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Frequency of Salmonella Typhimurium in Egg Shell and Determination of Antibiotic Resistance of Isolates

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Abstract: *Egg products are most often associated with outbreaks of Salmonella typhimurium (S. typhimurium) infection. Salmonella is the leading cause of foodborne illnesses and several outbreaks have been reported where eggs were the source of human infection. A total of 230 samples were randomly collected from various shops in Chaharmahal Va Bakhtiari province located in southwest of Iran. Conventional biochemical and serological test methods were used to identify the suspected salmonella. All the isolates were indicated the zoonotic potential of S. typhimurium. An antimicrobial susceptibility test was performed by using disk diffusion method. S. typhimurium was detected in the egg contents of 21 samples (9.13 %) and 19 samples (8.26%) out of 230 samples by PCR and culture methods respectively. The overall of isolated samples strains to antimicrobial agents was 86.1% for Ampicillin, 86.1% for Cephalexin and 45% for Kanamycin, have more resistant, respectively. The findings of the present study indicate pandemic multiple drug resistant S. typhimurium in egg shell samples; there is an urgent need to improve the hygienic level of consumed eggs.*

1. INTRODUCTION

Salmonella spp. is a major food-borne bacterial pathogen, with poultry and poultry products being a primary source of infection to humans. It has most often been associated with consumption of contaminated foods of animal origin, such as poultry, swine, dairy products and eggs [1]. *Salmonella* is a rod-shaped, motile, aerobic and facultative anaerobic, non-spore forming and gram-negative organism. It can grow from 5°C up to 47°C with an optimum at 37°C. *Salmonella* is a general name used for a group of more than 2000 closely related bacteria such as *Salmonella enteritidis*, *Salmonella typhimurium*, *Salmonella Derby*, *Salmonella Infantis*, *Salmonella Stanley* and *Salmonella Typhi*. *S. enteritidis* (anti-serum group D) and *S. typhimurium* (anti-serum group B) are the most commonly reported serotypes involving in human salmonellosis. According to the data provided by the Department of Health (DH), *S. typhimurium* was the commonest serotype isolated from human clinical specimens. *S. typhimurium* and *S. enteritidis* are the most frequently isolated serovar from foodborne outbreaks throughout the world and are responsible for half of human infections. 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 [2].

S. typhimurium infection of mice provides a well-characterized model for the pathogenesis of human typhoid fever. Orally ingested bacteria penetrate the intestinal mucosa and migrate via the lymph nodes to the spleen and liver to cause systemic disease. The administration of antimicrobial agents in chickens creates selection pressure that favors the survival of antibiotic resistant pathogens. Multidrug-resistant phenotypes have been increasingly described among *Salmonella* species worldwide. Prevalence of antimicrobial resistant *Salmonella* in broiler chicken and foods of animal origin has been reported from India. Most other reports of antibiotic resistance among *Salmonella* are from clinical isolates such as *S. typhimurium* [3, 4].

The discovery of a cluster in the *S. typhimurium* plasmid for the biosynthesis of fimbriae was the serendipitous result of a search for genes. The *pef* (plasmid encoded fimbriae) locus contains four genes (*pefBACDI*) named after the homology of their products with those of other fimbrial operons, and additional ORFs (*orf5*, *orf6*, *orf7*, *orf8*, *orf9*, and *orf11*) whose function cannot be deduced from sequence analysis. In *S. typhimurium*, *pef* genes carried on a multicopy plasmid determine the formation of surface filamentous structures. Transposon insertions in *pefA*, *pefC*, *orf5* and *orf6*, but not in *orf8* (*srgA*), abolish the formation of fimbriae. The similarity between the *pefA* genes of *S. enteritidis* and *S. typhimurium* plasmids is 76% in nucleotide sequence, and 82% in the deduced amino acid sequence [5, 6].

The aim of this study was to determining the prevalence of *Salmonella* on egg shell using conventional microbiology detection compared to that detected using *pef*A gene of *S. typhimurium* by PCR technique and in vitro susceptibility of the isolates to various antibiotics.

2. MATERIALS AND METHODS

2.1. Egg samples

Overall, a total of 230 chicken eggs samples were randomly collected from various shops in Chaharmahal Va Bakhtiari province located in southwest of Iran over a period of three months (August - October 2014). Each sample assessed for the total bacterial viable count and coliform count by pour plate method. Also, detection of *Salmonella* was performed.

2.2. Conventional microbiology detection

The collected samples were incubated in sterile conditions on Salmonella Shigella Agar (SSA) environment for 24 h at 37°C.

2.3. Antimicrobial susceptibility testing

The antimicrobial drugs, including Ampicillin, Chloramphenicol, Gentamycin, Kanamycin, Cephalexin, Tetracycline and Norfloxacin were used. Mueller Hinton agar was used as growth media. The results were interpreted after 24 h of incubation at 37°C, as sensitive, intermediately sensitive, and resistant according to the zone diameter around each antibiotic disk.

2.4. DNA preparation and PCR assay

Genomic DNA was extracted using DNA extraction kit (Qiagen, Germany), according to manufacturer's instructions and assayed on 2% agarose gel electrophoresis and measured at 260 nm optical density according to the method described by Sambrook and Russell. *Salmonella* specific *pef*A gene sequences of *Salmonella* 479 bp, *pef*A-1 TTC CAT TAT TGC ACT GGG TG *pef*A-2 GGC ATC TTT CGC TGT GGC TT were used as primers in this study. PCR was performed in a final volume of 25 µl containing 25 mM MgCl2, 10 mM of dNTPs, 1.5 U of Taq DNA polymerase , 10 pmol of each primer, 5 µL of 10X PCR buffer and 80 ng of DNA template. The mixture was subjected to 30 cycles of amplification in a thermal cycler. The first cycle was preceded by denaturation for 2 min at 95°C. Each cycle consisted of denaturation for 30 s at 95°C, annealing for 30 s at 64°C, and elongation for 30 s at 72°C. The last cycle was followed by a final elongation for 5 min at 72°C. The PCR products were analysed on a 1.5% (w/v) agarose gel electrophoresis.

3. RESULTS

Performing microbiological tests showed that the egg shell contents of 230 samples 19 (8.26%) samples were suspected *Salmonella* genus. The quality of the extracted DNA from samples was examined by electrophoretic analysis through a 2% agarose gel. The plasmid encoded fimbriae (*pef*) gene of *S. typhimurium* was successfully amplified with the *paf*A-1 and *pef*A-2 primers. Agarose gel electrophoresis of the PCR amplified products is shown in Fig. 1. From 230 *Salmonella* samples which assayed by PCR in this research, only 21 samples (9.13%) were positive (479 bp fragment).

The results of the standard disc diffusion tests and antibiogram of selected isolates are given in Table 1. Antibiogram studies revealed that *S. typhimurium* isolates were totally resistant to Ampicillin, Cephalexin and Kanamycin. Isolates were highly sensitive to Norfloxacin (100%), Chloramphenicol (87.3%) and Gentamycin (70.6%). *S. typhimurium* isolates showed poor susceptibility to Tetracycline, and Kanamycin.

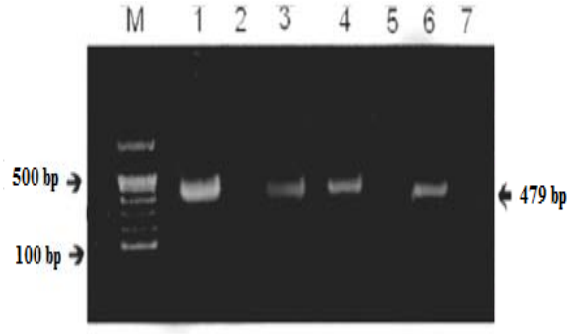

Fig. 1 :Agarose gel electrophoresis of the products amplified with PCR using the specific primers for *S. typhimurium* in egg shell samples. M; DNA ladder, Lane 1, 3, 4 and 6; PCR products of the positive samples, Lane 2 and 5; negative samples, Lane 7; negative control sample

Table 1: Antibiogram studies by disc diffusion method

Antimicrobial drugs	Complete resistance (%)	Intermediate resistance (%)	Susceptible (%)	Total
Ampicillin (15 µg/disc)	86.1	13.9	0	100
Chloramphenicol (30 µg/disc)	0	42.7	87.3	100
Cephalexin (30µg/disc)	86.1	13.9	0	100
Kanamycin (30µg/disc)	45	27.5	27.5	100
Gentamycin (10µg/disc)	0	29.4	70.6	100
Tetracycline (30µg/disc)	13.5	42.6	43.9	100
Norfloxacin (10µg/disc)	0	0	100	100

4. DISCUSSION

Salmonella is a genus of the family Enterobacteriaceae [7]. Before 1983, the existence of multiple *Salmonella* species was

taxonomically accepted. Since then, as a result of experiments indicating a high degree of DNA similarity, all *Salmonella* isolates were classified in a single species, *Salmonella Choleraesuis.* This species was subsequently sub classified into seven subgroups based on DNA similarity and host range. Subgroup I contains almost all the serotypes pathogenic for humans .In 1999, Euzeby proposed to designate "*Salmonella enteric*" as a "neotype species" and replace type species of the genus *Salmonella* from *S. choleraesuis* to *S. enterica* [8]. There are more than 2435 known serotypes of *Salmonella* and many of these serotypes are well documented human pathogens [9]. *S. typhimurium* and *S. enteritidis* are the most frequently isolated serovar from foodborne outbreaks throughout the world and are responsible for half of human infections [10]. 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. Persistence of *Salmonella* in the environment is an important characteristic in its prevalence. Organism penetrates inside the egg from the ovary directly or through contamination of the egg shell [11].

PCR is a sensitive method with a superior ability to detect *Salmonella spp*. in the presence of other competing bacteria [1]. In current study, microbiological tests showed that 19 of 230 the egg shell infection to *Salmonella* (8.26%) in other hand PCR test showed that 21of 230 the egg shell infection to *S. typhimurium*. In other study, *S. enteritidis* is the most frequently isolated serovar from the egg shells and egg contents in some countries such as Turkey [12]. Although some authors mentioned that the most frequent isolate from eggs in Isfahan, Iran was Enterobacter erogenes, *E. coli*, *Klebsiella pneumoniae*, *Buttiauxella agrestis*, *Cedecea lapagei*, *Cedecea davisae*, *Erwinia herbicola* and *Psedumonas aeruginosa* were the most common isolated species [13]. Even though the original population of *S. typhimurium* 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. typhimurium* and in undesirable circumstances such as salted egg yolk products have been previously documented [2].

Resistant bacteria are routinely isolated from a variety of foods, including poultry meat and eggs [12]. Antibiogram patterns for *Salmonella* isolates are summarized in Table 1. Most of the 100 isolates in this study were susceptible and intermediate resistance. Antimicrobial agents for many *Salmonella* isolates exhibited resistance were Ampicillin 86.1%, Cephalexin 86.1% and Kanamycin (45%). In similar study, Musgrove *et al.*, (2005) demonstrated the susceptibility of various *salmonella* to several antimicrobial drugs and reported high resistance to Nalidixic acid 63%, Tetracycline 63%, Streptomycin 63%, Ampicillin 61% and Kanamycin 61% [12]. In our assay no samples were resistance to Norfloxacin. Although, pan et al studiy on resistance antibiotic isolates *salmonella* of shell egg showed that 17.5% of samples were resistance to Norfloxacin [14].

According to our results, it seems that *S. typhimurium* is the most prevalent serotype of sell egg content contaminant in the Southwest area of Iran and the PCR method could be used as a reliable method of identifying *Salmonella* serovars; in addition, this study confirmed that the shell egg is a significant reservoir of foodborne pathogens such as *S. typhimurium*.

In summary, the results of standard disc diffusion method indicate the limited therapeutic value of Ampicillin, Cephalexin, and Kanamycin against *S. typhimurium*.

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