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Prevalence assessment of *Salmonella* serovars in apparently healthy pet dogs in Tehran, Iran

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ABSTRACT

Salmonellosis is considered to be a zoonotic disease, the transmission of which through oral-fecal contact is unavoidable because pet care has been popular recently. On the other hand, excessive use of human antibiotics to treat animals resulted in the emergence of antibiotic-resistant *Salmonella* serotypes. This study aimed to assess the prevalence of bacteria and antibiotic resistance to select the appropriate antibiotic for disease control. In this study, the presence of *Salmonella* serovars in the fecal samples of 256 pet dogs was investigated by enrichment and selective culture. Moreover, the existence of virulence and antibiotic resistance genes, as well as phenotypic antimicrobial resistance, were assessed. Of the total of 256 fecal samples, 21 samples (8.2%) of pet dogs were positive for *Salmonella*, including S. *Typhimurium*, S. *Enteritidis*, S. *Infantis*, and S. *Senftenberg*. Based on our findings, all serovars carried virulence genes *invA, invF, sitC, fimA* and *S. Typhimurium* resistant to ampicillin (100%), tetracycline (50%), oxytetracycline (75%), florfenicol (50%) and lincospectin (100%). While *S. enteritidis*, *S. infantis,* and *S. senftenberg* were sensitive to ampicillin, amikacin, gentamicin, and ciprofloxacin. *S. Infantis* was also sensitive to all antibiotics. In conclusion, our findings suggest that pet dogs are potential sources of *Salmonella* strains that carry resistance and virulence genes. Thus, healthy pet dogs could play an important role in human salmonellosis.

Keywords

antibiotic resistance gene, pet dog, Salmonella, virulence gene

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Abbreviations

S. Typhimurium: Salmonella typhimurium
S. Infantis: Salmonella infantis
S. Enteritidis: Salmonella enteritidis
S. Senftenberg: Salmonella senftenberg

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Introduction

In developed countries, dogs are one of the most popular pet animals and the relationship between humans and pets has changed dramatically [1]. Direct contact of dogs and humans with food and feces transmits bacteria to humans that pose a greater potential risk to children than adults [2]. One of the most important zoonotic bacteria is *Salmonella* [2, 3]. *Salmonella* is a gram-negative bacterium that includes endotoxins, enterotoxins, siderophores, flagella, and virulence plasmids. In humans and animals, this bacterium can cause gastroenteritis, pneumonia, abortion, and lethal sepsis. The *Salmonella* genus contains more than 2659 serovars [4].

Dogs are generally resistant to infection and and serve as carriers for human salmonellosis without any clinical symptoms. Indeed, most of antibiotic resistance genes identified in human infection are correlated with dog salmonellosis [5, 6]. On the other hand, pet dogs could be an important source of antibiotic-resistant serovars. Therefore, these animals are considered a public health, particularly for children, the elderly, and immunocompromised individuals [7-9].

Salmonella serovar prevalence in dogs is influenced by several variables: First, environment, if they contact wild animals or infected animals. Second, raw food was already reported to have a high-risk factor in *Salmonella* serovars prevalence. Third, in microbiota alteration, normal microbiota could inhibit the gut tract from pathogen colonization while microbiota change can provide an environment for pathogen replacement [3, 10, 11].

Generally, *Salmonella* virulence factors, such as the invA, invF, sitC, and fimA, are chromosomal, while antibiotic resistance genes are located on plasmids. For example, β-lactams, aminoglycosides, tetracyclines, trimethoprim, and sulfonamide resistance-related genes (*blaCMY-2, blaCMY-9, aac(3)-Ia, aac(3)-IIa, tetA, tetB, dhfrI, dhfrII, sulI, sulII*) [12-15].

Salmonella is of high importance in public health and human diseases. Furthermore, the desire to have pet dogs is increasing in Iran. however, no recent research has evaluated the prevalence of *Salmonella* serovars in healthy dogs in Iran.

Therefore, the aim of this study was the assessment of the presence of *Salmonella* serovars in healthy pet dogs in Tehran, Iran. Moreover, the virulence factors and antibiotic resistance genes (mentioned above) were also evaluated.

Results

The prevalence of Salmonella serovars in healthy pet dogs

Isolation of *Salmonella* serovars was confirmed based on the cultural and biochemical methods. Out of the specimens collected from 256 dogs, 21 samples (8.2%) were positive for *Salmonella* (17 samples from Tehran University Veterinary Hospital and 4 samples from Khavarmiane Veterinary Hospital).

Isolated *Salmonella* serovars were serotyped with O and H antisera. Serotyping revealed four differents serovars: *S. Typhimurium* (n = 4); *S. Infantis* (n = 4); *S. Enteritidis* (n = 10) and *S. Senftenberg* (n = 3) (Table 1).

Detection of Salmonella virulence genes

The results of PCR amplification of the extracted DNA from 21 isolates on *invA, invF, sitC,* and *fimA* virulence genes showed that all samples (100%) had *invA* gene. Moreover, *invF, sitC,* and *fimA* genes were detected in 19 samples (90.47%). All virulence genes were detected in *S. Typhimurium* and *S. Infantis* (Table 2). All the samples of *S. enteritidis* serovar showed all virulence genes except one which was *sitC*-negative. In the *S. Senftenberg* serovar, two isolates were positive for *sitC* and one was positive for *invF* and *fimA* virulence genes (Table 3).

Antibiotic resistance genotype

The results of the detection of antibiotic resistance genes are shown in Table 4. The prevalence of antibiotic resistance genes was examined in different strains. All isolates of *S. Typhimurium* were positive (100%) for *blaCMY-2, tet A,* and *sul I*. For the other genes, fewer isolates were positive. Furthermore, in *S. infantis*, the most prevalent resistance genes were *blaCMY-2, aac(3)-Ia, dhfrI, sul II,* and *tet A*, while the least prevalent genes were *sulI, dhfr II, tet B,* and *aac(3)-IIa*. In *S. enteritidis*, the most prevalent resistance genes included *aac(3)-IIa, tet B,* and *dhfrII*, while *tetA* and

Table 1.
Salmonella serovars isolated from dogs (n=21)

Salmonella Serovar	serogroup	H1	H2	number	percentage (%)
Salmonella typhimurium	*B (1,4,5,12)*	i	1,2	4	19.04
Salmonella infantis	*C1(6,7)*	b	1,2	4	19.04
Salmonella enteritidis	*D (1,9,12)*	g.m	---	10	47.61
Salmonella senftenberg	*E4(1,3,19)*	g.s.t	---	3	14.28
Total				21	

Table 2.
Distribution of the virulence genes (n=21)

Virulence gene	number	percentage (%)
invA	*21*	100
invF	*19*	90.47
SitC	*19*	90.47
fimA	*19*	90.47

Table 3.
Presence of virulence genes in *Salmonella serovars*

Salmonella Serovars	virulence genes (%)			
	invA	invF	sitC	fimA
Salmonella typhimurium	4 (100%)	4 (100%)	4 (100%)	4 (100%)
Salmonella infantis	4 (100%)	4 (100%)	4 (100%)	4 (100%)
Salmonella enteritidis	10 (100%)	10 (100%)	9 (90%)	10 (100%)
Salmonella senftenberg	3 (100%)	1 (33.33%)	2 (66.66%)	1(33.33%)

aac(3)-Ia had a low prevalence. The lowest abundance of *blaCMY-2, blaCMY-9, aac(3)-IIa, tet B, dhfrI,* and *sulII* genes were detected in *S. Septenberg.*

Antimicrobial resistance phenotypes

According to the results, *S. Typhimurium* was resistant to ampicillin (100%), Tetracycline (50%), Oxytetracycline (75%), Florfenicol (50%), and Lincospectin (100%). On the other hand, all isolates belonging to *S. Enteritidis, S. Infantis* and, *S. Senftenberg* were sensitive to Ampicillin, Amikacin, Gentamicin, and Ciprofloxacin. *S. Infantis* was also sensitive to all antibiotics (Table 5).

Table 4.
Distribution of the antimicrobial resistance genes in *Salmonella* serovars

Salmonella Serovars	Antimicrobial resistance genes									
	blaC-MY-2	*blaC-MY-9*	*aac(3)-Ia*	*aac(3)-IIa*	*tetA*	*tetB*	*dhfr I*	*dhfr II*	*Sul I*	*Sul II*
Salmonella Typhimurium	*4(100%)*	3(75%)	3(75%)	0(0%)	4(100%)	2(50%)	3(75%)	1(25%)	4(100%)	0(0%)
Salmonella Infantis	*4(100%)*	2(50%)	3(75%)	1(25%)	3(75%)	2(50%)	4(100%)	1(25%)	2(50%)	3(75%)
Salmonella Enteritidis	*9(90%)*	6(60%)	6(60%)	4(40%)	5(50%)	6(60%)	7(70%)	4(40%)	6(60%)	9(90%)
Salmonella Senftenberg	*0(0%)*	0(0 %)	2(66.6%)	0(0 %)	2(66.6%)	1(33.3%)	2(66.6%)	1(33.3%)	2(66.6%)	2(66.6%)

Discussion

Salmonella is one of the main causes of food poisoning, diarrhea, and gastroenteritis in humans [16]. Acute gastroenteritis is one of the most prevalent diseases in regions with low public health [17]. Salmonellosis is known as a common disease between humans and animals. Since keeping pets, especially dogs has become popular in recent years, the possibility of disease transmission through regular contact with feces (fecal-oral transmission) of animals is inevitable. In Iran, a significant percentage of gastroenteritis in children is related to *Salmonella* [18-20]. In recent years, the incidence of non-typhoid *Salmonella* has increased dramatically due to the emergence of many *Salmonella* serotypes [21, 22].

The *Salmonella* serovars have been isolated from 0%-79% of healthy pet dogs in diverse regions of the world [5, 6, 23, 24]. There are few studies on the infection of dogs with *Salmonella* in Iran. The first study in Tehran on outdoor dogs was carried out by Shimi et al. in 1976, it was shown that 15.8 % of dogs were infected with the serotypes of *Salmonella Derby* and *Newport* [25]. Zahraei Salehi et al. in 2013 found that 10.5% of dogs in Garmsar region were infected with *S. Reading* serotype [26]. Nimrodi et al. investigated dog feces specimens from ten rural areas of Mazandaran, Iran, and reported that 50%, 35%, and 15% of the isolates were *S. Enteritidis, S. Typhimurium*, and *S. Dublin*, respectively. The most frequent serovar in the latter study was *S. Enteritidis* [27]. In the present study, the prevalence of *Salmonella* serovars was 8.2% in Tehran. Four serovars were isolated, with *S. Enteritidis* (47.61%) and *S. Typhimurium* (19.04%) predominating as the major serovars associated with human disease. This difference in the prevalence of *Salmonella* first can be due to geographical variation [5, 23, 28] and then differences in the sample sizes, fe-

Table 5.
Antimicrobial resistance /susceptibility phenotypes of isolated *Salmonella* serovars

Antimicrobials	strains	*S. Typhimurium*			*S. Infantis*			*S. Enteritidis*			*S. Senftenberg*		
	No. of strains	4			4			10			3		
		S	I	R	S	I	R	S	I	R	S	I	R
AM		0	0	4	4	0	0	10	0	0	3	0	0
FOX		4	0	0	4	0	0	10	0	0	3	0	0
CPM		4	0	0	4	0	0	10	0	0	3	0	0
CEF		4	0	0	4	0	0	10	0	0	3	0	0
GEN		4	0	0	4	0	0	10	0	0	3	0	0
AMK		4	0	0	4	0	0	10	0	0	3	0	0
T		1	1	2	2	2	0	9	0	0	0	0	3
OTC		0	1	3	4	0	0	0	1	9	0	0	3
DOX		3	1	0	4	0	0	9	1	0	1	2	0
FLO		0	2	2	4	0	0	10	0	0	1	0	2
LS		0	0	4	4	0	0	9	0	1	3	0	0
ENR		4	0	0	4	0	0	10	0	0	3	0	0
CIP		4	0	0	4	0	0	10	0	0	3	0	0
TS		4	0	0	4	0	0	9	0	1	2	0	1

S denotes susceptible, I denotes intermediate resistance, and R denotes resistance. Antimicrobials: AM (ampicillin), FOX (Cefoxitin), CPM (cefepime), CEF (Ceftiofur), GEN (Gentamicin), AMK (Amikacin), T (Tetracycline), OTC (Oxytetracycline), DOX (Doxycycline), FLO (Florfenicol), LS (Lincospectin), ENR (Enrofloxacin), CIP (Ciprofloxacin), TS (Trimethoprim-Sulfamethoxazole).

cal sampling conditions, and isolation and detection methods employed. There have been many reports of different *Salmonella* serotypes being isolated worldwide from the feces of healthy dogs. About 53 serotypes were isolated, most of which were related to *S. Typhimurium, S. Anatum, S. Panama, S. Krfeld, S. Bronx, S. Newport, S. Indiana, S. Kentucky, S. Saintpaul,* and *S. Virchow* [29, 30]. Unlike developing countries where the pet dogs are fed a commercial diet, the main dog food in Iran is cooked homemade food, such as rice and chicken. Nadi, et al. in a study on 1425 stool samples (obtained from *Salmonella* outbreaks, 2013-2019) revealed that *S. Enteritidis* and *S. Senftenberg* were major Salmonellosis agents in Iran with frequencies of 26.3% and 21.3%, respectively [31]. A study conducted by Chantharothaiphaichit that healthy household dogs multidrug-resistant *Salmonella Enterica* [32].

In the world, as well as in Iran, *S. Enteritidis* is the major salmonellosis agent with the food source [33, 34]. Also, several studies have shown that a Salmonellosis agent was detected in cooked poultry and cooked meat [35, 36]. According to previous studies, food is one of the main sources of *Salmonella* infection in pet dogs, which can infect humans. Moreover, in our research, all isolates were positive for *invA* virulence gene. This gene is an international standard for identifying *Salmonella* (Malorny, Hoorfar, Bunge, & Helmuth, 2003). A previous study in Iran reported that the frequency of virulence genes in 13 positive *Salmonella* samples was reported as follows: *invA* (100%), *invF* (23.1%), and *sitC* (0%). However, due to the lack of serotyping, these results are not reliable [13]. In England and Iraq, all isolates carried *sitC* and *fimA* [37,38] which is consistent with our finding.

Diarrhea is the most common symptom of human salmonellosis [39,40]. Therefore, the assessment of antibiotic resistance to *Salmonella* serovars in dogs is especially important. The genotype and phenotype of antibiotic resistance of serovars have been investigated in our research. All isolates of *S. Typhimurium* were resistant to third-generation Ampicillin. We also found that *S. Typhimurium, S. Enteritidis,* and *S. Senftenberg* were resistant to the Tetracycline group except for *S. Infantis*. Several studies have shown that Tetracycline/Oxytetracycline resistance in *Salmonella* serovars is common [40,41]. Fortunately, The first antibiotic choice for non-typhoid salmonellosis in humans is ciprofloxacin [42], to which all isolates were susceptible in the present study. Similar results were

reported with our study on *Salmonella* isolates from around the world [31, 43-45]. In conclusion, in the present study, it was shown that *S. Enteritidis,S. Typhimurium, S. Infantis,* and *S. Senftenberg* are the main serovars respectively in apparently healthy pet dogs in Tehran. The prevalence of *Salmonella* in the feces of pet dogs was evaluated to be 8.2%. Food is a possible contamination source in dogs. Isolated serovars have the potential to cause infection in humans. In this study, despite resistance to some antibiotic susceptibility to antimicrobials of choice for the treatment of human salmonellosis detected. Thus, our finding provided promising information on the prevalence of *Salmonella* serovars and their antibiotic resistance in pet dogs which can contaminate their owners. Regular monitoring of pet dogs can play an important role in controlling human Salmonellosis.

Materials & Methods

Sample collection

All animals were handled according to animal care rules of the Faculty of Veterinary Medicine, University of Tehran, Tehran. In this study, we used 206 fecal samples of pet dogs (age under 4 years, during 2000-2001) collected from the small animal hospital of Teheran University and 50 samples (age under 4 years, during 2020-2021) from Khavarmiane Veterinary Hospital, Tehran. The health conditions of the animals were checked and they did not show any specific symptoms of the disease. Rectal swabs were collected and transported under refrigeration to the microbiology laboratory of the Faculty of Veterinary Medicine, University of Tehran.

Salmonella serovars isolation and serotyping

Salmonella isolation was using a standard method (ISO 6579: 2002). Briefly, each rectal swab was enriched for 24 h at 37 °C in 1:10 vol/vol buffered Peptone water 2.5% (Merck, Germany). Then, 100 µl of the culture suspension was spotted on MacConkey agar (Merck, Germany) and incubated at 37 °C for 24 h. Next, Colonies were selected for inoculation onto Salmonella Shigella agar (SS agar, Merck, Germany) at 37 °C for 24 h. *Salmonella* suspicious colonies were biochemically confirmed by applying oxidase and catalase tests, triple sugar iron agar (TSI) test and IMViC group tests. After biochemical confirmation, the isolates were serotyped by specific antisera according to the manufacturer's instructions (BD Difco, USA).

DNA extraction

The *Salmonella* serovars DNA was extracted via the boiling method and the DNA samples were stored at -20 °C until analysis [46].

Primers

In this study, 14 primers were purchased from the Sina Clone company (Tehran, Iran). Four virulence-related genes, including invA, invF, sitC, and fimA (Table 6) and ten antibiotic resistance genes were examined and confirmed at NCBI and Primer-BLAST sites (Table 7).

Conventional PCR Assays

The PCR was run in 25 µl reaction mixture using the PCR master mix (Amplicon, Denmark). A total volume of 25 µl of reaction mixture contained 1µM primer, 3 µl template DNA, 7.5 µl sterile distilled water, and 12.5 µl master mix. Initial denaturation for detecting *invA, invF, sitC,* and *blaCMY-9* genes was performed at 94 °C for 5 min followed by 34 cycles of amplification. The amplification cycle included the following 3 steps: 94 °C for 1 min (denaturation), 60 °C for 1 min (annealing), and 72 °C for 1 min (extension). The polymerase chain reaction for other genes was similar to the previous steps except that the annealing temperatures for *fimA, aac(3)-Ia, dhfrI,* and *dhfrII* genes was 55 °C for 1 min, for *blaCMY-2* was 56 °C for 1 min, *aac(3)-IIa* 52 °C for 1 min, and for *tetA, tetB, sulI*, and *sulII* genes was 72 °C for 1 min. After 34 amplification cycles, the samples were retained at 72 °C for 5 min to ensure complete strand extension. The standard strain of *Salmonella* (microbial collection of the Faculty of Veterinary Medicine, Tehran university) was used as positive control and distilled water was used as negative control.

PCR Product analysis

Analyzing the PCR products completed by using 1% agarose gel-stained with 0.5 µg/mL ethidium bromide. The PCR products were visualized by a UV transilluminator and photographed using a digital camera.

Antibiotic susceptibility

The antibiotic susceptibility of all isolates was tested according to the Clinical and Laboratory Standards Institute protocols [47]. The antibiotics selected to test Salmonella serovars. include Ampicillin (10 µg), Cefoxitin (30 µg), Cefepime (30 µg), Ceftiofur (30 µg), Gentamicin (10 µg), Amikacin (30 µg), Tetracycline (30 µg), Oxytetracycline (30 µg),

Table 6.
Primers used for the detection of *Salmonella* virulence genes

Virulence factor	Target virulence gene	Sequence 5' to 3'	Product size (bp)	References
Invasion factor F	*inv*F	F: AAGGGATCCATGTCATTTTCTGAAAGCGACAC R: GTTGTAGGGAAAGCTTCTCCAGTAATG	918	[13]
Invasion factor A	*inv*A	F: GTG AAA TTA TCG CCA CGT TCG GGC AA R: TCA TCG CAC CGT CAA AGG AAC C	284	[35]
Salmonella iron transporter C	*sit*C	F: CAGTATATGCTCAACGCGATGTGGGTCTCC R: CGGGGCGAAAATAAAGGCTGTGATGAAC	250	[13]
fimbrial protein A	*fim*A	F: CCT TTC TCC ATC GTC CTG AA R: TGG TGT TAT CTG CCT GAC CA	85	[35]

Doxycycline (30 µg), Florfenicol (30 µg), Lincospectin (100 µg), Enrofloxacin (5 µg), Ciprofloxacin (5 µg), and Trimethoprim-Sulfamethoxazole (240+52 µg). The antibiotic discs were purchased from Padtan Teb company, and zone diameters were assessed and categorized as susceptible, intermediate, or resistant according to company guideline tables.

Table 7.
Primers used for the detection of antibacterial resistance genes in *Salmonella serovars*

Antimicrobial agent	Target resistance gene	Sequence 5' to 3'	Product size (bp)	References
β-lactam	*blaCMY-2*	F: TGGCCGTTGCCGTTATCTAC R: CCCGTTTTATGCACCCATGA	870	[13]
	blaCMY-9	F: TCAGCGAGCAGACCCTGTTC R: CTGGCCGGGATGGGATAGTT	847	
Aminoglycoside	*aac(3)-Ia*	F: TGAGGGCTGCTCTTGATCTT R: ATCTCGGCTTGAACGAATTG	436	[13]
	aac(3)-IIa	F: CGGCCTGCTGAATCAGTTTC R: AAAGCCCACGACACCTTCTC	439	
Tetracycline	*tetA*	F: GCGCCTTTCCTTTGGGTTCT R: CCACCCGTTCCACGTTGTTA	831	[13]
	tetB	F: CCCAGTGCTGTTGTTGTCAT R: CCACCACCAGCCAATAAAAT	723	
Trimethoprim	*dhfrI*	F: CGGTCGTAACACGTTCAAGT R: CTGGGGATTTCAGGAAAGTA	220	[13]
	dhfrII	F: AGTTTGCGCTTCCCCTGAGT R: CTTAGGCCACACGTTCAAGTG	194	
Sulfonamide	*sulI*	F: TCACCGAGGACTCCTTCTTC R: CAGTCCGCCTCAGCAATATC	331	[13]
	sulII	F: CCTGTTTCGTCCGACACAGA R: GAAGCGCAGCCGCAATTCAT	435	

Authors' Contributions

AAK, RY, and TZS conceived and designed research. AAK, RY, TZS, IAT, and BB conducted experiments. AAK, BB, RY, and TZS analyzed data. AAK and BB wrote the manuscript. RY and TZS edited the manuscript. All authors read and approved the manuscript.

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Competing Interests

The authors declare that they have no conflict of interest.

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