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DETECTION OF *Escherichia coli* AND *Salmonella* FROM RETAIL QUAIL MEAT THROUGH OPTIMIZED MULTIPLEX PCR

Amna Kanwal[1,*], Ali Ahmad Sheikh[1], Masood Rabbani[1], Tanveer Hussain[2], Iqra Safdar[1], AyeshaTabassum[1], Asfa Rasool[1], Javed Muhammad[1] and Mawra Gohar[1]

[1]University Diagnostic Lab., University of Veterinary and Animal Sciences, Lahore, Pakistan;
[2]Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, Pakistan.
[*]Corresponding author's e- mail: aminah.kanwal@yahoo.com

The conventional and multiplex PCR was developed for rapid and simultaneous detection of multiple food-borne pathogens in single reaction mixture. Conventional PCR was optimized with annealing temperature at 50°C, each primer concentration of 10 pmol, DNA template 200 ng and 10 µl Fermentas master mix with 35 PCR cycles. Similarly multiplex PCR was optimized with annealing temperature 56°C, each primer concentration of 20 pmol, DNA template 200 ng using 23 µl Qiagen multiplex master mix and 35 PCR cycles. A total 100 slaughtered quails from various chain stores (n=40), retail market (n=40) and University of Veterinary and Animal Sciences quail farm (n=20) were processed for conventional and multiplex PCR for direct detection of *Escherichia coli* and *Salmonella*. Prevalence of *Escherichia coli* and *Salmonella* through conventional culture method and biochemical testing was detected to be 82.5% and 66.6% respectively. While through conventional and multiplex PCR recovery of *E. coli* was 90% and *Salmonella* was 82%. 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 *Escherichia coli* and *Salmonella* in quail meant collected from chain stores, retail market and UVAS quail farm.

Keywords: Food-borne pathogen, Multiplex PCR, conventional culturing methods, *E. coli*, *Salmonella*

INTRODUCTION

Food-borne pathogens are of major public health concern throughout the world. Raw and under-cooked poultry meat is a rich source of *E. coli, Salmonella, Shigella* and *Campylobacter*. These pathogens can be transmitted to humans by consuming contaminated food and can lead to the risk of food-borne illness (Sackey *et al.,* 2001; Nzouankeu *et al*., 2010; Hara-Kudo *et al*., 2012). *Salmonella, E. coli*, and *Shigella* are major pathogens regarding public health significance that belongs to Enterobacteriaceae family and residing in intestinal tract of warm blooded animals as normal flora. Such pathogenic strains of *E. coli* have special adhesion fimbriae, through which they can bind with intestinal mucosa, damaging the absorptive surface of intestine, leading to diarrhea (Vincent *et al.,* 2010). *Salmonella*, being the normal microbiota of poultry and animals, is one of the major causes of food-borne illness throughout the world. *Salmonella typhimurium* is major pathogenic *Salmonella* transmitted through food (Boonmar *et al.,* 1998; Forshell and Wierup, 2006). During 1988 to 2007, total 4093 food-borne outbreaks were recorded globally, among them *Salmonella enteritidis* outbreaks were frequently occurred in European Union while outbreaks due to *E. coli* were at top in Canada (Greig and Ravel, 2009).

Quail meat is an admirable source of vitamin B6, niacin, thiamin, pantothenic acid and riboflavin. Quail meat is considered better than other species of poultry meat due to its delicacy and low level of cholesterol and also due to high meat yield, little shrinkage during cooking, fast cooking and serving (Hamad *et al.,* 2012). Pakistan is exporting meat to various countries and during 2012 Pakistan has earned $123.61 million through meat export. In order to increase export of quality meat, it is necessary that meat should be free from food borne pathogen (Naeem, 2012).

Polymerase chain reaction (PCR) is a powerful diagnostic tool for sensitive, specific and selective detection of food borne pathogens even in lower amount (Malorny *et al*., 2003). Multiplex PCR can be used for the simultaneous amplification of two or more genome by using multiple primers in same reaction mixture (Henegariu *et al.,* 1997). Multiplex PCR amplification is an efficient, sensitive and quick method for the simultaneous detection of pathogens in meat (Vantarakis *et al.,* 2000). Conventional detection and identification methods of food-borne bacteria, commonly used in clinical laboratories, are laborious and time consuming. Other drawbacks include high cost and poor yield of bacteria (Liu *et al.,* 2012).

MATERIALS AND METHODS

***Sample Collection*:** A total of 100 slaughtered quails were purchased from various chain stores (n=40), retail market (n=40) and UVAS quail farm (n=20). The samples were

Table 1. Primers sequences used for the amplification of *malB* and *invA* gene

Gene	Sequence5'- 3'	Length	Annealing Temperature	Amplicon size bp
malB	Forward GACCTCGGTTTAGTTCACAGA	21	50°C	585
promoter	Reverse CACACGCTGACGCTGACC	18		
invA gene	Forward TATCGCCACGTTCGGGCAA	19	50°C	275
	Reverse TCGCACCGTCAAAGGAAC	18		

transported to University Diagnostic Lab, University of Veterinary and Animal Sciences, Lahore for isolation and identification of *E. coli* and *Salmonella.* For pre-enrichment 1g of quail meat was inoculated in 9ml of Tryptose Soy Broth (TSB) medium and incubated 37°C for 24 hours.

***Revival of positive controls*:** Whole experiment was run in parallel with positive control of American type culture collection of *E. coli* (ATCC 25922) and *Salmonella* (ATCC 14028) and was revived in University Diagnostic Laboratory (UDL), University of Veterinary and Animal Sciences, Lahore by culturing on TSB for preliminary enrichment. After initial enrichment, *E. coli* was purified on McConkey's agar and *Salmonella* was purified on XLD Agar. Bacteria were confirmed through microscopy, gram staining and biochemical testing (Chowdhury *et al.,* 2011).

***PCR primers*:** Primers were designed from Gene Link e-oligo (USA). *E. coli* and *Salmonella* in quail meat was detected using genus specific primers against *malB* gene and *invA* gene respectively and sequence of each primer are listed in Table 1 (Wang *et al*., 1997).

***Optimizing condition for conventional PCR*:** Optimization of PCR was majorly based on DNA concentration, primer concentration and concentration of commercially available PCR master mix (Fermentas thermo scientific, USA). The conventional PCR was performed in a total volume of 25 µl. PCR conditions were optimized by changing following parameters, annealing temperature (50°C, 50.7°C, 51.9°C, 53.8°C, 56.1°C, 58°C, 59.2°C and 60°C), template concentration (200 ng, 300 ng and 400 ng), primers concentration (8 pmol, 10 pmol and 20 pmol), and master mix (Fermentas Thermoscientific, USA) was checked with 8 µl, 10 µl and 12 µl concentration. The thermocycler (Bio-Rad, CFX™, Singapore) was set for number of PCR cycles 25, 30, 35 and 40 cycles (Henegariu *et al*., 1997). After PCR reaction, product was separated and visualized through 2% gel electrophoresis.

***Optimization of conditions for multiplex PCR for simultaneous detection of Escherichia coli and Salmonella*:** The PCR amplification was performed in a total volume of 50 µl and during optimization the following parameters including DNA concentration (150 ng, 200 ng, 250 ng, 300 ng), each primers concentration (10 pmol, 15 pmol, 20 pmol and 25 pmol), and QIAGEN multiplex PCR master mix (20 µl, 23 µl, 25 µl and 28 µl). The thermocycler (Bio-Rad, CFX™, Singapore) was set for annealing temperature at 56°C, and number of cycles were 35 for simultaneous amplifying of target genes of three bacterial types using Qiagen multiplex master mix.

***Detection of E. coli and Salmonella through Biochemical tests, conventional PCR and multiplex PCR*:** After initial enrichment *E. coli* was purified on EMB agar and *Salmonella* was purified on XLD Agar. Confirmation was done through microscopy, gram staining and biochemical testing (CIPARS, 2006; Quinn *et al*., 2011)**.** DNA from quail meat was extracted using Qiagen mini prep DNA Isolation kit. Optimized PCR (25 µl) mixture included10 pmol of each primer, 4 µl of DNA template (50 ng / µl), 10 µl of Qiagen master mix and 9 µl PCR grade ultra-pure water was used for detection of individual *Salmonella* and *E. coli*. The amplification condition was initial denaturation at 95°C for 5 min, then 35 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, extension 72°C for 1 minute and final extension at 72°C for 10 minutes**.** Same samples were processed through optimized multiplex PCR. 50 µl reaction mixture included 10 pmol of forward and reverse primer used against each bacteria, 4µl of each DNA template (50 ng/ µl), 23µl of Qiagen multiplex PCR master mix (USA) and 11µl PCR grade ultra-pure water. The amplification condition was initial denaturation at 95°C for 15 minutes, then 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 90 seconds, extension at 72°C for 90 seconds and final extension at 72°C for 10 min. The PCR products (10 µl of each) were separated by electrophoresis in 2% Agarose gel containing ethidium bromide as staining dye (1 µg/ ml) as shown in Fig. 1.

RESULTS AND DISCUSSION

Current study was based on many experiments for establishing optimized conventional and multiplex PCR, further evaluating the results by comparing with conventional cultural methods. Optimization was done by ATCC cultures of *E. coli* and *Salmonella.*

***Optimization of PCR conditions*:** During optimization of conventional PCR best results were obtained with reaction mixture of 25 µl reaction mixture including Fermentas Thermoscientific master mixture (10 µl), primer against both

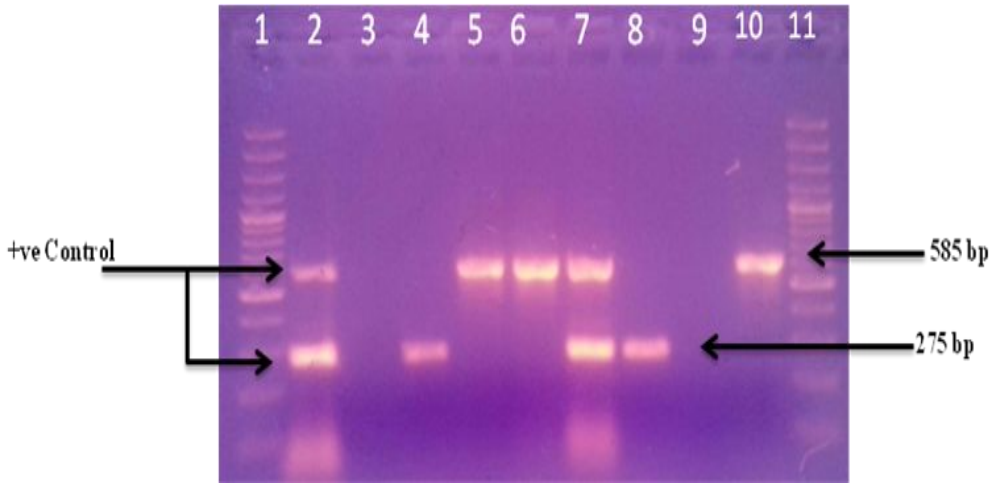

Figure 1. Agrose gel electrophoresis showing *malB* gene (*E. coli*) and *invA* gene (*Salmonella*)
Lane 1&11: DNA ladder (100 bp), Lane 2: Positive control for *malB* gene (585 bp) and *invA* gene (275 bp) Lane 3: negative control, Lane 4-10: quail meat samples.

for *E. coli* and *Salmonella* were of 10 pmol concentration and DNA template 4 µl (50 ng/ µl).

Touchdown PCR was carried out to optimize annealing temperature in the range of 50°C - 60°C for 1 minute per cycle. After positive results with this range, another gradients PCR with different annealing temperature of each row (50°C, 50.7°C, 51.9°C, 53.8°C, 56.1°C, 58°C, 59.2°C and 60°C) was run to get exact annealing temperature and to remove spurious bands and best annealing temperature was found as 56.1°C in both conventional and multiplex PCR.

An optimized multiplex PCR was developed in total reaction mixture containing Qiagen master mix 25 µl, each primer 2 µl (10 pmol), Q solution 6.7 µl, PCR grade water 1.1 µl and DNA template 4 µl for each bacteria (50 ng/µl). During PCR, conditions were standardized as 95°C for 15 minutes followed by 95°C for 90 sec, 56°C for 90 sec and 72°C for 90 sec with total 35 cycles and then final extension 72°C for 10 mint. In current study it was noted that increasing primer concentration compared to DNA template resulted in primer dimers and same issues was noted in another study that higher primer concentrations may result in increase of nonspecific product and may enhance the probability of generating primer-dimer (Malorny *et al*., 2003). DNA template with similar concentration was also reported in another study and further it was observed that multiplex PCR with 50 ng of DNA template gives specific and efficient amplification (Kim, 2012).

***Recovery rate of E. coli and Salmonella*:** In the present study, comparison among three different detection methods: conventional culture methods, conventional PCR and multiplex PCR was done in order to detect the food-borne pathogens (*E. coli* and *Salmonella*) form retail quail meat. Total 100 quail meat samples were taken from retail market (n=40), chain stores (n-40) and UVAS quail farm (n=20). Through conventional culture methods, *E. coil* was recovered from 82.5% of samples. Recovery rate of *E. coli* from chain stores, retail market and UVAS quail farm was 32 (80%), 35 (87.5%) and 16 (80%), respectively. In comparison same samples were tested through conventional and multiplex PCR and high prevalence (90%) of *E. coil* was recorded. Prevalence of *E. coli* through conventional and multiplex PCR in chain store, retail market and UVAS quail farm was 90%, 95% and 90%, respectively.

Salmonella recovered through conventional culture methods were 66.6% percent. Recovery rate of *Salmonella* from chain stores, retail market and UVAS quail farm was 26 (65%), 28 (70%) and 13 (65%) respectively. In comparison same samples were tested through conventional and multiplex PCR and high prevalence (82%) of *Salmonella* was recorded. Prevalence of *Salmonella* through conventional and multiplex PCR in chain store, retail market and UVAS quail farm was 90%, 95% and 90%, respectively. The results were same for the conventional PCR and multiplex PCR as given in Table 2. In comparison to conventional method for detection of *E. coli* and *Salmonella* in food samples, multiplex PCR was found time saving and specific as same samples showed through conventional method 80% and 65% positivity in quail meat collected from chain store. In another study conducted by (Hamad *et al*., 2012) results showed that *E. coli* prevail with different ratio in different organs. *E. coli* prevail in liver (24.32%), lungs (14.52%) and in intestine it was noted as (26.32%). In same

Table 2. Comparison of different methods for detection of food borne pathogens (*E. coli, Salmonella)*

No. of samples	Sample location	Bacteria	Conventional culturing method Positive (%)	Conventional PCR Positive (%)	Multiplex PCR Positive (%)
40	Chain stores	*E. coli*	80	90	90
		Salmonella	65	80	80
40	Retail market	*E. coli*	87.5	90	90
		Salmonella	70	85	85
20	UVAS quail farm	*E. coli*	80	90	90
		Salmonella	65	80	80
Total *E. coli*			82.5	90	90
Total *Salmonella*			66.6	82	82

study *E. coli* was found 18.2% in total 37 samples which showed significantly less count as compared to current study. Present study showed that *E. coli* and *Salmonella* both prevail dominantly and emphasized food-borne issues globally. In another study multiplex PCR was established to amplify PCR products of different sizes from five food-borne pathogenic bacteria including *E. coli* and *Salmonella* with detection limits approximately 10^5 CFU / ml (Kim, 2012). A single tube PCR and enrichment method was used to detect *Salmonella* spp., *Shigella* spp., enteroinvasive *E. coli*, and enterohemorrhagic *E. coli*, helped to simplify food analysis protocol, the method offers rapid report to food suppliers and help the quick shipment of safety-confirmed food products to markets (Hayashi *et al.,* 2013).

Statistical analysis of the present study showed that there is no significance difference ($p>0.05$) while using conventional culture method and conventional or multiplex PCR for detection of food-borne pathogen. Similarly no significant difference ($p>0.05$) was observed in recovery rate of *E. coli* and *Salmonella* in quail meant collected from chain stores, retail market and UVAS quail farm. Recently PCR is considered a reliable tool for the detection of food-borne bacteria. The method is considered rapid and reliable with high accuracy over conventional methods.

***Specificity*:** During specificity total 20 confirmed negative samples for *E. coli* and *Salmonella* was run with same optimized condition and none of sample was found positive as compared to those samples that were already positive for *E. coli* and *Salmonella*. Specificity was recorded as 100 per cent.

***Conclusions*:** We were able to develop, evaluate the specificity of multiplex PCR through standardizing the various parameters for direct detection of multiple food-borne pathogens in a single reaction without culturing them conventionally and minimize the wastage of time during detection of food-borne pathogens.

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