A Study on Latent Equine Salmonellosis Based on Phenotypic and Molecular Methods in Kurdistan Province of Iran

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Abstract

BACKGROUND: Equine salmonellosis is an important infection with a wide variety of consequences including development of acute salmonellosis in the cases of predisposing factors, nosocomial infections, public health risk, and environmental contaminations.

OBJECTIVES: The aim of this study was to evaluate the fecal shedders of Salmonella spp. in the horses of Kurdistan province of Iran using phenotypic and molecular approach.

METHODS: A total of 130 fresh feces were randomly collected from horses in four age groups and both sexes in four seasons from all over Kurdistan province. The samples were analyzed for the isolation of Salmonella spp. with culture and biochemical method. An invA-based polymerase chain reaction (PCR) method was also carried out for detection of Salmonella spp. in pooled fecal samples, simultaneously. The isolates were further serotyped and the antimicrobial profile of the isolates was determined using Kirby-Bauer method.

RESULTS: The results showed 1.53% (n=2) and 7.69% (n=10) by bacteriological methods and PCR method, respectively. There was no significant relation between the frequencies of Salmonella shedders and age, sex and season (P≥0.05). The two isolates were recognized as Salmonella Typhimurium, showing 100% resistance against ampicillin, tetracycline, streptomycin, sulphonamide, and chloramphenicol, and 50% resistance against gentamycin.

CONCLUSIONS: Rapidity and accuracy of PCR versus phenotypic method makes it an appropriate procedure for the surveillance programs regarding Salmonella detection in feces. Approximately high prevalence of subclinical form in equine salmonellosis or Salmonella fecal carriers in the studied region is instigated to seriously apply strategies to manage and control the distribution of infection to susceptible hosts.

KEYWORDS: Culture, Horse, Kurdistan, PCR, Salmonella

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Introduction
The Salmonella spp. is identified in several species which causes one of the most important zoonotic diseases worldwide (Koochakzadeh et al., 2015, Juffo et al., 2017). Salmonella spp. is one of the important causes of diarrhea in foals which are more prevalent enteric pathogens in foals between 1 and 3 month of age (Olivo et al., 2016). Salmonella can cause acute and chronic diarrhea disease, neonatal bacteremia, or subclinical colonization in apparently healthy horses (manship et al., 2019). Salmonella spp. is a bacillus of 0.7-1.5×2-5 μm diameters, facultative anaerobic and non-sporulating (Bustos et al., 2016). In addition to the sole host-adapted serovar, Salmonella enterica serovar abortus-equí, some ubiquitous serovars of the bacterium, including Salmonella enterica serovar Typhimurium (ST) and Salmonella enterica serovar enteritidis (S. enteritidis), are frequently isolated from horses (Ahmed et al., 2012; Zahraei Salehi et al., 2012; Juffo et al., 2017).

Salmonella enterica subspecies enteric serovar abortus-equí (S. abortus-equí) is frequently reported as a cause of abortion in mares and neonatal septicemia and polyarthritis in Asian and African countries (Bustos et al., 2020, Glandolfo et al., 2018). It is well known as the etiological agent of equine abortion (Wang et al., 2019). Although S. enterica can produce life-threatening colitis in horses, certain serotypes are more commonly associated with the clinical disease (Leon et al., 2016). Various factors may affect microbial balance often leading to disturbances that may result in debilitating conditions such as colic and laminitis. Among those factors are high carbohydrate nutrition, medical substances, animal-related factors, pathological conditions and stress (Uzal and Diab 2015; Garber et al., 2020).

The detrimental impact of the carrier state, either persistently or transiently, is due to veiled shedding of the bacteria and therefore the likelihood of prolonged propagation of the infection to the susceptible hosts. Further, zoonotic potential of the bacterium is assumed as a serious challenge in public health sector (Martelli et al., 2018).

Although selective media are incorporated in phenotypic protocols of Salmonella isolation, the problems related to the sensitivity and specificity of the methods may limit their usage in the epidemiological investigations. Moreover, these procedures are time-consuming and laborious (Singer et al., 2006). Recently, development of genotypic techniques for direct detection of Salmonella in feces and food samples is regularly reported (Zahraei Salehi et al., 2005; Singer et al., 2006; Moganedi et al., 2007). Molecular assays provide more efficiency in less time in comparison with the phenotypic methods of Salmonella isolation (Païno et al., 2013).

Nowadays, emergence and dissemination of antimicrobial resistance is a matter of worrisome worldwide not only for public but also for veterinary medicine. Current studies have documented multi-drug resistance (MDR) among Salmonella serovars isolated from human and animals, which may impede the effectiveness of antibiotic therapy (Ahmed et al., 2012; Zahraei Salehi et al., 2012; Leona et al., 2018). S. enterica is an important cause of health care-associated infection in veterinary hospitals with outbreaks of multi-drugs resistant (Burgess et al., 2018).

The most controversial antimicrobial agents prescribed in the cases of equine Salmonellosis are ceftiofur and gentamycin. Given that they do not necessarily kill all the Salmonella organisms in the gut, latent carrier state may be the outcome of antibiotics utilization following recovery. Moreover, the imbalance and disruption in normal intestinal microbial flora following the consumption of antibiotics may also prompt the colonization of Salmonella in the gut. This is because of the combat between
pathogens and normal flora for the nutrients and attachment sites (Leona et al., 2018). Popularity of horses and horse-racing as a recreation and favorable sport and paucity of information regarding Salmonella colonization in horses in the West of Iran encouraged us to aim this study towards a timely manner survey on the prevalence of latent equine salmonellosis, with the focus on the serotypes and antimicrobial resistance patterns of the isolates in Kurdistan province of Iran.

**Materials and Methods**

A total of 130 horses, categorizing in four age groups, were randomly enrolled in the research. Table 1 shows the season and gender variables in relation to the age groups. An approximate of 50 gr feces was collected from each animal in the sterile glass bottles in rectal palpation and delivered to the laboratory within maximum of five h under cold conditions.

The specimens were screened for Salmonella based on the phenotypic procedure introduced by López-Martín et al., (2016). The cell culturing, immunoassay and polymerase chain reactions are the current methods to detect these pathogenic agents (Zhang 2019). Initially, the homogenization of an approximate of 5 gr individual fecal sample was carried out in 90 mL Buffered Peptone Water (BPW, Merck, Germany) and followed by the incubation at 37°C for 18-24 h. Further, 25 μL of the pre-enrichment media was inoculated into 10 mL Rappaport Vassiliadis Enrichment (RV, Merck, Germany) broth and incubated for 15-18 h at 41.5-42°C. A loop-full of the previous enrichment medium was streaked onto Hektoen Enteric (HE, Merck, Germany) broth and incubated for 15-18 h at 41.5-42°C. A loop-full of the previous enrichment medium was streaked onto Hektoen Enteric (HE, Merck, Germany) agar, then, incubated for 24 h at 37°C. Finally, a presumptive colony to Salmonella (green colony with dark center) from each plate was purified on MacConkey (MAC, Merck, Germany) agar. The biochemical identification was based on the Gram staining, TSI, IMViC, and Urea reactions. The isolates were transferred to the Faculty of Veterinary Medicine, Tehran University (Tehran, Iran) for serotyping using commercial antisera.

In parallel, molecular detection of Salmonella was fulfilled from each pooled fecal sample. The overnight suspensions of the individual fecal samples incubated in Tetrathionate (TT, Merck. Germany) broth were used to prepare a 10⁻¹ aliquot. The aliquot was transferred to a microtube containing 500 μL Brain and Heart Infusion (BHI, Merck, Germany) broth. Following the incubation at 37°C for 3 h, the tubes were centrifuged at 12000 g for 5 min and the supernatants were discarded. Adding 200 μL deionized distilled water, the pellets were vortexed, boiled for 15 min, and centrifuged same as the previous step. The supernatant was collected as DNA repertoire (Alinovi et al., 2003).

The PCR (BIORad T100, USA) for detection of InvA gene was carried out in 25 μL volume containing 12.5 μL 2X ready-to use PCR master mix (CinnaGen, Iran), 9.1 μL of deionized distilled water, 2 μL (50 ng) of template DNA, and 0.7 μL of each primer. The sequence of the primer pair was ST139:5ʹ-GTGAAATTATCGCCACGTTCGGGCAA-3ʹ and ST141:5ʹ-TCATCGCACCGTCAAGGGAACC-3ʹ. The thermal condition of the reaction was the same as introduced by Rahn et al., (1992). The used positive and negative controls were Salmonella Typhimurium ATCC1730 and DNA-free master mix, respectively. The PCR products were electrophoresed on 1.2% agarose gel (CinnaGen, Iran). Further, PCR-positive samples were phenotypically re-analyzed for the Salmonella confirmation once more.

Likewise, agar disk diffusion method was applied to determine the antimicrobial resistance profile of the isolates, in accordance with the CLSI guidelines (CLSI 2013). The used antibiotic disks included ampicillin (A), chloramphenicol (C), enrofloxacin (E), florfenicol (F), gentamicin (G), kanamycin (K),
nalidixic acid (Na), nitrofurantoin (Ni), streptomycin (S), sulphamethoxazole (Su), and tetracycline (T).

The statistical association between the proportional morbidity of *Salmonella* and the variables was analyzed using SPSS software (version 21.0). Based on Kolmogorov-Smirnov normality test, parametric *t*-test, and non-parametric Mann-Whitney U test were used to investigate the significant differences between groups for the measured analyses. A P-value less than 0.05 was considered as statistical significance.

**Results**

Of the 130 fecal samples, two *Salmonella* isolates were recovered in routine bacteriological method, representing an overall incidence of 1.53%. In comparison, *Salmonella* was detected in 10 fecal samples (7.69%), producing a 284 bp amplicon in PCR reaction (Figure 1). As the positive samples in phenotypic method were also detected in molecular approach, it can be implied that the sensitivity of culture method was 20% of the PCR method. The two *Salmonella* isolates were recognized as ST based on the Kauffman-White Scheme (1,4,5,12:i:1,2).

Besides, despite the re-cultivation of PCR-positive samples, no *Salmonella* spp. was isolated. Generally, the highest prevalence of *Salmonella* fecal shedding was among ≥1-5 ≤ years-old animals, male sex, and in summer. Despite this, no statistical association was observed between the frequencies of fecal carriers and variables including age, gender, and season (*P* ≥0.05). **Table 2** represents the proportional morbidity of *Salmonella* in regards to the analyzed variables.

Antimicrobial susceptibility testing revealed resistance to ampicillin (it is from the same family of penicillin that is used to treat or prevent many different types of bacteria such as *Salmonella*), tetracycline, streptomycin, and sulphamethoxazole, and chloramphenicol in the both isolates, whilst resistance to gentamicin in only one isolate. No resistance was observed against nalidixic acid, nitrofurantoin, enrofloxacin, florfenicol, and kanamycin.

**Table 1.** Demographic information of the studied population

<table>
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<tr>
<th>Age groups</th>
<th>Sex</th>
<th>Season</th>
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<tr>
<td></td>
<td>Female</td>
<td>Male</td>
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<tr>
<td>Foal &lt; 1 year 14 (10.8%)</td>
<td>8</td>
<td>6</td>
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<tr>
<td>≥ Juvenile ≤5 71 (54.6%)</td>
<td>24</td>
<td>47</td>
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<tr>
<td>5 &gt; Adult ≤10 37 (28.5%)</td>
<td>9</td>
<td>28</td>
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<tr>
<td>10 &gt; Senior 8 (6.2%)</td>
<td>3</td>
<td>5</td>
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<tr>
<td>Total 130 (100%)</td>
<td>44</td>
<td>86</td>
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<tr>
<th>Age</th>
<th>Phenotypic method</th>
<th>Genotypic method</th>
<th>Prevalence</th>
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<tr>
<td>Foal &lt; 1 year</td>
<td>1 (0.76%)</td>
<td>1 (0.76%)</td>
<td>0.615</td>
</tr>
<tr>
<td>≥ Juvenile ≤5</td>
<td>8 (6.15%)</td>
<td>8 (6.15%)</td>
<td>0.097</td>
</tr>
<tr>
<td>5 &gt; Adult ≤10</td>
<td>1 (0.76%)</td>
<td>1</td>
<td>0.130</td>
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<tr>
<td>10 &gt; Senior</td>
<td>0</td>
<td>0</td>
<td>0.130</td>
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<th>Sex</th>
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<td>Male</td>
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<table>
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<tr>
<th>Season</th>
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<td>Summer</td>
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<td>Autumn</td>
<td>4</td>
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<td>Winter</td>
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Figure 1. Agarose gel electrophoresis of PCR products with invA gene primers (284 bp). M: 100 bp DNA Ladder (CinnaGen, Iran), PC: positive control (Salmonella Typhimurium ATCC1730). NC: negative control. Lanes 1-9: field samples.

Discussion

The carrier state of equine salmonellosis with ST is frequently documented (Ahmed et al., 2012; Hartnack et al., 2012, Haq et al., 2018). Fecal shedding of the bacterium is estimated to be for 14 months post-infection in the horse. A serious consequence is environmental contamination which leads to transmission of the infection to the susceptible hosts, particularly the foals. The environmental contamination with Salmonella may be underestimated by the certain culture techniques, which may impair the efforts to control the spread in veterinary hospitals (Lyle et al., 2015).

Besides, clinical manifestations of salmonellosis may also develop in the presence of predisposing factors in an individual infected horse. The nosocomial dispersion and acquisition of infection must also be considered through the introduction of an asymptomatic carrier, as Salmonella spp. may survive in the hospital area for at least a week (Hartnack et al., 2012). Likewise, the health risk associated with the carrier state of equine salmonellosis is prevailing. Because of the close contact between a horse and its owner and/or a veterinarian, the potential of human contamination is not far from expectation.

Isolation of the bacterium is the most reliable method for the diagnosis of Salmonellosis. Despite this, definitive identification of Salmonella in the phenotypic methods requires pre-enrichment and enrichment cultivation stages, which are time-consuming. Likewise, a significant limitation of the most culture methods for the Salmonella isolation is the requirement of presence an average number of 100 bacteria/gr of feces. The lower rate of the bacterial shedding in stool in subclinical state of equine salmonellosis has been reported (Mainar-Jaime et al., 1998). In addition, the isolation may fail because of the prior prescription of antibiotics (Juffo et al., 2017). Despite this, direct detection of Salmonella spp. through the phenotypic method conducts the implement of antibiogram assay as a magnitude step for the chemotherapy (Juffo et al., 2017). For the failure and limitation in scrutinizing shedders traditionally, molecular-based methods have been introduced for identification of Salmonella spp. in diverse samples (Ahmed et al., 2012; Traub-Dargatz et al., 2000; Zahraei Salehi et al., 2005). The benefit and privilege of PCR technique is greater sensitivity of this method compared to the culture method for detection of Salmonella in feces, particularly in
horses (Amavisit et al., 2001). Due to the correlation between invA gene and virulence of Salmonella isolates, it is considered as a proper candidate for the molecular detection of the bacterium. The gene encodes proteins in the inner membrane of Salmonella spp. which participate in invasion step of the bacterium to the epithelial cells (Darwin and Miller, 1999). Further, reduction of the Salmonella detection time from an average of three days in culture method to maximum 24 h in molecular procedure is the other beneficial point of PCR technique.

The results of the present study revealed the subclinical form in equine salmonellosis or carrier state in the West of Iran. The prevalence rate was 1.53% versus 7.69% in phenotypic and molecular methods, respectively. Higher sensitivity of PCR to the microbiological culture for Salmonella detection in feces was also reported by Cohen et al., (1996b). The overall prevalence of Salmonella fecal shedding in horse was cited as 2% and 40% in culture and PCR assays elsewhere (Amavisit et al., 2001). Higher (75%) and lower (0.6%) rates of equine fecal shedding of Salmonella was stated from veterinary teaching hospitals, respectively (Pusterla et al., 2010). Albeit, the number (one or multiple) and type (feces, lymph nodes, blood) of samples, the methodology used for Salmonella spp. detection, and differences in the population of the enrolled horses (general versus sick animals with clinical signs of Salmonellosis) may effectively influence the final prevalence rate detection of fecal shedding of Salmonella (Traub-Dargatz et al., 2000). It is noteworthy to emphasize that as regular and multiple stool sampling over a period of time is needed to definitively identify the transient carriers, conclusive data regarding the rate of equine subclinical salmonellosis is lacking in the present research.

The sole Salmonella serotype which was recognized was S. Tm, herein. But it is not absolute as only two out of 10 Salmonella samples were isolated in culture. As the used primers were not able to distinguish among the serotypes, it is highly recommended to accomplish the research by the molecular detection of Salmonella serotypes using serotype-specific primers. Some other studies have documented ST as the single or predominant serotype isolated from horses (Ahmed et al., 2012; Zahrara Salehi et al., 2012; Juffo et al., 2017). This serotype is responsible for both human and animal salmonellosis all around the world.

Recent increases in antimicrobial resistance among ST isolates, particularly multi-drug resistance, attract global concern towards the effectiveness of antibiotics in acute and peracute cases. Both S. Tm isolates represented the resistance pattern of ACSSuT, which is also reported from England, Canada, The Netherlands, and Japan (Weese et al., 2001; Vo et al., 2007; Ahmed et al., 2012). Multi-drug resistant ST definitive phage type 104 (DT104) is an important contributor of gastrointestinal infections in both human and farm animals with the same antimicrobial profile (Poppe et al., 1998; Izumiya et al., 1999). Horse is contributed as a potential source of human contamination with MDR ST DT104 (Weese et al., 2001; Vo et al., 2007; Ahmed et al., 2012). Although horse meat is not consumed in Iran, the infected horses may associate with the distribution of the bacterium to livestock through environmental contamination. Besides, horizontal transfer of resistance genes among and within commensal and/or pathogenic bacteria is a plausible way for distribution of these genes (Ahmed et al., 2012). Hence, phage typing of the ST strains isolated in the present study can be an important step in understanding the epidemiology of Salmonella spp. in horse population in the West of Iran. Generally, gentamicin and kanamycin are not appropriate candidates against intracellular bacteria such as Salmonella (Niwa et al., 2009) and florfenicol is not licensed for use in horse. Fluoroquinolones and nitrofuran metabolites may become
powerful choices in the cases of severe equine salmonellosis. Susceptibility of ST to ciprofloxacin and enrofloxacin was also reported elsewhere (Ahmed et al., 2012).

Although Salmonella spp. infects horses in all ages of, the individual susceptibility of young-aged horses for acquisition and establishment of infection is consistently elucidated in the literature (chandra and Gurpreet, 2018). This study also revealed that Salmonella fecal shedding in horse is typically highest in warmer months of the year. This coincided with the results of the present study. This may be related to the presence of more young foals and juveniles as a sensitive age group in this time of year for the reception and shedding of infection. Because of the lack of raining in the recent years in Iran, feeding of horses with high quality forage is not possible. Feeding restriction or using straw in the regimen of stallions rather than mares may be attributed to the higher prevalence of Salmonella fecal shedding in male horses. Dry silage may also lead to dental malfunctions, small colon impactions, and imbalance in normal microbiota in horses.

Conclusion
In brief, the results of the present study approved Salmonella spp. carrier state and subclinical form of salmonellosis among the horse population in the Kurdistan province of Iran. Because of the accuracy and rapidness of PCR in comparison with culture, it is highly recommended to be employed in the surveillance and epidemiological studies of Salmonella spp. in feces. Moreover, the hazard imposed by equine Salmonella carriers in veterinary and public health sectors should be considered. Continuous monitoring programs and utilization of biosecurity practices are affirmed with respect to restrain the introduction or dissemination of the infection.

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Conflict of Interest
The authors declared no conflict of interest.

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مقاله‌ای بر سالمونئوزیس اسب با روش‌های ملکولی و فنوتیپی در استان کردستان - ایران

شاهین فکوران 1، سید علی موسوی راد 2، الهام احمدی 3، 4

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زمینه مطالعه: سالمونئوز در اسب عفونت مهمی با طیف وسیعی از پیامدهای شاخص ایجاد سالمونئوزیس حاد در موارد وجو وفاکتورهای مستعدکننده عفونت‌های بیمارسایی مقدوی سالمونئوز در اسلان استان کردستان ایران به روش‌های فنوتیپی و ملکولی بود در مجموع تعداد 130 نمونه مدفوع تازه اسب در چهار گروه سنی مختلف (ده جنس و در چهار قسم مختلف از سراسر استان کردستان) جمع‌آوری شد.

روش گزارش متوجه به شناسایی سالمونئوز با روش کشت و بیوشیمی آنتی‌گونه نوی روش وکتور زنجیره‌ای پلیمر مبتنی بر زن invA در نمونه‌های ملکولی و فنوتیپی در نمونه‌های مفتوحی به صورت همبستگی انجام شد. سپس جدایی آن‌ها توسط سرپوشی سه و پروتئین آنتی‌بیوتیک آن‌ها با روش کنبی‌پویر به مساحت جداگانه تغییر داد.

نتایج: نتایج این مطالعه شوی 53/1% (و در مورد) و 69/7% (ده) در روش‌های پاتوژنیک و فنوتیپی را نشان داد ارتباط معنی‌داری بین فراوانی دفع‌کننده‌گان سالمونئوز با سن، جنس و طبقه اجتماعی مشاهده نشد (5). 4. در نمونه‌های مقدوی سالمونئوزیس شهد که 100% مقاومت بر در آمپیکورین، آنتی‌بیوتیک‌ها، سولفاتوراکسول و کاربامیپینکل و 50% مقاومت بر در آمپیکورین را نشان دادند.

نتیجه‌گیری نهایی: سرعت و دقت روش PCR در مقایسه با روش فنوتیپی، آن را به گزینه مناسب برای پیش‌بینی در شناسایی سالمونئوز در مفتوح‌های ملکولی و فنوتیپی در انسداد مورد مطالعه به طور جدی اعمال استراتژی هب به شناسایی و کنترل انتشار عفونت به مرز تغییر حساسیت است. در این مقاله از تعداد 130 نمونه اسب به مطالعه در این استان درآمد.

واژه‌های کلیدی: آنتی‌بیوتیک، اسب، حاملین مقدوی سالمونئوز، کشت