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Isolation, Molecular Identification, and Antibiotic Resistance Profile of *Salmonella* Typhimurium Isolated from Calves Fecal Samples of Dairy Farms in Hamedan

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ABSTRACT

Introduction: Calf diarrhea is one of the significant problems in dairy farms associated with treatment costs and reduced livestock production. Salmonellae are among the most common and the major causative agents of diarrhea in calves and humans. The present study was carried out to isolate and identify *Salmonella* in fecal samples of calves in industrial dairy farms of Hamedan and to determine antibiotic resistance profiles of the probable isolates. **Methods:** *Salmonella* were presumptively isolated based on the cultural characteristics and biochemical tests, and the identity of the isolates was further confirmed using genus- and serotype-specific PCR assays. Kirby-Bauer disk diffusion method was performed to determine antibiotic resistance profiles of the isolates. **Results:** Out of 120 stool samples collected from 8 industrial farms, 22 (18.33%) isolates possessing *rfbJ*, *fliC* and *fljB* genes were identified as *Salmonella* Typhimurium serotype. Antibiotic susceptibility test revealed all isolates (100%) were susceptible to gentamicin, amoxicillin-clavulanic acid, kanamycin, and ciprofloxacin and resistant against cotrimoxazole, cefazolin, and cefixime. **Conclusion:** To our knowledge, this study was the first report of *Salmonella* infection in Hamedan's dairy farms, indicating a relatively high prevalence rate of *S.* Typhimurium infection as the only detected serotype. Antibiotic resistance should also be considered a severe public health concern. Thus, effective hygiene measures should be adopted to prevent or reduce the infection, and monitoring antibiotic susceptibility is required to choose the drug of choice for treatment.

INTRODUCTION

Salmonella members are well-known zoonotic bacteria and causative agents of significant diseases, including intestinal fevers (typhoid and parathyroid fevers), food poisoning and septicemia in humans, and diarrhea and septicemia in cattle [1]. Enterocolitis caused by *Salmonella* can affect various animals regardless of the host age. Acute illness is associated with fever, anorexia, severe diarrhea (often containing blood), mucus, and epithelial casts. However, dehydration, weight loss, and abortion in pregnant animals may also occur [2].

Based on the somatic (O) and flagellar (H) antigens, more than 2500 serotypes have been identified in *Salmonella* genus according to the system devised by Kaufmann and White [3, 4]. Accordingly, PCR assays have been developed to detect these serotypes based on antigenic properties. For example, *Salmonella enterica* serotype Typhimurium can be identified using a multiplex PCR which detect *rfbJ*, *fliC*, and *fljB* genes involved in encoding O antigen (O4), phase-1 (H1) antigen (H:i), and phase 2 (H2) antigen (H: 1,2), respectively [5]. *Salmonella* serotypes infect many mammal species, birds, and reptiles. While some serotypes, such as *Salmonella* Dublin, are host-specific for cattle, other serotypes like *Salmonella enterica* serotype Typhimurium and *Salmonella enterica* serotype Enteritidis have a wide range of hosts. They may affect humans, ruminants, poultry, and other species [6]. Although the geographical distribution of various *Salmonella* serotypes in one region may differ from another, *Salmonella* Typhimurium is one of the most common serotypes of this genus and is widespread worldwide [7].

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Considering the economic losses in livestock, diarrhea in calves is a severe problem that imposes treatment costs, reduces calf growth, and may even result in the death of the affected hosts [8]. Young animals severely affected by salmonellosis are commonly recumbent and may die within days. The disease occurs in all beef and dairy breeds and is more common in areas where livestock are kept in intensive systems [1]. Infected calves may contaminate their environment by shedding large amounts of *Salmonella* in their feces, leading to infection in humans and other animals through contaminated water or food. People in close contact with farm animals or those who use livestock products are at higher infection risk [9, 10].

Most *Salmonella* carriers are subclinical shedders. These bacteria can also survive for nine months or more in the environment and infect humans and various animal species, mainly through the fecal-oral route [11]. Centers for Disease Control and Prevention (CDC) estimates that *Salmonella* causes foodborne illness more than any other bacteria, and salmonellosis is the most common foodborne disease in developing countries. However, the prevalence varies according to the country [12].

Since ingestion of contaminated food products of animal origins, particularly cattle or poultry, can lead to salmonellosis in humans; characterization, treatment, and/or prevention of this infection in animals aid in decreasing human cases [13].

Therefore, the present study investigated the *Salmonella* infection rate in industrial dairy farms of Hamadan, Iran, followed by determining the most common *Salmonella* serotype and antibiotic resistance patterns of the isolates.

MATERIAL AND METHODS

Collection of samples. One hundred twenty stool samples were collected from apparently healthy (n=30) and diarrheic (n=90) calves' rectum using sterile swabs ≤ one-month-old. The samples included both males (n=60) and females (n=60) animals from eight industrial dairy farms in Hamadan Province, west of Iran.

Isolation of *Salmonella*. The rectal swabs were cultured in Rappaport broth medium followed by incubation at 37 °C for 24-48 h. Then, they were subcultured on XLD (Xylose Lysine Deoxycholate, Merck, Germany), TSI (Triple Sugar Iron, Merck, Germany), SIM (sulfide, indole, motility, Merck, Germany), and Simmons' citrate (Merck, Germany) media for presumptive identification of *Salmonella* strains. After incubation at 37 °C for 18 h, the suspected colonies were harvested and stored in 30% glycerol at -70 °C until molecular examination [14, 15].

Molecular Identification of *Salmonella*. The identity of all isolates presumed to be *Salmonella* based on the previous steps was assessed using a *Salmonella* genus-specific PCR. For this purpose, bacterial DNAs were extracted using the boiling method [5]. For this, 5 ml of overnight cultures of isolates in tryptic soy broth (TSB) were centrifuged at 8000 rpm for 3 min, and the pellet was resuspended in sterile TE buffer (10 mM Tris, 1 mM EDTA, pH=8) followed by boiling at 100 °C for 10 min. The supernatants containing the extracted DNA were stored at -20 °C. The PCR assay was performed using the previously described primers, amplifying a 284 bp fragment of the *invA* gene (Table 1). The 20 µl PCR reactions contained 10 µl of a commercial PCR master mix, 5 µl of template DNA, 1 µl of each of the primers (50 pmol) and 4 µl distilled water. *Salmonella* Typhimurium ATCC 14028 DNA alongside distilled water were used as positive and negative controls, respectively, in all assays. The amplification was performed in a thermal cycler (Astec, Japan) programmed for pre-denaturation at 94 °C for 5 min, 34 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 45 sec and extension at 72 °C for 1 min followed by a final extension at 72 °C for 5 min. The PCR products were electrophoresed on a 1% agarose gel and photographed under UV light in a gel documentation system (UVITEC, France) [5].

Molecular Identification of *S.* Typhimurium serotypes. All *Salmonella* isolates were examined using a specific PCR to identify *S.* Typhimurium serotype. The primers in the multiplex-PCR method could simultaneously target the *rfbJ*, *fliC*, and *fljB* genes (Table 1). The 20 µl multiplex-PCR reactions contained 10 µl of a commercial PCR Mastermix, 5 µl of template DNA, 0.5 µl of each of the primers (50 pmol) and 2 µl distilled water. The amplification program included a pre-denaturation step at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 35 sec and extension at 72 °C for 1 min (35 cycles) with a final extension at 72 °C for 10 min. Finally, the PCR products were electrophoresed using the same procedure [16].

Table 1. Primer sequences used in the PCR reaction in this study

Primer	Sequence 5'-3'	Target gene	Amplicon Size (bp)	Reference
ST139	GTGAAATTATCGCCACGTTCGGGCAA	*invA*	284	[5]
ST141	TCATCGCACCGTCAAAGGAACC			
Rfbj-s	CCAGCACCAGTTCCAACTTGATAC	*rfbJ*	663	[16]
Rfbj-as	GGCTTCCGGCTTTATTGGTAAGCA			
Flic-s	ATAGCCATCTTTACCAGTTCCCCC	*fliC*	183	[16]
Flic-as	GCTGCAACTGTTACAGGATATGCC			
Fljb-s	ACGAATGGTACGGCTTCTGTAACC	*fljB*	526	[16]
Fljb-as	TACCGTCGATAGTAACGACTTCGG			

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Antibiotic susceptibility test. Kirby-Bauer disk diffusion method was applied to determine the antibiotic susceptibility of the *S.* Typhimurium isolates according to CLSI (2013) instructions [17]. The following antibiotic disks (Padtan Teb, Iran) were used for this purpose: cefazolin [30 µg], tetracycline [30 µg], ciprofloxacin [5 µg], amoxicillin-clavulanic acid [20/10 µg], gentamicin [30 µg], enrofloxacin [30 µg], streptomycin [30 µg], chloramphenicol [30 µg], amikacin [30 µg], kanamycin [30 µg], cefepime [30 µg], colistin [30 mg], fosfomycin [200 µg], cefoxitin [30 µg], nitrofurantoin [300 µg], azithromycin [30 µg], ceftazidime [30 µg], ofloxacin [5 µg], trimethoprim-sulfamethoxazole [1.25/23.75 µg], and cefixime [µg 5].

Statistical analysis. Statistical analysis of data was performed using SPSS software (version 16) and Chi-square test (X^2). The significance level was considered to be $P \leq 0.05$.

RESULTS

Isolation and identification of *Salmonella*. Based on the biochemical characteristics of the isolated colonies on/in the bacterial culture media, 29 isolates presumptively belonged to the *Salmonella* genus. However, the 284-bp DNA fragment was only detected in 22 isolates in the genus-specific PCR, confirming that only 22 isolates belonged to the *Salmonella* genus (Fig. 1).

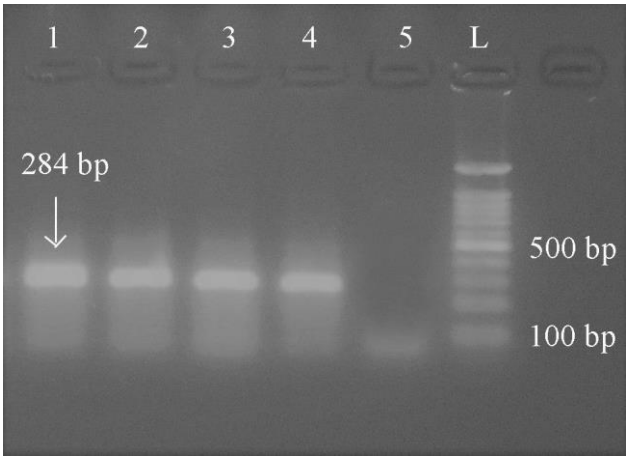

Fig. 1. Genus-specific PCR results for identification of *Salmonella* strains. Lanes 1-3: positive isolates for *invA* gene; lane 4: positive control (*Salmonella* Typhimurium ATCC 14028), lane 5: negative control (contained no DNA); lane L: a 100 bp DNA ladder.

Molecular Identification of *S.* Typhimurium serotype. Since Typhimurium serotype is known as one of the most common *Salmonella* serotypes in animals, all 22 identified *Salmonella* isolates were first screened by a serotype-specific multiplex-PCR assay to determine Typhimurium serotype. Multiplex PCR yielded the expected 663 bp, 183 bp, and 526 bp DNA bands, confirming the identity of *S.* Typhimurium in all 22 isolates with the antigenic profile of O4, H1-i, and H2-1,2 (Fig. 2).

The results revealed that 22 (18.33%) of the examined animals of both sexes (male n=12, female n=10) were infected with *S.* Typhimurium, encompassing 17 isolates from diarrheic calves and 5 from apparently healthy animals. Statistical analysis indicated no significant association between the prevalence of *S.* Typhimurium and calves sex (P= 0.638).

Antibiotic susceptibility test. The antibiotic susceptibility test results demonstrated that all *S.* Typhimurium isolates were sensitive to gentamicin, streptomycin, kanamycin, enrofloxacin, chloramphenicol cefepime, fosfomycin, cefoxitin, ceftazidime, ofloxacin, and amoxicillin-clavulanic acid. However, 100% of the isolates showed resistance against colistin, tetracycline, cotrimoxazole, azithromycin, cefazolin, cefixime, and nitrofurantoin.

DISCUSSION

Due to strains of animal origin, *Salmonella* infections in humans are widespread worldwide. These infections are among the most critical public health concerns in developing and developed countries. The wide range of hosts, the multiplicity of serotypes, and the presence of natural carriers make salmonellosis the most common infection of all foodborne microbial diseases in humans [18, 19]. As livestock and their products are the primary sources for transmitting *Salmonella* serotypes and causing food poisoning in humans, determining the epidemiological status of *Salmonella* infections in animals provides valuable data for taking appropriate controlling measures.

The results of the present study demonstrated that 22 (18.33%) out of 120 collected fecal samples harbored *Salmonella* bacteria. Furthermore, all the *Salmonella* isolates belonged to the Typhimurium serotype, indicating a relatively high prevalence rate for this

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zoonotic bacterial infection. Given that most *Salmonella*-infected cases (17 out of 22 cases: ~77%) were diarrheic calves, the potential key role of this bacterium as one of the main causative agents of diarrhea in the studied farms should be considered seriously.

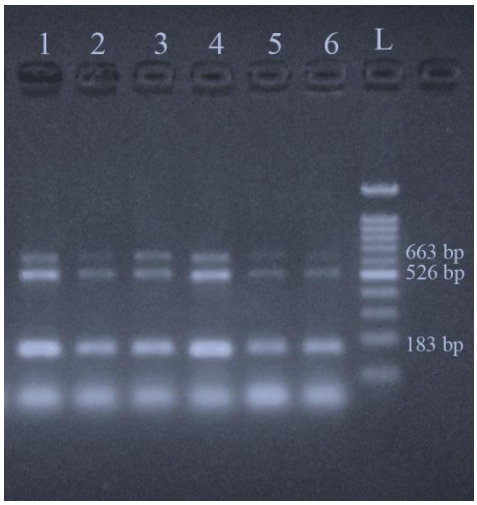

Fig. 2. Electrophoresis of multiplex PCR products for the identification of *S*. Typhimurium serotype. The corresponding DNA fragments of *fliC, fljB,* and *rfbJ* genes are visible. Lane L: a 100 bp DNA ladder; lane 1: positive control (*Salmonella* Typhimurium ATCC 14028), lanes 2-6: the isolates.

On the other hand, the increase in antibiotic resistance in *Salmonella* strains has become a global concern. A correct understanding is essential to select the appropriate antibiotics during treatment programs for livestock. Animals, mainly cattle and pigs are the primary source for *S.* Typhimurium isolates with the two antibiotic resistance patterns, tetra-resistant pattern (ASSUT: ampicillin-streptomycin-sulfonamides and tetracycline) and penta-resistant pattern (ACSSUT: ampicillin-chloramphenicol-streptomycin-sulfonamides-tetracycline) [20-24]. In humans, multidrug-resistant *Salmonella* strains have been the most common cause of hospitalization and mortality due to consumption of dairy products as the source of *Salmonella* infections in many cases [20, 22]. Consequently, antibiotic resistance is the next issue in controlling bacterial infections. Investigating antibiotic resistance among isolates provides beneficial information, particularly in the case of intestinal bacteria, e.g., *Salmonella*, a ubiquitous bacterium that can transfer antibiotic resistance genes to other bacterial species.

In the present study, the *S.* Typhimurium isolates were sensitive to some antibiotics, especially aminoglycosides, including gentamycin, streptomycin, and kanamycin. However, showed resistance to several high-consumed antibiotics such as colistin, tetracycline, cotrimoxazole, azithromycin, cefazolin, nitrofurantoin, and cefixime, suggesting a switch to a treatment regimen in these areas. Besides, a similar antibiotic resistance profile among *Salmonella* isolates may indicate that a specific type of *Salmonella* is prevalent in the studied farms due to antibiotic regimens administered in the area [25].

Regarding the importance of *Salmonella* infections, investigation on this bacterial infection in animals has also received attention in several parts of Iran. Keyvanfar *et al.* (1997) tested 2014 fecal samples of diarrheic calves around Shiraz and found 38 (1.89%) infected with *Salmonella* with the highest resistance against streptomycin. At the same time, 94.73% of them were resistant to one or more antibiotics [26]. In Markazi province (2012), of 1124 fecal cow samples, 36 (3.2%) contained *Salmonella* bacteria. Similar to our findings, the most effective antibiotics in that study were kanamycin and chloramphenicol [27]. In 2013, 19 *Salmonella* isolates belonging to *S.* Typhimurium and *S.* Dublin serotypes were obtained from feces, water, and milk samples taken from two dairy farms in Northeast Iran. In this study, *S.* Typhimurium isolates constituted the majority of the isolates and also showed multidrug resistance [28]. Examing 420 calves (2014) for *Salmonella* in 18 farms from Kermanshah province over one year revealed infection in only two (0.47%) animals [29]. Investigating enteropathogens by PCR in diarrheic female calves under three months of age in Shahrekord suburb dairy husbandries revealed higher prevalence rates for *Salmonella* (36.6%) and *Escherichia coli* (24.4%) than other characterized pathogens [30].

The reported prevalence rates of *Salmonella* infection in various districts of Iran are different. This difference can be due to several reasons, including improper keeping conditions of calves, non-observance of hygienic standards in farms, and lack of effective prevention programs, including vaccination of pregnant cows and colostrum intake. In addition, variations in antibiotic susceptibility profiles of *Salmonella* isolates might be due

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to the prevalence of a specific strain in the region, use of similar and repetitive antibiotic regimens, as well as the lack of complete and correct antibiotic treatment choices in affected farms [25, 31, 32].

The present study results showed that the infection rate of *Salmonella* in the industrial farms of Hamadan was relatively high, and this frequency was higher than in other regions of Iran. Therefore, it is necessary to take more efficient control measures to prevent economic and health losses due to *Salmonella* infection. On the other hand, since 100% of the isolates were resistant to some commonly used antibiotics, performing antibiotic susceptibility tests to determine the drug of choice for treatment is highly recommended.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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