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

H. STAJI¹, S. REZAEI¹, M. RASSOULI¹ & S. NAMROODI¹

¹Department of Pathobiology, Faculty of Veterinary Medicine, Semnan University, Semnan, Iran; ²Graduated student, Faculty of Veterinary Medicine, Semnan University, Semnan, Iran; ³Department of Environmental Sciences, Faculty of Fisheries and Environmental Sciences, Gorgan University of Agricultural Sciences & Natural Resources, Gorgan, Iran

Summary

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 humans 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 Mirzaei 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
Prevalence and genetic characteristics of Salmonella strains in wild Mallard ducks (Anas ...)

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

<table>
<thead>
<tr>
<th>Salmonella serotype</th>
<th>Primer</th>
<th>Target gene</th>
<th>Sequence (5´–3´)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em> Typhimurium</td>
<td>ST139-s</td>
<td>invA</td>
<td>GTGAAATTATCGGCCACGTTCGGGCAA TCATCGACCCGTAATAAGGAACC</td>
<td>284</td>
</tr>
<tr>
<td></td>
<td>ST141-as</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rbj-s</td>
<td>rfbJ</td>
<td>CCAGCCACAGTCCAACCTTGATAC GGCTCCGGCTTTATGGTAAGCA</td>
<td>663</td>
<td></td>
</tr>
<tr>
<td>Rbj-as</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flic-s</td>
<td>fliC</td>
<td>ATAGCCATCTTACCAGTCCCCC GCTGCAACTGTACCGATAGCTGCC</td>
<td>183</td>
<td></td>
</tr>
<tr>
<td>Flic-as</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fljb-s</td>
<td>fljB</td>
<td>ACGAATTGGTGACGGCTTGTAACC TACCGTGATAGTGACACTCGT</td>
<td>526</td>
<td></td>
</tr>
<tr>
<td>Fljb-as</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Salmonella</em> Enteritidis</td>
<td>ST11</td>
<td>random sequence</td>
<td>GCCAACCATGCTAAATGCGCA GGTAGAAATTGCCAGCGGTACTGG</td>
<td>429</td>
</tr>
<tr>
<td>ST14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>Spv**</td>
<td>GCCGTACAGGCTTTATAGA ACCTACAGGGGACACAAAC</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>S4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEFA2</td>
<td>sefA***</td>
<td>GCAGCGGTACTATTCGCGAC TACCGTGACGACATCGTG</td>
<td>310</td>
<td></td>
</tr>
<tr>
<td>SEFA4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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*.
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 fljB 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

<table>
<thead>
<tr>
<th>Isolated serotype</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. Typhimurium</td>
<td>10/247</td>
<td>4.0%</td>
</tr>
<tr>
<td>S. Enteritidis</td>
<td>6/247</td>
<td>2.4%</td>
</tr>
<tr>
<td>S. Infantis</td>
<td>2/247</td>
<td>0.8%</td>
</tr>
<tr>
<td>Total</td>
<td>18/247</td>
<td>7.2%</td>
</tr>
</tbody>
</table>

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

![Multiplex polymerase chain reaction for detection of S. Typhimurium](image1)

**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.

![Multiplex polymerase chain reaction for detection of S. Enteritidis](image2)

**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.
Prevalence and genetic characteristics of Salmonella strains in wild Mallard ducks (Anas ...)

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 spreader's 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. Enteritidis 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 (Wattieu 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 fliB 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, fliB 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 worthful 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-
Prevalence and genetic characteristics of Salmonella strains in wild Mallard ducks (Anas ...}

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.

ACKNOWLEDGEMENTS
The authors would like to thank Mrs. Behnaz Raeisian for collaboration in Salmonella isolation from faecal samples and Mrs. Nooshin Raei for collaboration in livestock and human cases with salmonellosis. This work was supported by Faculty of Veterinary Medicine in Semnan University, Semnan, Iran.

REFERENCES
Hoelzer, K., A. I. Moreno Switt & M. Wiedmann, 2011. Animal contact as a source of


Prevalence and genetic characteristics of Salmonella strains in wild Mallard ducks (Anas ...}


Paper received 04.04.2016; accepted for publication 10.06.2016

Correspondence:

Hamid Staji
Department of Pathobiology,
Faculty of Veterinary Medicine,
Semnan University, Semnan, Iran
email: hstaji@semnan.ac.ir