



PREVALENCE AND GENETIC CHARACTERISTICS OF *SALMONELLA* STRAINS IN WILD MALLARD DUCKS (*ANAS PLATYRHYNCHOS*) IN SEMNAN SUBURB, IRAN

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Summary

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Wild birds serve as major reservoirs for transmission of *Salmonella* to domestic animals and humans. Given the zoonotic potential of salmonellosis, the main goal of this research was to investigate the prevalence and molecular epidemiology of *S. enterica* infections in wild Mallard ducks. Faecal samples (n=247) from wild Mallard ducks were tested for the prevalence of *Salmonella* spp., and genotypes of strains were then differentiated by multiplex PCR. From the 247 faecal samples, 18 (7.29%) were positive for *Salmonella* spp. Biochemically the most predominant serovars were *S. Typhimurium* and *S. Enteritidis* (10 and 6 cases each, respectively), whereas only 2 serovars belonged to *S. Infantis*. Among the 10 *S. Typhimurium* serovars, nine strains were positive for *rfbJ*, *fljB*, *invA*, and *fliC* genes based on multiplex PCR assay and one strain contained only the *invA* gene. In *S. Enteritidis* serovars, PCR generated amplification products for *spv* and *sefA* genes, and a random sequence in all samples. The two *S. Infantis* contained the random sequence specific for *Salmonella* genus. With respect to the circulation of virulent *Salmonella* in wild ducks of Semnan suburbs, more work to assess the correlation of strains from wild life with human and livestock strains is needed.

Key words: Mallard ducks, multiplex PCR, *Salmonella*, Semnan

INTRODUCTION

Salmonella is an enteric, facultative intracellular and zoonotic pathogen which is widely distributed in the environment, wild life, pets and animals we use for food. Diseases in both animals and hu-

mans worldwide are caused by different serotypes and strains of the bacterial species *Salmonella enterica* subsp. *enterica* (Albufera *et al.*, 2009; Pan *et al.*, 2010). Through the ages, wildlife has been a sig-

nificant source of infectious diseases transmissible to humans (Mirzaie *et al.*, 2010). Today, zoonosis with a wildlife reservoir constitutes a superior public health problem, affecting all continents. *Salmonella* spp., are commonly found in the intestines of wild birds and these animals can acquire these pathogens from contaminated environments and extend it directly to humans or indirectly by contaminating commercial livestock operations (Kruse *et al.*, 2004; Mirzaie *et al.*, 2010). The importance of *Salmonella* strains lies in the fact that they are found relatively commonly in different hosts and wild birds may function as effective spreaders of these pathogens (Tizard, 2004), but little is known about the relationship between wild bird *Salmonella* strains and human and livestock strains in regions of Iran, particularly with respect to the virulence genes they contain and their serotype profiles. As some bird species like colonial water birds, predators, passerines and siskins can play an important role as reservoirs of *Salmonella* spp., and distributing these agents to the environment and other hosts via droppings, contact or other routes of transmission (Peighambari *et al.*, 2010), the aim of this study was isolation and prevalence assessment with genotyping of *Salmonella* strains from wild Mallard ducks in Semnan suburbs, Iran.

MATERIALS AND METHODS

Samples, isolation, identification & serotyping of Salmonella

Two hundred forty-seven fresh faecal samples of Mallard ducks (*Anas platyrhynchos*) were collected during a 6-month period in winter 2015 from protected areas in suburbs of Semnan, Iran. Semnan city is the administrative center of

the Semnan province (35°34'31" North, 53°23'39" East). This city consists of two major geographical areas; the mountainous to the north, and the fertile outskirts and plains to the southern deserts; so Semnan is significant for its variable climate. Faecal samples were transported immediately to the microbiology laboratory on ice packs, and *Salmonella* isolation was performed as described by Mirzaie *et al.* (2010) as followed. Faecal samples were cultured in selenite F medium, incubated at 37 °C for 18 h, then each sample was inoculated on Brilliant Green agar (BG) and Salmonella-Shigella agar (SS) plates. The plates were incubated at 37 °C for 24 h. Doubtful colonies morphologically alike *Salmonella* were subcultured for biochemical examination. Recognition of the biochemical characteristics was done using triple sugar iron (TSI) agar, lysine-iron agar (LIA) medium, urea agar, motility in SIM agar, Simmon's citrate agar, and lactose, sucrose, maltose, and mannitol broth media.

The *Salmonella* isolates were cultured onto TSI slant medium and grown overnight at 37 °C, and afterward were tested using antisera O (B, D, E, C) and H based on slide and tube agglutination tests to determine O and H antigens, respectively (Mirzaie *et al.*, 2010). Briefly, each suspect culture was mixed with a drop of polyvalent antisera and incubated for up to 2 min at room temperature. Positive reactors were then tested separately with different somatic O monovalent and flagellar H monovalent antisera to determine the serogroups and serotypes of the isolates.

DNA extraction & multiplex PCR

Prior to genome extraction, isolated *Salmonella* strains were grown up in Luria Bertani agar (LB) plates and incubated at 37 °C for 24 h, next, genomic DNA was

Table 1. Primers used for the detection of *Salmonella* Typhimurium (Zahraei Salehi *et al.*, 2007) & *Salmonella* Enteritidis (Pan & Liu, 2002) isolates

<i>Salmonella</i> serotype	Primer	Target gene	Sequence (5'–3')	Product size (bp)
<i>Salmonella</i> Typhimurium	ST139-s	<i>invA</i>	GTGAAATTATCGCCACGTTTCGGGCAA	284
	ST141-as		TCATCGCACCGTCAAAGGAACC	
	Rfbj-s	<i>rfbJ</i>	CCAGCACCAAGTTCCAACCTTGATAC	663
	Rfbj-as		GGCTTCCGGCTTTATTGGTAAGCA	
	Flic-s	<i>fliC</i>	ATAGCCATCTTACCAGTTCCCCC	183
	Flic-as		GCTGCAACTGTTACAGGATATGCC	
	Fljb-s	<i>fljB</i>	ACGAATGGTACGGCTTCTGTAAACC	526
	Fljb-as		TACCGTCGATAGTAACGACTTCGG	
<i>Salmonella</i> Enteritidis	ST11	random se- quence*	GCCAACCATTGCTAAATTGGCGCA	429
	ST14		GGTAGAAATTCCCAGCGGGTACTGG	
	S1	<i>Spv</i> **	GCCGTACACGAGCTTATAGA	250
	S4		ACCTACAGGGGCACAATAAC	
	SEFA2	<i>sefA</i> ***	GCAGCGGTTACTATTGCAGC	310
	SEFA4		TGTGACAGGGACATTTAGCG	

randomly cloned sequence specific for the genus *Salmonella*; ** *Salmonella* plasmid virulent gene; *** *S. Enteritidis* fimbrial antigen gene.

extracted from isolated strains with the Rapid One-Step Extraction (ROSE) method described by Osmundson *et al.* (2013). Multiplex PCR was conducted with 2 independent sets for DNA amplification and consolidation of *S. Typhimurium* and *S. Enteritidis* (primers sequences used in the study are submitted in Table 1). For *S. Typhimurium*, PCR was performed in a 25 µL volume containing 40 ng of total *S. Typhimurium* extracted DNA, 1.5 mM MgCl₂, 0.5 µM of primer, 1 U of Smartaq DNA polymerase and 200 mM dNTP mix in 1× PCR buffer. Thermal programme and electrophoresis conditions were as previously described (Zahraei Salehi *et al.*, 2007). For *S. Enteritidis*, PCR was performed in a total volume of 25 µL amplification mixture consisting of 2.5 µL of 10× 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 DNA that extracted as template and 9.6 µL of distilled water with thermal program and electrophoresis condition described by Pan & Liu (2002).

RESULTS

Bacterial colonies appearing red-pink opaque coloured in BG agar and transparent or translucent colourless with black centres in SS agar were chosen for more biochemical tests. Colonies showing alkaline/acid with H₂S production in TSI agar, lysine decarboxylation in bottom and negative deamination on slant part of LIA agar with H₂S, negative urease activity and autotrophy reaction in citrate agar with the following sugar fermentation characteristics: lactose (–), sucrose (–), maltose (+), and mannitol (+) were diagnosed biochemically as *Salmonella*.

Table 2. Distribution of *Salmonella* serotypes within faecal samples of wild Mallard ducks

Isolated serotype	Number	Percentage
<i>S. Typhimurium</i>	10/247	4.0%
<i>S. Enteritidis</i>	6/247	2.4%
<i>S. Infantis</i>	2/247	0.8%
Total	18/247	7.2%

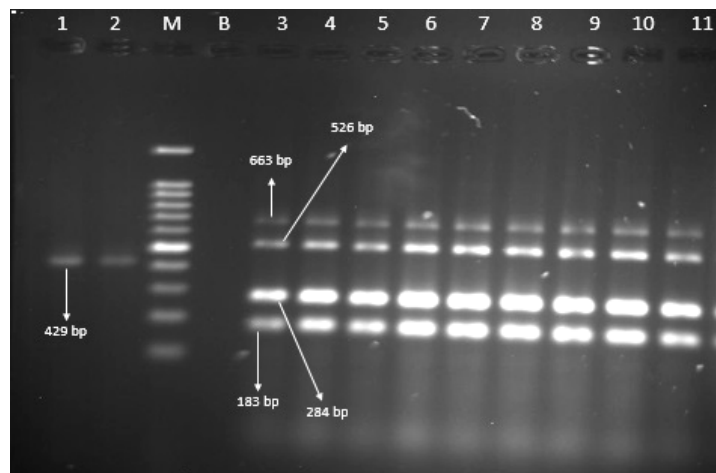


Fig. 1. Multiplex polymerase chain reaction for detection of *S. Typhimurium*. Lane M: 100 bp marker; lane B: negative control; lanes 3–11: *S. Typhimurium* isolates; lanes 1–2: *S. Infantis* isolates harbouring random sequence specific for *Salmonella* genus.

Based on the mentioned isolation protocol eighteen *Salmonella enterica* strains (7.2%) were isolated from 247 faecal samples of wild Mallard ducks living in Semnan suburbs (Table 2). Serotyping of the isolates showed that *Salmonella* Typhimurium (4%) was the most common serotype identified, then *Salmonella* Enteritidis (2.4%) and *Salmonella* Infantis (0.8%), respectively.

Genotyping of the collected *Salmonella* strains revealed that nine *S. Typhimurium* strains harboured *invA*, *rfbJ*, *fliC* and *fliB* orf's (open reading frames) and only one *S. Typhimurium* isolate was negative for the *invA* gene (Fig. 1). All six strains belonging to *S. Enteritidis* serotype

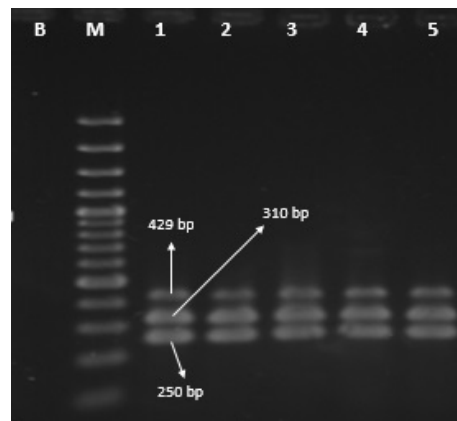


Fig. 2. Multiplex polymerase chain reaction for detection of *S. Enteritidis*. Lane M: 100 bp marker, lane B: negative control; lanes 1–5: *S. Enteritidis* isolates.

were positive for random sequence, *spv* and *sefA* genes (Fig. 2). The two serologically identified *Salmonella* Infantis were positive for the random sequence which is particular for the genus *Salmonella* and no more genotyping work was performed for them (Fig. 1).

DISCUSSION

Free-living birds are propounded to be potential carriers of zoonosis and to play a role in the ecology and circulation of several pathogens such as *Salmonella* (Krawiec *et al.*, 2015). Wild birds not only function as effective spreaders of this infectious agent to humans and to different animal species through contamination of the environment, but also cases of suspected bird to human transmission of the bacterium have been reported (Alley *et al.*, 2002; Handeland *et al.*, 2002). Many studies have been done in different countries to check the outbreak of *Salmonella* in wild birds and a wide variety of serovars has been reported (Hoelzer *et al.*, 2011). It's suggested that certain serotypes and strains of *S. enterica* subsp. *enterica* are associated with different groups of wild birds (Refsum *et al.*, 2002; Pennycott *et al.*, 2010). Mallard ducks may pose a so far underestimated risk to human and animal health by transmitting *Salmonella* spp. via their faecal deposits to various environmental sources. The results of our study showed that *S. Typhimurium* and *S. Enteritidis* are the most frequently serotypes among wild Mallard ducks in Semnan suburb in parallel with results of other studies introducing these serotypes of *Salmonella* as predominant strains circulating in wild birds (Čížek *et al.*, 1994; Kapperud *et al.*, 1998). According to the Centers for Disease Control and Prevention, *S. Typhimurium* and *S. En-*

teritidis are the two serovars associated most commonly with human disease, and therefore of importance to public health (CDC, 2004). Previous studies indicate that the prevalence of *Salmonella* infection among wild birds is variable and factors like migration patterns, season or feeding behaviour can influence the prevalence of the agent in such hosts (Kirk *et al.*, 2002; Kobayashi *et al.*, 2007; Skov *et al.*, 2008; Gaukler *et al.*, 2009). The present study showed a *Salmonella* prevalence of 7.2% in Mallard ducks. It was evaluated as a high risk, because some authors indicate that a prevalence in wild birds, especially colonial water birds has increased significantly in recent years, and these animals can easily transmit this pathogen to other animals and hosts by contaminated faeces, since they often gather in very large numbers (Peighambari *et al.*, 2010). Activities such as hunting, human behaviours and populational factors can also influence the epidemiology of this infectious agent (Kruse *et al.*, 2004).

Serotyping of our *Salmonella* isolates from the free-living Mallard ducks in Semnan suburb, showed that 4% of strains belonged to *S. Typhimurium* serotype, 2.4% – to *S. Enteritidis* and about 0.8% – to *S. Infantis*. Subtyping of *S. enterica* has been typically carried out by serotyping, a method in which surface antigens are recognised on the basis of agglutination reactions with particular antibodies (Wattiau *et al.*, 2011). Despite several studies showing the prevalence and serotypes of *S. enterica* isolated from various hosts (Tizard, 2004; Coburn *et al.*, 2007; Pennycott *et al.*, 2010), serotyping presents no information about the phyletic relationships inside the different *S. enterica* subspecies for epidemiological investigations and for such purposes, the use of methods

that can reveal the genotype of causative strains at a taxonomic level far more particular than that obtained by serotyping, is needed (Wattiau *et al.*, 2011). By the PCR method, *in vitro* DNA amplification is a powerful tool in microbiological diagnostics and several markers such as virulence chromosomal genes and genes involved in the synthesis of flagellin have been used to detect *Salmonella* in environmental and natural samples as well as food and faecal samples (Malorny *et al.*, 2003; Jamshidi *et al.*, 2010). All of our *Salmonella* isolates were positive for *invA* gene which contains sequences unique to this genus, encoding a protein in the inner membrane of bacteria responsible for invasion to the epithelial cells of the host (Shanmugasamy *et al.*, 2011). The amplification of this gene now serves as an international standard for detection of *Salmonella* genus with potential diagnostic applications (Malorny *et al.*, 2003). In the present study, *S. Typhimurium* strains were monitored for the presence of *fliC* and *fljB* genes encoding phase-1 and phase-2 flagella and *rfbJ* gene coding for CDP-abequose synthase (Lim *et al.*, 2003; Dilmaghani *et al.*, 2010; Jarvik *et al.*, 2010). Nine of our isolates were harbouring all three genes, while one isolate was negative for all three genetic markers. Lim *et al.* (2003) designed this triplex PCR based on detection of *flic*, *fljB* and *rfbJ* indicating that this method is useful for specific detection of *S. Typhimurium* and discrimination of this serovar between other *S. enterica* serovars. Our results confirm that serotyping of *Salmonella* isolates in epidemiological investigations may not be sufficient enough, so molecular and genetic typing seems to be necessary. Also, *Salmonella* strains diagnosed as *S. Enteritidis* were evaluated for the presence of a random sequence, *spv* and *sefA* genes in a

Triplex PCR assay (Table 1). All the six serologically identified *S. Enteritidis* isolates were positive for the presence of the related genes confirming the virulence of strains and results of the serotyping, because *spv* and *sefA* genes are related to virulence and important for discrimination of *S. Enteritidis* from non-*Enteritidis* strains. The results of the present study showed that *Salmonella* strains circulating in wild Mallard ducks living in the Semnan suburb are potentially virulent for other hosts such as human and livestock because they contained virulence genes. Their importance and risk is higher considering that most published studies elsewhere in livestock emanate from small epizootics and are of either dead birds at feeding stations or infected birds in or around farms where the livestock was infected with *Salmonella* (Hatanaka *et al.*, 2003). Waterfowl isolates could rapidly spread to other ducks because of their colonial and gathering characteristics.

It would be worthwhile understanding the relationships between wild and domesticated hosts of different regions in greater detail for epidemiological surveillance and for assessment of the risk of wild birds as reservoirs or vectors of *Salmonella* infections (Hughes *et al.*, 2008), as few studies have been conducted in wild-life species in Iran on this subject. Recognition of *Salmonella* can be performed via both molecular and serotyping methods. Although serotyping offers a reliable method for differentiating strains of the agent, the molecular identification of this bacterium seems to be necessary for epidemiological researches, evaluation of virulence potential of the isolates and confirmation of serotyping results because of the high sensitivity and specificity of these techniques (Mirzaie *et al.*, 2010). Genotyping of *Salmonella* strains from diffe-

rent hosts and sources in the area by methods like DNA microarrays, pulse-field gel electrophoresis is recommended to understand the relationship of the strains from wild birds, livestock and human cases with salmonellosis (Staji *et al.*, 2015).

CONCLUSIONS

Molecular characterisation of the *Salmonella* isolates collected from wild ducks living in Semnan suburbs, Iran, showed that *S. Typhimurium* and *S. Enteritidis* are probably the most prevalent serotypes in these hosts. The multiplex PCR could be used as a reliable method of identifying and genotyping *Salmonella* serovars. According to this study wild ducks may play an important role as significant reservoirs of zoonotic pathogens such as *Salmonella* for livestock and humans. More genotyping work would be necessary to understand the relationships of *Salmonella* serotypes between wild and domesticated animal hosts and humans in a defined region.

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