Molecular Detection of *Salmonella enteric* Serovar Enteritidis in Chicken-Related Samples Collected from Egypt

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Abstract: *Salmonella enterica* serovar Enteritidis considered to be the most predominant serovar in poultry and eggs moreover, S. *Enteritidis* is a leading cause of salmonellosis in humans. Therefore, *S.* Enteritidis has been targeted by a number of control programs. The present study is aimed to investigate *Salmonella* Enteritidis in chicken related samples collected from Egypt using conventional methods and Polymerase Chain Reaction (PCR) and *sefb* gene specific primers specific for *S.* Enteritidis. During the summer of 2012, a total of 1100 chicken related samples (450 cloacal swabs, 400 chicken eggs and 250 chicken meats) were collected from 12 poultry farms located in different Egyptian governorates. The collected samples were identified by standard bacteriological methods as well as PCR using selective broth culture, Rappaport- Vassiliadis (RV) using specific primer for *S.* Enteritidis. The highest rate of isolation of *S.* Enteritidis 4.44% was obtained from cloacal swabs and chicken eggs (4%), followed by chicken meats 2% . PCR using selective broth culture (RV) and specific primer for *S.* Enteritidis could detect all the bacteriolgically positive samples, in addition, to 5 cloacal samples (1.11%) previously identified as negative samples with bacteriological examination. The PCR-RV using primers specific for *S.* Enteritidis could detect more positive samples of *S.* Enteritidis than conventional methods for rapid detection of foodborne pathogens.
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Key words: *Salmonella enterica***,** PCR-RV**,** Chicken related samples**,** *S.* Enteritidis.

1. Introduction

Salmonella enterica infections are the most significant public health concern worldwide, contaminated poultry, meat, and eggs are important vehicles of Salmonella infections, especially when the bacterium is in the egg contents (Aarestrup *et al.,* 2007; CDC 2010; Sugawara *et al.,* 2012). This contamination problem was recently highlighted in salmonellosis outbreak caused by *Salmonella enterica* serovar Enteritidis (Benenson and Chin. 1995, Nayak et al., 2008; Melendez *et al.,* 2010). The most commonly identified serovars associated with human infections in the United States are *Salmonella enterica* serovars *Typhimurium*, Enteritidis, Newport and Heidelberg (CDC 2008). Centers for Disease Control and Prevention (CDC, 2006) reported that *Salmonella* Enteritidis was the second most commonly identified cause of infection, representing 16.6% of the cases (CDC, 2008). More recently, the CDC's Foodborne Diseases Active Surveillance Network reported that *S.* Enteritidis caused 19.2% of all *Salmonella* infections in 2009 (CDC 2008). Studies have shown that contaminated shell eggs and egg products are the most important sources of *S.* Enteritidis (Braden 2006; CDC, 2010). Therefore, *S.* Enteritidis considered to be the most predominant serovar in poultry and eggs moreover, *S.* Enteritidis developed into a leading cause of salmonellosis in humans. Because of these problems, *S.* Enteritidis has been targeted by a number of control programs.

There have been significant shifts in the predominant *Salmonella* serovars associated with poultry and human infections. *S. enterica* serovar Enteritidis and *S.* Heidelberg are the most commonly detected serovars in chickens over the last 25 years also among the top five serovars associated with human infections (CDC, 2008; Foley *et al.,* 2008). While *S.* Typhimurium remains the most common cause of human infections (CDC 2008; Moussa 2010; Sugawara *et al.,* 2012).

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

sensitivity for samples with low level of contamination (Naravaneni and Jamil, 2005; Yan and Sekaran, 2010). 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*. Therefore, the investigation of *Salmonella enteric* serovar Enteritidis in chicken related samples from Cairo, Egypt using conventional and molecular techniques (PCR using specific primer for *Salmonella enteric* serovar Enteritidis SefB127L-SefB661R, based on the *sefb* gene (Wang *et al.,* 2010) and selective broth culture) is the major strategy of this study.

2. Materials and Methods

Bacteria and Reagents

The reference strains used in this study were *Salmonella* Typhimurium ATCC-14028, *Salmonella* Typhimurium NCIMB-50076, *Salmonella* Typhi ATCC-9992, *Salmonella* Enteritidis ATCC**-13076, *Escherichia coli* (O157:H7) ATCC-35150, *Enterococcus fecalis* NCIMB-50029. 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

During the summer of 2012, a total of 450 cloacal swabs were collected from poultry farms to be subjected for isolation of *Salmonella enteric*. Moreover, a total of 450 chicken eggs and 250 chicken meats were collected from different retail establishment markets in Cairo, Egypt we also used 10 negative control field samples collected from young birds a few hours after hatching, these birds coming from breading flocks 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*

The standard microbiological techniques for detection of different *Salmonella* serovars conducted according to ISO 6579. 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). 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 bacteriologicaly positive strains were grown in 10 ml Tryptic Soya Broth (TSB) at 37 °C for 24 hours. The whole genomic DNA were extracted from overnight cultures and from the field samples enriched in RV broth was carried out by the same method reported by Sugawara *et al*. (2012).

Polymerase chain reaction

Oligonucleotide primers

The PCR primers reported by Wang et al., (2010) from the *sefb* gene of *S. enterica* serovar Eenteritidis (accession number L11009) were Sef.B127L (5'-AGATTGGGCACTACACGTGT-3') and SefB661R (5'-TGTACTCCACCAGGTAATTG-3') which produced a DNA fragment of 535 bp.

DNA amplification

PCR amplifications were performed in a final volume of 50µl in micro-amplification tubes (PCR tubes). The reaction mixtures consisted of 5 µl of the DNA template, 5 µl 10x PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM MgCl2, 50 mM KCl, 20 mM (NH4)2SO4), 1 µl dNTPs (40µM), 1µl (1U Ampli Taq DNA polymerase), 1µl (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 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 at56°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.

3.Results

Bacteriological examination:

Out of 450 examined cloacal swabs, the bacteriological examination revealed 30 strain of *Salmonella* serovars (6.66%). Serotyping of the recovered strains revealed twenty strains (4.44%) *S.* Enteritidis, Four strains (0.89%) *S.* Typhimurium, five strains (1.11%) *S.* Agona and one strain (0.22%) *S.* Kentucky. The bacteriological examination of the chicken egg samples revealed 16 strains (4%) *S.* Enteritidis and 2 strains (0.5%) *S.* Typhimurium. While only 5 strains (2%) of *S.* Enteritidis and one strain (0.4%) of *S.* Typhimurium were isolated from

chicken meats as shown in Tables (1& 2).The highest rate of isolation of *S.* Enteritidis 4.44% was obtained from cloacal swabs and chicken eggs (4%), followed by chicken meats 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 *sefb* gene (specific for *Salmonella enteric* serovar Enteritidis). All *Salmonella enteric* serovar Enteritidis were positive for amplification of 535 bp fragments of *sefb* gene, while all non *sefb* genes 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 535 bp fragments specific for *sefb* gene was observed. In addition, 5 cloacal samples (1.11%) (1.11%) previously identified as negative samples with bacteriological examination were positive with PCR using the two primer pairs Table 2 and Figure 1. The negative control field samples were negative for the PCR assay and no amplification could be detected with the primer pairs.

Table (1): Salmonella serovars recovered from Chicken related productp

Samples	*S.* Enteritids	*S.* Typhimurium	*S.* Agona	*S.* Kentucky	Total
Cloacal swabs	20 (4.44%)	4 (0.89%)	5 (1.11)	1 (0.22%)	30 (6.66%)
Chicken eggs	16 (4%)	2 (0.5%)	0	0	18 (4.5%)
Chicken meats	5 (2%)	1 (0.4%)	0	0	6 (2.4%)

Table (2): Comparison between standard microbiological techniques (SMT) and PCR for detection of *Salmonella enterica* serovar Enteritids:

samples	Number of samples	SMT		PCR	
		Number	Percent	Number	Percent
Cloacal swabs	450	20	4.44 %	25	5.56 %
Chicken eggs	400	16	4 %	6	10%
Chicken meats	250	5	2 %	8	13,33%

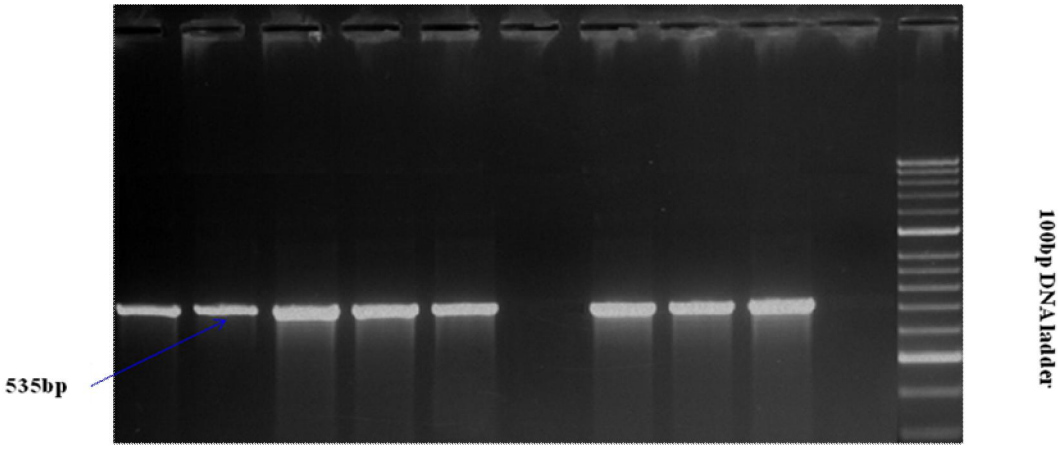

Figure (1): Agarose gel electrophoresis showing amplification of 535 bp fragments of *sefb* gene specific for *Salmonella enterica* serovar Enteritidis

4.Discussion

Salmonella is the most important pathogen causing food-borne outbreaks around the world (CDC, 2008; CDC, 2010; Sugawara *et al.,* 2012). 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 (Naravaneni R and Jamil Moussa *et al.,* 2010). When a foodborne

outbreak is suspected, faster the source of a pathogen can be identified, the sooner the public can regain confidence in the food supply (Aktas *et al.,* 2007) *Salmonella enterica* serovar Enteritidis considered to be the most predominant serovar in poultry and eggs moreover, *S.* Enteritidis is a leading cause of salmonellosis in humans. Therefore, *S.* Enteritidis has been targeted by a number of control programs. The present study is aimed to investigate *Salmonella* Enteritidis in chicken related samples collected from Egypt using conventional methods and Polymerase Chain Reaction (PCR) and *sefb* gene specific primers specific for *S.* Enteritidis. *Salmonella* isolation revealed a total percentage of 4.9% out of 1100 examined field samples, the highest rate of isolation of *S.* Enteritidis 4.44% was obtained from cloacal swabs and chicken eggs (4%), followed by chicken meats 2% as shown in Tables (1& 2). These results indicated that poultry and poultry products are the major source of *Salmonella* foodborne diseases (CDC, 2008; CDC, 2010; Sugawara *et al.,* 2012). The results of bacteriological examination revealed that *S.* Enteritidis was dominating among the recovered *S. serovars* indicating 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, commonest serotypes causing disease in humans Patrick et al., 2004**;** Aktas *et al.,* 2007; Dimitrov *et al.,* 2007**;** Nayak *et al.,* 2008).

To overcome the drawbacks of the conventional methods, PCR using selective broth culture was used for the detection of different *Salmonella* Enteritidis, targeting specific sequence for members of *sefb* gene. The specificity of the oligonucleotide primers revealed positive amplification of 535 bp fragments of *sefb* gene with all the recovered *Salmonella* Enteritidis, in addition to the standard positive strains, while all non *Salmonella* Enteritidis were negative as shown in Figure (1), which indicate the specificity of such sequence to *Salmonella* Enteritidis[&]

The sensitivity of PCR assay targeting *Salmonella* Enteritidis 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 were positive by PCR-RV, PCR, and amplification of 535 bp fragments specific for *Salmonella* Enteritidis were observed, in addition, 5 cloacal samples (1.11%) previously identified as negative samples with bacteriological examination were positive with PCR using the two primer pairs as shown in Table 2 and Figure 1. 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 detected more positive samples of *Salmonella* species than conventional methods, these results confirm the conclusion of Fratamico *et al.,* (2003); Myint *et al.,* (2006); Sugawara *et al*., (2012) , 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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