ANTIMICROBIAL SUSCEPTIBILITY AND RESISTANCE GENES IN *SALMONELLA ENTERICA* SEROVAR ENTERITIDIS ISOLATED FROM TURKEYS

Türkyılmaz, S.[1,3*], Hazımoğlu Ş.[2] and Bozdoğan B.[3]

Adnan Menderes Üniversitesi, Aydın, TURKEY

[1]Veteriner Fakültesi, Mikrobiyoloji AD
[2]Sağlık Bilimleri Enstitüsü
[3]ADU BILTEM Epidemiyoloji Birim

*Corresponding author: Süheyla Türkyılmaz, Tel.: +90 256 247 07 00, Fax: +90 256 247 07 20
E-mail: suhturkyilmaz@yahoo.com

ABSTRACT

Salmonella enterica subsp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis), a cause of food poisoning in humans was isolated from turkeys slaughtered in Izmir, Turkey. It was identified by classical techniques and polymerase chain reaction (PCR), Its antimicrobial susceptibility was determined, and the common resistance genes in antibiotic-resistant strains were demonstrated by PCR. Of the 587 cloacal swabs, 86 (14.7%) were *Salmonella* positive and of these, 11 (1.9%) were serovar Enteritidis. Antimicrobial susceptibilities were tested by agar dilution MICs. All strains were susceptible to cefoxitin, ciprofloxacin and amikacin. Of the 11 strains tested 9 (81.8%), 6 (54.6%), 6 (54.6%), and 3 (27.3%) were susceptible to gentamicin, ampicillin, chloramphenicol, and tetracycline respectively. Among 11 *S.* Enteritidis isolates 2 were susceptible to all antimicrobials tested, and 7 strains were multi-drug resistant. Of 8 tetracycline resistant strains, 5 and 3 carried *tet*(A), *tet*(B) genes, respectively. Of 5 ampicillin resistant strains, 3 had *bla*TEM, and 2 had *bla*OXA. Of 5 chloramphenicol-resistant strains one had *cat3*, 3 had *floR,* and known resistance genes were negative by PCR in one resistant strain. Of the 2 gentamicin-resistant strains, one had *aadB*, and one had *aacC* genes. This study verified the presence of *S.* Enteritidis among turkey flocks in Izmir. Antibacterial resistance among isolated strains was relatively high. Further surveys are necessary to detect presence of salmonellae in poultry and turkey meat products that could present a potential health threat.

Keywords: *Salmonella* Enteritidis, turkey, multiplex-PCR, antimicrobial susceptibility profiles, resistance genes

INTRODUCTION

Salmonella enterica subsp. *enterica* serovar Enteritidis (*Salmonella* Enteritidis) is an important cause of human salmonellosis and food poisoning (1). *S.* Enteritidis has become prevalent in humans and on poultry farms as a result of vertical and horizontal transmission in and between large poultry organisations (2).

Antimicrobial resistance is a growing public health problem which causes increased morbidity and mortality among humans and animals. Antimicrobials are used for treatment of infected animals , to protect them from infectious diseases, and to provide a faster growth rate. Overuse of antimicrobials may cause the selection, emergence and dissemination of resistant pathogens that are transmitted to humans through various routes, including the consumption of contaminated food. The resistance determinants of these organisms can be transferred also to the human gut bacterial flora, so becoming a reservoir of resistance genes for pathogenic bacteria. Treatments of infections due to resistant pathogens are more difficult and food borne diseases associated with antibiotic resistant bacteria have become a major public health issue (3, 4). Turkeys are a common source of *Salmonella* infection for the consumer. The isolation rates of *Salmonella* from turkeys and their environment are often higher than those from layers and broilers (5).

The pathogenic ability of *Salmonella* depends on many virulence factors which are either chromosomal or plasmidic. Among these virulence factors, the chromosomally located gene, *inv*A, encodes an inner membrane protein and provides the bacteria with the capability to invade epithelial cells as seen in cultured epithelial cells (6). There is little published information on the antimicrobial susceptibility profiles and antimicrobial resistance patterns of indigenous *S.* Enteritidis isolates. This information could help limit the spread of infections and provide data about the best choice for treatment.

Resistance genes of antibiotic-resistant *Salmonella* spp. isolated from food animals have not been studied in Turkey. The aim of this study was to isolate salmonellae from turkeys slaughtered in İzmir province, to identify *S.* Enteritidis by classical techniques and polymerase chain reaction (PCR), to determine antimicrobial susceptibility profiles and antimicrobial resistance genes in antibiotic-resistant isolates.

MATERIALS AND METHODS

Bacteria

A total of 587 cloacal swab samples were taken from turkeys at different ages (110-130 day old) from a turkey abattoir in Izmir province from March 2006 through March 2007. The cloacal swabs were transported in Carry-Blair transport medium (Oxoid) to the laboratory and cultured on the same day. All isolates identified as *Salmonella* were frozen for further studies. For all experiments, reference strain *S.* Typhimurium (ATCC 14028) served as positive control and quality control strains. *Escherichia coli* strain (ATCC 25922) and *Staphylococcus aureus* (ATCC 29213) as negative control were included.

Isolation and Identification

Salmonellae were isolated by standard methods (7). Nine ms of buffered peptone water (Oxoid) were inoculated with swabs. Samples were incubated for 18 h at 37°C, then 0.1 ml of the pre-enriched cultures were transferred to Rappaport-Vassiliadis (Oxoid) and Selenite broths (Difco) and incubated at 42 °C and 37 °C, respectively. After 24 and 48 h of incubation, 10µl of culture from each enriched broth was streaked on *Salmonella Shigella* (SS) agar (Difco) and xylose lysine deoxycholate (XLD) agar (Difco) and incubated at 37 °C for 24 h. The plates were examined for the presence of typical *Salmonella* colonies on SS and XLD agars (8). Suspected colonies were confirmed by conventional biochemical methods and also with a commercial kit (API 20E Kit System, Bio-Mérieux, France), following standard procedures for confirmation of genus level identifications. All colonies were tested serologically using specific *Salmonella* polyvalent O, monovalent O:9, and type-specific H antisera (Bio-Rad Laboratories, Inc. Richmond, CA, USA) (9).

Extraction of DNA, Primers and Multiplex PCR

For DNA extraction, a few colonies grown on selective agar was transferred to a 1.5 ml tube containing 100µl sterile lysis solution (1 ml 1M Tris-HCl, 22.5 µl IGEPAL CA-630 –Sigma-, 22.5 µl Tween 20 –Biorad-, 220 µl Protease K (10mg/ml), 8,8 µl DiH_2O). The tubes were vortexed and incubated for 10 min at 60 °C, 10 min at 95 °C and cooled to 4 °C.

Molecular identification was done by PCR. *Salmonella* spp. (*inv*A) and *S.* Enteritidis (*sefA*) specific primer sets used in the multiplex-PCR assay were designed by Cortez *et al*. The sequences of forward and reverse primers were as follows: *inv*A-1 (5'-TTGTTACGGCTATTTTGACCA-3') *inv*A-2 (5'-CTGACTGCTACCTTGCTGATG-3'), *sef*A-1 (5'-GCAGCGGTTACTATTGCAGC-3'), and *sef*A-2 (5'-TGTGACAGGGACATTTAGCG-3'), respectively. Multiplex PCR were done as described previously. This primer set generated amplicons of 521 bp and 330 bp, from *inv*A and *sef*A, respectively using the method described (10).

Detection of Minimal Inhibition Concentration

Minimal inhibitory concentration (MIC) tests were carried out on Mueller-Hinton agar (Acumedia, USA) containing serial two-fold serial dilutions of the antibiotics. Twelve concentrations of tetracycline, ampicillin, chloramphenicol, gentamicin, cefoxitin, ciprofloxacin, and amikacin ranging from 0.06 to 128 mg/L were tested. The inocula were prepared by diluting overnight Brain Hearth Infusion Broth (Difco, USA) cultures to yield ~10^4 cfu per spot before testing. The plates were spot-inoculated with a Steers-type multipoint inoculator (AQS Manufacturing, UK) and incubated for 18 h at 37°C. Breakpoints indicated by CLSI (11) used for determination of susceptibility and resistance were assessed daily. Quality control strains, were assessed daily (each strain three times a week) from pure subcultures, and the inhibition zones were within the limits as defined by the CLSI.

Resistance Genes

Common resistance genes were detected for tetracycline, ampicillin, chloramphenicol and gentamicin by PCR using the conditions described previously. All primers used are shown in Table 1 (12, 13, 14, 15, 16, 17).

RESULTS

Isolation, Identification and PCR

Salmonella strains were isolated from 86 out of 587 (14.7 %) cloacal swab samples. All strains were positive for the *inv*A gene , specific for *Salmonella* genus. Of 86 isolates, 11 (1.9 %) were positive for the *sef*A gene, and were designated as serovar Enteridis. The presence of these genes was tested by multiplex PCR as shown in Figure 1. These strains were also positive by *inv*A PCR

All 86 *Salmonella* isolates were positive by *Salmonella* polyvalent O antisera.. *Salmonella* isolates were tested with monovalent O:9 antisera and 11 isolates were positive, and all were in serogroup D_1. All 11 strains reacted also with "g,m" from phase 1 antisera and with "1,7" from phase 2 antisera in the agglutination test. Thus, the 11 strains had the following antigenic formula: "O9, 12:g,m:1,7" which corresponds to *S*. Enteridis serovar. These 11 isolates were also positive by *sef*A PCR.

Antimicrobial susceptibility profiles

All strains were susceptible to cefoxitin, ciprofloxacin and amikacin. Of 11 strains tested 9 (81.8%), 6 (54.6%), 6 (54.6%), and 3 (27.3%) were susceptible to gentamicin, chloramphenicol, ampicillin, and tetracycline respectively. Of the tetracycline resistant strains, 3, 1, 3, and 1 strain had MICs of 16, 32, 64 and 128 mg/L respectively. Five ampicillin resistant strains all had MICs 128 mg/L. Five were resistant to chloramphenicol with MICs 32 mg/L (4 strain), and 128 mg/L (1 strains). Two were resistant to gentamicin with MICs 16 mg/L (Table 2). The distribution of resistance and susceptibility rates of isolated *Salmonella* strains are shown in Figure 2. Of 11 strains 9 (81.8%) were antibiotic resistant isolates. Two strains were found to be resistant to only one antimicrobial, while 7 were multi-drug resistant. Two strains were susceptible to all antibiotics used.

Resistance genes

Presence of known resistance genes was studied among resistant strains. Of 8 tetracycline resistant strains, 5 and 3 carried *tet*(A), and *tet*(B) genes, respectively. Of 5 ampicillin resistant strains 3 had *bla*TEM and 2 had *bla*OXA. Of 5 chloramphenicol resistant strains one had *cat3*, 3 had *floR* .Known resistance genes were undetected by PCR in one resistant strain. Of the two gentamicin resistant strains, 1 had *aadB* and 1 had *aacC* genes (Table 2).

DISCUSSION

Salmonellae can be isolated from numerous animal species including turkeys because these strains are part of the normal flora in these species. For this reason the intestinal tract is the primary reservoir of zoonotic Salmonella. *S.* Enteritidis is now the most common cause of salmonellosis. Many studies show different rates of *Salmonella* isolates in cloacal swabs and stool samples in turkeys world-wide, with a wide range of rates reported. In some studies the rates are as low as 3.3% (18) and in others as high as 53.8 % (19). In Turkey only a few studies have shown the presence of *Salmonella* in turkeys, Aksakal *et al*. found isolation rates of 5% in eastern Turkey (20). In another study from north-eastern Turkey, no *Salmonella* was isolated from 42 samples (21). In this study, the isolation rate of *Salmonella* serovars from cloacal swabs was 14.7%. The differences in isolation rates may be due to differences in isolation and sampling methods used, and geographic characteristics of regions where the studies were done.

Increasing rates of antimicrobial resistance among bacteria including *Salmonella* is a growing health care problem that needs continuous survey. Antimicrobial resistance of *Salmonella* has double importance. Firstly for the treatment of poultry in which they cause infections, and also for human infections that they cause (1,2). One of the main factors for development of antimicrobial resistance is the over-use of antimicrobials. Of the most common antimicrobial resistance in *Salmonella*, quinolone, aminoglycoside, beta-lactam and tetracycline resistance can be cited. The resistance levels show wide differences among reports. Blackburn *et al*. reported that all *Salmonella* strains isolated from turkeys were resistant to gentamicin (22). Resistance rates were low among *Salmonella* isolated in Denmark. Pederson *et al*. stated that 1.7 %, 1.7 %, 8.7 %, and 9 % of strains isolated from Danish turkeys between 1995 and 2000, were resistant to gentamicin, trimethoprim/sulphamethoxazole, tetracycline and streptomycin, respectively (23). However high level resistance data were reported from Canada. Poppe *et al*. found that 25.8 %, 38 %, 14.3 %, 1.7 %, 2.4 %, and 27.7 % of isolated strains in Canada from turkeys were resistant to gentamicin, tetracycline, ampicillin, trimethoprim/ sulphamethoxazole, chloramphenicol, and kanamycin, respectively (24). Aksakal also reported that 20.0 %, 25.0 %, 35.0 %, 35.0 %, 65.0 % of *Salmonella* strains isolated from turkey stools in the Van province of Turkey were resistant to trimethoprim/sulphamethoxazole, ampicillin, gentamicin, tetracycline, and penicillin G, respectively (20). In the study of 11 *S.* Enteritidis strains tested 2 (18.2%), 5 (45.5%), 5 (45.5%), and 8 (72.7%) were resistant to gentamicin, chloramphenicol, ampicillin, and tetracycline respectively. None of the isolate was resistant to ciprofloxacin, cefoxitin and amikacin. Also an analysis of multidrug resistance levels was seen to be important among *Salmonella* in our study, since 7 (63.6%) strains were multi-drug resistant.

Tetracycline resistance genes were detected among all 8 tetracycline-resistant isolates. The distribution was as follows: 5 *tet*(A), 3 *tet*(B). Roberts reported *tet*(B) and *tet*(C) tetracycline resistance genes to occur most frequently in salmonellae (25). However Pierano *et al*. reported that they found 25 *tet*(A), 23 *tet*(D) and 10 tet(C) (26). The most common tetracycline resistance determinant was also *tet*(A) in chickens (27, 28, 29) as well as in humans (28). Tetracycline resistance was also the most common resistance in our study like previous reports. The high level resistance to tetracycline may be due to this antibiotic being one of the most commonly used antibiotic for animal production. Among 5 ampicillin resistant isolates, 3 contained *bla*TEM and 2

strains carried *bla*OXA. None of the isolates was positive for *bla*PSE-1. Resistance to ampicillin in *Salmonella* is usually mediated by TEM type β-lactamases which was the most common gene found among turkey isolates in the present study (26). Of the 5 chloramphenicol-resistant isolates, the resistance genes, *cat3* and *floR* were found in 1 and 3 isolates, respectively. One isolates were negative for *cat1*, *cat2* and *cat3* genes as well as for *floR* gene. Gentamicin resistance is usually due to inactivation of these antibiotics by enzymes. In this study of the 2 gentamicin-resistant isolates, 1 carried *aadB* and the other carried the *aacC* gene. In most studies *aadB*-associated gentamicin resistance is more common (30, 31).

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Table 1: Primers used in PCR for the detection of resistance genes

Gene	Primer*	Nucleotide Sequence (5' to 3')	Annealing Temperature	Resistance Mechanism	Size (bp)	Encoded Resistance**	Reference
tetA	F R	GCTACATCCTGCTTGCCTTC CATAGATCGCCGTGAAGAGG	55	Efflux	210	TET	13
tetB	F R	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG	53	Efflux	659	TET	13
tetC	F R	CTTGAGAGCCTTCAACCCAG ATGGTCGTCATCTACCTGCC	56	Efflux	417	TET	13
tetG	F R	GCTCGGTGGTATCTCTGCTC AGCAACAGAATCGGGAACAC	59	Efflux	468	TET	12
tetS	F R	CATAGACAAGCCGTTGACC ATGTTTTTGGAACGCCAGAG	58	Ribosomal protection	667	TET	17
*bla*TEM	F R	CATTTCCGTGTCGCCCTTAT TCCATAGTTGCCTGACTCCC	55	β-lactamase	793	AMP	16
*bla*PSE-1	F R	AATGGCAATCAGCGCTTCCC GGGGCTTGATGCTCACTACA	59	β-lactamase	586	AMP	12
*bla*OXA	F R	ACCAGATTCAACTTTCAA TCTTGGCTTTTATGCTTG	55	β-lactamase	590	AMP	14
aadB	F R	GAGCGAAATCTGCCGCTCTGG CTGTTACAACGGACTGGCCGC	61	Aminoglycoside adenyltransferase	319	GEN	16
aacC	F R	GGCGCGATCAACGAATTTATCCGA CCATTCGATGCCGAAGGAAACGAT	58	Aminoglycoside acetyltransferase	488	GEN	15
cat1	F R	CCTATAACCAGACCGTTCAG TCACAGACGGCATGATGAAC	56	Chlorampenicol acetyltransferase	491	CHL	16
cat2	F R	CCGGATTGACCTGAATACCT TCACATACTGCATGATGAAC	56	Chlorampenicol acetyltransferase	567	CHL	16
cat3	F R	CCCACAATTCACCGTATTCC GAACCTGTACTGAGAGCGGC	58	Chlorampenicol acetyltransferase	310	CHL	EU715370.1
floR	F R	AACCCGCCCTCTGGATCAAGTCAA CAAATCACGGGCCACGCTGTATC	60	Efflux	548	CHL	16

*F, forward; R, reverse;
**TET: Tetracycline, AMP: Ampicillin GEN: Gentamicin CHL: Chloramphenicol

Table 2: Distribution of resistant strains and resistance genes among S. Enteritidis strains

Drug**	No of isolates having MIC(mg/L)												Breakpoint (mg/L)	No of resistant isolates and (%)	Genes tested	No of isolates
	0,06	0,125	0,25	0,5	1	2	4	8	16	32	64	128				
TET	0	0	0	0	0	1	2	0	3	1	3	1	≥ 16	8 (72.7)	*tetA* *tetB* *tetC* *tetG* *tetS*	5 3 - - -
AMP	0	0	0	0	0	0	3	1	2	0	0	5	≥ 32	5 (45.4)	*bla*TEM *bla*OXA *bla*PSE-1	3 2 -
CHL	0	0	0	0	0	1	2	3	0	4	0	1	≥ 32	5 (45.4)	*floR* *cat3* *cat2* *cat1* unknown	3 1 - - 1
GEN	0	0	0	0	0	2	7	0	2	0	0	0	≥ 16	2 (18.2)	*aadB* *aacC*	1 1
CEF	9	2	0	0	0	0	0	0	0	0	0	0	≥ 32	0	-	
CIP	9	1	1	0	0	0	0	0	0	0	0	0	≥ 4	0	-	
AMI	0	0	0	6	4	1	0	0	0	0	0	0	≥ 64	0	-	

**TET: Tetracycline, AMP: Ampicillin, GEN: Gentamicin, CHL: Chloramphenicol, CEF: Cefoxitin, CIP:Ciprofloxacin, AMI: Amikacin

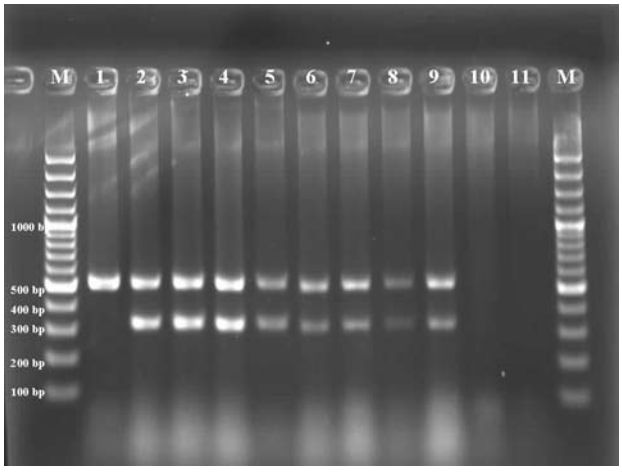

Figure 1.
Salmonella specific genes invA and S. Enteritidis specific sefA were determined by PCR (10). Salmonella spp. is all positive for invA and only Salmonella Enteritidis isolates were positive for sefA. Lane M = 100 bp Marker Lane 1 = Salmonella Typhimurium (positive control) Lanes 2-9 = sefA positive S. Enteritidis strains from our study. Lane 10. E. coli (negative control) Lane 11= Negative control without DNA.

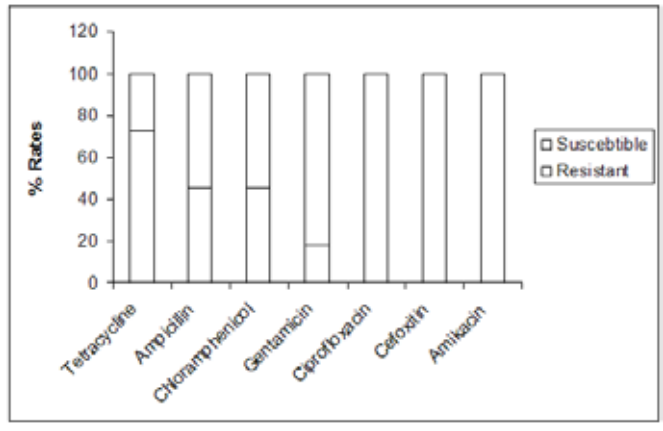

Figure 2.
The distribution of resistance and susceptibility rates of isolated S. Enteritidis strains