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Molecular Characterization of Extended- Spectrum β- Lactamase Resistance Genes among *Salmonella* Isolates from Poultry, Poultry Products and Human Patients in Saudi Arabia

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Thirty seven *Salmonella* isolates from poultry, poultry products and human patients in Saudi Arabia were examined for antimicrobial susceptibility and characterized by pulsed-field gel electrophoresis (PFGE) and plasmid profile. Cephalosporin-resistant isolates were examined for the presence of genes encoding β_lactamases by PCR and sequencing. Seven different PFGE types were observed. One type was recovered in all sources; two types were found only in human patients, two only in poultry , one only in poultry products, and one both in poultry and poultry products. Seven isolates were susceptible to all antimicrobial agents tested, whereas 29 were resistant to three or more antimicrobials. Most resistance was observed among the isolates from human patients. Of the 17 isolates from human patients, 12 displayed resistance to ampicillin and the cephalosporins ceftiofur and cephalothin. All 12 isolates tested negative for *bla*CMY-1, *bla*CMY-2, and *bla*ACC, but positive for *bla*SHV, of which five were sequenced to *bla*SHV-2. Plasmid profiling and hybridization revealed that the *bla*SHV gene was located on plasmids of approximately 70 kb. Five plasmid profiles were found among these 12 isolates. The plasmid profiling confirmed the PFGE-type and was able to further subdivide the strains nine of these 12 isolates contained also *bla*TEM, of which four representatives were sequenced to *bla*TEM-1H. One isolate contained *bla*CTX-M-15, *bla*SHV-2, and *bla*TEM-1H, with the *bla*CTX-M-15, and *bla*TEM-1H genes located on a 64-kb transferable plasmid. This study showed a high frequency of resistance among *salamonella.* isolated from humans and poultry, with a lower frequency in poultry and poultry products. The clonal relatedness among the isolates from three sources could indicate a recent spread of the isolates.

Key words: β- Lactamase Resistance Genes, *Salmonella* isolates, poultry products, patients.

Salmonella is one of the most common causes of human gastroenteritis worldwide. More than 2,500 different serovars of *S. enterica*have been identified, and most of them have been described as the cause of human infections; however, most reports have mentioned *S. enterica* sero var Typhimurium and *S. enteric* serovar Enteritidis as the most common causes of human salmonellosis worldwide[13,14,25]. However, in some regions, other serovars have been reported to be of even greater importance[2], and changes in the importance of different serovars over time seem to take place[2,25]. The occurrence of different serovars in one country can be of global importance because of travel and trade of breeding animals and food products worldwide. Knowledge regarding the occurrence and molecular epidemiology of different serovars in different countries and geographic regions may assist in the recognition and tracing of new emerging pathogens. *Salmonella* resistant to multiple antimicrobial agents have emerged worldwide. However, it is not resistanceto all antimicrobial agents that is of equal importance. Fluoroquinolones are in many cases the drug of

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choice for treatmentof gastrointestinal infections in humans. Thus, resistance to this class of antimicrobial agents is associated with increased mortality and morbidity[1,12] and thus especially unwanted. *Salmonella* isolates resistant tooxyiminocephalosporins due to the production of extended spectrum β lactamases (ESBLs) haveemerged worldwide since 1992.[3] this has also caused concern because cephalosporins are drugs of choice for the treatment of salmonellosis in children, to which fluoroquinolones must notbe administered. *S. enterica* serovarresistant to oxyiminocephalosporins due to the production of SHV-2 was reported[16] However, there isonly limited information on the occurrence of antimicrobial resistance and molecular diversity of *Salmonella* isolates. Applied genotypic typing methods. Pulsed-field gel electrophoresis (PFGE) has been widely used in the molecular epidemiological investigation of *Salmonella* spp[21]. The plasmid profiling is among methods used to determine and characterize possible genetic relationships between the different serovars of the genus *Salmonella*[30]. The main objectives of this study were to determine the genetic relatedness of isolates of *Salmonella* isolates from humans and poultry products. The potential of antibiogram, PFGE and plasmid analysis were examined to trace the clonal relationship and to discern the possible transmission of *Salmonella* isolates from different sources. Also this study was conducted to determine the occurrence of antimicrobial resistance and molecular variation of *Salmonella* from humans, poultry products,is Saudi Arabia . The implication of the findings in relation to global spread of new serovars is discussed.

MATERIALS AND METHODS

A total of thirty four isolates of *Salmonella* strains were collected from patients at the clinical microbiology laboratory from Riyadh Hospital in Riyadh, Saudi Arabia between 2011 and 2013.The isolates originated from humans patients[17], poultry[13] and from poultry products[7]. The isolates from poultry and poultry products were collected from different market in Riyadh, Saudi Arabia .The isolates were initially identified and serotyped in the originating sources under study.

Serotyping

Serotyping of *Salmonella* sp. isolates was performed on the basis of somatic O and phase 1 and phase 2 flagellar antigens by agglutination tests with antisera according to the WHO Collaborating Centre scheme[28]

Antimicrobial susceptibility testing

Susceptibility to antimicrobial agents was performed as MICdeterminations using a commercially prepared, dehydrated panel(Sensititre®). The following antimicrobial agents were used: amoxicillin-clavulanic acid, ampicillin, apramycin, ceftiofur,cephalothin, chloramphenicol, ciprofloxacin, colistin, florfenicol, gentamicin, nalidixic acid, neomycin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim. All plates were inoculated following the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI), and CLSI breakpoints were used for interpretation of the MIC results[23].

Detection of resistance genes

Twelve ampicillin- and ceftiofur-resistant isolates wereanalyzed by PCR to detect the presence of blaTEM, blaCTX,blaCMY-1, blaCMY-2, blaSHV, and blaACC. PCR amplification wasperformed using primer pairs 5'ATGAGTATTCAACATTTCCG-3' and 5'ACCAATGCTTAATCAGTGAG-3' (blaTEM)[24], 5' ATGTGCAGYACCAGTAARGTKATGGC-3', and 5' TGGGTRAARTARGTSACCAGAAYCAGCGG-3' (blaCTX)[17], 5'TGGTGGATGCCAGCATCCA-3' and 5'GGTCGAGCCGGTCTTGTTGAA-3', (blaCMY-1)[11], 5'-GCACTTAGCCACCTATACGGCAG-3'and 5'GCTTTTCAAGAATGCGCCAGG-3' (blaCMY-2)[11], and 5'-AGCCTCAGCAGCCGGTTAC-3' and 5'-GAAGCCGTTAGTTGATCCGG-3' (blaACC-1)[11], and5'-TTATCTCCCTGTTAGCCACC-3' and 5'-GATTTGCTGATTTCGCTCGG- 3' (blaSHV).11 The amplicon sizes of blaTEM,blaCTX, blaCMY-1, blaCMY-2, blaSHV, and blaACC were 964 bp,593 bp, 915 bp, 758 bp, 854 bp, and 818 bp, respectively.

All PCRs were conducted with the buffer supplied by the manufacturers for each primer (HT Biotechnology, UK). All reaction mixtures (75μl) were amplified using the following programs: 3 min at 94°C; 25 cycles of 1 min at 94°C, 1 min. at 50°C, and 1 min at 72°C; 10 min at 72°C (*bla*TEM), 5 min at 94°C; 30 cycles of45 sec at 94°C, 45 sec at 45°C, and 45 sec at 72°C; 10 min at72°C (*bla*CTX),

5 min at 94°C, 25 cycles of 1 min at 94°C, 1min at 58°C, and 1 min at 72°C; 10 min at 72°C (*bla*CMY-1), 5min at 94°C; 25 cycles of 45 sec at 94°C, 45 sec at 58°C and1 min at 72°C; 10 min at 72°C (*bla*CMY-2) and 3 min at 95°C;35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C;10 min at 72°C (*bla*SHV) and 5 min at 94°C; 30 cycles of 45 sec at 94°C, 45 sec at 55°C, and 1.5 min at 72°C; 10 min at72°C (*bla*ACC). Nine out of 12 PCR amplicons positive for *bla*TEM, five outof 20 amplicons positive for *bla*SHV, and one amplicon against *bla*CTX were selected for sequencing. Prior to sequencing, theamplicons were purified using the GFX™ PCR DNA kit (AmershamBiosciences). Sequencing and sequence analysis of all genes were performed on DNA sequencer (PerkinElmer, Applied Biosystems) using the same primers as in thePCR analysis. The resulting nucleotide sequences were comparedto sequences obtained from the GenBank database and http://www.lahey.org/studies/webt.html. The software Vektor Sequence analysis\eBiotoolsNTI suite 8 (InforMax, Inc.) was used for alignment.To distinguish between the genes *bla*CTX-M-15, and *bla*CTX-M-28, another amplification of *bla*CTX was performed andsequenced using two other primer pairs 5'-CCATGGTTAAAAAATCACTGCG-3' (*bla*CTX-M-15 front P1) and 5'-TGAAGTGGTATCACGCGGATC-3' (*bla*CTX-M-15 front P2) and5'-CCGTTTCCSCTATTACAAACCG-3' (*bla*CTX-M-15 end P2)and 5_-ATGTGCAGYACCAGTAARGTKATGGC-3' (*bla*CTXM U1). The following program was used for both PCR amplifications:3 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at57°C, and 1 min at 72°C; 10 min at 72°C with amplicon sizesof 557 bp and 680 bp, respectively. The nucleotide sequencesof the genes detected in the study were matched to known genesin the GenBank database.

Plasmid analysis and conjugation

Plasmid DNA of the 12 isolates showing a positive reaction against *bla*SHV, including the one also showing a positive reaction against *bla*CTX, was purified by an alkaline lysismethod, using Nucleobond® AX (Macherey-Nagel Inc., Easton,PA). The plasmid DNA was detected on a 0.8% agarosegel electrophoresis for 2 hr at 120 V. The molecular sizes ofthe bands were determined by reference to plasmidsof known band sizes (*E. coli* 1RS:.Conjugation was performed by the filter mating method[32] using a mutated plasmid-free, amoxicillin-sensitive andnalidixid acid- and rifampicin-resistant *Salmonella SRT* as recipient for conjugation of the ceftiofur-resistant isolate showing a positive ampliconagainst*bla*CTX and other isolates showing positive amplicons against *bla*SHV. Transconjugates were isolated on brain heart infusion(BHI) agar containing cephalothin (42 μg/ml), nalidixic acid (60 μg/ml), and rifampicin (60 μg/ml). The transconjugate was investigated by PCR, plasmid profiling, and pulsed-field gel electrophoresis (PFGE) to confirm the presenceof the original plasmid, the *bla*TEM-1H and the *bla*CTX genes, and to match the PFGE pattern with the one of the recipients.

Pulsed-field gel electrophoresis

PFGE was performed using XbaI according to CDC PulseNetprotocols[31]. To perform the phylogenetic analysis of the types, Cluster analysis usingthe Dice correlation for band matching with a 0.7% position tolerance was used to generate a dendrogram describing the relationship among the different types and subtypes.

RESULTS

Antimicrobial resistance and PFGE typing

Only 7 (20.5%) of the 37 isolates were susceptible to allantimicrobial agents tested, whereas 29 (85.2%) were resistant to three or more antimicrobial agents. All isolates were susceptibleto apramycin, ciprofloxacin, florfenicol, colistin,neomycin, and spectinomycin. A higher frequency of resistancewas found toward streptomycin(64.8%) and sulfonamides(62.1%) (Table 1). Resistance was also observed to ampicillin (32.4%), ceftiofur (27%), chloramphenicol (33.4%), gentamicin(29.7%), nalidixic acid (35.1%), and tetracyclines(29.7%). In poultry, no ceftiofur, cephalothin, or gentamicin resistancewere found. In poultry product, no resistance to ampicillin, ceftiofur,cephalothin, chloramphenicol, were observed.Of the 17 human isolates from, 7 displayed resistanceto ampicillin, cephalothin, and ceftiofur. No difference in susceptibility between the isolates frompoutry products and human isolates was observed.7 different PFGE types of *Salmonella*(1, 2, 3, 4, 5,6, 7) were observed among the isolates(Table 1) and their phylogeny is shown

Table 1. Occurrence of Antimicrobial Resistance and PFGE Types among 37 Salmonella isolates From Saudi Arabia

Source	No_ of isolates	PFGE Types	No_ of isolates with antimicrobial resistance (%)							
			AMP	CEF	CHL	GEN	NAL	STR	SUL	TET
Human patient	17	1	4	4	4	4	4	4	4	4
		2	5	1	2	4	5	6	5	5
		4	0	3	3	1	4	9	8	0
		5	0	0	0	1	0	2	3	0
		7	0	0	0	0	0	0	0	0
Poultry	13	2	0	0	0	0	0	0	0	0
		3	1	0	0	0	0	0	0	0
		4	0	0	0	0	0	0	0	0
		5	0	2	3	0	0	2	1	0
		7	0	0	0	0	0	0	0	0
Poultry Products	7	2	0	0	0	0	0	0	0	0
		3	0	0	0	0	0	0	0	0
		3	2	2	0	0	0	1	2	2
		4	0	0	0	1	0	0	0	0
		5	0	0	0	0	0	0	0	0
		7	0	0	0	0	0	0	0	0

AMP, Ampicillin; CEF, ceftiofur; CHL, chloramphenicol; GEN, gentamicin; NAL, nalidixic acid; STR, Streptomycin; SUL, sulfamethoxazole; TET, tetracycline.

Table 2. PFGE and Plasmid Profiles of 12 *bla*SHV-positive isolates

Number of isolates	β- lactamase genes	PFGE type	Plasmid profile
31	*bla*SHV, *bla*TEM	1	1
33	*bla*SHV, *bla*TEM	1	1
34	*bla*SHV, *bla*TEM	2	2
36	*bla*SHV, *bla*TEM	2	2
37	*bla*SHV, *bla*TEM	2	3
28	*bla*SHV	2	3
19	*bla*SHV, *bla*TEM	5	4
20	*bla*SHV, *bla*TEM	4	5
32	*bla*SHV, *bla*TEM	5	4
18	*bla*SHV, *bla*TEM, *bla*CTX	3	4
25	*bla*SHV	7	5
26	*bla*SHV	6	4

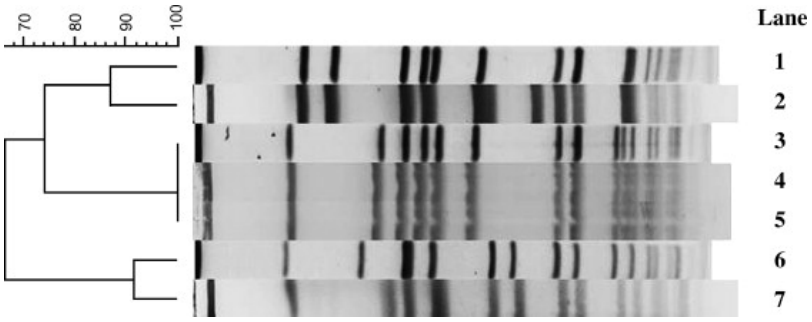

Fig. 1. Phylogeny of the PFGE types of *Salmonella* isolates

in Fig. 1. Types 1, 2, 4,5and 7 were found only in human patients whereas types 2,3, 4,5,7were found only in poultry. Types 2,3, 4, 5 and 7 were found in poultry products, whereas types 2,3,4,5 and 7 were common to both poultry and poultry products.

Detection and identification of Extended- Spectrum β- Lactamase Resistance Genes

All 12 ampicillin- and ceftiofur-resistant isolates gave negativereactions for *bla*CMY-1, *bla*CMY-2, and *bla*ACC genes butwere positive for *bla*SHV. Five of them were sequenced to*bla*SHV-2. nine of the 12 isolates also gave positive reactionsfor *bla*TEM, and five representative isolates were sequenced.

A comparison with GenBank revealed 100% identityto the sequence of *bla*TEM-1H for one isolate. The *bla*TEM-1H-positive isolate of *Salmonella*sp.was also positive for *bla*CTX-M-15. The *bla*CTX-M-15gene was located on a 70-kb transferable plasmid, which alsocarried the *bla*TEM-1H gene. The genes*bla*SHV-2 and *bla*CTXM-15 were containing isolates of *Salmonella*sp.

Plasmid analysis and conjugation

Five different plasmid profiles were found among the 12 isolatesinvestigated (Fig. 2). The plasmid profiles confirmed inmost cases the PFGE-type (Table 2). However, the four isolatesof PFGE type 2 were assigned to two different plasmid profiles(two of plasmid profile 2 and two of profile 3). The *bla*CTX-M-15 gene was located on a plasmid of approximately70 kb, giving rise to a new plasmid profile. The plasmid with *bla*CTX-M-15 was transferable by conjugation, whereas notransconjugants were obtained from the isolates only containing*bla*SHV.

DISCUSSION

*Salmonella*sp showed in a hospitalpoultry food poisoning where the isolates recovered were allsusceptible to the antimicrobial tested[10]. Puthucheary SD[29] recently reported the isolation of *Salmonella* from the environment of one dairy without any history of clinical salmonellosis. This isolate was also susceptible to all antimicrobial agents tested. *Salmonella* was reported the most frequent serovars isolated from poultry products of *Salmonella*[33]; the bacteria was mainly recovered in turkey meat.9 Finally, a study on *Salmonella* sp. contamination of restaurantpoultry meat products collected over a period of 1 year found that *Salmonella* sp were detected in red meat and fowl[15]. This study suggests this *Salmonella* serovar have the potential for causinglocal or global infection. My investigation found major differences in the occurrence of antimicrobial resistance between human infection and food products. There was high frequency of resistance among *Salmonella* sp isolated from humans in Riyadh, SA. With a lower frequency in, poultry and poultry products .seventeen ampicillin- and ceftiofur-resistant isolateswere recovered from human where the dominant resistance profile was ampicillin, ceftiofur, chloramphenicol, gentamicin, streptomycin, and sulfamethoxazole. This resistanceprofile was not or less seen in poultry or poultry products. Resistance totetracyclines was observed among isolates from poultry andpoultry products, but not from human. A similar predominant patternof resistance to nalidixic acid, streptomycin, sulfamethoxazole,and tetracycline were found in isolates from human and poultry.This high frequency of resistance might be because thisserovar easily acquires resistance, or because the natural reservoirswere exposed to large amounts of antimicrobial agents. Itis tempting to speculate that the hypothesis might be morerelevant for this isolates that showed the higher resistance (Table 2). All *Salmonella* isolates were related, as determined by PFGE.

A total of nine isolates contained *bla*TEM (*bla*TEM-1H). *bla*SHV-2 was found in all 8ceftiofur-resistant isolates from human. The gene could not be transferred by conjugation from the nine isolates where it was attempted,but hybridization to plasmid profiles indicated that thegene was located on plasmids of approximately 70 kb. Genesof the SHV family are commonly reported from Enterobacteriaceae worldwide[34], but seem especially to be commonly found in *Klebsiella* spp.[6,17, 27]

The *bla*CTX-M-15 gene was present in one isolate also containing*bla*SHV-2 and *bla*TEM-1H. The *bla*CTX-M-15 gene was locatedon a 70-kb transferable plasmid, which also carried the*bla*TEM-1H gene. The *bla*CTX-M-15 gene has previously been associatedwith mobile elements, most commonly insertion sequences,such as

ISEcp1, or integron-associated *bla*CTX-M.[19],[20] Co-carriage of ESBL genes is not a new finding, but it is reportedas uncommon.[19,20] It has been reported that *bla*CTX-M-14and *bla*CTX-M-13 were carried together with either *bla*SHV-12 or *bla*SHV-11.5 Isolate containing the *bla*CTX-M-14gene was found to also contain *bla*SHV-12[16]. *bla*CTX-M2 and *bla*SHV-2 was observed in a *S.* Virchowisolate.11 Co-carriage of *bla*CTX-M-2 and *bla*SHV-5 geneshas also been reported in *Klebsiella*spp.[4,27].the study investigation indicated that some of these *Salmonella*isolates were certainly acquired in the same hospital. They all had the same PFGE type 2 with thesame resistance and plasmid profile and contained both *bla*SHVand *bla*TEM. In conclusion, this study showed a high frequency of resistance among *Salmonella* sp isolated from humans and a lower frequency in poultry and poultry products. PFGE typing suggests that all of the isolates from the different sources are clonally related, which could indicate that they resistance.

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