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Prevalence of Tetracycline Resistance Genes among Clinical Isolates of *Salmonella enterica*

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ABSTRACT

Aims The use of antibiotics in food-producing animals has elevated concerns regarding their potential affect on human health. Resistant Salmonella may be transmitted through the food chain to humans. The aim of this study was to determine the prevalence of tetracycline resistance genes among tetracycline-resistant Salmonella enterica from Iran.

Materials & Methods In this experimental study, A total of 4369 stool specimens were collected via rectal swab from hospitalized children under the age of 5 with watery diarrhea, with or without blood, mucus and stomach cramps. Antimicrobial susceptibility profiles of Salmonella isolates were performed and Minimum inhibitory concentration (MIC) of tetracycline was assessed. Bacteria were grown on blood agar at 37°C overnight, and genomic DNA was extracted. For evaluating of PCR products used of 1.5% agarose gel in TBE buffer at for 80min.

Findings High level of resistance was observed against minocycline (78.5%), tetracycline (76.0%), nalidixic acid (66.6%) and streptomycin (42.0%). The MIC for 46.0% of the isolates was 256µg/ml, while 15.6% showed the MIC of 128µg/ml and the remaining revealed 64µg/ml MIC to tetracycline. Among the 33 tetracycline-resistant isolates the tet(A) or tet(B) genes were detected in 10 (23.8%) and 5 isolates (11.9%), respectively. The tet(A)and tet(B) genes were identified in 2 out of the 42 tetracycline-resistant Salmonella isolates (4.8%). The tet(C) or tet(D)genes were not found among tetracycline-resistant isolates.

Conclusion Resistance to Salmonella strains is increasing. The predominant tetracycline-resistant gene is tet(A) followed by tet(B).

Keywords *Salmonella enterica*; Antimicrobial Resistance; Tetracycline

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[1] General outbreaks of infectious intestinal disease ... [2] An Outbreak of Salmonella typhimurium infections in ... [3] A regional outbreak of S. Enteritidis phage type 5, traced back to the flocks of an egg ... [4] Host transmission of Salmonella enterica serovar typhimurium is controlled by virulence factors ... [5] Natural antibiotic susceptibility of Salmonella enterica ... [6] Characterization of seven outbreaks of hemorrhagic hepatopathy syndrome in commercial pullets following the administration of a Salmonella enteritidis ... [7] Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial ... [8] Trends in antibiotic resistance among diarrheal pathogens isolated in Thailand ... [9] Supplement 2002 (no. 46) to the Kauffmann-White ... [10] Simple and rapid detection of Salmonella strains by direct PCR amplification ... [11] Genetic characterization of antimicrobial resistance in Canadian isolates of Salmonella serovar ... [12] High prevalence of integron-mediated resistance in clinical isolates of Salmonella ... [13] Characterisation of antimicrobial resistance patterns and class 1 integrons among ... [14] Multidrug resistance in Salmonella enterica serovar typhi isolated from patients with typhoid fever ... [15] Antimicrobial resistance in Salmonella spp. recovered from patients admitted to six ... [16] Susceptibility of Escherichia coli and Enterococcus faecium isolated from pigs and broiler ... [17] Antimicrobial susceptibility and occurrence of resistance genes among Salmonella ... [18] Salmonella enteritidis and antibiotic resistance patterns ... [19] Characterization of the first extended-spectrum ... [20] Quinolone resistance among Salmonella enterica and Escherichia coli of animal ... [21] Prevalence and characterization of Salmonella infantis isolates ... [22] Detection and characterization of extended-spectrum ... [23] An outbreak of multidrug-resistant ... [24] Ciprofloxacin-resistant Salmonella enterica serotype ... [25] Epidemiological characteristics and molecular typing of Salmonella ... [26] Salmonella typhi in the democratic republic of the congo ... [27] Is it time to change fluoroquinolone breakpoints for ...

Introduction

The genus Salmonella is included of over 2,400 different serotypes that infect a wide range of hosts, including poultry, cattle, rodents and humans. *S. enterica* serovars are well host-adapted and causes a wide range of diseases from self-limiting gastroenteritis to life-threatening bacteremia and enterocolitis. Diarrheal illnesses caused by Salmonella spp remain a serious public health issue in industrializing countries, and is still a significant cause of morbidity and mortality in developed as well as undeveloped countries. The use of antibiotics in food-producing animals has elevated concerns regarding their potential effect on human health. Resistant Salmonella may be transmitted through the food chain to humans [1, 2]. A transmission cycle of *S. enteric* between environmental sources, vegetable food- or animal-feed plants, animals, food and humans, has been recognized [3, 4]. Salmonella spp. show the remarkable ability (intrinsic, acquired, and adaptive) to resist a wide spectrum of antimicrobial agents including ampicillin, broad-spectrum cephalosporins, aminoglycosides, quinolones, trimethoprim and chloramphenicol, and resulting in widespread nosocomial outbreaks in hospital throughout the world [5, 6]. Moreover, the widespread and empirical use of broad-spectrum antibiotics in human, agricultural and aqua cultural settings has led to the emergence of multidrug resistant (MDR) Salmonella spp. that presents a major challenge to clinical therapy and contributes significantly to increased morbidity and mortality.

Tetracycline resistance in Salmonella is attributed to production of an energy-dependent efflux pump, is encoded by the tet genes in Salmonella genomic island I including tet(A), tet(B), tet(C), tet(D), and tet(G) to remove the antibiotic from within the cell [7, 8]. Other mechanism of resistance, such as modification of the ribosomal target and enzymatic inactivation of tetracycline, have been documented in other bacterial species but have yet to be reported in Salmonella [7].

The aim of this study was to determine the prevalence of tetracycline resistance genes among tetracycline-resistant *Salmonella enterica* from Iran.

Material and Methods

Bacterial strains: In this experimental study, A total of 4369 stool specimens were collected via rectal swab from hospitalized children under the age of 5 with watery diarrhea, with or without blood, mucus and stomach cramps. Each specimen inoculated into Cary Blair transport medium and incubated at 41.5+1˚C for 18-24h. Enriched broth cultures were then plated on selective agar plates, including Salmonella-Shigella agar and xylose-lysine-deoxycholate agar (Merck; Hamburg; Germany) media and incubated at 37˚C for 18-24h. Presumptive Salmonella colonies were subcultured, and identity was confirmed with API 20E strips according to the manufacture's specification (bioMérieux Vitek, Inc.; Hazelwood; MO). A practical slide agglutination test, with commercial antisera was performed antisera according to the Kauffmann-White scheme [9] for serological typing of Salmonella spp. Finally, isolates were confirmed by PCR amplification targeting the invasion gene regulator (hilA) gene (Table 1) [10, 11].

Table 1) Characteristics of primers used in PCR amplification

Gene	**Primer sequence** (5' to 3')	**Amplicon size** (bp)	**Annealing temp.** (°C)
hilA	F-GGAACGTTATTTGCGCCATGCTGAGGTAG R-GCATGGATCCCCGCCGGCGAGATTGTG	784	56
tetA	F-GTGAAACCCAACATACCCC R-GAAGGCAAGCAGGATGTAG	927	55
tetB	F-CCTTATCATGCCAGTCTTGC R-ACTGCCGTTTTTTCGCC	659	53
tetC	F-CTTGAGAGCCTTCAACCCAG R-ATGGTCGTCATCTACCTGCC	418	55
tetD	F-TGGGCAGATGGTCAGATAAG R-CAGCACACCCTGTAGTTTTC	787	53

Antimicrobial susceptibility: Antimicrobial susceptibility profiles of Salmonella isolates was performed by Kirby-Bauer disk diffusion method using Mueller-Hintonagar plates. The standard procedure of the Clinical and Laboratory Standards Institute (CLSI) were strictly followed throughout the testing procedure. *Escherichia coli* (ATCC 25922) was used for quality control in each run.

The antibiotics tested were (µg/disc): co-trimoxasol [12], gentamicin [13], tetracycline [14], minocycline [14], streptomycin [13], amoxicillin [12], ciprofloxacin [5] and nalidixic acid [14].

Minimum inhibitory concentration of tetracycline: Minimum inhibitory concentration (MIC) of tetracycline was assessed by disk diffusion and agar dilution methods and the resistance break points were specified in

accordance with the guidelines of the CLSI (M7-A7) guidelines.

Detection of tet determinants: Bacteria were grown on blood agar at 37°C overnight, and genomic DNA was extracted using High Pure Purification kit (Qiagen GmbH; Germany) according to the manufacturer's protocol for Gram-positive bacteria. The Concentration of DNA was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Del.; USA). Thermal cycler (Biorad T100; USA) according following protocol: 10min for initial denaturation at 95°C, In the following 30 cycle including denaturation for 1min at 95°C, annealing (annealing Tm for each primer is indicated in Table 1), extension for 1min at 72°C and a final incubation for 5min at 72°C. For evaluating of PCR products used of 1.5% agarose gel in Tris/Borate/EDTA (TBE) buffer at for 80min and then Gel Documentation system was used for visualizing of gel. A 50bp plus DNA ladder (fermentas) was used as a size reference (figure 1-4).

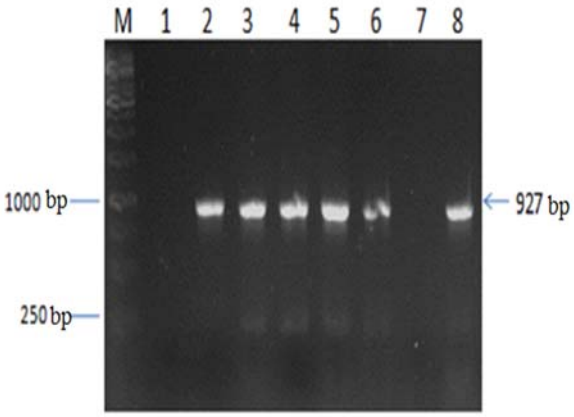

Figure 1) PCR detection of tet A

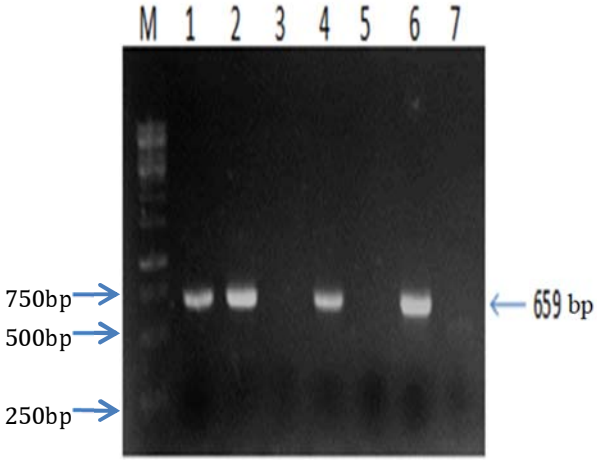

Figure 2) PCR detection of tet B

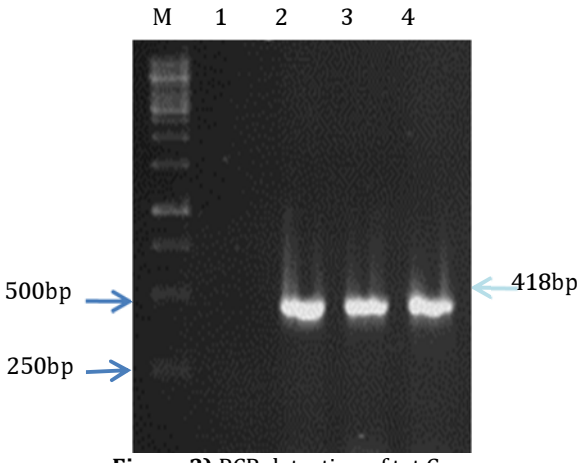

Figure 3) PCR detection of tet C

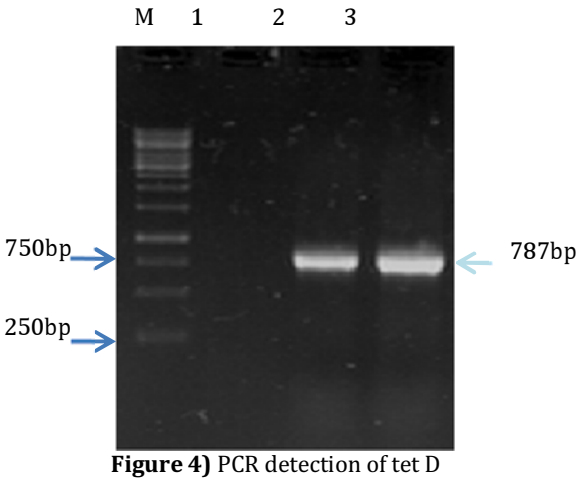

Figure 4) PCR detection of tet D

Findings

Serological typing of Salmonella spp.: 42 Salmonella spp. were isolated from stool samples of children with diarrhea. Identification of the 42 isolates showed that all of them were *S. enterica* and 6 (14.3%) of the total strains identified as *S. enterica* serogroup C, 2 (4.7%) of the isolates belong to serogroup B, 8 (19.04%) of the isolates belong to serogroup D and 26 (61.9%) of the total strains identified as S. enterica spp. based on serotyping with commercial antisera.

Percentage of distribution of antibiotic resistance among Salmonella isolates: Higher resistance was observed against minocycline (78.5%), tetracycline (76.0%), nalidixic acid (66.6%), and streptomycin (42.0%). The isolates showed lower resistance against amoxicillin (38.0%), co-trimoxazole (25.0%), and ciprofloxacin (7.0%). No isolate was found to be resistant against gentamicin.

Result of MIC among tetracycline resistant Salmonella isolates: MIC results of the tetracycline resistant isolates showed a high level of resistance (MIC≥16µg/ml) to tetracycline. The MIC for 46.0% of the isolates was 256µg/ml,

128µg/ml for 15.6% of the isolates and 64µg/ml for the rest.

Phenotypes of antibiotic resistance among the *S. enterica* isolates: In addition, 18 different phenotypes of antibiotic resistance were identified among the *S. enterica* isolates, 14 of them conferring resistance to at least three different families of antibiotics (Table 2).

PCR result: Regarding the PCR result, among the 33 tetracycline-resistant isolates the tet(A) or tet(B) genes were detected in 10 (23.8%) and 5 isolates (11.9%), respectively. The tet(A)and tet(B) genes were identified in 2 out of the 42 tetracycline-resistant Salmonella isolates (4.8%). The tet(C) or tet(D) genes were not found among tetracycline-resistant isolates.

Table 2) Antibiotic resistance phenotypes and genes detected in 42 resistant (to at least one antibiotic) Salmonella isolates

No. of isolates	Serotype	Antibiotic-resistance phenotype*	Resistance genes	MIC (µg/ml)
BS 1	spp.	SXT/NAL/MN/TET		64
BS 2	spp.	-		64
BS 3	spp.	STR/AMX/MN/SXT/TET		sensitive
BS 4	spp.	STR/NA/MN/SXT/TET		64
RS 1	spp.	STR/AMX/MN/SXT/TET	tet(B)	128
RS 2	spp.	STR/NAL/TET		64
RS 3	spp.	STR/AMX/NAL/MN/SXT/CIP/TET		256
RS 4	spp.	STR/AMX/MN/SXT/TET		sensitive
RS 5	spp.	-		256
RS 6	C	MN	tet(B)	64
RS 7	D	STR/AMX/MN/SXT/TET		256
RS 8	D	STR/AMX/MN/SXT/TET		256
RS 9	spp.	STR/NAL/MN/SXT/TET	tet(A)	64
RS 10	spp.	STR/AMX/NAL/MN/SXT/TET		256
RS 11	spp.	STR/AMX/NAL/MN/SXT/CIP/TET		sensitive
RS 12	C	STR/NAL/MN/SXT/TET	tet(A)	256
RS 13	D	STR/AMX/AUG/NAL/MN/SXT/TET		64
RS 14	D	TET	tet(A)	128
RS 15	B	-		256
RS 16	spp.	STR		sensitive
RS 17	spp.	STR/AMX/AUG/MN/SXT	tet(A), tet(B)	256
RS 18	spp.	MN	tet(A)	64
RS 19	spp.	STR/NAL/MN/SXT/TET		64
RS 20	spp.	STR/AMX/NAL/MN/SXT/CIP/TET		sensitive
RS 21	spp.	-		256
RS 22	spp.	STR/AMX/NAL/MN/SXT/CIP/TET/GM		sensitive
RS 23	spp.	NAL/MN/TET	tet(B)	256
RS 24	D	AMX/MN/TS/TET	tet(A),	256
RS 25	spp.	NAL/MN/TET		64
RS 26	C	AMX/NAL/MN/TE	tet(A), tet(B)	128
RS 27	spp.	NAL/MN/TET		sensitive
RS 28	D	NAL/MN/TET		256
RS 29	spp.	NAL/MN/TET	tet(A),	64
RS 30	D	AMX/MN/SXT/TET		256
RS 31	spp.	NAL/MN/TET		sensitive
RS 32	spp.	NAL/MN/TET	tet(A),	256
RS 33	B	AMX/NAL/TET		sensitive
RS 34	C	NAL/MN/TET		128
ses 1	D	NAL/MN/TET		64
ses 2	C	AMX/NAL/TET	tet(A),	256
ses 3	C	STR/NAL/MN/TET		sensitive
lasa	spp.	TET		128

*AMX: amoxicillin; AUG: amoxicillin-clavulanic acid; GM: gentamicin; CIP: ciprofloxacin; MN: minocycline; STR: streptomycin; TET: tetracycline; SXT: trimethoprim-sulfamethoxazole; NAL: nalidixic acid

Discussion

In the present study, we have shown the widespread occurrence of resistance to several groups of antibiotics in clinical isolates of *S. enterica*. The most frequent resistance phenotypes of the isolates detected were to minocycline, tetracycline, nalidixic acid, streptomycin, and amoxicillin. These results may not be unexpected, as high frequency of farmers or persons have been widely used antimicrobial drugs in clinics and animal foods resulted in the development of antimicrobial resistance. We acknowledge that our

antibiotic resistance data are contrary to a study [15], which showed that Salmonella clinical isolates have low levels of resistance to tetracycline. As tetracycline is widely used in food-producing animals as a growth enhancer in Iran and other countries resulted in the increase of tetracycline resistance of Salmonella isolates [15, 16].

In this study, high levels of resistance to nalidixic acid were also found in accordance with the previous studies in Iran [15], probably due to the use of these agents in the treatment of invasive gastrointestinal infections [17]. In addition, two of Salmonella isolates were resistant to ciprofloxacin whereas previous reports from Iran showed that all Salmonella isolates were susceptible to ciprofloxacin [12, 18, 19]. This result was probably associated with the use of ciprofloxacin in poultry farms in Iran for treatment and prophylaxis may have contribute in distribution of resistant isolates via the food chain [20]. Several studies have shown that *S. enterica* resistant to quinolones was found in healthy poultry [21, 22]. A previously study demonstrated that decreased susceptibility to ciprofloxacin in multidrug-resistant strain DT104 was related to mortality [23]. The high susceptibility rates to flouroquinolones are important as this antibiotic act as alternatives in the treatment of resistant cases [14]. Reduced resistance to ciprofloxacin was found comparatively lower as compared to 81% in the USA [24], 100% in Turkey [25] and 15.4% in the Democratic Republic of Congo [26]. Although previous reports have suggested that quinolones should not be used in the treatment of invasive Salmonella infections in order to avoid decreasing susceptibility to quinolones and diminish the risk of treatment failure [27] but these antibiotics and extended spectrum cephalosporins are the drugs of choice in treatment of invasive salmonellosis [13]. Thus, in order to avoid the emergence of multidrug resistant strains of Salmonella the use of antimicrobial drugs should be carefully monitored.

Among the tetracycline-resistant strains, the results showed that the predominant tetracycline-resistant gene was tet(A) followed by tet(B) that are in accordance with a previously report from Iran [15]. However, another study in Brazil demonstrated that among tetracycline-resistant strains tet(B) was the predominant tetracycline resistance gene, followed by tet(A) gene. But according to the MIC results, the presence of both genes tet(A) and tet(B) has increased MIC to a similar extent. In some isolates, although neither of these two genes was present, the MIC has increased, which can be due to the presence of other resistance genes in these isolates.

Taken together, these results highlight evidence that high number MDR strains of *S. enterica* isolates can confer a serious public health problem. We maintain optimism that the determination of multidrug resistant *S. enterica* isolates in human samples and different types of animals should eventually be developed toward reducing the potential transmission of these bacteria through the food chain.

It is necessary to design intervention approaches to control the spread of antibiotic-resistant Salmonella, and applicably manage the use of antibiotics in animal husbandry to inhibit the further development and spread of antibiotic resistance and protect food safety.

Conclusion

The predominant tetracycline-resistant gene is tet(A) followed by tet(B). Among the 33 tetracycline-resistant isolates the tet(A) or tet(B) genes are in 10 (23.8%) and 5 isolates (11.9%), respectively. The tet(A) and tet(B) genes are in 2 out of the 42 tetracycline-resistant Salmonella isolates (4.8%). The tet(C) or tet(D) genes do not exist among tetracycline-resistant isolates.

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Ethical Permissions: There are no ethical permissions.

Authors Contribution: Bahroudi M. has done all the affairs.

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