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Full Length Research Paper

Rapid detection of *Salmonella enterica* in food of animal origins collected from Riyadh, King Saudi Arabia

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The present study is aimed to investigate *Salmonella* species in food of animal origin collected from Riyadh, King Saudi Arabia (KSA) using conventional methods and polymerase chain reaction (PCR), targeting *fimA* gene specific for members of genus *Salmonella*. *Salmonella* isolation revealed 20 *Salmonella* serovars (8%) out of 250 examined samples. Nine strains (6, 92%) were recovered from 130 examined minced meats and 5 *Salmonella* strains (8.33%) were recovered from 60 local frozen chickens. Moreover, 6 *Salmonella* strains (10%) were isolated from 60 examined local chicken cuts. PCR using selective broth culture, Rappaport-Vassiliadis (RV) was used for the detection of different *Salmonella* species, targeting the *fimA* gene. All samples revealed positive results with bacteriological examination were positive by PCR -RV, and amplification of 120 bp fragments specific for *fimA* gene were observed, in addition, to 4 samples (1.6%) previously identified as negative samples with bacteriological examination were positive with PCR using the two primer pairs. The results revealed that the PCR-RV using primers specific for *fimA* gene could detect more positive samples of *Salmonella* species than conventional methods for rapid detection of food borne pathogens.

Key words: *Salmonella* species, *fimA* gene, polymerase chain reaction- Rappaport-Vassiliadis (PCR-RV), frozen chickens, minced meats.

INTRODUCTION

Salmonella is one of the most important Foods borne pathogens in human worldwide (Patrick et al., 2004; Soumet et al., 1999). The centers for disease control and prevention (CDC) reports an estimated 1.4 million cases of salmonella food poisoning occur annually in the United States. Food of animal origins especially poultry and poultry products have been identified as the principal sources of *Salmonella* leading to food borne illness in human (Myint et al., 2006; Fratamico, 2003).

It is estimated that 16 million new cases of typhoid fever occur each year round the world, mostly in developing country (D'Aoust, 1994; Parry et al., 2002; Dimitrov et al., 2007). The infection is characterized by a variety of clinical manifestations ranging from high-grade fever to complications including "encephalopathy, peritonitis, perforation and hemorrhage". The commonest serotypes causing disease in humans are *Salmonella enteritidis* and *Salmonella typhimurium* (Baggesen et al., 2002; Aktas et al., 2007). Egg associated Salmonellosis is an important public health problem in the United States and several European countries. *S. enteritidis* silently infects the ovaries of healthy appearing hens and contaminates the egg before the shells are formed and if the eggs are eaten raw or undercooked, the bacterium can cause illness.

The process of isolation and identification of *Salmonella* with traditional biochemical standard methods is laborious and time consuming. It may take up to 10

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Table 1. Reference standard strains used for evaluation of the specificity of PCR.

No.	Bacterial species	Source
1	*Salmonella typhimurium*	ATCC-14028
2	*S. typhimurium*	NCIMB-50076
3	*Salmonella typhi*	ATCC-9992
4	*Salmonella heidelberg*	WHO***
5	*Salmonella enteritidis*	WHO***
6	*Salmonella enteritidis*	ATCC** -13076
7	*Escherichia coli* (O157:H7)	ATCC-35150
8	*E. coli*	NCIMB* -50034
9	*Enterococcus fecalis*	NCIMB-50029

*NCIMB: National Collection for Industrial and Marine Bacteria.** ATCC: American Type Culture Collection.*** WHO: World Health Organization.

days and show poor sensitivity for samples with low level of contamination (Stone et al., 1994), so there is a need for the development of rapid, sensitive and accurate method for specific identification of Salmonella food-borne pathogen to overcome the drawbacks of the conventional methods. Molecular techniques such as polymerase chain reaction (PCR) especially by using selective broth culture have been invaluable tools for the detection of different *Salmonella species*. The *fimA* gene in *S. typhimurium* encodes the major fimbrial subunit (Clegg and Gerlach, 1987; Nichols et al., 1990). This gene had been cloned and sequenced from *S. typhimurium* (Swenson et al., 1991), and a particular region was found to be specific for Salmonella. The nucleotide sequence of the *fimA* gene is available on GenBank. Therefore, the investigation of *Salmonella* species in food of animal origin collected from Riyadh, King Saudi Arabia (KSA) using conventional and molecular techniques (PCR using specific primer for *fimA* gene specific for members of genus salmonella and selective broth culture) is the major strategy of this study.

MATERIALS AND METHODS

Bacteria and reagents

The bacterial reference strains used in this study were illustrated in Table 1. The materials, chemicals and reagents used in this study were purchased from Sigma Chemicals Co. (St. Louis, MO. USA) unless otherwise specified. PCR reagents were purchased from Promega (Madison, WI, USA).

Samples collection

During the summer of 2009, a total of 250 samples of poultry, minced meat and poultry products were collected from different retail establishment markets in Riyadh, KSA including, 130 minced beef meat, 60 local whole frozen chickens, and 60 local chicken cut samples (Liver, framed and Giblets). Ten negative control field samples were collected from young birds, a few hours after hatching; these birds coming from breading flocks are continuously monitored for *Salmonella* by standard microbiological techniques.

All samples were transported to the laboratory under refrigerated conditions where they were processed and bacteriologically examined immediately.

Isolation and identification of *Salmonella*

For the isolation and identification of *Salmonella*, the technique recommended by the International Organization for Standardization (ISO) 6579 (1998) was used. Briefly, 25 g of each sample was homogenized in 225 ml of buffered peptone water (BPW) (Oxoid, England) using a laboratory blender (Stomacher 400, Seward, England). After incubation at 37°C for 16 to 20 h; 0.1 ml was inoculated into a tube containing 10 ml of Rappaport-Vassiliadis (RV) magnesium chloride-malachite green broth (Oxoid, England) and was incubated at 42°C for 18 to 24 h. Another 1 ml from same pre-enrichment culture was inoculated into 10 ml of selenite cystine (SC) broth (Difco, USA) and was incubated at 37°C for 18 to 24 h. Each selective enrichment broths were streaked onto brilliant green-phenol red-lactose-sucrose (BPLS) agar (Merck, Darmstadt, Germany) and xylose lysine deoxycholate (XLD) agar (Merck, Darmstadt, Germany). Presumptive positive colonies (non lactose fermentative with suitable colony morphology) were identified morphologically, biochemically, serologically by slide agglutination test using polyvalent and monovalent somatic (O), virulence (Vi) and tube agglutination test for flageller (H) antigens (Difco Laboratories, Detroit, Michigan, USA) and enzyme linked immuno-sorbant assay (ELISA) (Reveal Salmonella test kits systems, Neogen Corporation). One milliliter of BPW which had been incubated at 37°C was saved for the PCR-non selective test (PCR-NS) and 1 ml of the 37°C RV broth for the PCR-RV test.

Extraction of DNA

The standard and bacteriollogically positive strains were grown in 10 ml tryptic soya broth (TSB) at 37°C for 24 h. The overnight cultures were centrifuged at 3000 RPM for 5 min and the supernatant were decanted carefully. The bacterial pellets were washed three times with phosphate buffer saline pH 7.2 and resuspended in 400 µl tris- EDTA buffer (pH 8.0) and heated in water bath at 100°C for 20 min. There were left to cool at room temperature and centrifuged at 14,000 rpm for 10 min. An aliquot of 5 µl of the supernatant was used as template DNA in the PCR. While the extraction of DNA from the field samples enriched in RV broth was carried out by the same method reported by Oliveira et al. (2003).

Polymerase chain reaction

Oligonucleotide primers

Primers were designed for *S. typhimurium* base on the *fimA* gene sequence. The *fimA* gene in *S. typhimurium* encodes the major fimbrial subunit (Clegg and Gerlach, 1987; Nichols et al., 1990). This gene has been cloned and sequenced from *S. typhimurium* (Swenson et al., 1991), and a particular region was found to be specific for Salmonella. The nucleotide sequence of the *fimA* gene is available from GenBank and the sequence was retrieved for designing the primer (GenBank accession no. M18283). The sequence of the primers designed is: Forward primer, 5'- CCT TTC TCC ATC GTC CTG AA -3'; reverse primer, 5'- TGG TGT TAT CTG CCT GAC CA -3'.

Table 2. Salmonella isolated recovered from minced meat, frozen chickens and chickens cut samples obtained from supermarkets in Riyadh.

Sample	Number of sample			*Salmonella* serotypes
	Tested	Positive	Percent	
Minced meat	130	9	6.92	*S. anatum, S. saintpaul, S. newport and S. derby*
Frozen chickens	60	5	8.33	*S. typhimurium, S. enteritidis, S. agona* and *S. infantis.*
Chicken cuts	60	6	10	*S. typhimurium, S. enteritidis, S. infantis* and *S. agon*
Total number	250	20	8	7 different serotypes from *Salmonella species*

DNA amplification

PCR amplifications were performed in a final volume of 50 µl in micro-amplification tubes (PCR tubes). The reaction mixtures consisted of 5 µl of the DNA template, 5 µl 10× PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)2SO_4$), 1 µl dNTPs (40 µM), 1 µl (1U Ampli Taq DNA polymerase), 1 µl (25 pmol) from the forward and reverse primers of both primer pairs and the volume of the reaction mixture was completed to 50 µl using distilled-deionized water (DDW). The thermal cycler was adjusted as follows: Initial denaturation at 94°C for 5 min., followed by 35 cycles of (denaturation at 94°C for 1 min., annealing at 56°C for 1 min. and extension at 72°C for 1 min) . Final extension was carried out at 72°C for 10 min and the PCR products were stored in the thermal cycler at 4°C until they were collected.

Agarose gel electrophoresis

The PCR products were tested for positive amplification by agarose gel electrophoresis previously reported by Sambrook et al. (1989) using suitable molecular weight markers.

RESULTS

Bacteriological examination

Out of 250 examined field samples, the bacteriological examination revealed 20 *Salmonella* serovars (8%). Nine strains (6, 92%) were recovered from 130 examined minced imported meat (3 of *Salmonella anatum* and *Salmonella newport* were isolated with 2.31% for each, followed by two strains (1.54%) *S. saintpaul* and only one strain was identified as *S. derby* (0.76%). While from frozen local chickens, 5 strains (8.33 %) were recovered and identified as follows; two strains as *S. enteritidis*, (3.33 %), 4 strains as *S. typhimurium, Salmonella agona, Salmonella infantis* (1.67% for each). Meanwhile, 6 strains (10%) were recovered from chicken cuts and were identified as 4 strains of *S. enteritidis* and *S. typhimurium*, (3.33% for each), and the other two strains were *S. infantis*. and *S. agona* (1.67% for each) as shown in Table 2.

Molecular typing using PCR

The specificity of the oligonucleotid primers were carried out by testing of all the recovered *Salmonella* strains in addition to the standard positive and standard negative strains with PCR using the primer pairs targeting the *fimA* gene (specific for all members of *Salmonella* species) . All *Salmonella* serovars were positive for amplification of 120 bp fragments of *fimA* gene, while all non *Salmonella* serovars were negative as shown in Figure 1.

All the examined field samples with bacteriological examination as well as the negative control field samples were tested by PCR using the same primer pair after selective enrichment on RV broth. All bacteriologically positive samples (100%) were positive by PCR and amplification of 120 bp fragments specific for *fimA* gene was observed. In addition, 4 samples (1.6%) previously identified as negative samples with bacteriological examination were positive with PCR using the two primer pairs (Table 3 and Figures 2 and 3). The negative control field samples were negative for the PCR assay and no amplification could be detected with the four primer pairs.

DISCUSSION

Salmonella is the most important pathogen causing food-borne outbreaks around the world (Aktas et al., 2007). Poultry are one of the most important reservoirs of *Salmonellae* that can be transmitted to humans through the food-chain. The most common serotypes isolated from humans are *S. typhimurium* and *S. enteritidis*. Traditional detection methods for Salmonella are based on cultures using selective media and characterization of suspicious colonies by biochemical and serological tests which may take up to 10 days to confirm the results (Stone et al., 1994). When a food borne outbreak is suspected, the faster the source of a pathogen can be identified, the sooner the public can regain confidence in the food supply (Bhagwat and Lauer, 2004) . Therefore, the present study is aimed to investigate *Salmonella* species in food of animal origin collected from Riyadh, King Saudi Arabia (KSA) using conventional and molecular techniques (PCR using specific primer for *fimA* gene specific for members of genus salmonella and selective broth culture). *Salmonella* isolation revealed a total percentage of 8% out of 250 examined field samples; a high incidence of *Salmonella* serovars isolation was recovered from chicken cuts (10%),

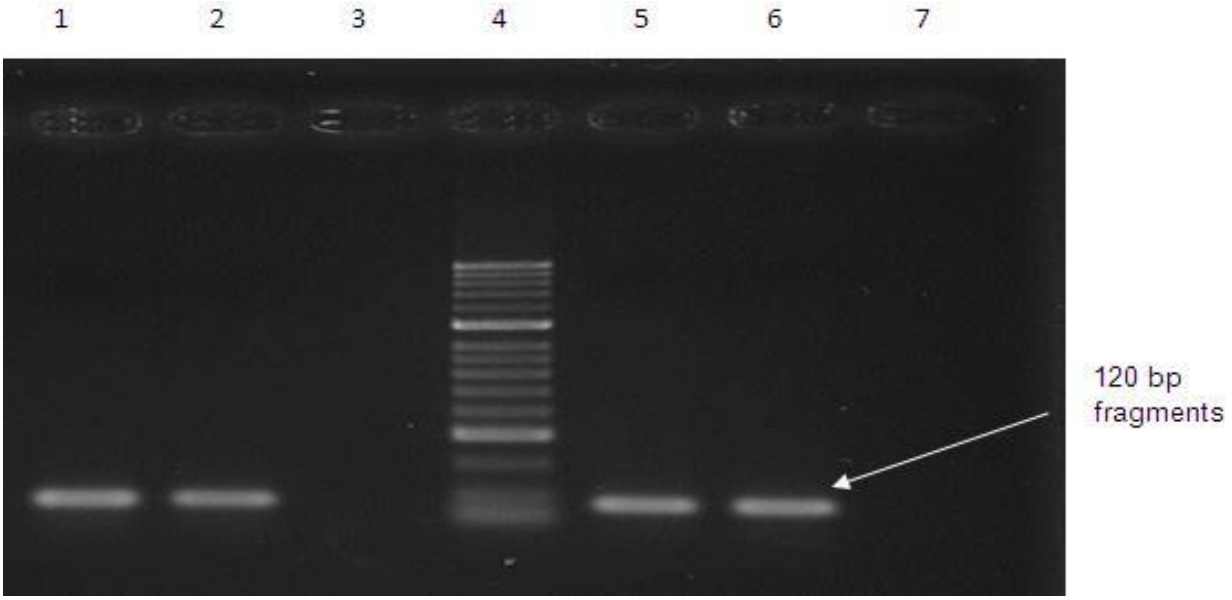

Figure 1. Agarose gel electrophoresis showing the specificity of the *fimA* gene. Amplification of 120 base pair fragments were observed in all standard salmonella strains, lanes 1, 2, 5 and 6, while the other non salmonella strains showing negative results, lanes 3 and 7. lane 4 shows hyper ladder II.

Table 3. Comparison between SMT and PCR for detection of *Salmonella* serovars.

Types of sample	Origin	Number of sample	SMT		PCR	
			Number	Percent	Number	Percent
Minced meat	Imported source	130	9	6,92	10	7.69
Frozen chickens	Local source	60	5	8.33	6	10
Chicken cuts	Local source	60	6	10	8	13,33
Total	-	250	20	8	24	9.6

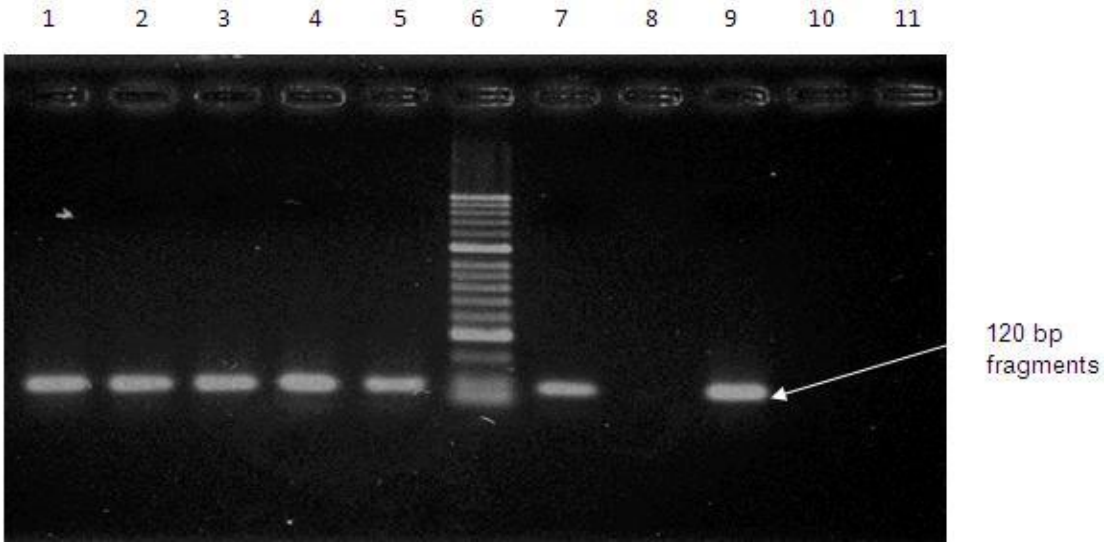

Figure 2. Agarose gel electrophoresis showing amplification of 120 bp fragments of *fimA* gene from minced meat contaminated with different salmonella serovars.

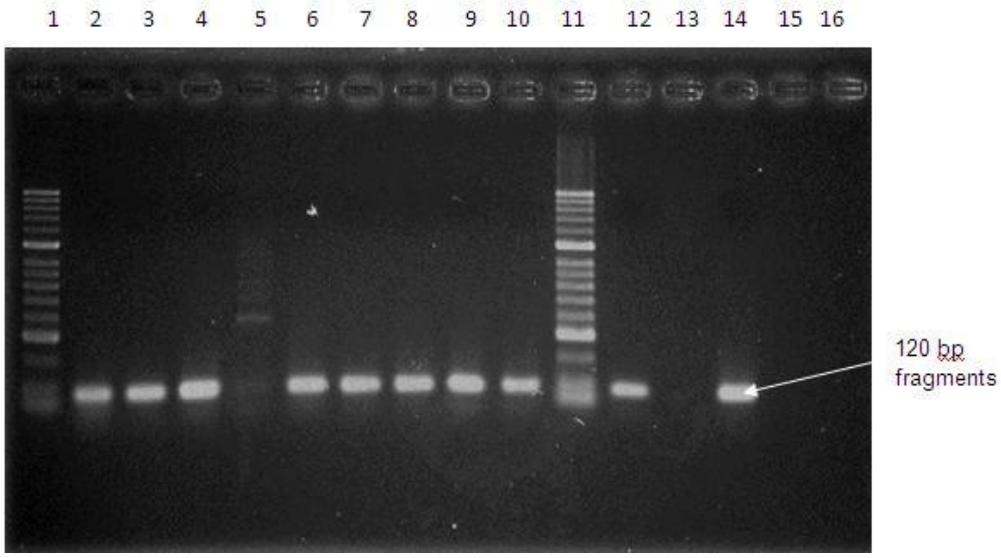

Figure 3. Agarose gel electrophoresis showing amplification of 120 bp fragments of *fimA* gene from frozen chickens and chicken cuts contaminated with different *Salmonella* serovars.

followed by local frozen chickens and imported minced meat (8.33%) and (6.92%), respectively as shown in Table 2. These results indicated that poultry and poultry products are the major source of *Salmonella* food borne diseases (Altekruse et al., 1999; Humphrey, 2002; Schlundt, 2002); it also confirm the conclusion of Moussa et al. (2010) who found that the high incidence of *Salmonella* serovars isolation occur among chicken cuts (10%), followed by local frozen chickens in food of animal origins collected from Riyadh, KSA. Moreover, the *Salmonella* serovars (6, 92%) recovered from 130 examined minced imported meat were (3 strains of S. *anatum and* S. *newport* with a percentage 2.31 for each, followed by two strains (1.54%) *S. saintpaul* and only one strain was identified as *S. derby* (0.76%) as shown in Table 2. These results indicated the ability of imported chickens to introduce different *Salmonella* species to the local area that can cause new and devastating outbreaks (Altekruse et al., 1999; Moussa et al., 2010). The results of bacteriological examination revealed that *S. enteritidis* was dominating among the recovered *S. serovars* with incidence of 3.33 % in both frozen chickens and chicken cuts, followed by *S. typhimurium*, (3.33%) and (1.67%) in frozen chickens and chicken cuts, respectively. These results indicate the ability of chickens and chicken products to be one of the most important source of salmonella foodborne out breaks as it could transmit *S. typhimurium* and *S. enteritidis*, the commonest serotypes causing disease in humans (Baggesen et al., 2002; Aktas et al., 2007).

To overcome the drawbacks of the conventional methods, PCR using selective broth culture was used for the detection of different *Salmonella* species, targeting specific sequence for members of genus *Salmonella* in the *fimA* gene. The specificity of the oligonucleotide primers revealed positive amplification of 120 bp fragments of *fimA* gene with all the recovered *Salmonella* strains, in addition to the standard positive strains, while all non *Salmonella* serovars were negative as shown in Figure 1, which indicate the specificity of such sequence to all members of *Salmonella* species (Rasmussen et al., 1994; Cohen et al., 1993, Naravaneni and Jamil, 2005).

The sensitivity of PCR assay targeting *fimA* gene combined with RV selective enrichment broth (PCR-RV) for the detection of *Salmonella* species in the collected field samples were tested in this study. All samples revealed positive results with bacteriological examination been positive by PCR-RV as shown in Table 3, and amplification of 120 bp fragments specific for *fimA* gene were observed; in addition, 4 samples (1.6%) previously identified as negative samples with bacteriological examination were positive with PCR using the two primer pairs (Table 3 and Figures 2 and 3). While all the negative control field samples were negative for the PCR assay and no amplification could be detected with the primer pairs. The recorded results confirmed that the PCR-RV assay could detect more positive samples of *Salmonella* species than conventional methods; these results confirm the conclusion of Oliveira et al. (2003) and Moussa et al. (2010); they concluded that, the PCR test combined with RV selective enrichment is more sensitive in detecting *Salmonella* serovars than bacteriological methods.

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