3) was found between these two methods. According to findings of this study, PCR was proved more reliable and specific and PCR might improve the detection of *S. aureus* in different food samples (Alarcon *et al*., 2006).

These samples were also processed for the detection of *B. cereus* through conventional biochemical testing, conventional PCR and multiplex PCR. Prevalence of *B. cereus* was 65% from 100 quail meat samples when conventional culturing method was used. Forty samples were taken from chain store and 25 samples (62.5%) were positive. While 40 samples were taken from retail market and 25 samples (62.5%) were positive. Out 0f 20 samples collected from UVAS quail farm 15 samples (75%) were positive. The highest prevalence was observed in the samples that were collected from the UVAS quail farm. Prevalence of *B. cereus* was 72% from quail meat samples when detection was done through conventional and multiplex PCR. Out of 40 samples from chain store 27 samples (67.5%) were positive. While 40 samples were taken from retail market and 29 samples (72.5%) were positive. Out 0f 20 samples collected from UVAS quail farm 16 samples (80%) were positive. Highest prevalence was observed in the samples that were collected from the UVAS quail farm. In another study, total 68 food samples were processed for the detection of *B. cereus.* For detection specific media was used i.e. Polymyxin pyruvate egg yolk mannitol bromocresol purple agar. *B. cereus* was found 36.7% while enterotoxigenic *B. cereus* was 29.41%. All the isolates were screened for the presence of different genes. Enterotoxigenic *B. cereus* showed presence of *hbla* gene while no gene was found in non-enterotoxigenic *B. cereus* (Das *et al*., 2009).

There was little difference of results. Through PCR more samples were positive because PCR is more sensitive and specific. Although this molecular technique requires expertise but it was time saving as compared to conventional

testing. PCR has advantage over conventional culturing method due to the identification of bacteria at specie level. While conventional culturing method also requires more time for bacterial identification at specie level. According to the recent report, if the enrichment time is shortened then it is possible to enrich a sample and detection of pathogens might be done by real time PCR (Josefsen *et al*., 2004).

***Conclusion*:** This study may be helpful for the rapid and simultaneous detection of Gram positive foodborne pathogens (*S. aureus* and *B. cereus*) from commercially available quail meat through conventional and multiplex PCR.

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