Molecular characterization a *Salmonella Typhimurium* isolate from Caspian pony

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Abstract
Typhoid disease or salmonellosis is a common sickness in horses. In several epidemiological studies in hospitalized horses, several serotypes of *Salmonella* often are predominant in nosocomial infections. Transportation, overcrowding, dehydration, oral antimicrobial therapy and infections are the risk factors which may activate latent or subclinical salmonellosis. In this study, the occurrence of typhoid due to *Salmonella* serogroup B was considered in a Caspian ponies flock kept in a husbandry center of ponies around Tehran. During transportation of 19 ponies, two pregnant ponies aborted and four cases died because of acute septicemia. Pathological and bacteriological follow up showed salmonellosis. A multiplex polymerase chain reaction (m-PCR) assay was used for detection and identification of *Salmonella* to confirm pathological and bacteriological studies. *Salmonella* Typhimurium was isolated from bone marrow, mesenteric lymph nodes, liver and intestinal contents of died pony. *Salmonella* was not isolated from stools of other ponies. Pulsed Field Gel Electrophoresis (PFGE) and antibiotic susceptibility test were also performed. PFGE pattern was similar to the other collected isolates which have existed since more than 30 years ago in Iran. Because of importance of salmonellosis in ponies, Using of rapid methods are recommended to confirm the presence of *Salmonella*. Results showed that m-PCR permit to evaluate samples more rapidly than other methods and also can detect multiple genes simultaneously like virulence factors which declare virulence of the isolates and have surveillance significance.

Keywords**:** Caspian Pony; *Salmonella* Typhimurium; Transportation; Multiplex PCR; PFGE

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INTRODUCTION

In several epidemiological studies in hospitalized horses, several *Salmonella* serotypes such as *Salmonella Typhimurium*, S. *Newport*, S. *Anatum*, S. *Agona*, Heidelberg and *S. Ohio* have been commonly isolated and one or two serotypes often are predominant in nosocomial infections. *Salmonella enterica* serovar Typhimurium is the most frequently isolated serovar worldwide and is considered as one of the most virulent serotypes, affecting horses of all ages. (Gay, 1995; Walker *et al*., 1995; Mainar-Jaime *et al*., 1998; Ernst *et al*., 2004). Typhoid disease or salmonellosis is a common disease of hospitalized horses. The spectrum of disease associated with *Salmonella* infections ranges from fecal shedding without clinical signs to septic shock and death. Many of the risk factors for salmonellosis in horses have been elucidated through descriptive studies of outbreaks on breeding farms and in hospitalized horses. In addition, a limited number of case-control studies have compared affected horses with unaffected horses to investigate potential risk factors. Stress factors that have been associated with salmonellosis in horses include transportation, antimicro-

bial administration, intestinal surgery, changes in diet, parturition, anesthesia and anthelmintic treatment (House *et al*., 1999).

In addition, dosage of bacteria and virulence are important factors in incidence of disease. Diagnosis and isolation of bacteria 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. Nowadays, advanced PCR based on oligonucleotide primers called m-PCR has been developed. This technique is more quickly and sensitive than bacterial culture (Aabo *et al*., 1993). The aim of this study was molecular characterization of *Salmonella enterica* serovar Typhimurium isolated from Caspian ponies.

MATERIALS AND METHODS

Samples: In the present study, during transportation of 19 Caspian ponies, two pregnant ponies aborted and four cases died because of acute septicemia. Samples were obtained in the pathology laboratory of veterinary faculty and included intestinal content, mesenteric lymph node, liver and bone marrow which were collected from aborted and died Caspian ponies and feces from other live ponies. Samples freshly were placed into plastic bag with ice pack and quickly (less than 2 h) transported to the Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran. Samples were inoculated into selenite-cystein broth (Merck, Darmstadt, Germany) for overnight enrichment at 37ºC, and then plated on MacConkey agar (Merck) for primary selection. Presumptive *Salmonella* isolates were confirmed using conventional biochemical tests [triple sugar iron (TSI), urease test, MR-VP, Indole product and citrate utilization test] and serological agglutination (Bacto-*Salmonella* O and H antisera; DifcoTM; Becton Dickinson and Company, Franklin Lakes, MI, USA).

Oligonucleotide primers: For m-PCR assay, four primer sets were selected. ST139 and ST141, specific for *Salmonella* genus (Rahn *et al*., 1992) and the RfbJ, FliC and FljB, specific for the *rfb*J, *fli*C and *flj*B genes of *Salmonella* Typhimurium or other *Salmonella* serovars with similar antigenic properties (Lim *et al*., 2003). ST139 and ST141 primers are specific to detect *Inv*A gene (one of Salmonella virulence genes which indicate to "invasion"). Also RfbJ, FliC and FljB primers are specific to detect O:4 (one of *Salmonella* LPS genes), H:i and H:1,2 (two of *Salmonella* flagella genes which indicate to motility), respectively. Primer sets, inv-A, RfbJ, FljB and FliC chosen, have been successful to detect *Salmonella* Typhimurium (Zahraei Salehi *et al*., 2007). The primers sequences and their corresponding genes are shown in Table 1.

DNA extraction and amplification: 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 at 14,000 rpm. Polymerase chain reaction was performed with 10 µl of DNA sample, 5 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.5, 1 µM of each primer, 200 µM dNTPs (Fermentas, Latvia) and 1 U of Taq DNA polymerase (Fermentas, Latvia) in a final vol-

Table 1. Primers sets used for detection of Salmonella serovars and identification of *Salmonella Typhimurium*.

Primers	Target gene	Amplicon (bp)	Reference
ST139-s: 5'-GTGAAATTATCGCCACGTTCGGGCAA-3' ST141-as: 5'-TCATCGCACCGTCAAAGGGAACC-3'	*inv*A	284	Rahn *et al*., 1992
Rfbj-s: 5'-CCAGCACCAGTTCCAACTTGATAC-3' Rfbj-as: 5'-GGCTTCCGGCTTTATTGGTAAGCA-3'	*rfb*J	663	Lim *et al*., 2003
Flic-s: 5'-ATAGCCATCTTTACCAGTTCCCCC-3' Flic-as: 5'-GCTGCAACTGTTACAGGATATGCC-3'	*fli*C	183	Lim *et al*., 2003
Fljb-s: 5'-ACGAATGGTACGGCTTCTGTAACC-3' Fljb-as: 5'-TACCGTCGATAGTAACGACTTCGG-3'	*flj*B	526	Lim *et al*., 2003

ume of 25 µl. Amplifications were performed in a DNA thermocycler (Techne, TC-512, Cambridge, UK). The m-PCR protocol consisted of the following steps: The initial denaturation step of 5 min at 95ºC; 30 cycles, with considering of 1 min at 95ºC, 1min at 65ºC and 30 s 72ºC; and a final extension step of 7 min at 72ºC. The PCR products were subjected to electrophoresis in 1.2% (w/v) agarose gel, stained with ethidium bromide and photographed under UV transilluminator. In each PCR run, a negative (distilled water) and a positive (*Salmonella* Typhimurium ATCC14028) control tubes were included (Zahraei Salehi *et al*., 2007).

Pulsed Field Gel Electrophoresis (PFGE): Pulsed-field gel electrophoresis was performed according to the procedures developed by the Centers for disease Control and Prevention (CDC) for molecular subtyping of *Escherichia coli* O157:H7, non-typhoidal *Salmonella* serovars and *Shigella sonnei* and as previously described (Centers for Disease Control and Prevention, 2004). Briefly, agarose-embedded DNA was digested overnight in a water bath at 37°C with 50 U of *Xba*I (Fermentas, Latvia). The restriction fragments were separated by electrophoresis in 0.5X Tris-borate-EDTA (TBE) buffer at 14°C for 20 h in 6 V/Cm using a CHEFF DR II electrophoresis system (Gene Navigator, Pharmacia, Sweden) with pulse times of 2.2 to 63.8 s. The gels were stained with ethidium bromide (1 µg/ml) and destained with the buffer remained in the electrophoresis apparatus for 60-90 min and then images were captured with trans illuminator. Also isolates presenting DNA smear patterns were retested. The size standard used for all gels was *Xba*I-digested DNA from *Salmonella* Braenderup strain H9812 (ATCC no BAA-664), the universal size standard used by all PulseNet laboratories.

Antibiotic susceptibility: Antibiotic susceptibility test was performed by the standard disk diffusion method in Mueller-Hinton agar, and the results were interpreted in accordance to the criteria of the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory

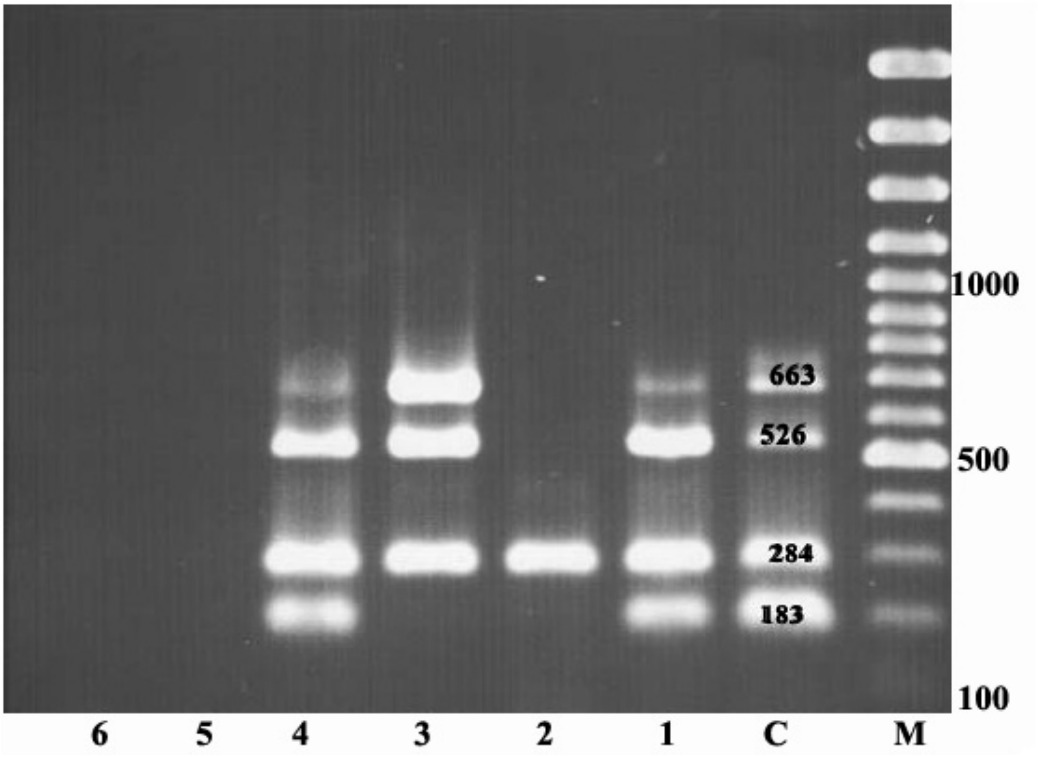

Figure 1. Multiplex PCR result of some serotype of *Salmonella* for confirming of *S*. Typhimurium. 183 bp: *fli*C; 284 bp: *inv*A; 526bp: *flj*B: 663bp: *rfbJ*; M: 100 bp DNA ladder (Fermentas, Latvia); C: Positive control (*S.* Typhimurium ATCC14028); 1: *S.* Typhimurium isolated from pony (samples); 2: *S*. Enteritidis (wild type); 3: S. Paratyphi B (wild type); 4: *S*. Typhimurium (wild type); 5: *E. coli* (wild type); 6: negative control. Numbers have been explained on the basis of bp.

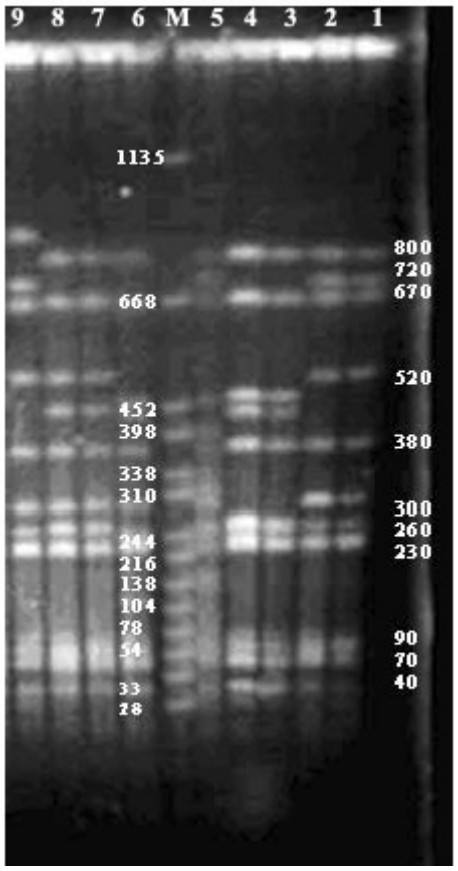

Figure 2. PFGE by *Xba*I enzyme digestion of some *S*. Typhimurium isolates. 1: ponie's isolate 2: cat's isolate, 3,4,7,8: chicken's isolate, 5,6: cow's isolate, 9: *S. Typhimurium* with ATCC 14028 and M: *S*. Braenderup H9812 Marker (*Salmonella* PFGE marker according to PulseNet protocol).

Standards, 2001). The strains were screened for resistance to the following antibiotics: cephalexin (CN, 30 µg), oxytetracycline (T, 30 µg), trimethoprim (TMP, 5 µg), lincospectin (LP, lincomycin/spectinomycin 15/200), enrofloxacin (NFX, 5 µg), trimethoprim sulfamethoxazole (SXT), nalidixic acid (NA, 30 µg), nitrofurantoin (FM, 300 µg), ampicillin (AM,10 µg), chloramphenicol (C: 30 µg), kanamycin (K, 30 µg), streptomycin (S, 10 µg) and ceftiofur (CFTIO, 30 µg) (Bahar afshan Co, Tehran, Iran).

RESULTS

Isolation and identification of *Salmonella* Typhimurium: Cultured samples were positive for *Salmonella enterica* serovar Typhimurium. Also the samples were positive with serotyping (1,4,5,12:i:1,2). Four amplified product (663, 526, 284 and 183 bp) were found in all specimens that had serovar Typhimurium, they corresponded to the *rfb*J, *flj*B, *inv*A and *fli*C genes of this serovar respectively. In *Salmonella enterica* serovar Enteritidis (1, 9, 12; g, m:-) only one PCR product (284 bp) was amplified from the *inv*A gene. In *Salmonella enterica* serovar Paratyphi B (1,4,5,12:b:1,2) three positive bands (284, 526 and 663 bp) were amplified corresponding to the *inv*A, *flj*B and *rfb*J genes respectively (Fig. 1).

Pulsed Field Gel Electrophoresis (PFGE): Eleven bands were presented in PFGE pattern of the isolates. Bands sizes were almost 40, 70, 90, 230, 260, 300, 380, 550, 670, 730 and 780 kb. This pattern was like to the other PFGE patterns isolated from cat (2006; 1979), sparrow (2005) and parrot (2005) in Iran which their information were documented in surveillance system of our laboratory. Comparison between PFGE pattern of one isolate with the standard size and *S. Typhimurium* with ATCC 14028 was presented in Figure 2.

Antibiotic susceptibility: The isolates presented resistance against cephalexin, oxytetracycline and streptomycin but were sensitive to oxytetracycline (T), trimethoprim (TMP), lincospectin (LP, lincomycin/spectinomycin), enrofloxacin (NFX), trimethoprim sulfamethoxazole (SXT), nalidixic acid (NA), ampicillin (AM), chloramphenicol (C), kanamycin (K) and ceftiofur (CFTIO).

DISCUSSION

Salmonellosis is a commonly encountered infectious disease of horses (Walker *et al*., 1995). *Salmonella* are among the most frequent causes of acute diarrhea in horses and the incidence seems to be increasing (van-Duijkeran *et al*., 1995). *Salmonella* Typhimurium is the most common serotype identified and is considered as one of the most virulent serotypes affecting horses of all ages. A number of other serotypes with apparent varying degrees of virulence have also been reported to cause salmonellosis in the horses such as *S. Enteritidis*, *S. Newport*, *S. Anatum*, *S. Java*, *S. Saintpaul*, *S. Kerfeld*, *S. Thompson*, *S. Heidelberg*, *S. Hadar*, *S. Infantis*, *S. Derby*, *S. Oranienburg*, *S. Hindmarsh* (Ernst *et al*., 2004; van Duijkeren *et al*., 2002; Mainar-Jaime *et al*., 1998; Daniel *et al*., 1997; van-Duijkeran *et al*., 1995; Walker *et al*., 1995; Traub-

Dargatz *et al*., 1990). Recently, the multiresistant *S*. Typhimurium phage type DT104 was the most common phage type isolated from horses correspond with those found in human, pigs and cattle and have highly zoonotic significance (van Duijkeren *et al*., 2002).

The predominance of one or two *Salmonella* serotypes in herd and hospital of horses in the most outbreaks of salmonellosis, suggests that many of these outbreaks reflect nosocomial infections. In addition, some case-control studies have compared affected with control horses to investigate potential risk factors. Stress factors that have been associated with salmonellosis in horses include transportation, antimicrobial administration, intestinal surgery, changes in diet, food deprivation, dehydration, colic, gastrointestinal tract disease, anesthesia and anthelmintic treatment (Ernst *et al*., 2004; House *et al*., 1999; Mainar-Jaime *et al*., 1998).

Owen *et al*. 1983 declared that transportation has a major role in reactivating the *Salmonella* infection in ponies. Diarrhea due to a reactivation of the *Salmonella* infection occurred greater than 3 days after stress, although maximal shedding of organisms occurred within 24 h (Owen *et al*., 1983).

In this study, it seems that transportation of ponies, changing in diet and probably deprivation during transportation, have been the most important risk factors and in accordance to predominance of *S*. Typhimurium, the outbreak has had nosocomial identity. Following of source of the infection in water supplies and environments of the herd, *Salmonella* was not detected. There were not any food sources for examination but it seems that only food materials could be the source of initial infection. PFGE pattern of the isolates revealed similarity with the other collected isolates which have existed since more than 30 years ago in Iran (unpublished data). This similarity indicates that the strain probably present for several years in this region. So, according to permanent and reliable profiles generated in PFGE method, we concluded and confirmed that main reason of such epidemic with systemic property in pony, is stress factors such as transportation, changing in diet and probably deprivation during transportation that introduce optimum conditions for Salmonella invasion or reactivation of latent Salmonella in carriers.

Antibiotic susceptibility test indicated that the isolate does not have any noticeable antibiotic resistance pattern. So, it seems that pathogenesis of this strain is related to risk factors or susceptibility of pony (as host) to *S*. Typhimurium.

The greatest difficulty in identifying of the outbreaks is detection of the first source of infection for determination and separation of nosocomial infection with one source from those acquired prior to admission (House *et al*., 1999). Therefore, sensitivity and rapidity of the methods which are applied for *Salmonella* detection are important. The limit of detection for most culture techniques is around 100 *Salmonella* organism/g of the feces. Subclinical infections in horses are more difficult to detect because less organism are shed and shedding may be intermittent (Mainar-Jaime *et al*., 1998).

Advanced PCR based techniques, such as multiplex PCR, are logically the most sensitive methods which could be applied for detection of even one organism/g of feces. Amavisit *et al*. (2001) indicated, while the sensitivity of the PCR assay was less than culture of feces for *Salmonella*, its sensitivity on fecal samples obtained from horses, was much greater than culture method. They detected *Salmonella* DNA in 40% of fecal samples using the PCR assay while *Salmonella* was isolated by culturing from only 2% of the samples. Also, this method performs in less than 5-7 h and actually decreases the time of identification (Amavisit *et al*., 2001). Due to correlation between *inv*-A virulent gene and clinical signs such as fever, bloody diarrhea and prolapsed rectum in ponies, it seems m-PCR by presenting multiple virulent genes (Such as *inv*-A), confirms the virulent properties of an isolate and is useful for molecular characterization of the isolate which causes an epidemic.

Therefore, in accordance to low prevalence of *Salmonella* shedding in horses about 1.7%, low drainage of *Salmonella* bacteria from feces (Mainar-Jaime *et al*., 1998), virulence and zoonotic importance of *Salmonella* and for molecular characterization of an isolate which causes *S. Typhimurium* epidemics, we need powerful method to detect it in *equidae*. M-PCR is suggested to be employed as a rapid and sensitive method for identifying the source of *Salmonella* especially in outbreaks of *S*. Typhimurium and even in carrier status in *equidae*.

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