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

Shahin Fakour*[1], Seyed Ali Musavi Rad[1], Elham Ahmadi[2]

[1] *Department of Clinical Sciences, Sanandaj Branch, Islamic Azad University, Sanandaj, Iran*
[2] *Department of Pathobiology, Sanandaj Branch, Islamic Azad University, Sanandaj, Iran*

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 ($P \geq 0.05$). The two isolates were recognized as *Salmonella* Typhimurium, showing 100% resistance against ampicillin, tetracycline, streptomycin, sulphamethoxazole, 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*

Correspondence

Shahin Fakour, Department of clinical sciences, Faculty of Veterinary Medicine, Branch Sanandaj Islamic, Azad university, Sanandaj, Iran Tel: +98 (918) 88717317, Email: fakours@yahoo.com

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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-equi, 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-equi (*S. abortus-equi*) 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 (Paião *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) 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 ST139:5′-GTGAAATTATCGCCACGTTCGGGCAA-3′ and ST141:5′-TCATCGCACCGTCAAAGGGAACC-3′. 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 Man-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 to 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 ($P \geq 0.05$). 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

Age groups	Sex		Season			
	Female	Male	Spring	Summer	Autumn	Winter
Foal < 1 year 14 (10.8%)	8	6	2	1	7	4
1≥ Juvenile ≤5 71 (54.6%)	24	47	9	23	21	18
5 > Adult ≤ 10 37 (28.5%)	9	28	4	5	19	9
10 > Senior 8 (6.2%)	3	5	1	1	4	2
Total 130 (100%)	44 (33.8%)	86 (66.2%)	16 (12.3%)	30 (23.1%)	51 (39.2%)	33 (25.4%)

Table 2. Prevalence of *Salmonella* fecal shedding in related to age, sex and season in the present study.

Variables		Prevalence		P-value
		Phenotypic method	Genotypic method	
Age	Foal < 1 year	1 (0.76%)	1 (0.76%)	0.615
	1≥ Juvenile ≤5	0 (0%)	8 (6.15%)	
	5 > Adult ≤ 10	1 (0.76%)	1	
	10 > Senior	0	0	
Sex	Female	0	1	0.097
	Male	2	9	
Season	Spring	0	1	0.130
	Summer	0	5	
	Autumn	2	4	
	Winter	0	0	

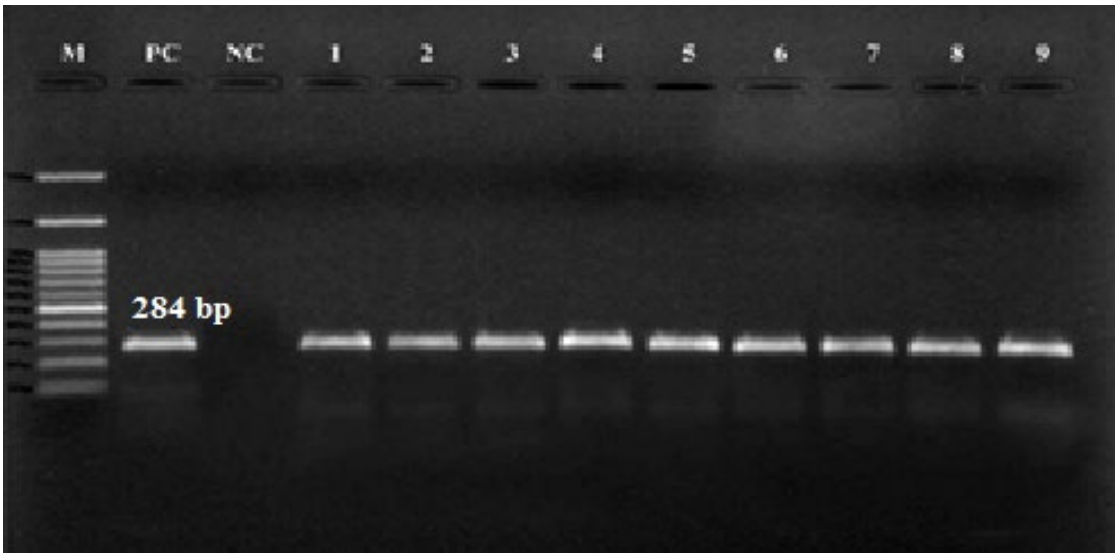

Figure 1. Agarose gel electrophoresis of PCR products with *invA* gene primers (284 bp). M: 100 bp DNA Ladder (CinnaGen, Iran), PC: positive control (*Salmonella Typhimurium* ATCC1730). NC: negative control. Lanes 1-9: field samples.

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; Zahraei 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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مطالعه‌ای بر سالمونلوزیس اسب با روش‌های ملکولی و فنوتیپی در استان کردستان – ایران

شاهین فکور*[۱]، سید علی موسوی راد[۱]، الهام احمدی[۲]

[۱] گروه علوم درمانگاهی، واحد سنندج، دانشگاه آزاد اسلامی، سنندج، ایران.

[۲] گروه پاتوبیولوژی، واحد سنندج، دانشگاه ازاد اسلامی، سنندج، ایران.

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زمینه مطالعه: سالمونلوز در اسب عفونتی مهم با طیف وسیعی از پیامدها شامل ایجاد سالمونلوزیس حاد در موارد وجود فاکتورهای مستعدکننده، عفونت‌های بیمارستانی، مدفوعی *سالمونلا* در اسبان استان کردستان ایران به روش‌های فنوتیپی و مولکولی بود. در مجموع تعداد ۱۳۰ نمونه مدفوع تازه از اسب در چهار گروه سنی مختلف، هر دو جنس و در چهار فصل مختلف از سراسر استان کردستان جمع‌آوری شد..

روش کار: نمونه‌ها به منظور جداسازی *سالمونلا* به روش کشت و بیوشیمیایی آنالیز شدند. نوعی روش واکنش زنجیره‌ای پلیمراز مبتنی بر ژن *invA* برای شناسایی *سالمونلا* در نمونه‌های مدفوع نیز به صورت همزمان انجام شد. سپس جدایه‌ها تعیین سروتیپ شده و پروفایل آنتی‌میکروبی آن‌ها با روش کربی-بوئر مشخص شد

نتایج: نتایج این مطالعه شیوع ۱/۵۳٪ (دو مورد) و ۷/۶۹٪ (ده مورد) بترتیب در روش‌های باکتریایی و ملکولی را نشان داد. ارتباط معنی‌داری بین فراوانی دفع‌کنندگان *سالمونلا* با سن، جنس و فصل مشاهده نشد($P\geq$۰/۰۵). دو جدایه به عنوان *سالمونلا* تیفی‌موریوم شناسایی شدند که ۱۰۰٪ مقاومت برعلیه آمپی‌سیلین، تتراسایکلین، استرپتومایسین، سولفامتوکسازول و کلرآمفنیکل و ۵۰٪ مقاومت برعلیه جنتامایسین را نشان دادند.

نتیجه‌گیری نهایی: سرعت و دقت روش PCR در مقایسه با روش فنوتیپی، آن را به گزینه مناسبی در برنامه‌های غربالگری در شناسایی *سالمونلا* در مدفوع معرفی می‌نماید. شیوع نسبتا بالای حاملین مدفوعی اسب در منطقه مورد مطالعه، به طور جدی اعمال استراتژی‌ها به منظور مدیریت و کنترل انتشار عفونت به میزبانان حساس را ایجاب می‌کند.

واژه‌های کلیدی: آنتی‌بیوگرام، اسب، حاملین مدفوعی، *سالمونلا*، کشت

نویسندهٔ مسئول: شاهین فکور، گروه علوم بالینی، دانشکده دامپزشکی، واحد سنندج، دانشگاه آزاد اسلامی، سنندج، ایران. ایمیل: fakours@yahoo.com