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Original Article

Molecular inspection of contamination with *Salmonella* Enteritidis in Tabriz city aviaries

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Summary
The aim of this study was to investigate the occurrence of *Salmonella* Enteritidis infection between the birds in Tabriz city aviary by molecular method. In this study, the presence of paratyphoid infection in the pet bird market of Tabriz city was investigated for the first time. One hundred six fecal samples were obtained from aviaries in Tabriz urban area. The samples were then transferred to tetrathionate broth culture media. After 24 hours of incubation, samples were transferred to MacConkey agar plates. Samples showing no fermentation of lactose and containing transparent colonies were transferred to salmonella shigella agar plates. In the next stage, the resulting dark colonies, which were determined to be salmonella bacteria, were entered to a multiplex polymerase chain reaction test. Findings showed that 7 (6.6%) out of 106 samples were contaminated with salmonella bacteria. In addition, results of multiplex PCR confirmed *Salmonella* Enteritidis as the source of *Salmonella* Enteritidis contamination in 2 (1.88%) samples. This finding shows that a slight degree of contamination with enteritidis infection is present in birds in Tabriz city aviaries. It can be concluded that there is a slight rate of infection by *Salmonella* Enteritidis in Tabriz city aviaries. Our findings show that this contamination is latent, and necessary measures should be taken to confront it.
***Keywords**:* Tabriz aviary, Bacterial infection, *Salmonella* Enteritidis, PCR

Introduction

Salmonellosis is among the most important zoonotic diseases, which are common to happen among different species of animals, as well as between human beings (Grimes, 1987). The disease can be transmitted through contact with infected droppings of birds (Nakamura et al., 1994). Moreover, the exposure of the vulnerable to contaminated dust and feces may contribute to the spread of the disease (Harbaugh et al., 2006). Keeping an infected bird with other birds is known to be a common cause of infection. Humans, flies, beetles, and parasites are considered the most important carriers of the infection (Doyle and Erickson, 2006). *Salmonella* can survive long periods on wooden surfaces, as well as inside, feces, and dust. In addition, it has been reported that *Salmonella* may remain active in bird droppings for up to 28 months (Ryan, 1985). In most cases, the incubation period is reported to be circa one week. Clinical signs of *Salmonella* infection in pet birds can range from mild enteritis to severe diseases, including anorexia, diarrhea, lethargy, dehydration, and stasis in the crop. In addition, acute sudden death may happen without clinical signs that may indicate systemic infection

(Orosz et al., 1992). It has been reported that non-selective and selective enrichment in combination with the polymerase chain reaction (PCR) test has better sensitivity in the detection of *Salmonella* (Bennett et al., 1998, Carli et al., 2001). Researchers believe that performing a polymerase chain reaction test with pre-enrichment in a broth medium is a fast and useful method of detection that increases the number of living *Salmonella* bacteria in the sample and thus increases the sensitivity of the test (Carli et al., 2001).

Materials and methods

During the implementation stage of this project, 106 fresh fecal samples of pet birds were collected. First, an initial assessment of the birds' keeping place was performed. Stool samples were then randomly taken using sterile forceps and added to a microtubule containing 5cc of Peptone water solution. The birds were ordinarily kept individually or in pairs, except for the budgerigar and finch, which were kept in colonies (10 to 30 birds in each cage). For them, several fresh stool samples were taken together, and the colony was considered as one sample. According to the bird shop owners, some of the sick birds were treated using unknown medicine. After filling each microtube, the characteristics of the bird were recorded in a form. In the next step, the samples were incubated for 18 to 24 hours at 37 °C (Sareyyüpoğlu et al., 2008). Then, the samples were transferred to tetrathionate broth media. The media was incubated for 24 hours at 37 °C (Winter et al., 2010). After this step, samples were transferred to MacConkey agar medium.

During this study, *Salmonella* enterica subsp. Enterica serovar Enteritidis with ATCC code 13076 was obtained from the collection of microorganisms of the Faculty of Veterinary Medicine, University of Tehran, and was used as a standard sample for positive control. Then, the suspicious colonies were transferred to the *Salmonella*-Shigella agar (SS agar) medium. The pellets were incubated, and the suspected colonies were taken to the PCR test (Anderson et al., 2005). The dark colonies formed in the SS agar culture medium were transferred to tubes and boiled in 100 °C water for 10 minutes, then all tubes were centrifuged at 3500 rpm for 5 minutes (Ahmed and Dablool, 2017).

A solution containing three pairs of primers was used to perform multiplex PCR (Table 1). The sequence of these primers was extracted from the study of Pan and Liu (2002) and was obtained from Sinaclone Company (Iran). In this study, a ready Master Mix (Reddy) made by Sinaclone Company (Iran) was used. The volume used in the PCR in this study was 12.5 microliters.

Salmonella (ATCC 13076) was used as the positive control, and the PCR products were treated with electrophoresis so that the product was placed on a gel inside the tank, and after 50-60 minutes of 90 volts treatment, when the loading buffer traveled two-thirds of the length of the gel, the system was cut off, and then the gel was washed with water and then placed on a trans-illuminator (Biotap, Finland), and the results were observed.

Table 1. Specification of primers used in PCR test

Target	Primer	Sequence	Product size (bp)
ST	ST11	5'–GCCAACCATTGCTAAATTGGCGCA-3'	429 bp
	ST14	5'- GGTAGAAATTCCCAGCGGGTACTGG –3'	
Spv	S1	5'- GCCGTACACGAGCTTATAGA – 3'	250 bp
	S4	5' - ACCTACAGGGGCACAATAAC –3'	
***Sef*A**	*SEF*A2	5' - GCAGCGGTTACTATTGCAGC –3'	310 bp
	*SEF*A4	5' - TGTGACAGGGACATTTAGCG – 3'	

Results

During this study, an attempt was made to cover the common types of pet birds available in the bird market of Tabriz city.

According to Table 2, out of a total of 106 samples, 20 (18.8%) belonged to finches, 12 (11.3%) to budgerigars, 10 (9.4%) to cockatiels, 10 (9.4%) to Ornamental chicken, 8 (7.5%) to Mynah, 6 (5.6%) to lovebirds, 6 (5.6%) to pigeons, 6 (5.6%) to canaries, 5 (4.7%) to Alexandrine Parrot, 5 (4.7%) to African grey parrot, 4 (3.7%) to Green-cheeked parakeet, 4 (3.7%) to Ring-necked parakeet, 2 (1.9%) to Rosella, 2 (1.9%) to Senegal, 2 (1.9%) to Conure, and 1 (0.9%) to Turquoise parrot, Grey partridge , Chough, and Lorikeet.

Table 2. Name and abundance of birds in the current study

Name	Scientific name	abundance
Finch	*Taeniopygia guttata*	20
Budgerigar	*Melopsittacus undulatus*	12
Cockatiel	*Nymphicus hollandicus*	10
Ornamental Chicken	*Brahma chicken*	10
Mynah	*Acridotheres tristis*	8
Lovebird	*Agapornis roseicollis*	6
Pigeons	*Columbidae*	6
Canary	*Serinus canaria*	6
Alexandrine Parrot	*Psittacula eupatria*	5
African grey parrot	*Psittacus erithacus*	5
Green-cheeked parakeet	*Pyrrhura molinae*	4
Ring-necked parakeet	*Psittacula krameri*	4
Rosella	*Platycercus*	2
Senegal parrot	*Poicephalus senegalus*	2
Conure	*Pyrrhura molinae*	2
Turquoise parrot	*Neophema pulchella*	1
Grey partridge	*Perdix perdix*	1
Chough	*Pyrrhocorax*	1
Lorikeet	*Oreopsittacus. Neopsittacus.*	1
Total		**106**

Among the colonies that showed positive signs of Enterobacteriaceae in MacConkey agar medium, samples were isolated and introduced into the SS agar medium. Then, after 24 hours of culture in MacConkey agar medium at 37 °C, fermentation and colony formation were observed in 64 samples (60.3%) in the culture medium. In addition, in 49 samples (46.2%) the colony growth was positive; however, they did not ferment lactose. In 11 cases (10.4%) positive fermentation appeared without colony growth in the culture medium, and in 4 culture media (3.8%) fermentation and colony was not observed in the culture.

In the next stage of the experiment, 49 colonies observed in the MacConkey Agar culture medium were transferred to SS agar medium under the hood and next to the flame using a sterile loop. The media were then incubated for 24 hours at 37 °C and the colonies in which *Salmonella* was detected were entered into the PCR stage.

The results of culturing *Salmonella* susceptible colonies in SS agar medium showed that 7 dark colonies were formed, indicating the presence of colonies related to *Salmonella* family bacteria. Moreover, 10 pellets had sticky gray colonies. And in 3 culture media pink colonies were observed. In addition, no colonies were observed in 29 culture media. In addition, in some cases, both sticky gray colonies and pink colonies were observed in one culture medium. To determine the type of bacterial

species and to confirm the presence of *Salmonella* Enteritidis, these 7 samples were PCR tested.

A multiplex PCR test was performed to confirm the presence of Enteritidis strain in 7 samples that showed *Salmonella*-specific colonies. For this, the dark colonies formed in the SS agar culture medium were transferred using a loop to 500 µL of physiological serum in tubes and boiled. Then, the PCR was performed using the following setting for the thermocycler:

- Initial denaturation at 94 °C for 5 minutes
- 30 repetitive cycles for denaturation at 94 °C for 45 seconds
- Annealing of primer at 60 °C for 45 seconds
- Polymerization at 72 °C for 3 minutes
- Final expansion at 72 °C for 5 minutes

The overall results are presented in Table 4. According to Table 3, all seven samples were tested positive for *Salmonella*, indicating that all tested samples contained *Salmonella* bacteria. On the other hand, out of 7 samples, in samples 3 and 5, all three genes were identified, indicating that samples 3 and 5 had *Salmonella* Enteritidis. Also, Spv and SefA genes were not identified in samples 1, 2, 4, 6, and 7, so that these samples were found to be free of *Salmonella* Enteritidis. Additional information on the samples for which the PCR test was performed is presented in Table 4.

Based on the data in Table 4, the molecular method for the study of *Salmonella* Enteritidis infection in samples taken from Tabriz city aviaries showed that 2 samples (1.88%) of the 106 samples were infected with *Salmonella* Enteritidis.

Table 3. Results of PCR test on positive control, negative control, and samples

Gene / Sample No.	ST (429bp)	*Spv* (250bp)	*Sef*A (310bp)
Positive control *Salmonella Enteritidis* ATCC 13076	Positive	Positive	Positive
1	Positive	Negative	Negative
2	Positive	Negative	Negative
3	Positive	Positive	Positive
4	Positive	Negative	Negative
5	Positive	Positive	Positive
6	Positive	Negative	Negative
7	Positive	Negative	Negative
Negative control	Negative	Negative	Negative

Table 4. PCR results of different bird species fecal samples.

Sample No.	Bird
1	Ornamental chicken
2	Canary
3 (*Salmonella Enteritidis* positive)	Alexandrine Parrot
4	Alexandrine Parrot
5 (*Salmonella Enteritidis* positive)	Cockatiel
6	Ornamental chicken
7	African grey parrot

Discussion

In this study, the presence of paratyphoid infection in the pet bird market of Tabriz city was investigated for the first time. A number of bird shop owners stated that the birds were being treated with antibiotics and vitamin supplements;

however, the exact identification of the medicine administered to the birds was unclear. According to our observations, most of these drugs did not have a clear label and were stored in inappropriate conditions in terms of temperature and environment.

No fermentation was observed in 4 culture dishes and no colony was observed in MacConkey's agar medium. Given that in some bird shops, the bird shop owner stated that the birds were being treated with antibiotics, justifying such an observation, it could be said that these colonies belonged to birds that had received antibiotics that prevented the growth of any colonies in the culture medium.

The results showed that in 7 (6.6%) culture dishes containing SS agar medium, colonies related to *Salmonella* were observed. In the study of Peighambari et al. (2011) on the samples taken from the birds market of Tehran, the method of culture on SS agar was used. The results of their study showed that 2.8% of the samples were infected with *Salmonella.* This difference can be explained by the method used for sampling in the study of Peighambari and his colleagues, which was random. In the present study, more attention was paid to birds that showed clinical signs of the disease. Therefore, considering this point, these two studies can be considered consistent in terms of culture results.

During this project, out of 106 studied samples, 2 cases (1.88%) were found to be positive for *Salmonella* infection. Kobayashi et al. (2007) examined 328 cloaca samples taken from wild birds in Tokyo, Japan, and found that 19 samples (5.8%) were infected with *Salmonella* (Kobayashi et al., 2007). Considering that the samples taken in their study were from birds that were died due to falling, being hunted by predators and falling out of the cage (probably weaker birds), the lower level of contamination in the study of Kobayashi et al. (2007) is justifiable. *Salmonella* is not usually associated with specific clinical signs in wild birds. Therefore, the results of this study can be considered in line with the current study.

During the molecular study of Sareyyüpoğlu et al. (2008) on 185 fecal samples by a polymerase chain reaction, it was found that 5 samples (2.7%) were infected with *Salmonella* bacteria, which is consistent with the results of the present study.

During the study of Mirzaie and colleagues on sparrows around Tehran city, out of 470 samples tested, 18 samples were identified as infected with *Salmonella*, which is in line with the results of the current study.

Conclusion

According to the findings of this study, it can be concluded that the birds in Tabriz city aviaries have a slight infection of paratyphoid *Salmonella*. Moreover, observing the hygienic principles of keeping birds can play a role in reducing infection and preventing the transmission of this common disease to humans. The findings of this study showed that paratyphoid infection in birds in the bird market of Tabriz is the latent type and the necessary health measures should be taken when exposed to them. Further studies with a focus on other *Salmonella* species in the bird market of Tabriz are suggested. It also seems useful to study the risk factors affecting the contamination of any species. Moreover, the methods of controlling and prevention of *Salmonella* in birds should be considered in future studies. The effect of the use of acidifiers, probiotics, and antibiotics on resistance to avian salmonellosis can also be the subject of further studies. Finally, the use of serum monitoring methods such as Rapid and ELISA methods in the evaluation of avian salmonellosis is recommended.

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Conflict of interest statement

The authors declare that there is no conflict of interest in this project.

Ethical approval

Not applicable

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