Prevalence of *Salmonella* spp. in the quail egg interior contents: A provincial study

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Abstract:

BACKGROUND: Poultry products have been recognized as major sources of human gastrointestinal disease caused by *Salmonella* spp. and several outbreaks have been reported where eggs were the source of human infection. **OBJECTIVES:** This study was carried out to determine the prevalence of *Salmonella* spp. in the quail egg interior contents from retail stores of Semnan, Iran and to characterize the isolated *Salmonella* serovars via serotyping and Multiplex PCR techniques. **METHODS:** 140 packages of quail eggs (each package containing 12 eggs) were collected from different batches during summer 2010 and tested for the presence of *Salmonella* through conventional culture and serotyping methods. **RESULTS:** From these samples, *S. enteritidis* was detected in the egg contents of one package (0.71 %) out of 140 packages. This isolate was confirmed by Multiplex PCR generated amplification products for a random sequence that is specific for the genus *salmonella* and spv and *sefA* genes. **CONCLUSIONS:** According to our results, *S. enteritidis* is the most prevalent serotype of quail egg content contaminant in the Semnan area of Iran and the multiplex PCR method could be used as a reliable method of identifying *Salmonella* serovars.

Introduction

Salmonella infection is the most frequent foodborne gastrointestinal disease transmitted from animals to humans, mainly through beef, poultry meat, egg and milk (Gillespie et al., 2003) (Riyaz-Ul-Hassan et al., 2004). Poultry products have been recognized as major sources of human illness caused by this pathogen (Amavisit et al., 2001; Corry et al., 2002), and several outbreaks have been reported where eggs were the source of human infection (Amavisit et al., 2001; Berghold et al., 2003; Crespo et al., 2005; Jamshidi et al., 2010); in particular, raw or half cooked eggs (in mousse, mayonnaise, beverages and other food products) are at high risk for human (Moore and Madden, 1993; Gillespie et al., 2005).There are more than 2435 known serotypes of *Salmonella* and many of these serotypes are well documented human pathogens (Rahman, 2002) *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis are the most frequently isolated serovar from foodborne outbreaks throughout the world and are responsible for half of human infections (Herikstad et al., 2002; Bhattacharjee et al., 2011). In chicken it has been shown that both *S. Typhimurium* and *S. Enteritidis* infect the reproductive tract and contaminate forming eggs and thus can be present within the contents of intact egg shells (Keller et al., 1995,1997 ; Humphrey, 1994).

Since various *Salmonella* species have particular antigenic profile and can show different disease syndromes and host specificity, it is vital to discriminate *Salmonella* serovars from each other in order to insure that each pathogen and epidemiology is correctly recognized (Lim et al., 2003).

Bacteriological method for detecting pathogens typically involves culturing the organism in selective

media and identifying isolates according to their morphological, biochemical, and/or immunological characteristics. This method is sensitive and permits the specific detection of microorganism of interest in complex environments such as foods and certain clinical samples. However, the method is time consuming and usually requires 5-11 days (Riyaz-Ul-Hassan et al., 2004).

Rapid detection methods, such as DNA or RNA probing, immunodetection methods and nucleic acid hybridization have been developed, but they do not have enough sensitivity and specificity (Zhu et al., 1996).

The advent of DNA amplification by the PCR method and its application have significantly improved the specificity, sensitivity and time necessary for detection of microbial pathogens in the environment (Bej, 2003).

PCR has also become a valuable tool for investigating food-borne outbreaks and identifying pathogens (Riyaz-Ul-Hassan et al., 2004; Kapley et al., 2000; Daum et al., 2002; Jamshidi et al., 2010).

The highest level of n-3 polyunsaturated fatty acids, the best ratio of n-6/n-3 fatty acids and the lowest cholesterol content in quail egg yolks in comparison to other bird species, i.e. hens, pheasants, ostriches and ducks (Kazmierska, 2005) have led to an increasing interest in consumption of the quail egg all over the world and in Iran. Therefore, this study was undertaken for the first time to determine the prevalence of *salmonella* infection in the quail egg content samples, collected from retail stores in Semnan, Iran, using bacteriological and molecular techniques.

Materials and Methods

Sample collection: Quail Eggs were collected randomly from local supermarkets in Semnan city over a period of 3 months in summer, 2010 (from 22 May to 22 Aug 2010). In this regard, Semnan was divided to five main regions (north, south, west, east and center of city) according to their source of quail eggs. During the experiment, a total of 140 packages of eggs (containing 12 eggs in each package) in different batch numbers were analyzed for the presence of *Salmonella* in egg content. Every package from each store was transported to the laboratory on crushed ice. The samples were kept in the refrigerator at 4ºC for about 4 hours till testing. Experiment was done in a completely randomized design including four replications (time), five treatments (regions) and seven observations in each replicate. SAS package software (ver. 9.1) was used for statistical analysis in general linear model procedure and Duncan's multiple range test was used to compare treatment means (α=0.05).

Culturing method for isolation and identification of *Salmonella* spp.: In order to collect the egg contents, the surfaces of the eggs were sterilized by immersion in 70% alcohol for 2 min, flamed for removing residual alcohol, and then cracked with a sterile knife. Egg contents of each package were mixed thoroughly, and 25 mL of mixed egg content was inoculated into 225 mL of peptone buffered water 1% and incubated at 37ºC for 24 h. After pre-enrichment,0.1 mL of the cultures of all sample types were transferred to 10 mL of Rappaport-Vassiliadis (RV) medium (Merck) and 1 mL to 9 mL of tetrathionate broth (Merck) and incubated at 43?C and 37ºC, respectively for 24 h for selective enrichment. The cultures were then streaked onto Xylose lysine desoxycolate (XLD) (Merck) and Hektoen enteric agar (HE) (Merck), and incubated at 37ºC for 24 h. The plates were observed for typical *Salmonella*-like colonies and randomly, two colonies per plate were picked, purified and confirmed by routine biochemical and serological tests as recommended by Douglas Waltman (Douglas Waltman et al., 1998).

The suspected colonies were subjected to multiplex PCR assay for final confirmation and identification of *Salmonella* spp.

Multiplex PCR Amplification: The typical *Salmonella* colonies on selective media that had been confirmed as *Salmonella* strains by biochemical and serological tests were employed as templates for Multiplex PCR assay. *S. enteritidis* (ATCC-13076) was used as positive control and sterile distilled water was used as negative control. One of the bacterial colonies that was confirmed as *Salmonella* spp. by biochemical and serological tests, was cultured on LB agar overnight and after the growth of isolated *Salmonella* strain on LB agar, one colony was selected for DNA extraction. Genomic DNA was extracted from isolated strain with the AccuPrep®

Table 1. Sequence of oligonucleotides used as primers in the multiplex-PCR.

primers	Sequence (5´- 3´)	Target gene	Amplicon fragment(bp)
ST11	**GCCAACCATTGCTAAATTGGCGCA**	**Random sequence**	**429**
ST14	**GGTAGAAATTCCCAGCGGGTACTGG**	**Random sequence**	
***SEFA*2**	**GCAGCGGTTACTATTGCAGC**	***sefA***	**310**
***SEFA*4**	**TGTGACAGGGACATTTAGCG**	***sefA***	
S1	**GCCGTACACGAGCTTATAGA**	**spv**	**250**
S4	**ACCTACAGGGGCACAATAAC**	**spv**	

Genomic DNA Extraction Kit (BIONEER, Korea) according to the manufacturer's protocol.

A volume of 3 µL of the supernatant was used as template for amplification by Multiplex PCR assay. The sequence of the three pairs of primers for the Multiplex PCR is shown in Table 1. While the ST11 and ST14 primers are specific for the genus *Salmonella*, the S1 and S4 primers and the *SEFA*2 and *SEFA*4 primers are specific for the *S. enteritidis* serovar, they can distinguish this serovar from non-enteritidis serovars of *Salmonella*. (Pan, 2002; Mirzaei, 2010).

Reactions with these primers were carried out in a total volume of 25 µL amplification mixture consisting of 2.5 µL of 10X reaction buffer (500 mM KCl, 200 mM Tris-HCl), 0.8 µL dNTPs (10 mM), 1 µL MgCl2 (50 mM), 1.25 µL of each primer (10 µM), 0.6 µL of Taq DNA polymerase (Fermentase) and 3 µL of extracted DNA as template and 9.6 µL of distilled water.

Amplification was performed in Techne TC-512 thermocycler (Techne, UK). The cycling conditions were as follows: 35 cycles of denaturation at 94ºC for 30s, annealing at 56ºC for 90s, elongation at 72ºC for 30s, and final extension period for 10 min at 72ºC. Amplified products were electrophoresed in 1.8% agarose gel and a 100-bp DNA ladder was used as a size marker.

After staining with ethidium bromide, the gel was visualized with UV gel documentation

apparatus (BIORAD, UK).

Results

Performing microbiological tests showed that the quail egg contents of one package (around 0.71%) of 140 packages were suspected *Salmonella* genus and according to serological tests, this isolate was identified as *S. enteritidis*. The M-PCR method confirmed this result by producing 429, 310 and 250 base pair amplification products from a random sequence (specific for the genus *Salmonella*), *sefA* and spv in both ATCC-13067 strain of *S. enteritidis* (used as positive control) and the isolated strain of *Salmonella* enteritidis from quail eggs (Figure 1).

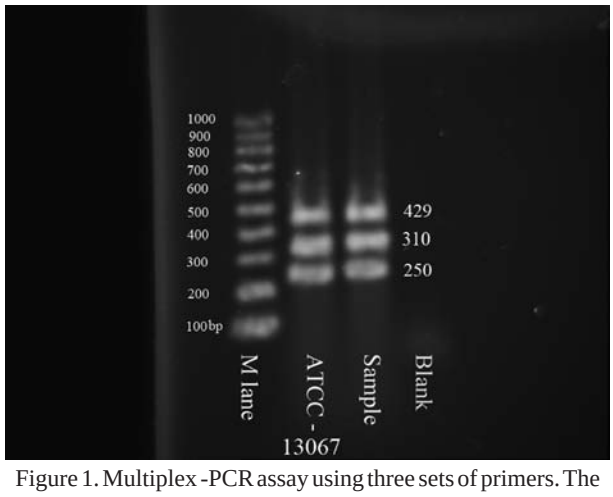

Figure 1. Multiplex -PCR assay using three sets of primers. The 429-bp amplified product from random sequence gene specific for *Salmonella* spp., 310 and 250bp from *sefA* and spv gene specific for *S.enteritidis*. Lane (1): 100-bp molecular weight marker; Lane (2): *S. enteritidis* as positive control; Lane (3): Positive sample for *S.enteritidis*; Lane (4): negative control.

Discussion

Prevalence levels of *Salmonella* spp. contamination of egg contents in other countries were reported to vary from zero in Poland (Radkowski, 2001) to 1.8% in India (Suresh et al., 2006). These reports are, for the most part,not related to quail eggs. In a study conducted by Ozbey (2008) for determining the presence of *Salmonella* spp. in 448 egg samples from chickens, ducks and quails, 3 samples of quail eggs (0.66%) were positive for *Salmonella* (Ozbey et al., 2008), which resembles our results. In another study

performed on 123 liquid whole quail eggs in Turkey, 5.69% *Salmonella* contamination was detected (Erdogrul, 2004), which is much higher than its contamination found in this study. This difference may be affected by local factors.

Similar to the results of this study, *S.enteritidis* is the most frequently isolated serovar from the eggshells and egg contents in some countries such as Turkey (Musgrove et al., 2005; Erdogrul, 2004). Although some authors mentioned that the most frequent isolate from eggs in Iran was S.typhimurium (Jamshidi et al., 2010),in this study it is *S.enteritidis*.

In chicken it has been shown that both *S. typhimurium* and *S. enteritidis* infect the reproductive tract and contaminate forming eggs but *S. enteritidis* persists after eggs are laid (Keller et al., 1995, 1997). It has been proved that a specific gene possibly alters *S. enteritidis* interaction with egg albumin components, but *S. typhimurium* does not have this protective gene (Clavijo et al., 2006). In addition to the reproductive tract, egg contamination by Salmonellae can occur through contact with *Salmonella*-contaminated equipment, personnel, environment, infected wild birds and rodents (Cox et al., 2000).

In the present study, ST11 and ST14 primers were used for specific detection of *Salmonella* at the genus level which target the randomly cloned sequence specific for the genus *Salmonella*. On the other hand, for specific detection of *S. enteritidis*, S1 and S4 primers which target *Salmonella* plasmid virulence gene (spv) and *SEFA*2 and *SEFA*4 primers which target *S. enteritidis* fimbrial antigen gene (*sefA*) were used, and detection of both of these genes is very important to distinguish *S. enteritidis* from *Salmonella* non-Enteritidis strains (Pan, 2002; Mirzaei, 2010).

It is believed that some *Salmonella* serovars (rough *Salmonella* isolates) which, because of developing new specificities on their cell surface and causing the diversity of O antigens can not be identified by traditional serotyping methods, can be identified by molecular typing methods (Malorny et al., 2007); but in our study, both molecular and conventional tests had the same results.

Even though the original population of *S. enteritidis* in liquid egg seems to be low, they have the ability to increase to a disease causing level. Temperature abuse of the egg product can lead to higher numbers of organisms that may not be completely eliminated by current pasteurization protocols; moreover, the increased resistance of *S.enteritidis* in undesirable circumstances such as salted egg yolk products have been previously documented (Cotteril and Glauert 1968; Palumbo et al., 1995); in this regard, Center for Disease Control and Prevention (CDC) believes that *S. enteritidis* is one of the most common serovars associated with human disease and is therefore of considerable importance to public health (CDC as reference)

According to our results, it seems that *S. enteritidis* is the most prevalent serotype of quail egg content contaminant in the Semnan area of Iran and the multiplex PCR method could be used as a reliable method of identifying *Salmonella* serovars; in addition, this study confirmed that the quail egg is a significant reservoir of food-borne pathogens such as *Salmonella*. Incidentally, new on-farm initiatives in food safety, such as the application of hazard analysis critical control point systems, will help reduce the risk of *Salmonella* transmission through quail eggs. Moreover, proper refrigeration, cooking, and handling should prevent most egg safety problems.

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تعیین میزان شیوع سالمونلا در محتویات داخلی تخم بلدرچین: یک مطالعه استانی

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چکیده

زمینه مطالعه: محصولات غذایی حاصل از طیور از مهمترین منابع ایجاد مسمومیت‌های غذایی سالمونلایی در انسان می‌باشندکه در بین آنها تخم ماکیان از اهمیت ویژه‌ای برخوردار است. **هدف:** این مطالعه به منظور تعیین میزان شیوع سالمونلا در محتویات داخلی تخم بلدرچین‌های عرضه‌شده در خرده فروشی‌های شهرسمنان و تشخیص سروار آن با استفاده از تکنیک‌های سروتایپینگ و مولتی پلکس PCR انجام‌شد. **روش کار:** ۱۴۰ بسته تخم بلدرچین (که هرکدام شامل ۱۲ عدد تخم بلدرچین بودند) از نقاط مختلف شهرسمنان و از شماره بهرهای مختلف در تایستان ۱۳۸۹ جمع‌آوری شدند و به منظور تشخیص سالمونلا به روش سنتی و سروتایپینگ مورد آزمایش قرار گرفتند. **نتایج:** از میان این نمونه‌ها سالمونلا انتریتیدیس از محتویات داخلی یک بسته از ۱۴۰ بسته (۰/۷۱٪ نمونه‌ها) جداشد. این سویه جداشده با استفاده از مولتی پلکس PCR که ژن‌های رندم، spv و sef A (اختصاصی سالمونلا و سالمونلا انتریتیدیس) را تکثیر می‌کرد مورد تائید قرار گرفت. **نتیجه‌گیری نهایی:** براساس نتایج حاصل از این تحقیق سالمونلا انتریتیدیس شایع ترین سروتیپ حاضر در محتویات داخلی تخم بلدرچین‌های سمنان می‌باشد و همچنین مولتی پلکس PCR به عنوان یک تکنیک قابل اعتماد می‌تواند در تشخیص سروارهای سالمونلا مورد استفاده قرار گیرد.

واژه‌های کلیدی: سالمونلا، تخم بلدرچین، روش سنتی، مولتی پلکس PCR، سمنان.

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