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Drug resistance and virulence-associated genes screening in *Salmonella enterica* isolated from Caspian pony, Iran

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Abstract

The most serious problem in public health is salmonellosis, a common disease in horse. The aim of this study was to investigate the shedding of *Salmonella* serotypes in healthy Caspian pony. We examined 143 pony's fecal samples collected from the north of Iran belonging to different ages and sexes. Samples were cultured, then identification of isolates were performed by common bacteriological methods and polymerase chain reaction (PCR). The PCR was also used to explore the presence of *fimA* and *salmonella secreted effector L (SseL)* genes as virulence factors in the isolates and all were assigned to antibiotic susceptibility test via disc diffusion method. Results showed two fecal samples (1.39%) contaminated with *Salmonella* and further examination demonstrated the isolates belonging to *S. enterica* serotype *typhimurium*. Both serotypes were isolated from female and <6 years of age group of ponies and we detected *fimA* and *SseL* genes in the isolates. Observing multiple drug resistance and virulence genes in isolates is of utmost importance from both clinical and public health perspectives. It is highly likely that we face instances of salmonellosis in animals or humans that lead to severe infections and fail to respond to treatment in future. This study revealed that the occurrence of *Salmonella* was low in ponies, however, regarding the presence of virulence factors with multidrug resistant trend in this zoonotic bacterium, establishment of good hygienic measurement to prevent the transmission of bacteria between animal and human is necessary.

Introduction

As a serious infectious disease of horse, salmonellosis is caused by various serotypes of *Salmonella enterica*. Fatal septicemia and severe diarrhea in foals as well as colitis in horses of all ages are important clinical manifestations of salmonellosis in equids.[1] Although more than 2,500 serotypes of *S. enterica* have been identified so far, only a few serotypes have been isolated from horses with clinical symptoms including *S. typhimurium*, *S. anatum*, *S. newport*, *S. Agona*, *S. heidelberg* and *S. ohio.*[2,3]

Currently, *Salmonella* infection is among the most significant health problems affecting humans and animals worldwide. The majority of the public health issues and economic losses stem from infected or diseased animals. These economic losses include abortion, treatment expenses, reduced feeding efficiency and death among others. *Salmonella* can be shed through the feces of sick animals. Even animals that have recovered or are carriers whether permanently or temporarily pose a significant danger due to the potential for shedding *Salmonella* through their feces. This increases the likelihood of long-term spread of infection in susceptible hosts. The potential for bacterial zoonosis is also a serious concern for public health.[4] Many risk factors have been identified in relation to *Salmonella* shedding through horse feces with excessive use of antibiotics particularly oxytetracycline and transportation stress being among the most important.

New studies show that the use of antibiotics to treat salmonellosis in horses causes the bacteria to remain in the intestine after the animal recovers. Antibiotics do not completely eradicate all *Salmonella* bacteria. In addition, they disrupt the normal flora of the intestines. Normal flora helps eliminate pathogens by competing with *Salmonella* for food in healthy animals. New studies have proven that the increase in drug resistance in *Salmonella* leads to delays in effectively treating the disease in both humans and animals. Therefore, it is helpful to determine the serotypes of *Salmonella* and conduct antimicrobial susceptibility tests to trace the origin of the infection.[5]

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The distribution of most *Salmonella* virulence genes is over the chromosome and clustered in pathogenicity islands and others are located on the plasmid.[6] In *Salmonella* serotypes a type1 fimbriae is expressed that is sensitive to mannose-receptor and play a role in attachment of bacteria to eukaryotic cells. Adhesion plays a crucial role in the successful colonization and pathogenesis of *Salmonella* serotypes.[7]

Eukaryotic cells use an essential process called Ubiquitination that regulate proteins degradation, localization and function. Intracellular pathogens including *Salmonella* can manipulate the ubiquitin system by releasing numerous effectors to the cell cytosol that impede the host cell Ubiquitination.[8,9] *Salmonella secreted effector L* (*SseL*) is one of these effectors that is needed for full virulence within infection of host. The *SseL* seems to be specific to some serotypes of *Salmonella*, indicating that its function could be vital to exclusive features of *Salmonella* virulence.[10] With its deubiquitinase activity, *SseL* prevents the destruction of *Salmonella* inside the cell and aids in the bacteria's multiplication within the cell.[11] Additionally, Geng *et al.* reported that the *SseL* protein leds to the destruction of macrophages and hampered the innate immune responses of the host animal.[9]

Nowadays, multidrug-resistant *Salmonella* isolates from horses have been frequently reported, especially among those serotypes that are associated with clinical disease.[3,12] Although, there are many reports on the emergence of antibiotic resistance to *Salmonella* in public health, little is known about drugs resistance in *Salmonella* isolates in ponies. The antibiotics in veterinary practice could contribute to the development of resistance of drug in *Salmonella*. Unprincipled consumption of antimicrobial agents, therefore, may increase zoonotic transmission of multidrug-resistance *Salmonella* and making animals susceptible to infection.[13] Cummings *et al.* conducted a review of drug resistance in *Salmonella* isolates from horses in the United States between 2001 and 2013.[3] The researchers discovered that the prevalence of drug resistance varied among different isolates ranging from zero for imipenem to 51.05% for chloramphenicol. Another study investigated drug resistance in *Salmonella* isolates from racing horses in Thailand in 2019 and found that the highest level of resistance was observed against streptomycin.[14] Similarly, Vaez *et al.* examined the drug resistance profile of *Salmonella* isolates from various livestock in Iran.[15] Their findings revealed that the isolates displayed the highest resistance against nalidixic acid,tetracycline and streptomycin.

Ponies are suitable for children to ride and sport, however, they can quickly become infected with *Salmonella* during the fecal shedding of multidrug-resistant *Salmonella* by direct contact with the feces of infected ponies. The purpose of the present investigation was to evaluate the profile of antibiotic resistance and virulence associated genes in *S. enterica* isolates from Caspian horse (pony) in Iran.

Materials and Methods

Area study and fecal sampling. Considering the distribution of Caspian pony, seven farms selected for sampling from three provinces from north of Iran including Gilan, Mazandaran, and Golestan (Fig. 1). Number of animals population in all facilities was < 30, therefore, all apparently healthy animals were sampled (Table 1). Using sterile disposable gloves (Faradamkala, Tehran, Iran), around 10.00 g fecal samples were obtained through the rectum. Samples were immediately added into the sterile stool bottle (Rapidtest, Tehran, Iran) and moved to the laboratory on ice. A total number of 143 pony fecal samples were collected from breeding and riding centers. The sampled ponies were further classified into male, female and four age groups: < 6, 6 - 12, 12 - 24 and > 24 months.

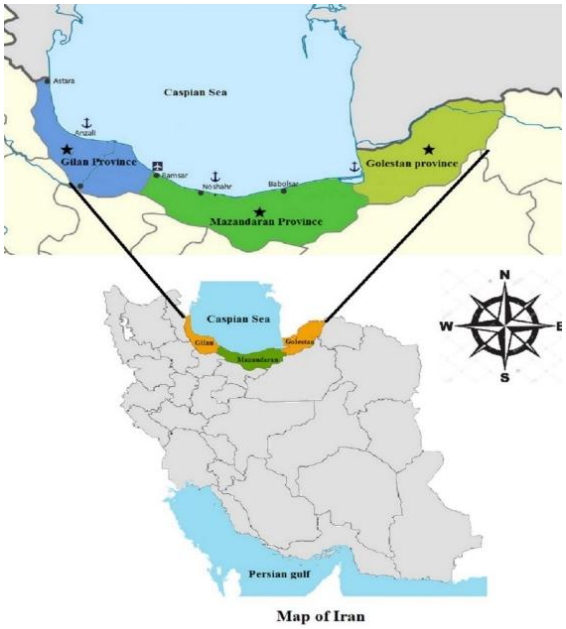

Fig. 1. The studied area (highlighted) and its location regarding Iran map.

Table 1. *Salmonella* isolates in pony fecal samples in different provinces.

Provinces	No. of farms sampled	Average No. of pony in province	No. of ponies sampled	No. of pony positive for *Salmonella* (%)
Gilan	4	84	78	2 (1.39)
Mazandaran	2	51	51	0 (0)
Golestan	1	14	14	0 (0)
Total	7	149	143	1.39

***Salmonella* isolation.** All fecal samples were suspended separately in 50.00 mL F-Selenite broth medium (Merck, Darmstadt, Germany) followed by incubation at 37.00 ˚C for 18 hr. Following enrichment, a loop of inoculum from the F-Selenite was streaked onto *Salmonella* and *Shigella* agar (SS agar; Merck) and Xylose Lysine Deoxycholate agar (XLD agar; Merck) plates followed by incubation at 37.00 ˚C for 24 hr.[16,17] The presumptive *Salmonella* colonies (transparent colonies with black center on SS agar and red colonies with black center on XLD agar) were picked up and identified through performing tests for Gram staining (negative bacilli), motility (+), utilization of citrate (+), production of indole (–), methyl red (+), Voges-Proskauer (–), reduction of nitrate (+), fermentation of Glucose (+), lactose (–) and Sucrose (–).[18,19] Using standard tube agglutination test via *Salmonella* O and H antigens, isolates were serotyped (Razi Institute, Karaj, Iran).

Molecular confirmation of *S. enterica* using polymerase chain reaction (PCR). Following conventional bacteriological tests for detection of *S. enterica* in pony fecal samples, a 660 bp fragment of *16S rRNA* was amplified via conventional PCR using a set of primers, forward: *5'* GGAACTGAGACACGGTCCAG *3'* and reverse: *5'* CCAGGTAAGGTTCTTCGCGT *3'*.[20] Using a commercial bacterial genomic DNA extraction kit, template DNA was extracted (Favorgen, Taipei, Taiwan) from a 1.00 mL aliquot of selective enrichment of *S. enterica* isolates in tryptic soy broth medium (Merck). The DNA extraction was performed according to the kit instruction and the extracted DNA was kept at – 80.00 ˚C until use. The PCR reaction volume was 25.00 µL containing 4.00 µL (85.00 ng) of genomic DNA, 12.50 µL of 2.00 X Master Mix (Pishgam, Tehran, Iran), 0.50 µL (10.00 pmol) of each forward and reverse primer and 7.50 µL of diethyl pyrocarbonate (DEPC) water. Corbett Thermo-cycler (Corbett, Sydney, Australia) was set for 5 min at 95.00 ˚C for initial denaturation, 28 cycles each 1 min 94.00 ˚C for denaturation, 1 min at 55.00 ˚C for annealing and 1 min at 72.00 ˚C for extension and final extension at 72.00 ˚C for 10 min. The PCR product was run on 2.00% agarose along with a DNA ladder (50 bp). Purified PCR products were sequenced (Sanger sequencing method) by Macrogen, Seoul, South Korea. The DNA sequences were analyzed by the NCBI-BLAST program.

Detection of virulence-associated genes in *S. enterica*. The presence of *fimH* and *SseL* genes were screened in *S. enterica* using specific primers (Table 2). To amplify *fim*A and *SseL* genes, a single PCR cycling condition was used separately. The final volume of PCR reaction system was 25.00 µL that contained 12.50 µL of 2.00 X Master Mix (Pishgam), 6.50 µL DEPC water, 4.00 µL DNA template and 1.00 µL of each primer for both genes separately. The PCR reaction was set for initial denaturation at 94.00 ˚C for 10 min, 28 cycles of 94.00 ˚C for 1 min, 55.00 ˚C for 1 min, 72.00 ˚C for 1 min and final extension at 72.00 ˚C for 10 min.[21]

Antimicrobial susceptibly test. Antimicrobial susceptibly was conducted using disk diffusion method and an overnight culture of each bacterial isolates (1.50×10^8 CFU mL^{-1}) streaked separately on Muller Hinton agar plates (Merck). Antibiotic disks were used as per manufacture instructions (Padtan Teb, Tehran, Iran) containing neomycin 30.00 µg, nitrofurantoin 300 µg, ciprofloxacin 5.00 µg, sulfamethoxazol 15.00 µg, oxacillin 1.00 µg, streptomycin 10.00 µg, tetracycline 30.00 µg, chloramphenicol 30.00 µg, cefazolin 30.00 µg, cephalexin 30.00 µg, ceftazidime 30.00 µg, and doxycycline 30.00 µg. The zone of inhibition was measured via digital caliper (Insize, Derio, Spain) in millimeter and findings were evaluated based on Clinical and Laboratory Standards Institute (CLSI) instructions.[22]

Statistical analysis. The data collected were analyzed using ANOVA and Tukey's HSD test in SPSS software (version 24.0; IBM Corp., Armonk, USA), with statistical significance determined at $p < 0.05$.

Results

Detection of *S. enterica* in fecal samples. Two *S. enterica* belonging to *typhimurium* serovars were isolated from the 143 pony fecal samples (1.39%). The isolates were detected in female ponies with age < 6 month in Gilan province and out of the 143 ponies, 141 (98.61%) did not shed *Salmonella* in feces. *Salmonella* isolation from feces of female pony foal was significantly ($p < 0.05$) higher than in male pony (Table 2). Also, *Salmonella* prevalence in pony with age < 6 months was significantly ($p < 0.05$) higher than in other age groups (Table 3). Molecular analysis showed specific amplicon of 671 bp in two isolates fecal samples (Fig. 2). The op394072 was filed under accession number of *16S rRNA* gene in *S. enterica* that is available in gene bank (ncbi.gov.com).

Presence of virulence-associated genes in isolated *Salmonella*. Both virulence-associated genes (*fimA* and *SseL*) were detected in isolated *S. enterica* serovar *typhimurium*. Amplification of *fimA* gene showed a fragment of 85 bp (Fig. 3A) and also a fragment of 637 bp for *SseL* gene (Fig. 3B).

Table 2. The primers used for amplification of *fimA* and *salmonella secreted effector L* (*SseL)* genes in isolated *Salmonella* from Caspian pony fecal samples.

Genes	Sequence 5-3	Product size (bp)
fimA	F: CGTCGTCATAAAAGGAAAAA R: GAACAAAACACAACCAATAGC F: CCTTTCTCCATCGTCCTGAA R: TGGTGTTATCTGCCTGACCA	85
SseL	F:GTTCAGCGACAACCGACCTTTCTA R: CCGGGTTTGGGTGTTAAATAGTGC	637

Table 3. Number of ponies sampled for shedding of *Salmonella* in feces and identified fecal positive specimens (in parentheses).

Provinces	Sex	Age (months)				Total
		< 6	6 - 12	12 - 24	> 24	
Gilan	Male	8 (0)	8 (0)	3 (0)	11 (0)	30 (0)
	Female	10 (2)	7 (0)	8 (0)	23 (0)	48 (0)
	Total	18 (2)	15 (0)	11 (0)	34 (0)	78 (0)
Mazandaran	Male	4 (0)	5 (0)	6 (0)	7 (0)	22 (0)
	Female	7 (0)	4 (0)	7 (0)	11 (0)	29 (0)
	Total	11 (0)	9 (0)	13 (0)	18 (0)	51 (0)
Golestan	Male	1 (0)	0 (0)	0 (0)	3 (0)	8 (0)
	Female	3 (0)	2 (0)	3 (0)	2 (0)	6 (0)
	Total	4 (0)	2 (0)	3 (0)	5 (0)	14 (0)

Antimicrobial sensitivity. Two isolates were resistant to oxacillin, streptomycin, tetracycline, chloramphenicol, cefazolin, cephalexin, ceftazidime and doxycycline. They are moderately resistant against neomycin and ceftriaxone, however, sensitive to nitrofurantoin, ciprofloxacin and trimethoprim. This finding showed that both isolates were pathogenic enough to put both animals and humans at the risk of infection or disease. Considering the sensitivity of these isolates to the mentioned antibiotics, their use could be effective in treating infected animals.

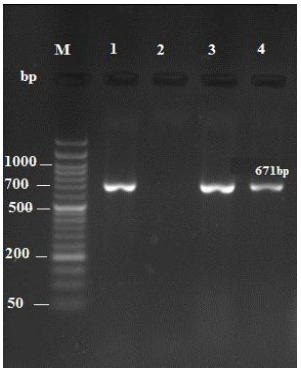

Fig. 2. Agarose gel image of amplified fragment of *16S rRNA* of *Salmonella enterica* (671 bp); M: 50-bp DNA ladder. Lane 1: Positive control, lane 2: Negative control, and lanes 3 - 4: Positive samples for *16S rRNA*.

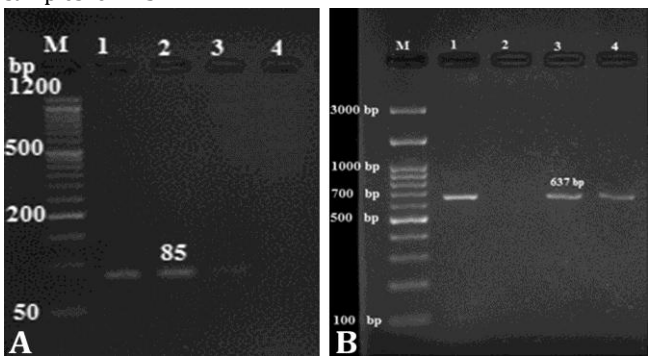

Fig. 3. Agarose gel image of amplified fragment of **A)** *fimA* gene (85 bp). M: 50-bp DNA ladder, lanes 1 - 2: Positive samples, lane 3: Positive control and lane 4: Negative control for *fimA*, and **B)** *SseL* gene (637 bp). M: 50-bp DNA ladder, lane 1: Positive control, lane 2: Negative control, and lane: 3 - 4: Positive samples for *salmonella secreted effector L (SseL).*

Discussion

There is no accurate information on *Salmonella* infection in Caspian pony, thus, understanding the epidemiology of salmonellosis and control of its infection seems necessary. Salmonellosis is a zoonotic disease and could modulate the prevalence of infection in other animals and human. Isolation of *S. typhimurium* from 1.39% fecal samples of Caspian pony was in agreement with results of others researcher that reported 1.00% to 20.00% equine fecal positive for *Salmonella*.[23,24]

Inconsistency in the prevalence of *Salmonella* in equids could be due to variation in climate circumstances, husbandry management in different countries and type of animals (healthy or sick) sampled in various studies. In our study the other reason for differences might be low-population and distribution limitation of Caspian pony in three provinces with the same climate situation.

Up to the last three decades, serovar *S. typhimurium* was the rare serovar isolated from horse feces.[25] There is no available information on *Salmonella* serotypes that were likely predominant in Caspian pony feces in the past, and isolation of *S. typhimurium* from the feces of the animals examined in this study supported the hypothesis that there has been a shift in the pattern of the change of *Salmonella* serotypes pattern over time. This was an important point that should be taken into consideration. Moreover, studies conducted in horse feces in other regions of the world support the notion that serotype changes can occur. Transmission of *Salmonella* infection between Caspian pony and humans might be another possible reason of change in patterns of serovar occurrence in equids, however, further investigations are required. However, given the close relationship between humans, particularly children, and Caspian ponies, it is not surprising that *Salmonella* serotypes can be transmitted between humans and animals. In this regard, we suggested that the common serotype of *Salmonella* found in riders, owners or any one whom comes into contact with Caspian ponies should be checked.

Isolation of *Salmonella* from 1.39% healthy ponies demonstrated the prevalence of salmonellosis in Caspian pony in Iran, also Zahraei Salehi *et al.* isolated *S. enterica* serovar *typhimurium* from two pregnant ponies aborted and four cases died because of acute septicemia.[2] Our study showed that two female ponies with age < 6 months were excreting *Salmonella*. The reason for the sex for the shedding was not clear and it might be due to the large number of female ponies examined.

We investigated the presence of *fimA* and *SseL* genes as virulence-associated genes in isolated *S. Typhimurium* from Caspian pony. As shown in Figures 2 and 3, in all isolates of *Salmonella* PCR amplified fragments of 85 bp and 637 bp were related to *fimA* and *Ssel* genes, respectively. The *fimA* gene has been implicated in *Salmonella* pathogenicity and

encodes the major structural subunit of type I fimbrial protein.[26] Coombes *et al.* demonstrated that *SseL* virulence gene was specific for some serotype of *Salmonella* like *typhimurium*.[8] The *SseL*, a virulence determinant, is translocated into host cells throughout intracellular infection and is needed for full virulence during animal infections.[27-29] The existence of both virulence-associated genes in isolated *S. typhimurium* from Caspian pony can be a potential risk factor for infection. Hence, considering the close contact between human and ponies, there is a possibility of transmission of virulent serotypes of *Salmonella*. In line with this, an outbreak of salmonellosis (Typhoid fever) among the people who had contact with ponies was reported in Leipzig of Germany in June 2004.[30] The presence of these two virulence factors in *Salmonella* isolates from Caspian Ponies could be correlated with the severity of the disease. The preliminary sampling data also confirmed that both animals exhibited symptoms such as fever, lethargy, weakness, watery diarrhea and anorexia at the time of sampling. Based on these findings, it can be predicted that Caspian Ponies are at risk of contracting acute and multidrug resistant strains. So, it is necessary to consider *Salmonella* infection in this animal as a significant health problem.

Tracking the antimicrobial susceptibility resistance profile among *Salmonella* isolates from animals is useful for antimicrobial usage in clinical practice and evaluating the potential risk to public health. Our results showed isolated *Salmonella* were resistant against a lot of antibiotics that we tested. These antibiotics are wildly used for treating infectious disease of horse in Iran. All *Salmonella* isolates obtained from ponies in current study were sensitive to nitrofurantoin, ciprofloxacin and trimethoprim. These finding supported recommending these antibiotics as the safest choice for treatment of salmonellosis in Caspian pony. Zahraei Salehi *et al.*[2] isolated *S. typhimurium* from Caspian pony and showed isolates were resistant against cephalexin, oxytetracycline and streptomycin, however, were sensitive to oxytetracycline, trimethoprim, lincospectin, enrofloxacin, nalidixic acid, ampicillin, chloramphenicol, kanamycin and ceftiofur. Our results showed that during the past decade antibiotic resistance trend in isolated *Salmonella* from Caspian pony was distinctly changed and resistant against many drugs. In agreement of our study, Soza-Ossandon *et al.* reported *S. typhimurium* was a highly dominant serotype that was responsible for salmonellosis in equine hospitals.[24] Also, they showed most of isolates were resistant to amoxicillin, ampicillin, ciprofloxacin, chloramphenicol, streptomycin, gentamicin, trimethoprim/sulfamethoxazole and Tetracycline. In another study, Leon *et al.*[5] documented that *S. enterica* serotype Newport was the most abundant isolate in equine hospital in USA. These researchers proved the presence of antibiotics resistant genes in *S. Newport* for gentamicin, streptomycin, sulfonamides, trimethoprim, phenicols, tetracyclines and macrolides.

Although prevention is challenging due to the presence of bacteria in the environment and the feces of apparently healthy animals, it is recommended to promptly isolate and identify *Salmonella* when ponies are experiencing stress or malnutrition. One preventive measure is to separate sick animals from healthy ones. It is essential for owners to have sufficient knowledge about the zoonotic nature of *S. enterica*. Individuals who come into contact with ponies, especially children, should adhere to hygiene practices such as regular hand washing.

In conclusion, the dominant serotype of *Salmonella* in Caspian pony from the north part of Iran was *typhimurium*. Although the presence of two virulence-associated genes was investigated in the isolates and considering that there were many virulence genes in *Salmonella*, public hygiene is essential to prevent the transmission of bacteria. Based on our antibiotic susceptibility test, the drug resistant trends is changed among *Salmonella* serotype and everyday appear multi-drug resistant strains of this bacterium. Thus, antimicrobial resistance of *Salmonella* serovars remains to be an important challenge to control *Salmonella* infection worldwide.

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Conflict of interest

The authors declare no potential conflict of interest.

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