Detection of virulence and antibacterial resistance genes in Salmonella isolates from diarrhoeic dogs in Iran

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SUMMARY
This study investigated the presence of some virulence and antibacterial resistance genes in Salmonella isolates from diarrhoeic dogs in Iran. Seventy diarrhoeic dogs presented to the Islamic Azad University Veterinary Teaching Hospital (IAUVTH), Iran, were sampled purposively. Rectal swabs were collected and cultured for isolation and identification of Salmonella using standard biochemical and serotyping methods. Polymerase chain reaction (PCR) was used to detect 3 virulence genes: invA, invF and sitC, and 12 antibacterial resistance genes which encode for resistance to β-lactam (blaTEM and blaSHV), tetracycline (tetA and tetB), trimethoprim (dfrA1 and dfrB1), aminoglycosides (aac(3)-Ia and aac(3)-IIa), sulfonamide (sul1 and sul2), and chloramphenicol (cat1 and cat2). Of 70 rectal swabs cultured, 13 (18.6%) were positive for Salmonella. Of the 13 positive samples/isolates, all (100%) were positive for invA while 3 (23.1%) were positive for invF. Of the isolates was positive for sitC. Out of the 13 isolates, 11 (84.6%) were positive for antibacterial resistance genes while 2 (15.4%) were negative for all the tested resistance genes. Of the 11 positive isolates, 7 (63.6%) were positive for blaTEM and blaSHV, 4 (36.4%) for aac(3)-Ia and aac(3)-IIa, 8 (72.7%) for tetA and tetB, 3 (27.3%) for dfrA1, dfrB1, sul1 and sul2 while 1 (9.1%) of the isolates was positive for cat1 and cat2. The isolates exhibited 5 multidrug resistant genotype patterns. Three (27.3%) of the isolates exhibited blaTEM+blaSHV+tetA+tetB, blaTEM+blaSHV+aac(3)-IIa+aac(3)-Ia and tetA+tetB+cat1+cat2 patterns while 1 (9.1%) of the isolates exhibited blaTEM+blaSHV+aac(3)-IIa+aac(3)-Ia+tetA+tetB and tetA+tetB+cat1+cat2 patterns. This study has shown that salmonellae associated with canine diarrhoea in Iran harbour several virulence and antibacterial resistance genes.

Keywords: Salmonella, virulence, antibacterial resistance genes, diarrhoeic, dogs, Iran.

RÉSUMÉ
Détection de la virulence et des gènes de résistance antibactérienne sur des isolats de Salmonella provenant chiens diarrhéiques en Iran

Cette étude a examiné la présence de gènes de virulence et de résistance antibactérienne sur des isolats de Salmonella provenant chiens diarrhéiques en Iran. Soixante-dix chiens diarrhéiques présentés à l’hôpital universitaire d’enseignement vétérinaire islamique Azad (IAUVTH), Iran, ont été échantillonnés à cet effet. Des écouvillons rectaux ont été recueillis et cultivés pour l’isolement et l’identification de Salmonella en utilisant des méthodes biochimiques et un sérotypage standard. La technique PCR a été utilisée pour détecter les 3 gènes de virulence (inv A, inv F et sit C) et 12 gènes de résistance aux antibactériens qui codent pour la résistance à la β-lactame (blaTEM et blaSHV), à la tétracycline (tet A et tet B), au triméthoprime (dfrA1 et dfrB1), aux aminoglycosides (aac(3)-Ia et aac(3)-IIa), aux sulfamides (Sul1 et Sul2) et au chloramphénicol (cat1 et cat2). Sur les 70 prélèvements rectaux cultivés, 13 (18,6%) étaient positifs pour Salmonella. Sur les 13 échantillons positifs / isolats, tous (100%) étaient positifs pour inv A tandis que 3 (23,1%) étaient positifs pour inv F. Aucun des isolats n’était positif pour sit C. Sur les 13 isolats, 11 (84,6%) étaient positifs pour tous les gènes de résistance antibactérienne tandis que 2 (15,4%) étaient négatifs pour tous les gènes de résistance testés. Parmi les 11 isolats positifs, 7 (63,6%) étaient positifs pour inv A, 4 (36,4%) pour aac(3)-Ia et aac(3)-IIa, 8 (72,7%) pour tet A et tet B, 3 (27,3%) pour dfrA1, dfrB1, sul1 et sul2 et 1 (9,1%) a été positif pour cat1 et cat2. Les isolats présentaient 5 modèles de génotype multi résistants.

Mots-clés: Salmonelles, virulence, résistance antibactérienne, diarrhée, chiens, Iran.

Introduction

Salmonella, a member of the family Enterobacteriaceae is distributed worldwide, and has been implicated in diarrhoea of animals including companion animals [33, 44, 45]. Pathogenicity of Salmonella isolates has been attributed to the presence of pathogenicity island 1 (SPI-1) in all Salmonella serovars [21]. The SPI-1 contains plasmids in which virulent genes such as invasion gene A (invA) which encodes for type 3 secretion system protein is located [21]. Other virulent genes such as invF, and Salmonella iron transporter gene C (sitC), one of the genes encoding for iron acquisition are also not uncommon in Salmonella serovars [37, 41, 42].

Frequent use of antimicrobial agents such as β-lactams, aminoglycosides, potentiated sulfonamides and tetracyclines in animals and humans, led to selection pressure and development of resistance in bacterial organisms by acquisition of resistance genes [18]. Reports have shown that companion animals are potential reservoirs of salmonellae harbouring antimicrobial resistance genes [18, 33, 44]. These genes encode for resistance to numerous antimicrobial agents resulting in compromise to antibacterial therapy and hence increased virulence [18, 33]. Faecal shedding of Salmonella by healthy carrier animals constitutes an important source of environmental contamination [5, 26]. Animals with clinical conditions such as diarrhoea usually have immune suppression which favours faecal shedding of Salmonella [33, 38].

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Diarrhoeic animals defaecate frequently and uncontrollably, thus they tend to spread Salmonella more than the non-diarrhoeic animals [33]. Faecal shedding of salmonellae harbouring virulent and antibacterial resistance genes by animals pose serious health threat to other animals and humans following zoonotic transmission of the organisms [16, 18, 33]. Diarrhoeic companion animals are particularly important in transmission of Salmonella since humans especially the veterinarians and pet owners have direct close contact with them [18, 26, 29]. The general public are often infected with the organisms when they make indirect contact with the primary contacts and/or diarrhoeic animals via ingestion of contaminated vegetation [44]. By horizontal transfer, enteric bacterial organisms in infected individuals easily acquire resistance genes from infective organisms since these genes are located on plasmids, transposons and other mobile genetic elements [10]. Therefore, diarrhoeic companion animals play significant role in dissemination of salmonellae harbouring antibacterial resistance genes; and subsequently this affects the ecology of antimicrobial resistance [18, 33].

Antibacterial resistance genes housed by a bacterial organism determines its phenotypic resistance to antibacterial agents. Often times, bacterial organisms may not exhibit phenotypic resistance to a particular antibacterial agent despite the presence of the gene encoding for it. This situation may result in treatment failure and further complicate antibacterial therapy. Thus, detection of antibacterial resistance genes is usually more useful in empirical treatment of bacterial-associated infections.

In available literature, majority of studies on Salmonella in companion animals were done in non-diarrhoeic dogs [1, 2, 7, 17, 23-25, 37, 38]. Very few studies on Salmonella have been conducted in diarrhoeic dogs [20, 33]. Only a study [2] investigated the presence of some virulence genes in the Salmonella isolates, but none investigated the presence of any antimicrobial resistance gene in the isolates. Moreover, the only study on Salmonella isolates from dogs in Iran is that of Zahraei Salehi et al. (2013) [44]. The study reported the genetic relatedness and antimicrobial resistance phenotype of Salmonella isolates from clinically healthy Shepherd dogs reared in Garmasar province. No study has been conducted to detect virulence and/or antimicrobial resistance genes in Salmonella isolates from any diarrhoeic companion animal in Iran. The objective of this study therefore was to detect the presence of some virulence and antimicrobial resistance genes encoding for resistance to the commonly used antimicrobials in Salmonella isolates from diarrhoeic dogs in Iran.

Material and Methods

SAMPLING

This cross-sectional study was conducted between February and April, 2014. A total of 70 household diarrhoeic dogs of varied breed, sex and ages presented to IAUVTH, Iran, for diagnosis and treatment were sampled. Prior to administration of any drug, rectal swab was collected from the dogs using sterile swab sticks. The swabs were transported aseptically in ice-packs to the Veterinary Microbiology Laboratory, Shahrekord Branch, Islamic Azad University, Iran and processed within 6 hours (hr) of collection.

ISOLATION AND IDENTIFICATION OF SALMONELLA ISOLATES

The swabs were inoculated into buffered peptone water for pre-enrichment, incubated at 37°C for 24 hr aerobically. Enrichment of the BPW culture was done by inoculating a loopful into Rapaport-Vassiliadis and subsequently into tetrathionate broth and incubated at 42°C for 18 hr. Then, a loopful from the tetrathionate broth culture was sub-cultured onto each of xylose lysine tergitol 4 (XLT4) (Merck, Germany), brilliant green sulfa (BGS) (Merck, Germany) and bismuth sulphate (BS) (Merck, Germany) agar, incubated at 37°C for 24 hr. Typical presumptive Salmonella colonies of each isolate were then sub-cultured on Mac Conkey agar (MCA) (Merck, Germany), incubated at 37°C for 24 hr. Three non-lactose-fermenting colonies of each isolate were then sub-cultured onto tryptic soy agar (TSA) (Merck, Germany), incubated at 37°C for 18 hr. Identification of the isolates as Salmonella was done by biochemical tests (urease and triple sugar iron agar tests) and serotyping of the somatic (O) and capsular (Vi) antigens using Salmonella O antiserum poly A-1 following standard procedures.

DETECTION OF VIRULENCE GENES IN THE SALMONELLA ISOLATES

DNA of the isolates was extracted using bacteria DNA extraction kit (Cinagen, Tehran, Iran) following the manufacturer’s instructions. The presence of 3 virulence genes: invA, invF and sitC were investigated using specific primers designed as previously described (Table I). Positive controls from the collection of the Shahrekord Branch, Islamic Azad University, Iran, were included in each PCR reaction, while sterile distilled water was used as the negative controls. Analysis of the PCR products was performed in 1.5% horizontal agarose gel electrophoresis stained with ethidium bromide under UV light.

DETECTION OF ANTIBACTERIAL RESISTANCE GENES IN THE SALMONELLA ISOLATES

The presence of antimicrobial resistance genes encoding for β-lactam (blaCMY-2 and blaCMY-3), aminoglycoside (aac(3)-Ia and aac(3)-IIa), tetracycline (tetA and tetB), trimethoprim (dhfr1 and dhfrII), sulfonamide (sulI and sulII) and chloramphenicol (cat1 and cat2) resistance were investigated by PCR using specific primers designed based on GenBank accession numbers (Table II). Positive controls were obtained from the same source as above. Sterile distilled water was used as the negative controls. Analysis of the PCR products was also performed as above.

DATA ANALYSIS

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Data generated were subjected to descriptive statistics using Microsoft Excel version 2010 and expressed in percentages.

Results

Isolation Rate of Salmonella and Occurrence of Virulence Genes in the Isolates

Out of 70 rectal swabs cultured, 13 (18.6%) yielded positive growth of Salmonella. Of the 13 Salmonella isolates, all (100%) were positive for PCR of invA while 3 (23.1%) were positive for PCR of invF. None of the isolate was positive for PCR of sitC.

OCCURRENCE OF ANTIBACTERIAL RESISTANCE GENES IN THE ISOLATES

Out of the 13 isolates, 11(84.6%) were positive for PCR for antibacterial resistance genes while 2 (15.4%) were negative for PCR of all the tested resistance genes. Of these 11 positive isolates, 7 (63.6%) were positive for PCR of β-lactam resistance genes (blaCMY-2 and blaCMY-9), 4 (36.4%) for aminoglycoside resistance genes (aac(3)-Ia and aac(3)-IIa),

<table>
<thead>
<tr>
<th>Virulence factor</th>
<th>Target virulence gene</th>
<th>Primers Sequence</th>
<th>Amplicon size (base pair)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasion factor F</td>
<td>invF</td>
<td>F: 5’- AAGGGATCCATGTCATTTTTCGGAAGCCGACAC- 3’  R: 5’- GGTAGGAAACGTGTTCCAGTATG- 3’</td>
<td>918</td>
<td>[11]</td>
</tr>
<tr>
<td>Invasion factor A</td>
<td>invA</td>
<td>F: 5’- CTGCGCGGGGTTTGTTCCTTTCTATT- 3’  R: 5’- GTTTTTCCGCCCTCTTCATGCGCTACC- 3’</td>
<td>284</td>
<td>[41]</td>
</tr>
<tr>
<td>Salmonella iron transporter C</td>
<td>sitC</td>
<td>F: 5’- CAGTATATGCTCAACGGCGATGTGGGTCTCC - 3’  R: 5’- CGGGGCGAAAATAAAAGGCTGTGATGAAC - 3’</td>
<td>250</td>
<td>[41]</td>
</tr>
</tbody>
</table>

Table I: PCR primers used for detection of Salmonella virulence genes.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Target resistance gene</th>
<th>Primers Sequence</th>
<th>Amplicon size (base pair)</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactam</td>
<td>blaCMY-2</td>
<td>F: 5’- TGGCCGTTCCGTTATC- 3’  R: 5’- CCCGTTGTCGCACCCATGA- 3’</td>
<td>870</td>
<td>X91840</td>
</tr>
<tr>
<td></td>
<td>blaCMY-9</td>
<td>F: 5’- CTGCGGGGTTGTTGTTCCTTTCTATT- 3’  R: 5’- GTTTTTCCGCCCTCTTCATGCGCTACC- 3’</td>
<td>874</td>
<td>AB04958</td>
</tr>
<tr>
<td>Aminoglycoside</td>
<td>aac(3)-Ia</td>
<td>F: 5’- TGAGGGCTGCTCTTGATCTT- 3’  R: 5’- ATCTCGGGCGAAAGGTGTTCCACCTTTG- 3’</td>
<td>436</td>
<td>X15852</td>
</tr>
<tr>
<td></td>
<td>aac(3)-IIa</td>
<td>F: 5’- CGGCCTGCTGAATCTGTTCCACCTTTG- 3’  R: 5’- AAAGCCGACGACCTTTTGTC- 3’</td>
<td>439</td>
<td>X15343</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>tetA</td>
<td>F: 5’- GCGCGCTTCTTCTTGTTGTTGTGTTGC- 3’  R: 5’- CCACCCCGGCACCCGTGTTAATGC- 3’</td>
<td>831</td>
<td>X00006</td>
</tr>
<tr>
<td></td>
<td>tetB</td>
<td>F: 5’- CCCGCGCTTCTTGTTGTTGC- 3’  R: 5’- CCACCCAGCCGTCAGGGTGTTAATGC- 3’</td>
<td>723</td>
<td>V00611</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>dhfrI</td>
<td>F: 5’- CAGTGGGATGTTCCAAGGTAAGTTG- 3’  R: 5’- CTTAGGATGTTCCAAGGTAAGTTG- 3’</td>
<td>220</td>
<td>A832145</td>
</tr>
<tr>
<td></td>
<td>dhfrII</td>
<td>F: 5’- CAGTGGGATGTTCCAAGGTAAGTTG- 3’  R: 5’- CTTAGGATGTTCCAAGGTAAGTTG- 3’</td>
<td>194</td>
<td>A083409</td>
</tr>
<tr>
<td>Sulfonamide</td>
<td>sulI</td>
<td>F: 5’- CAGTTCGGGAGCCTCTTTCTTC- 3’  R: 5’- CAGTTCGGGAGCCTCTTTCTTC- 3’</td>
<td>331</td>
<td>X15024</td>
</tr>
<tr>
<td></td>
<td>sulII</td>
<td>F: 5’- CCTGTTGTTGCTCCGACACAAG- 3’  R: 5’- GAAGCCGACGCCCAGGGAATTTATG- 3’</td>
<td>435</td>
<td>M36657</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>cat1</td>
<td>F: 5’- CCTGTTGTTGCTCCGACACAAG- 3’  R: 5’- ATCCCAATGGGATGTTCCAAGGTAAGTTG- 3’</td>
<td>508</td>
<td>M64281</td>
</tr>
<tr>
<td></td>
<td>cat2</td>
<td>F: 5’- CAGCAGATGGAACCTTTGATTTG- 3’  R: 5’- ATCCCAATGGGATGTTCCAAGGTAAGTTG- 3’</td>
<td>547</td>
<td>A401047</td>
</tr>
</tbody>
</table>

Table II: PCR primers used for detection of antibacterial resistance genes.
8 (72.7%) for tetracycline resistance genes (tetA and tetB), 3 (27.3%) for trimethoprim (dhfrI and dhfrII) and sulfonamide (sul1 and sul2) resistance genes, and 1 (9.1%) of the isolates was positive for the PCR of chloramphenicol resistance genes (cat1 and cat2) (Table III). The isolates exhibited 5 multidrug resistant genotype patterns (Table IV). Three (27.3%) of the isolates exhibited bla<sub>CMY-2</sub> + <br> + tetA + tetB, <br> + aac(3)-Ia + aac(3)-Ia and tetA + tetB + dhfrI + dhfrII + sul1 + sul2 patterns while 1 (9.1%) of the isolates exhibited <br> + dtfrI + dtfrII + sul1 patterns 

and 0.09% Salmonella isolation rate in apparently healthy dogs in Iran [44], Turkey [25], Trinidad [38] and Japan [17], respectively. The higher Salmonella isolation rate in this study may be because all the sampled dogs (70) were diarrhoeic. Dogs in this study might have had immune suppression resulting in higher Salmonella shedding [38] than the apparently healthy dogs in the previous studies. It might also be that there was better environmental standard, lower level of contamination of kennel, food and water sources [20, 22] in the other study areas. The age, sex and breed of the dogs sampled in the various study areas could also have affected the isolation rates, since Salmonella shedding have been reported to be more in young and female dogs which usually have weaker immune response [20, 38]. The focus of this study however was not the predisposing factors for Salmonella shedding, but on isolation of the organisms from diarrhoeic dogs.

The 18.6% isolation rate in this study is however lower when compared with 23.5 and 43.7% Salmonella isolation rate in apparently healthy dogs in Sudan [24] and Nigeria [20], respectively. Variations in Salmonella isolation rate in the study areas could also be due to the reasons mentioned above. Management conditions might have been better in

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Tested genes encoding for resistance</th>
<th>Number (Percentage) of isolate positive (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>tetA + tetB</td>
<td>8 (72.2)</td>
</tr>
<tr>
<td>β-lactam</td>
<td>bla&lt;sub&gt;CMY-2&lt;/sub&gt; + bra&lt;sub&gt;CMY-9&lt;/sub&gt;</td>
<td>7 (63.6)</td>
</tr>
<tr>
<td>Aminoglycoside</td>
<td>aac(3)-Ia + aac(3)-Ia</td>
<td>4 (36.4)</td>
</tr>
<tr>
<td>Sulphonamide</td>
<td>sul1 + sul2</td>
<td>3 (27.3)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>cat1 + cat2</td>
<td>1 (9.1)</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>dhfrI + dhfrII</td>
<td>3 (37.3)</td>
</tr>
</tbody>
</table>

Table III: Occurrence of antibacterial resistance genes among Salmonella isolates from diarrhoeic dogs

<table>
<thead>
<tr>
<th>S/N</th>
<th>Resistance genotype pattern</th>
<th>Frequency</th>
<th>Percentage of isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>bla&lt;sub&gt;CMY-2&lt;/sub&gt; + bra&lt;sub&gt;CMY-9&lt;/sub&gt; + tetA + tetB</td>
<td>3</td>
<td>27.3</td>
</tr>
<tr>
<td>2</td>
<td>bla&lt;sub&gt;CMY-2&lt;/sub&gt; + bra&lt;sub&gt;CMY-9&lt;/sub&gt; + aac(3)-Ia + aac(3)-Ia</td>
<td>3</td>
<td>27.3</td>
</tr>
<tr>
<td>3</td>
<td>tetA + tetB + dhfrI + dhfrII + sul1 + sul2</td>
<td>3</td>
<td>27.3</td>
</tr>
<tr>
<td>4</td>
<td>bla&lt;sub&gt;CMY-2&lt;/sub&gt; + bra&lt;sub&gt;CMY-9&lt;/sub&gt; + aac(3)-Ia + aac(3)-Ia + tetA + tetB</td>
<td>1</td>
<td>9.1</td>
</tr>
<tr>
<td>5</td>
<td>tetA + tetB + cat1 + cat2</td>
<td>1</td>
<td>9.1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>11</td>
<td>100</td>
</tr>
</tbody>
</table>

Table IV: Frequency of multidrug resistance genotype patterns exhibited by the Salmonella isolates.
the present study area than in those with higher *Salmonella* isolation rates. Hence the lower *Salmonella* isolation rate recorded in this study.

In this study, the presence of three virulent genes *invA*, *invF* and *sitC* often expressed by *Salmonella* was investigated in the isolates. All the virulence genes targeted in this study have previously been shown to be required for full *Salmonella* virulence in a murine model [30, 41]. The *sitC* gene is essential for iron acquisition [30, 47]. None of the isolate tested in the current study possessed *sitC*. This finding is unusual and contrasts previous studies [25, 30] that investigated the distribution of the genes in *Salmonella* isolates from animals which reported 85-100% *sitC* detection rate. This result may suggests that *sitC* could be located on virulence plasmids which may not always be present and the virulence plasmids could be serovar specific, and not all plasmid-bearing serovars contain these virulence plasmids [30]. The serotyping done in this study was not enough to identify the serovars of the isolates which may belong to the uncommon serovars that lack *sitC*. This finding need to be further verified. Although the presence of other genes (such as *iroN*) encoding for iron acquisition [30, 41] was not verified in this study, it is possible that the isolates possessed them since iron is a limiting nutrient for bacterial growth.

Detection of *invA* in all the isolates proved that they were *Salmonella* species [25, 30]. The *invA* gene contains sequences unique to the genus *Salmonella* and is considered the international standard for its identification [28]. The 100% detection of *invA* as against 23.1% *invF* detection among the isolates, suggests that *invA* is the predominant invasive gene harboured by salmonellae associated with diarrhoea in dogs reared in Iran. This finding is not uncommon as *invA* has been reported to be contained in a virulence plasmid located in SPI-1 which occur in all *Salmonella* [21]. Detection of *invA* gene is the standard for detection of invasion gene in salmonellae [15]. The *Salmonella* *invA* and *invF* genes encodes for a protein in type 3 secretion system in the inner membrane which helps the organism to invade the host epithelial cell [11, 21, 23]. The 100% *invA* detection in this study is similar to the report of Abatcha et al. (2014) [2] who also detected *invA* in 100% of *Salmonella* isolates from healthy dogs in Malaysia. Other studies also reported 100% detection of *invA* in *Salmonella* isolates from animals [25, 30].

Detection of antibacterial resistance genes in 11 (84.6%) of the isolates revealed that high percentage of *Salmonella* associated with canine diarrhoea in Iran habours antibacterial resistance genes. This finding is of public health importance since humans are in close contact with these dogs. Bacterial flora in humans could acquire these genes from the isolates [18]. The consequence is compromise in antibacterial therapy in these individuals [18, 33]. Tetracycline resistance genes (*tetA* and *tetB*) were detected in 72.7% of the positive isolates. This result is not uncommon as *tetA* and *tetB* has been noted to be widely distributed among *Salmonella* isolates of animal origin [30, 34, 35, 39]. This suggests that the genes are the most predominant antibacterial resistance genes haboured by salmonellae associated with canine diarrhoea in Iran. Tetracycline is a broad spectrum antibacterial agent commonly used in treatment of animals including dogs. Thus the isolates could have acquired these genes from other enteric bacteria in the dogs via horizontal transfer of plasmids and transposons [10]. The *tetA* and *tetB* encode energy-dependent membrane-associated efflux proteins [10, 30, 36]. It is possible that in addition to *tetA* and *tetB*, the tetracycline-resistant gene positive isolates in this study possess mechanisms of tetracycline resistance other than efflux proteins. Other tetracycline-resistant genes are thought to confer resistance through ribosomal protection and enzymatic inactivation [10, 30]. However, the presence of these other genes was not verified in this study.

High rate (63.6%) of detection of both *bla*<sub>CMY-2</sub> and *bla*<sub>CMY-9</sub> suggests that high percentage of the *Salmonella* isolates associated with canine diarrhoea in Iran habour several genes encoding for β-lactam resistance. The *bla*<sub>CMY-2</sub> has been reported as the commonest gene that encodes for AmpC β-lactamases in *Salmonella* isolates from food-producing animals [6, 19, 43, 46]. The *bla*<sub>CMY-9</sub> has not been reported in any *Salmonella* isolates from companion animals, but Bortolaia et al. (2014) [6] reported that 86% of *Escherichia coli* isolates from dog in Western USA haboured the gene. Significantly too, detection of *bla*<sub>CMY-9</sub> in *Salmonella* isolates in this study might be the first report on occurrence of this gene in *Salmonella* isolates from companion animals. The gene has been reported in Klebsiella and *E. coli* isolates from humans [12].

The findings of this study is important because *bla*<sub>CMY-2</sub> and *bla*<sub>CMY-9</sub> encode for CMY-2 and CMY-9 β-lactamases, respectively, which mediate resistance to cephemycins and third-generation cephalosporins especially ceftriaxone, a drug commonly used for treatment of clinical salmonellosis in humans [6, 13, 43]. It has also been reported that CMY-2 may mediate resistance to carbapenems (last resort drugs) in combination with loss of outer membrane porins [6]. Therefore, the presence of these β-lactamases encoding genes in the isolates obtained in this study portends serious public health threat because of close contact of humans with the dogs. The *bla*<sub>CMY</sub> genes are plasmid-encoded in enteric bacteria and is often associated with transposons/intergrons which makes it to spread easily among enteric bacterial organisms [14, 19, 27]. Thus, transmission of the salmonellae from the diarrhoeic dogs to humans would result in transfer of these genes to the bacterial population in infected individuals. Consequently, this would lead to compromise in antibacterial therapy and further dissemination of the resistance genes.

The presence of the *bla*<sub>CMY</sub> genes could explain the multidrug resistance genotype patterns observed in this study [9, 12]. The fact that all the isolates haboured 2 or 3 of the tested genes encoding for resistance to different
classes of antibacterial agents, implies that they exhibited multidrug resistance genotype patterns. The multidrug resistance genotype patterns $bla_{CMY-1} + bla_{CMY-9} + tetA + tetB$, $bla_{CMY-2} + bla_{CMY-9} + aac(3)-Ia + aac(3)-IIa$ and $tetA + tetB + dhfrI + dhfrII + sul1 + sul2$ were most predominant having occurred in 3 (27.3%) of the positive isolates. It has been reported that chromosomal Salmonella genomic island 1 contains genes encoding for tetracycline, aminoglycosides, amphenicols and trimethoprim resistance [2, 14, 31]. Therefore, the genes encoding for tetracycline and aminoglycoside resistance in the isolates could have been chromosomally-encoded. This may also explain for the $bla_{CMY-2} + bla_{CMY-9} + aac(3)-Ia + aac(3)-IIa + tetA + tetB$ and $tetA + tetB + cat1 + cat2$ patterns observed in 1 (9.1%) of the positive isolates.

Interestingly, $tetA + tetB$ were present in 4 of the 5 resistance genotype patterns shown by the isolates. This could reflect high rate of spread and/or acquisition of genes encoding for tetracycline resistance among salmonellae colonizing dogs in Iran. Aminoglycosides particularly streptomycin is often combined with β-lactam especially penicillin to exert a broad spectrum activity for treatment of dogs in Iran. This could also explain the moderate to high acquisition and detection of $aac(3)$-Ia and $aac(3)$-IIa genes encoding for aminoglycoside resistance in 36.4% of the isolates. The $aac(3)$ genes in Salmonella genomic island encodes for aminoglycoside acetyltransferases which mediate resistance to gentamicin, kanamycin and tobramycin [2, 40]. Therefore, the high detection rate of these genes in this study may also be due to frequent use of gentamicin in treatment of bacterial infection in the study area. This could have necessitated acquisition of the $aac(3)$ genes at a high rate.

It is of interest also that genes encoding for trimethoprim ($dhfrI$ and $dhfrII$) and sulfonamides ($sul1$ and $sul2$) resistance were both detected in 3 (27.3%) of the positive isolates. This finding could be related to the fact that sulfonamide-trimethoprim combination is commonly used in small animal practice [4] including in the study area. Thus the 3 isolates were the same that harboured both genes encoding trimethoprim and sulfonamide resistance as revealed by the resistance genotype pattern. In Salmonella, $sul1$ and $sul2$, encode forms of dihydropteroate synthase that are not inhibited by the drug [14].

In the present study, $cat1$ and $cat2$ which encode chloramphenicol acetyltransferases which are among the enzymes that mediate chloramphenicol resistance in Salmonella [8, 31], was detected in 1 (9.1%) of the isolates which suggests that they are the least predominant antibacterial resistance genes harboured by salmonellae associated with canine diarrhea in Iran. This finding may be due to the fact that chloramphenicol has long been banned for treating animals and humans. Therefore, there may not have been need for the isolates to acquire the $cat$ genes.

In conclusion, this study has shown that Salmonella is shedded by a sizeable percentage (18.6%) of diarrhoeic dogs in Iran. A high percentage (84.6%) of the Salmonella isolates harboured some virulence and antibacterial resistance genes. The isolates are potential reservoirs and sources of transfer of antibacterial resistance genes especially the β-lactamase encoding genes $bla_{CMY-1}$ and $bla_{CMY-9}$ to other bacteria organisms. This study might be the first report on detection of $bla_{CMY-9}$ in Salmonella isolates from companion animals. The presence of the antibacterial resistance genes in the isolates portends serious threat to public health when the organisms are shedded in faeces since the dogs are in close contact with humans. The findings of this study would be vital in devising strategies for mitigation of spread of antibacterial resistance and empirical treatment of dogs in the study area. Further characterization of the isolates to verify the absence of $sitC$ and to detect some other virulence and antibacterial resistance genes is recommended.

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Conflict of interest

The authors acknowledge no conflict of interest in this study.

References

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