Archives of Razi Institute, Vol. 70, No. 2 (2015) 81-87

DOI: 10.7508/ari.2015.02.002

Original Article

Detection of *Salmonella* spp. from some wild captive herbivores in Iran and determination of serogroup, antibiotic susceptibility and presence of *invA* gene in the isolated strains

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Received 01 Jul 2014; accepted 15 Jul 2014

ABSTRACT

Salmonella spp. are zoonotic enteric bacteria able to infect humans, livestock and wildlife. The aim of this study was to investigate the prevalence of *Salmonella* (spp.) and to determine antibiotic susceptibility, serogrouping and presence of *invA* gene (*Salmonella* Invasion Gene A) in the detected strains in wild captive herbivores in Iran. The fecal samples of 103 animals from 8 different species were evaluated for presence of the *Salmonella*. Results indicated that 9.7% of animals were positive for *Salmonella*, all of these strains belonged to D serogroup, also all the *Salmonella* strains harbored *invA* gene. In vitro antibiotic activities of 10 antibiotic substances against the isolates were determined by disc diffusion test. The highest rate of resistance was against Amoxicillin (100%), Tetracycline (80%), Neomycin (60%), Lincospectin (50%) and Enrofloxacin (40%), Resistance to Furazolidone wasn't observed. In conclusion, these species can act as a reservoir for *Salmonella*. Also, since the study was conducted in some parts of Iran, a more accurate conclusion needs more distributed sampling. To our knowledge this is the first study which reports the fecal shedding of *Salmonella* from *Cervus elaphus*, *Capra aegarus*, *Oryx leucoryx*, *Ammotragus lervia* and *Lama glama* in Iran.

Keywords: *Salmonella*, wild captive herbivores, antibiogram, serogroup, *invA* gene, Iran

INTRODUCTION

Salmonella is the cause of salmonellosis both in animals and humans causing acute and chronic diarrhea and death (Oladapo *et al* 2013). The *Salmonella* genus consists of three different species, *Salmonella bongori*, *Salmonella enterica*, and *Salmonella subterranea*, which are further divided into eight different subspecies designated as subspecies I, II, IIIa, IIIb, IV, V, VI, and VII (Shelobolina *et al* 2004). Although approximately 2500 *Salmonella* serovars have been identified, most *Salmonella* infections belong to *S. enterica*, and are the cause of the two diseases called salmonellosis. Specifically, these two diseases are enteric fever (typhoid), resulting from bacterial invasion of the bloodstream, and acute gastroenteritis, resulting from a

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food borne infection/intoxication. These diseases include subspecies in the typhi and non-typhi *Salmonella* serovars, respectively (Mulvey *et al* 2006). Severe diseases caused by *Salmonella* spp. are common in humans and in livestock, companion, and zoo animals. These diseases result in significant morbidity and mortality rates and account for extensive economic losses each year (Ebani *et al* 2005). Also cause morbidity and mortality in zoo animals, which is frequently overlooked (Thomas *et al* 2001). The probable sources of *Salmonella* spp. in zoos are foods indiscriminately provided by zoo visitors and native rodents and wild small birds that gain access to the enclosures (Yokoyama *et al* 2007). Death of wild animals due to infectious diarrhea arising from *Salmonella* have been reported (Oludairo *et al* 2013, Falade *et al* 1976) with the animals expressing both symptomatic and asymptomatic infections (Palmgren *et al* 2006). This poses serious risk to staff, visitors to the zoo and the general public due to the possibility of zoonotic infection from contaminated fecal materials, environment and other objects for example cages railings etc. (Palmgren *et al* 2006). In some countries a relatively high prevalence of *Salmonella* in wildlife has been reported (Henzler *et al* 1992, Refsum *et al* 2002) and it has been documented that wildlife may contribute to the horizontal transmission of *Salmonella* (Tauni *et al* 2000). Diagnosis and isolation of *Salmonella* is very difficult in subclinical cases because of a few and or alternative fecal shedding. For laboratorial diagnosis, the amount of bacteria must be at least100 particles/gr. However, not all patients shedding *Salmonella* may be detected by bacterial culture of fecal samples (Aabo *et al* 1993). *In vitro* amplification of DNA by the PCR method is a powerful tool in microbiological diagnostics (Malorny *et al* 2003). *invA* gene have been used to determine the virulence of *Salmonella* strains, also the *invA* gene of *Salmonella* contains sequences unique to this genus and has been proved as a suitable PCR target, with potential diagnostic applications (Rahn *et al* 1992). The increased use of antimicrobial agents in animal and human medicine as a means of preventing and treating diseases is a significant factor in the emergence of antibiotic-resistant bacteria. Therefore, the antibiotic resistance developed as a result of antibiotic use in animal can be transferred to humans. Contamination of food with antibiotic-resistant bacteria can be a major threat to public health, causing community outbreaks of infectious diseases. There is also the hazard of therapeutic failure due to the increasing incidence of antimicrobial resistance among *Salmonella* species (Arslan *et al* 2010) The use of antimicrobial agents in any environment increases selective pressures that may favor the survival of antibiotic-resistant strains (Arslan *et al* 2010, Tambekar *et al* 2005). The aim of this study was to investigate the presence of *Salmonella* in wild captive herbivores fecal samples in Iran and Serogrouping and to determine antibiotic susceptibility patterns of the isolates.

MATERIALS AND METHODS

Sample collection and Isolation and identification of *Salmonella*. Fresh Fecal samples from 103 wild captive herbivores from 8 different species were collected (Table 1). The samples were transported on Amies media (Merck,Germany) to the laboratory and then cultured on Selenit F broth (Merck, Germany). After overnight incubation at 42 ^{o}C, each sample cultured on *Salmonella-Shigella* Agar (SSA) separately, plates were incubated at 37 for 24 h. Presumptive *Salmonella* isolates were confirmed using conventional biochemical tests [triple sugar iron (TSI), urease test, MR-VP, Indole product and citrate utilization test] (Quinn *et al* 1994) and serological agglutination (Baharafshan, Iran).

DNA extraction and PCR amplification for detection of invA gene. After confirming the isolates as *Salmonella* by biochemical tests, the isolates were sub-cultured on LB Agar, a single colony of each isolate on agar plate was picked and suspended in 200 µl of distilled water. After vortexing, the suspension was boiled for 5 min, and 50 µl of the supernatant was collected after centrifuging for 10 min at14000 rpm

(Zahraei *et al* 2012). All the strains which were Confirmed as *Salmonella* were screened by PCR using specific primers for *invA gene* (*Salmonella* Invasion Gene A) (S139: 5´GTGAAATTATCGCCACGTT CGGGCAA 3´and S141: 5´TCATCGCACCGTCAAA GGAACC 3´) as described by Rahn et al. (1992). The PCR method was conducted using 2.5 µl 10X PCR buffer, 1 µl MgCl2 (50 mM), 0.5 µl dNTP (10 mM), 0.2 µl *Taq* DNA polymerase (5u/µl), 3 µl DNA, 0.5 µl (10 µM stock) of each primers and 16.8 µl water. The thermal cycle included three steps as followed. Primary denaturation was performed at 94 °C for two minutes as the first step. In the second step, 35 cycles each included three sections as denaturation at 94 °C for 60 seconds, annealing at 64 °C for 30 seconds and extension at 72 °C for 30 seconds were performed. Eventually, final extension was conducted at 72 °C for 5 minutes as the third step. *S. typhimurium* (ATCC 14028) was used as positive control. To detect whether the specific 284 bp amplicon of *invA* gene exists, the PCR product was electrophoresed for 60 minutes on 1.2% agarose gel.

Antibacterial Susceptibility. Antibacterial susceptibility of the *Salmonella* strains was determined on Mueller-Hinton agar (Merk, Germany) by Kirby–Bauer method according to CLSI protocol (CLSI 2008). Ten antibacterial discs were used including Enrofloxacin, Furazolidone, Neomycin, Amoxicillin, Lincospectin, Tetracycline, Gentamicin, Ceftriaxone, Florfenicol and Trimethoprim- Sulfamethoxazole (Padtan Teb, Iran).

RESULTS

A total of 103 fecal samples from 103 wild captive herbivores belonging to 8 different species were investigated for the presence of the *Salmonella* spp. by Culturing and biochemical tests. The strains which were recognized as *Salmonella* spp. were objected to PCR test for presence of *invA* gene. The results showed that ten animals (9.7%) belonged to five species were carried *Salmonella* strains, also all of these strains were positive for *invA* gene. (Figure 1, Table 1). The results of serological agglutination for serogrouping indicated that these isolates belonged to D serogroup. All of the isolates were resistant to Amoxicillin; while, resistance to Lincospectin, Tetracycline, Enrofloxacin and Neomycin was observed in 50, 80, 40 and 60% of the isolates, respectively. The most effective antibacterials were Furazolidone, Trimethoprim-Sulfamethoxazole, Ceftiaxone and Gentamicin with the susceptibility rates of 100, 80 and 90, 70%, respectively. Noticeably, intermediate susceptibility to Florfenicol and Enrofloxacin was observed in majority of the isolates. All strains showed multiple resistance (Table 2).

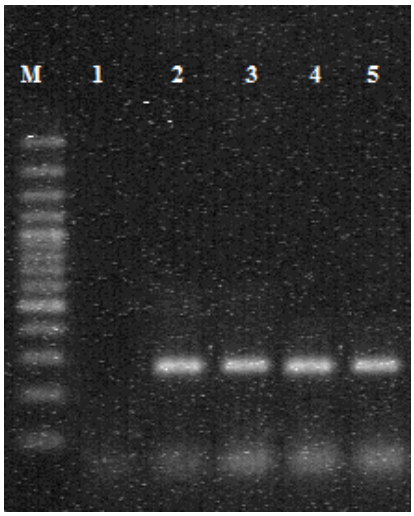

Figure 1. PCR results for detection of *invA* gene. M) Marker 100bp. 1) Control negative. 2). *invA* (284 bp) *S. typhimurium* (ATCC 14028) (control positive). 3,4 and 5) *invA* positive strains (284 bp).

DISCUSSION

Salmonella could cause infection in human beings if the bacteria are contracted either from the wildlife directly or contaminated objects around them. This is especially important in animal contact settings where contacts are made with wildlife and objects around them (Oludairo *et al* 2013). In this study, *Salmonella* were detected from 9.7% of animals. Many studies have been conducted on the presence of *Salmonella* in wild animals and some studies indicated wild animals can act as reservoirs of the infection in human livestock while in (Chiari *et al* 2013, Silva-Hidalgo *et al* 2012,

Table 1. Isolation of *Salmonella* from fresh fecal swabs of various species

Family	Common Name	*Nomenclature*	No. of samples tested	No. of positive samples	No. of positive samples for presence of *invA* gene	Location
Equidae	Onager	*Equus hemionus*	5	-	-	Tehran Eram zoo
Cervidae	Red deer	*Cervus elaphus*	14	1	1	Mashhad Vakil abad zoo
			17	1	1	Tehran Eram zoo
	Persian fallow deer	*Dama dama*	19	-	-	Tehran Eram zoo
			9	-	-	Karhkeh national park
			10	-	-	Mashhad Vakil abad zoo
Bovidae	Wild goat	*Capra aegarus*	9	4	4	Mashhad Vakil abad zoo
	Arabian oryx	*Oryx leucoryx*	6	1	1	Mashhad Vakil abad zoo
	Barbary sheep (aoudad)	*Ammotragus lervia*	8	2	2	Mashhad Vakil abad zoo
Camelidae	Llama	*Lama glama*	4	1	1	Mashhad Vakil abad zoo
	Bactrian camel	*Camelus bactrianus*	2	-	-	Mashhad Vakil abad zoo
Total	-	-	103	10	10	-

Table 2. Antibacterial susceptibility of ten *Salmonella* isolates in this study

Antibacterial agents	Resistant	Intermediate	Susceptible
Enrofloxacin	40%	50%	10%
Furazolidone	-	-	100%
Neomycin	60%	40%	-
Amoxicillin	100%	-	-
Lincospectin	50%	40%	10%
Tetracycline	80%	10%	10%
Gentamicin	10%	20%	70%
Ceftriaxone	10%	-	90%
Florfenicol	-	70%	30%
Trimethoprim- Sulfamethoxazole	10%	10%	80%

Arshad *et al* 2006). Our finding shows that all the isolates belonged to *Salmonella* serogroup D. This result could be due to high prevalence of *S. enteritidis* (belonging to serogroup D) in livestock in Iran (Madadgar *et al* 2008). While in Arshad et al. (2006), Silva-Hidalgo et al. (2012) and Chiari et al. (2013) studies the most dominant *Salmonella* serogroups isolated from wild animals were D, B and C1 Respectively. *Salmonella* may possess *invA* gene which is responsible for invasion of host cells and virulence. This gene encodes a protein in the inner membrane of bacteria which is responsible for invasion to the epithelial cells of the host (Darwin *et al* 1999).The gene may not also always be present in all *Salmonella* spp. While it may be possible to state that the species that showed the *invA* band may be virulent, penetrate and cause infection in host cells. The detection of the gene in the *Salmonella* isolated implies the organisms are virulent and will be able to penetrate host epithelia cells, causing infection. In the present study we used

S139 andS141 primers for detection of *invA* gene All he *Salmonella* strains were positive for *invA* gene. Absence of the gene in the confirmed *Salmonella* isolates can lead to lack of invasiveness by those isolates (Bacci *et al* 2006). This implies that the isolate that do not carry the gene may not be able to invade epithelial cells and may not be virulent. This finding is at variance with the reports of Oliveira et al. (2003), Zahreai et al. (2006), Jamshidi et al. (2008) and Amini et al. (2010), who all detected and reported *invA* gene in all *Salmonella* isolates they tested. The implication of the presence of *invA* gene in the isolates is that the organisms are actually able to cause infection in wildlife from which they were isolated, especially if host immunity is suppressed. Antibiotic usage is possibly the most important factor that promotes the emergence, selection and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine (Neu 1992, Witte 1998). In this study antibiotic susceptibility of the *Salmonella* strains were determined, multiple resistances were observed in all of the isolates. Some studies also indicates that multiple antibiotic-resistant strains of *Salmonella* were isolated from wild and domestic animals (Shetty *et al* 2012, Murugkar *et al* 2008). Our results indicated that the most effective antibiotic was Furazolidone, while in Shetty et al. (2012) and Murugkar et al. (2008) studies the most effective antibacterial drugs were Ceftriaxon and Ciprofloxacin respectively. This difference could be due to the variety of antibacterial drugs which were used in different area. There is little information available regarding for *Salmonella* infection wild captive animals in Iran. Due to the presence of *Salmonella* infection in wild herbivores, the results may indicate the possibility that wild captive herbivores have an important role for carriage of *Salmonella* strains. On the other hand, continuous monitoring of wild captive animals for early treatment of *Salmonella* infection and prevention of transmission to other animals and humans is necessary. And according to various antibiotic resistant *Salmonella*, antibiogram seems essential for effective treatment. To conclude more precisely about the excretion status of *Salmonella* strains in wild captive herbivores of Iran, a more perfect study with more samples in different parts of Iran should be conducted.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted review article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

This study was financially supported by the Department of Microbiology Faculty of Veterinary Medicine, University of Tehran, Tehran – Iran, with the grant No: 7504002/6/15.

Acknowledgments

Authors would like to express their gratitude to everyone who contributed to this research, in particular, technical assistance of Dr Ali Rasooli (Pasteur veterinary laboratory Neyshbour, Iran) and Mr. Asadi.

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