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Molecular Analysis, Serotyping and Antibiogram Pattern of Salmonella in Marketed Local, Industrial and Breeder Poultry Eggs in Tabriz City, Iran

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ABSTRACT: Food borne diseases are considered as the major problems for public health in the world and salmonella serotypes are the most important pathogens causing these types of diseases. Present study was carried out for the isolation of salmonella and determination of their antibiotic sensitivity and serovars from local, industrial and breeder poultry eggs. In this cross-sectional survey, 300 samples of local, industrial and breeder poultry eggs (each containing 100 samples) were collected from Tabriz city, Iran and transferred to the laboratory of food hygiene, Islamic Azad University of Tabriz. Isolation of salmonella from egg yolk was performed according to the Iranian National Standards. For definitive detection of salmonella, Biochemical tests, serotyping, and PCR tests were performed followed by Antibiogram test. From a total of 300 analyzed eggs, just 3 out of local samples (3%) were contaminated with *salmonella enteritidis*. 100 percent of isolates were sensitive to Ciprofloxacin, Gentamycin, Neomycin, Trimethoprim and Sulfamethoxazole. All of the isolates were found to be resistant to at least 5 of 9 evaluated antibiotics. According to the results of this study, the rate of contamination in local eggs supplied and distributed in Tabriz was %3 but the industrial and breeder poultry eggs were recognized safer for public use. According to these findings, it is therefore recommended to pay further attention to cold storage of local eggs.

Keywords: *Antibiogram, Egg, PCR, Salmonella, Serotyping*.

Introduction

Salmonella is a group of gram negative basils belonging to Enterobacteriaceae that are mostly pathogen, but the degree of their pathogenesis is different (Soltan Dallal *et al.*, 2014). The infective dose of Salmonella which may lead to disease with clinical symptoms or without symptom in human is 10^5 to 10^8 (in case of *Salmonella typhi*, it is about10^3) (Ranjbar *et al.*, 2009).

More than 2500 salmonella serotypes have been identified which are based on this classification and have been assorted to A, B, C, D …groups (Soltan Dallal *et al.*, 2014).

According to the reports by the Center for Disease Control and Prevention (CDC) as well as World Health Organization (WHO), every year 1.3 billon cases of acute Gastroenteritis occurs due to non-typhoid salmonellosis, which 3 million of them lead to death (Soltan Dallal *et al.*, 2014). Gastroenteritis is the most common salmonella infection in human that often

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caused by *salmonella entritidis* and *typhimurium* (Soltan Dallal *et al.*, 2014). The incubation period of disease is usually between 8-24 hours, depending on the number of bacteria entered the body this period might vary. The disease starts with symptoms such as nausea, vomiting, headache, stomachache, fever and ague according to Soltan Dallal *et al.*, 2014. Salmonellosis is the most common food born disease in developed and developing countries (Shapouri *et al.*, 2009). Vegetables and dairy products, meat products especially poultry, egg and their by-products are known as the most important contamination sources of salmonellas (Amir Mozafari *et al.*, 2013). In recent years, it has been observed that some contamination by these bacteria has occurred through new carries like eggs (Aminzare *et al.*, 2009). In 2002, CDC reported 29 epidemics of salmonella and the rate of salmonella infections was the highest in summer. The most likely to be in danger are under 20 and over 70 years old. The first report conceited with salmonella outbreak of food born was by Gartner in 1888 in Germany (Nosraty, 2012). In the USA, approximately 37.6 % of the isolated salmonella was from poultry source (Shapouri, 2009). During the past decades, antimicrobial resistance and multi-medicinal resistance of salmonella species have significantly increased. The reason for this increase is due to the addition of antibiotics to animals diet (Monadi *et al.*, 2015). Molecular methods namely PCR can provide a quick and accurate resaet for Salmonella detection from environmental, clinical and food samples (Monadi *et al.*, 2015). Local eggs as free-range eggs and breeder chicken eggs as double –yolk eggs have great popularity among consumers. They have an important role in transmission of salmonella serovars to human (Esmaili *et al.*, 2014). Therefore present study was carried out to isolate salmonella and determine their antibiotic susceptibility and serovars from local, industrial and breeder chicken eggs.

Materials and Methods

- *Sampling*

In this cross-sectional study, 100 local, 100 industrial and 100 breeder chicken eggs (totally 300 eggs) were randomly collected from east, west, north and south parts of Tabriz by multistage cluster sampling method. Samples were tested according to the instruction by Iranian National Standard method (ISO, No. 1810).

- *Culture*

In order to prepare the initial suspension, 25ml of the contents of disinfected whole egg was inoculated to 225ml peptone water and incubated at 37ºC for 24 to 48 hours. 0.1 ml of the prepared suspension was added to a tube containing 10 ml selenite cysteine broth. (Merk, Germany) and also, 1ml of suspension was transferred to a tube of 10ml tetrathionate broth containing brilliant green and iodine. They were kept in 41.5ºC for 24 to 48 hours in an incubator. The RVS (Rappaport - Vassiliadis salmonella Enrichment broth) was cultivated on shining green agar /phenol red and SS- agar (Salmonella Shigella Agar), the two mentioned environments were repeated precisely and were put at an incubator. Five suspicious colonies were cultured on nutrient agar and incubated. Typical colonies were chosen for biochemical and serological tests were used (ISO, 2005). Isolates were verified using polyvalent antiserum (SIFIN, Germany) to determine the species (A, B, C, D groups) (ISO, 2005).

DNA extraction**:**

PCR (Polymerase chain reaction) test was performed to confirm the isolates. Boiling method was used for the extraction of bacterial genome and then it was mixed with TEA buffer (containing tries base, acetic acid and EDTA). 300 µl of samples was transferred to sterile micro tubes. Using a sterile loop, colonies from each sample were

inoculated in buffer and then mixed followed by putting them in water bath for 25 minutes and 80 ºC. In the next step, samples were centrifuged at 10000 rpm for 12 minutes. The supernatants containing DNA were transferred to sterile micro tubes and keep in refrigerator for 10 to 15minutes. Finally, the absorbance of the samples was read at 260-280 nm. The used components and their volumes for PCR tests were according to Table 1. Cycling conditions of PCR test and primers sequences used in this study are presented in Tables 2 and 3, respectively.

After the preparation of 1% agarose gel, 1µl of samples were transferred into the holes in the gel. Loading dye was also injected into holes. After the gel electrophoresis with a voltage of 110V for 40 minutes, the bands were observed using a gel documentation system (UV TAK, France). As illustrated in Figures 1 and 2, the bands appeared in 1% agarose gel and the appeared length was matched with the size of the marker (Figure1).

Antibiogram:

The antibiotic susceptibility of salmonella isolates was analyzed using disk diffusion method (Padtan Teb Co., Iran). In this study, 9 antibiotic disks were used including nalidixic acid, gentamycin, ampicillin, tetracycline, streptomycin, nitrofurantoin, trimethoprim sulfamethoxazole, ciprofloxacin and neomycin. For this purpose, after preparation of the bacterial suspension equivalent to half McFarland, it was added to Mueller Hinton agar medium (Merck, Germany). Antibiotic disked were placed in the medium and plates were incubated at 37ºC for 24 hours. According to the observation for the inhibitory zone around the disks and instructions of the producer company, the sensitivity or resistance pattern of isolates were reported**.**

Table 1. Components and volumes for PCR

Row	Material	Sample1	Sample3
1	Master mix	12.5 µl	37.5 µl
2	Primer Forward	0.5 µl	0.5 µl
3	Primer Revers	0.5 µl	0.5 µl
4	Template DNA	2.0 µl	6.0 µl
5	Water	9.5 µl	38.5 µl

Table 2. Cycling conditions for PCR (Sandhye *et al.*, 2012)

Stage	Temperature	Time
Initial heat activation	94ºC	5min
Denaturation	94ºC	30s
Annealing	66ºC	30s
Extension	72ºC	15s
Final extension	72ºC	5min
Number of Cycles 45		

Table 3. Primers used in this study (Sandhye *et al.*, 2012)

Primer	Primer sequence (5′ →3′)	Product length (bp)
hilA-F	TTAACATGTCGCCAAACAGC	**216**
hilA-R	GCAAACTCCCGACGATGTAT	

Results and Discussion

The analyses of 300 marketed local, industrial and breeder eggs indicated that 3% of the local eggs were contaminated with *Salmonella enteritidis* (Table 4). All of the isolates showed agglutination reaction with D_1 antiserum (D_9) and all the isolated salmonella belonged to serum group D. This was confirmed by PCR. In antibiogram test, 100 % of the isolates were sensitive to ciprofloxacin, gentamycin, neomycin, trimethoprim and sulfamethoxasin. All of the isolates were resistance to at least five of the nine evaluated antibiotics (Table 5).

Table 4. The rate of bacteria isolated from local, Industrial and breeder eggs in Tabriz city, Iran

Breeder eggs	Industrial eggs	Local eggs	Bacteria
Escherichia coli	63(63 %)	58(58 %)	1(1%)
Klebsiella	21(21 %)	30(30%)	10(10%)
Salmonella	-	-	3(3 %)
Proteus mirabilis	-	1(1%)	-
Proteus vulgaris	9(9 %)	11(11%)	15(15**%)**
Citrobacter	2(2%)	-	-
Enterobacter	1(1%)	-	-

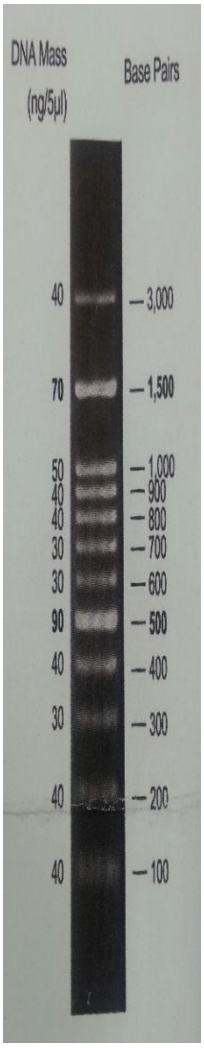

Fig. 1. Size markers

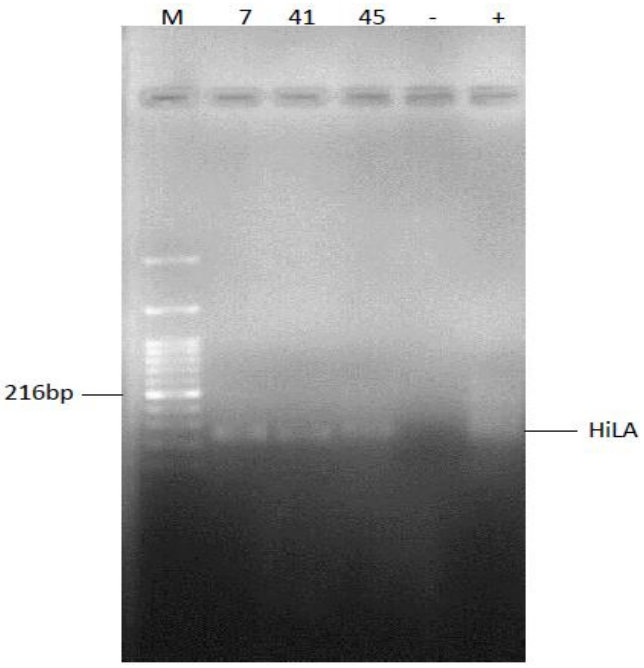

Fig. 2. The electrophoresis of hilA gene (216bp) on 1% agarose gel

Table 5. Distribution of Salmonella isolates susceptible to 9 antibiotics

Susceptibility of Salmonella isolates						
	Resistant		Intermediate		Susceptible	
	Number	(%)	Number	(%)	Number	(%)
Ciprofloxacin (5 µg)	-	-	-	-	3	100
Nitrofurantion (300 µg)	2	66.66	1	33.33	-	-
Gentamycin (10 µg)	-	-	-	-	3	100
Nalidixic Acid (30 µg)	2	66.66	1	33.33	-	-
Ampicillin (10 µg)	2	66.66	1	33.33	-	-
Tetracyclin (30 µg)	1	33.33	2	66.66	-	-
Streptomycin (10)	2	66.66	1	33.33	-	-
Neomycin (30 µg)	-	-	-	-	3	100
Trimethoprim	-	-	-	-	3	100
Sulfamethoxa (23/75 µg)						

Due to the presence of nutrients like protein and several vitamins, egg is a complete and important food and is one of the most frequently used products. In recent years, several outbreaks of salmonella have occurred due to the consumption of raw or medium cooked eggs, specifically in individuals consuming raw eggs. Among salmonella, serovars, the subgroup of entritidis has been known as a threat to public health for decades and contaminated eggs were identified as the major transmission ways of this pathogen to human. The reason for this issue is the ability of organism to survive the temperature of 42ºC (Esmaili *et al.*, 2014). During years of 1975 to 2003, 70% of *Salmonella entritidis* outbreaks have occurred due to the consumption of egg or products containing egg. Furthermore, it is reported that most of the salmonella contamination in eggs belonged to the groups C and D. According to the results of Aminzare *et al.* (2009) in Urmia, Iran, salmonella contamination was identified in 6% of the local eggs that belonged to group D. In 2013, CDC reported that dealing with local poultry may increase the risk of salmonella infection (Esmaili *et al.*, 2014). In a study in Arkansas, U.S., the contents and shell of 1200 eggs were analyzed for salmonella contamination. The external contamination with *Salmonella Heidelberg* was detected in 12 eggs, while there was not salmonella contamination in the content (Aminzare *et al.*, 2009). Poppe (1992) in a study on 1404 eggs from laying flocks in Canada detected salmonella serovare entritidis in egg shells (Poppe *et al.*,1992). In Europe, the rate of this contamination is reported between nil to 13.3 % (Little *et al., 2004)* and this rate was found about 6 % in India (Amir Mozafari *et al.*, 2013). In a study on 157 eggs with contaminated shell, salmonella was only isolated from the contents of 10 eggs (6.36 %) (Little *et al.*, 2004). Suresh *et al.*, (2006) detected salmonella in the content of one-third of the eggs (Suresh *et al.*, 2006). Davis and Roy (1996) reported the salmonella contamination in 11.7 % of breeder farms and the majority of species was *salmonella entritidis*. From a total number of 384 pakistanian eggs, the rate of outbreak of the infection in egg shell, contents and storing trays were 38.88 %, 15 %, and 43.9 %

respectively (Shahzad *et al.*, 2012). In a study conducted in Tehran, Iran, 300 food samples including egg, beef and chicken were analyzed for the presence of salmonella and totally 30 strain consisting of eight serologic variated were isolated. The most isolated serotype in egg, beef and chicken samples were *S. enteretidis*, *S. paratyphi* A and *S. typhimurium*, respectively (Shapouri *et al.*, 2009). Namaei *et al.*, (2006) reported 3 eggs (0.6 %) that were contaminated with salmonella from a total of 500 eggs in Birjand, Iran. The highest resistance was observed for erythromycin, nalidixic acid and sulfamethoxazole and the highest sensitivity was found to ciprofloxacin, cephalexin and gentamycin (Amir Mozafari *et al.*, 2013). Jamshidi *et al.*, (2010), using multiplex PCR and culture methods, found that 4 samples (1.6 %) out of the total of 250 samples have been contaminated. Jahantigh (2010) reported that 7 samples (at least 4 %) of the local eggs in Zabol city, Iran were contaminated with *salmonella* entritidis which was mostly sensitive to norfloxacin. In the present study, the rate of contamination of local eggs with salmonella was reported about 3 % and the isolated serotype was *S. entritidis*. Antibiotic resistance analysis also showed that 100% of the isolates were sensitive to ciprofloxasin, gentamycin, neomycine, trimethoprim and sulfamethoxazole. All of the isolates were resistance to at least 5 out of 9 tested antibiotics. The findings in the present study indicate that 3% of local eggs in Tabriz were contaminated with salmonella. These results are in accordance with the previous reports. Contamination of egg contents usually occurred through the yolk sac. However transmission of bacteria might have occurred through cracked shell to the inside of egg (Namaei *et al.*, 2006). In numerous studies, *Salmonella enteritidis* was the most common isolated salmonella (Namaei *et al.*, 2006). Regarding the high quality of local eggs and the acceptability of the product by consumers, villagers are more likely to be exposed to salmonellosis. The, lack of accurate monitoring and controlling as well as inappropriate initial storing condition of local eggs in rural areas threatens the health of rural population by salmonellosis and other zoonotic diseases. Detection of the contaminated samples and their elimination before consumption by the consumers is another logical way to control this pathogen. Due the fact that the appearance of contaminated eggs is similar to the healthy eggs, the consumption of the raw eggs because of the hygienic problems must he prevented (Esmaili *et al.*, 2014).

Conclusion

The obtained results in this study indicated and proved the presence of salmonella in local eggs in Iran and its high antibiotic resistance. Therefore, this might threaten the consumer health. Regarding the fact concerned with the resistant of some strains in eggs, excessive application of antibiotics in poultry industry must be limited.

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