Original Article

Isolation and Molecular Characterization of Multidrug-Resistant *Salmonella, Shigella* and *Proteus* from Domestic Birds

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Abstract

The increase in multidrug-resistant enteric bacteria, especially *Salmonella*, *Shigella* and *Proteus* in poultry has become a significant public health threat. Therefore, this work was aimed to investigate whether domestic birds have a role in the emergence and transmission of multidrug-resistant (MDR) bacteria in Taif province. *Salmonella*, *Shigella* and *Proteus* were isolated from different types of healthy domestic birds (n=42) collected from different places. Bacterial isolates were recovered from cloacal swabs of birds by non-selective and selective pre-enrichment technique. One hundred and sixty six bacterial isolates were screened for antibiotic susceptibility such as Cefaclor, Oxacillin, Ampicillin, Chloramphenicol, Cephalexin, Neomycin, Colistin, Ciprofloxacin, Oxytetracycline, Norfloxacin, Lincomycin, Gentamycin, Amoxicillin, Enrofloxacin and Piperacillin. The MDR bacterial isolates were identified using morphological, biochemical characteristics, and confirmed by API 20E strips, serological tests and 16S rRNA analysis. Forty-eight percent of the collected birds were positive for drug-resistant pathogenic bacteria including *Salmonella*, *Shigella* and *Proteus*. Phenotypic characterization was proved by PCR amplification of *invA* and *mdh* genes. The MDR bacterial isolates were found to harbour the plasmid-encoded multi-drug resistance. These results revealed that poultry might serve as a reservoir of multidrug-resistant *Salmonella*, *Shigella* and *Proteus*, and could be a possible mean of distribution of these pathogens to human.

Keywords: Antibiotic resistant bacteria, domestic birds, *invA* gene, *mdh* gene, *Proteus*, *Salmonella*, *Shigella*

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Introduction

Salmonellosis and shigellosis are endemic infections and have appeared as a global health affair being a primary reason of disease and death in humans (Foley et al., 2008; Yah, 2010). *Proteus* species is the major source of diseases acquired outside the hospital, wherever many of these diseases finally need hospitalization (De Champs et al., 2000). Gastroenteritis caused by *Salmonella* and *Shigella* has the highest bad outcome on progress of children (Webb and Starr, 2005).

The major causative agents responsible for the high endemicity of typhoid fever and other related infections are *Salmonella* sp., mostly *Salmonella* Typhi, which is responsible for most typhoidal infections; *Salmonella* Typhimurium and *Salmonella* Enteritidis, which have been attributed to most cases of non-typhoidal illnesses (Akinyemi et al., 2007). A great incidence of infections caused by *Salmonella* sp. is common. *Salmonella* sp. occurs primarily as major components of the gut flora of domestic animals, making such animals a common medium of transmission of the infection with these organisms and facilitating cross-contamination of food and drinking water sources (Kim et al., 2005; Gyles, 2008). More specifically, *Salmonella* sp. is increasingly associated with poultry production and this has been a major factor for the rise of *Salmonella* infections over the years (Bada-Alambedji et al., 2006). A common number of 1.3 billion yearly cases of *Salmonella*–affected people gastroenteritis rises from eating of polluted foods such as raw beef, fish, shell fish, milk and eggs (Esaki et al., 2004).

Shigella species isolated from chicken or human has identical biological and serological characteristics (Xu et al., 2004). Antibiotic treatment reduces the infection time of *Shigella dysenteriae* and, therefore, is suggested, for the management of moderate to severe dysentery (David and John, 2010). Rapid emergence of resistance allows the need for continuous monitoring of sensitivity patterns (Zafar et al., 2009). Antibiotic-resistant *Salmonella* and *Shigella* are of global concern because they affect both developed and developing countries (Dubois et al., 2007).

The sub-therapeutic practice of antibiotics in fowl has become a popular practice and there is an increasing frame of scientific indication to the effect that the increasing incidence of antibiotic-resistant bacteria is closely associated with the heavy use of these antibiotics in poultry and other related agricultural practices (Kilozo-Nthenge et al., 2008). This constitutes a significant public health risk due to possible cross-contamination with antibiotic-resistant bacteria of drinking water and food denoted for public intake, which always results in human illnesses, mostly typhoid fever, non-typhoidal illnesses, diarrhoea, with serious clinical consequences (Sutiono et al., 2010). The growing incidence of multidrug-resistant *Salmonella* Typhi has become a global phenomenon and antibiotic-resistant bacteria are increasingly isolated from a wide group of sources in the clinical environments, poultry, cattle food, retail meat and drinking water sources. In different parts of Nigeria, there are some scientific evidence of the growing rate of recovery of antibiotic-resistant *Salmonella* Typhi from poultry and local bird (Enabulele et al., 2010; Ajayi and Egbebi, 2011). The increased use of antibiotics in poultry is a cause for concern as it poses risks of the emergence of antibiotic-resistant bacteria.

In order to decrease pollution and disease caused by *Salmonella*, *Shigella* and *Proteus* infections, the incidence of *Salmonella*, *Shigella* and *Proteus* pollution, and antimicrobial susceptibility data in poultry should be examined. Therefore, the present work was planned to isolate and characterize *Salmonella*, *Shigella* and *Proteus* from domestic birds in Taif region, Saudi Arabia. In sight of the dangers posed by the use of antibiotics, this study was also proposed to examine the incidence of antibiotic-resistant *Salmonella*, *Shigella* and *Proteus*.

Materials and Methods

Sample collection: Sterile swabs immersed with sterilized saline solution were injected in the cloacae of 42 different types of healthy domestic birds (Table 1) at Taif province, Saudi Arabia, and placed in sterile vials. Afterward, the cloacal swabs were transported immediately to the laboratory in shielding fizzle box with frost and examined by culture for *Salmonella*, *Shigella* and *Proteus*.

Isolation of bacteria: Cloacal swab sampling was performed by inoculating each sample directly into buffered peptone water and incubating them for 24 hours at 37°C (non-selective pre-enrichment). The non-selective pre-enrichment culture was then subcultured into Rappaport Vassiliadis Soy (RVS) media and incubated for a further 24 hours at 41.5°C. Immediately after enrichment, the organisms were inoculated onto Xylose Lysine Desoxycholate (XLD) agar and Brilliant Green (BA) agar plates for isolation of strains of bacteria. All plates were incubated for 24 hours at 37°C and bacterial strains were investigated for characteristic colonial morphology for *Salmonella*, *Shigella* and *Proteus* on the XLD or BA agar (Post, 1997). The bacterial isolates were inoculated onto Nutrient agar for morphological, biochemical and further tests.

Antibiotic susceptibility test: The standard method of Kirby-Bauer by disk diffusion was employed to investigate the antibiotic susceptibility profiles of the bacterial isolates (NCCLS, 2012) for some antimicrobial agents such as Cefaclor (30 µg), Oxacillin (1 µg), Ampicillin (10 µg), Chloramphenicol (30 µg), Cephalexin (30 µg), Neomycin (30 µg), Colistin (10 µg), Ciprofloxacin (5 µg), Oxytetracycline (30µg), Norfloxacin (10 µg), Lincomycin (2 µg), Gentamycin (10 µg), Amoxicillin (25 µg), Enrofloxacin (5 µg) and Piperacillin (100 µg) (Table 2). These antibiotics were obtained from the BIO-RAD LABORATORIES, SGH. This antibiotic group was selected to contain antibiotics with possible ability against Enterobacteriaceae. Moreover, these antibiotics were selected basically on their significance in treatment of animal or human infections by Enterobacteriaceae, their practice as feedstuff additives to support growth in animals, and their function to provide a wide range

for different antibiotics classes.

Plates of Mueller-Hinton agar were streaked with bacterial isolates inoculated and incubated in TSB to a turbidity of 0.5 McFarland values. The ready antibiotics disks (4x50, BIO-RAD) were placed on the inoculated agar plates. The plates were then incubated for 24 hours at 37°C. Clear zones of growth inhibition were measured in millimeters by a ruler (NCCLS, 2012).

Identification of antibiotic-resistant isolates: Suspected colonies of antibiotic-resistant bacterial isolates were selected for identification by biochemical tests as described by Olutiola et al. (2001). Biochemical tests such as methyl red, indole, citrate, Voges Proskaurs and triple sugar iron agar were achieved for identification of bacterial isolates at the genus level. Bacterial isolates were confirmed by API 20E kits following the manufacturer's recommendations.

Table 1 Types, names, location and status of collected birds

No.	Names of birds	No. of birds	Location	Status
1	Chicken-1	2	East of Taif	Open farm, Municipality
2	Pigeon (Zajel)-2	2	East of Taif	Closed farm
3	Pigeon (Zajel)-3	2	East of Taif	Closed farm
4	Pigeon (Zajel)-4	2	East of Taif	Closed farm
5	Chicken-5	2	East of Taif	Open farm, Municipality
6	Chicken-6	2	East of Taif	Open farm, Municipality
7	Farmyard pigeons-7	2	South of Taif	Market
8	Quail-8	2	South of Taif	Market
9	Chicken-9	2	South of Taif	Market, Municipality
10	Chicken-10	2	South of Taif	Market
11	Chicken-11	2	South of Taif	Farm
12	Farmyard pigeon-12	2	South of Taif	Farm
13	Farmyard pigeon-13	2	North of Taif	Farm
14	Farmyard pigeon-14	2	North of Taif	Farm
15	Chicken-15	2	North west of Taif	Indonesian
16	Chicken-16	2	North west of Taif	Dutch
17	Quail-17	2	West of Taif	Farm
18	Chicken-18	2	West of Taif	Farm
19	Qmari-19	2	West of Taif	Farm
20	Farmyard pigeon-20	2	West of Taif	Farm
21	Duck-21	2	West of Taif	Farm

Table 2 Available commercial antibiotics used for susceptibility of bacterial isolates

No.	Antibiotics	Antibiotic code	Antibiotic disc concentration	Antibiotic class
1	Cefaclor	CEC	30 µg	Cefalosporins
2	Oxacillin	OX	1 µg	Penicillin
3	Ampicillin	AM	10 µg	Penicillin
4	Chloramphenicol	C	30 µg	Chloramphenicol
5	Cephalexin	CL	30 µg	Penicillin
6	Neomycin	N	30 µg	Aminoglycosides
7	Colistin	CT	10 µg	Polymyxin
8	Ciprofloxacin	CIP	5 µg	Fluoroquinolone
9	Oxytetracycline	T	30 µg	Tetracyclines
10	Norfloxacin	NOR	10 µg	Fluoroquinolones
11	Lincomycin	L	2 µg	Glycerol
12	Gentamycin	CN	10 µg	Aminoglycosides
13	Amoxicillin	AX	25 µg	Phenols
14	Enrofloxacin	ENR	5 µg	Fluoroquinolone
15	Piperacillin	PRL	100 µg	Beta-lactam/beta-lactamase inhibitor

Serotyping of bacterial isolates: The bacterial isolates identified by biochemical tests were further characterized by serological tests using polyvalent serum against O *Salmonella* antigens. Slide agglutination test using polyvalent somatic (O) antisera kit (Remel Europe Ltd) was used to confirm positive isolates of *Salmonella*. Saline suspension of 18-hour-old culture of an isolate (one drop) was mixed with Polyvalent O antiserum (one drop) on a glass slide. The slide was sloped in a back-and-forth motion for 1-2 min and any degree of agglutination was taken as a positive reaction (Brenner, 1998).

Molecular identification of the most multidrug-resistant (MDR) bacteria

Preparation of genomic DNA: A loopful of bacterial isolates was taken with a sterilized needle, and suspended in 0.5 ml of sterilized saline in a 1.5 ml Eppendrof tube. Centrifugation was performed at 10,000 rpm for 10 min. The pellet was suspended in 0.5 ml of InstaGene Matrix (Bio-Rad, USA) and incubated for 30 min at 56°C and then heated for 10 min at 100°C. Then, the supernatant was employed for PCR.

PCR amplification of mdh and invA genes: To investigate the presence of *malic acid dehydrogenase* (*mdh*) gene (261bp), the primers *mdh*–F, 5´-TGCCAAC GGAAGTTGAAGTG-3´ and *mdh*–R, 5´CGCATTCCA C CACGCCCTTC-3´ were used (Lin and Tsen, 1999). The presence of *invA* gene of *Salmonella*, S139 and S141 primers, 5´-GTGAAATTATCGCC ACGT TCG GGCAA–3´and 5´–TCATCGCACCGTCAAAGGAA CC–3´ were used (Rahn et al., 1992).

PCR reactions were achieved in a total volume of 50 µl in PCR Eppendrof: 2.5 µl of each primer, 8 µl of dNTP, 5 µl of 10 x buffer, 3 µl Mg Cl2, 0.2 µl Taq DNA polymerase, 5 µl DNA containing suspension and 23.8 µl water to complete the volume.

PCR conditions were as follows: 94°C for 5 min, followed by denaturation at 94°C for 25 sec, annealing at 54°C for 45 sec and then extension at 72°C for 1 min for 30 cycles, and further extension at 72°C for 7 min. PCR products were subjected to 1% agarose gel electrophoresis at a constant voltage of 100 for 25 min using 50 bp DNA markers. The gel was UV visualized.

Table 3 Antibiotic susceptibility test of 166 selected bacterial isolates from domestic birds. Antimicrobial susceptibility was achieved according to CLSI procedures (Clinical and Laboratory Standards Institute, 2012).

Antibiotic discs, codes, concentrations	No. of bacterial isolates (%)		
	Resistant	Intermediate	Sensitive
Cefaclor, CEC (30 µg)	24(14.5)	0.0(0.0)	142(85.5)
Oxacillin, OX (1 µg)	164(98.8)	2(1.2)	0.0(0.0)
Ampicillin, AM (10µg)	90(54)	58(34.9)	18(10.8)
Chloramphenicol, C (30 µg)	120(72.3)	46(27.7)	0.0(0.0)
Cephalexin, CL (30 µg)	24(14.5)	0.0(0.0)	142(85.5)
Neomycin, N (30 µg)	52(31.3)	102(61.4)	12(7.2)
Colistin, CT (10 µg)	2(1.2)	22(13.3)	142(85.5)
Ciprofloxacin, CIP (5 µg)	90(54)	60(36)	16(9.6)
Oxytetracycline, T (30 µg)	164(98.8)	2(1.2)	0.0(0.0)
Norfloxacin, NOR (10 µg)	16(9.6)	6(3.6)	144(86.7)
Lincomycin, L (2 µg)	166(100)	0.0(0.0)	0.0(0.0)
Gentamycin, CN (10 µg)	32(19.3)	10(6)	124(74.7)
Amoxicillin, AX (25 µg)	76(45.8)	8(4.8)	82(49.4)
Enrofloxacin, ENR (5 µg)	18(10.8)	6(3.6)	142(85.5)
Piperacillin, PRL (100 µg)	48(28.9)	6(3.6)	112(96.6)

Table 4 Antibiotic resistance profiles of multidrug-resistant bacterial isolates

Bacteria isolates	Antibiotic resistance profiles	No. of antibiotics
Shigella dysenteriae X3-1	[1]OX,T,L	3
Shigella dysenteriae X4-1	OX,T,L	3
Salmonella Typhimurium X5-2	OX,AM,C,T,L	5
Salmonella Typhi X6-1	OX,AM,C,CT, T,L,AX	7
Salmonella Typhi X6-2	OX,AM,C, CIP,T,L,AX	7
Proteus vulgaris X9-1	OX,AM,C,N,CIP,T,L,CN,ENR,PRL	10
Salmonella Typhimurium X9-2	CEC,OX,AM,CL,N,CIP,T,L,CN,AX,PRL	11
Proteus vulgaris X10-1	OX,AM,C,N, T,L,AX,PRL	8
Shigella dysenteriae X11-1	OX,AM,C,T,L,AX,PRL	7
Shigella dysenteriae X13-2	OX,AM,C,T,L	5
Shigella dysenteriae X14-2	OX,AM,C,T,L	5
Proteus vulgaris X17-1	OX,AM,C,CL,N,CIP,T,NOR,L,CN,AX,ENR,PRL	13
Shigella dysenteriae X17-2	CEC,OX,AM,C,CL,L,AX	7
Proteus vulgaris X18-1	CEC,OX,AM,C,CL,N,CIP,T,L,AX	10
Shigella dysenteriae B4-1	OX,C,N,CIP,T,L	6
Salmonella Typhimurium B5-1	OX,AM,C,N,CIP,T,L	7
Salmonella Typhimurium B5-2	OX, C,N,CIP,T,L	6
Salmonella Typhimurium B6-1	OX,AM,C,CIP,T,L,AX	7
Salmonella Typhimurium B6-2	OX,AM,C,CIP,T,L,AX	7
Shigella dysenteriae B8-1	OX,C,CIP,T,L	5
Shigella dysenteriae B8-2	OX,C,CIP,T,L	5
Shigella dysenteriae B10-1	OX,AM,C, N,CIP,T, L,AX,PRL	9
Shigella dysenteriae B11-1	OX,AM,C,T,L,AX,PRL	7
Shigella dysenteriae B12-1	OX,AM,T,L	4
Shigella dysenteriae B12-2	OX,T,L	3
Shigella dysenteriae B18-1	OX,AM,C,N,CIP,T,L,CN,AX,PRL	10
Shigella dysenteriae B18-2	OX,AM,C,N,CIP,T,L,CN,AX,PRL	10

[1]CEC, Cefaclor; OX, Oxacillin; AM, Ampicillin; C, Chloramphenicol; N, Neomycin; CIP, Ciprofloxacin; T, Oxytetracycline; NOR, Norfloxacin; L, Lincomycin; CN, Gentamycin; AX, Amoxicillin; ENR, Enrofloxacin; PRL, Piperacillin

[2]Z, Zajel; [3]F, Farmyard

Table 5 Characteristic tests of multidrug-resistant bacterial isolates

Characteristic tests	*Salmonella* sp.	% of isolates	*Shigella* sp.	% of isolates	*Proteus* sp.	% of isolates
Gram Staining	G-, short bacilli	9.9	G-, short bacilli	19	G-, rod-shaped	5
Motility	+	9.6	-	18	+	4.8
Catalase Test	+	10	-	18	+	4.8
Oxidase Test	-	9.8	-	18.5	-	4.7
Indole Test	-	9.7	+	18	+	4.8
Methyle Red Test	+	10	+	18	+	4.8
Voges-Proskauer Test	-	9.6	-	18.6	-	4.8
Citrate Test	-	9.6	-	18.8	-	4.9
Lactose Test	-	9.6	-	18	-	4.8
Glucose O/F	+	9.7	+	18	+	
Gelatin Hydrolysis Test	-	9.9	-	18	+	4.8
Casein Hydrolysis test	+	9.6	-	18	+	4.9
Nitrate Reduction Test	+	10	+	18	+	5
Urea Hydrolysis test	-	9.6	-	18.5	+	4.9
Grow with KCN	-	9.6	-	18	+	4.8
Tryptophan deaminase	-	9.6	-	18	+	4.8
H_2S production	+	9.8	-	18.2	+	4.9
Lysine decarboxylase	+	9.8	-	18.1	-	4.8
Ornithine decarboxylase	+	9.7	-	18.1	-	4.9
Acid and gas from:						
Glucose	+	9.6	+	18	+	4.8
Mannitol	+	9.6	+	18	-	4.8
Maltose	+	9.6	+	18	+	4.8
Sorbitol	+	9.6	-	18.1	-	4.8
Sucrose	-	9.6	-	18.1	+	4.8
Lactose	-	9.6	-	18	-	4.9

*+, presence; -, absence

Table 6 Prevalence of *invA* and *mdh* genes of multidrug-resistant bacterial isolates

Isolates	*invA* (~284 bp)	*mdh* (~261 bp)
Shigella dysenteriae X3-1	-*	+
Shigella dysenteriae X4-1	-	+
Salmonella Typhimurium X5-2	+	+
Salmonella Typhi X6-1	+	+
Salmonella Typhi X6-2	+	+
Proteus vulgaris X9-1	-	+
Salmonella Typhimurium X9-2	+	+
Proteus vulgaris X10-1	-	+
Shigella dysenteriae X11-1	-	+
Shigella dysenteriae X13-2	-	+
Shigella dysenteriae X14-2	-	+
Proteus vulgaris X17-1	-	+
Shigella dysenteriae X17-2	-	+
Proteus vulgaris X18-1	-	+
Shigella dysenteriae B4-1	-	+
Salmonella Typhimurium B5-1	+	+
Salmonella Typhimurium B5-2	+	+
Salmonella Typhimurium B6-1	+	+
Salmonella Typhimurium B6-2	+	+
Shigella dysenteriae B8-1	-	+
Shigella dysenteriae B8-2	-	+
Shigella dysenteriae B10-1	-	+
Shigella dysenteriae B11-1	-	+
Shigella dysenteriae B12-1	-	+
Shigella dysenteriae B12-2	-	+
Shigella dysenteriae B18-1	-	+
Shigella dysenteriae B18-2	-	+

*+, presence; -, absence

PCR of 16S rRNA gene: One microliter of DNA suspension was added in 20 µl of PCR reaction using the following primers (Gutell et al., 1990): 27F (5´-AGA GTT TGA TCM TGG CTC AG-3´) and 1492R (5´-TAC GGY TAC CTT GTT ACG ACT T-3´). The PCR reaction was carried out as described above (Section: PCR amplification of *mdh* and *invA* genes). Thirty-five PCR cycles were performed at 94°C for 45 sec, 55°C for 1 min, and 72°C for 1 min. DNA fragments were amplified ~1,400 bp. PCR products were cleaned up by using Montage PCR Clean up kit (Millipore).

Sequencing of 16S rRNA gene: The PCR products (~1,400 bp) were sequenced by using the following primers (Gutell et al., 1990): 518F (5´-CCA GCA GCC GCG GTA ATA CG-3´) and 800R (5´-TAC CAG GGT ATC TAA TCC-3´). Sequencing was achieved by Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) was used to resolve the sequencing products.

From the nucleotide sequence databases, selected sequences of other microorganisms with greatest similarity to the 16S rRNA sequences of the bacterial isolates were taken and aligned using CLUSTAL W (1.81) Multiple Sequence Alignment creating phylogenetic tree. The 16S rRNA gene sequences of the bacterial isolates, which are described in this study, were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases having accession numbers: AB855726 (*Shigella dysenteriae* X11-1), AB855727 (*Shigella dysenteriae* X17-2), AB855728 (*Shigella dysenteriae* B10-1), AB855729 (*Shigella dysenteriae* B11-1), AB855730 (*Shigella dysenteriae* B18-1), AB855731 (*Shigella dysenteriae* B18-2), AB855732 (*Salmonella typhimurium* X9-2), AB855733 (*Salmonella typhimurium* B5-1), AB855734 (*Salmonella typhimurium* B6-1), AB855735 (*Salmonella typhimurium* B6-2), AB855736 (*Salmonella typhi* X6-1), AB855737 (*Salmonella typhi* X6-2), AB855738 (*Proteus vulgaris* X6-2), AB855739 (*Proteus vulgaris* X10-1), AB855740 (*Proteus vulgaris* X17-1) and AB855741 (*Proteus vulgaris* X18-2).

Isolation of plasmids: Plasmid of the MDR bacterial isolates was investigated by the method described previously (Sambrook and Russell, 2001) using alkaline lysis method. The plasmid preparations were subjected to 0.8% agarose gel electrophoresis before visualization under UV transilluminator. *Escherichia coli* V517 MTCC 131 (harbors eight different plasmids with known molecular weight) was used as a standard plasmid marker to detect the molecular weight of plasmids.

Statistical analysis: All analyses were carried out according to one-way analysis of variance (ANOVA) and evaluated by post hoc comparison of means using lowest significant differences (LSD) using SPSS 11.0 software. They were considered significant at $p<0.05$ level. The tests were achieved in triplicate.

Table 7 Plasmid patterns of multidrug-resistant bacterial isolates

Isolates	Plasmids (Molecular size, kb)
Shigella dysenteriae X3-1	~3 kb
Shigella dysenteriae X4-1	~3 kb
Salmonella Typhimurium X5-2	~14 kb
Salmonella Typhi X6-1	~120 kb
Salmonella Typhi X6-2	~120 kb
Proteus vulgaris X9-1	~4 kb, 5kb
Salmonella Typhimurium X9-2	~140 kb, 14 kb
Proteus vulgaris X10-1	~5kb
Shigella dysenteriae X11-1	~92 kb
Shigella dysenteriae X13-2	~4 kb
Shigella dysenteriae X14-2	~4 kb
Proteus vulgaris X17-1	~4 kb, 5kb
Shigella dysenteriae X17-2	~7 kb
Proteus vulgaris X18-1	~4 kb, 5kb
Shigella dysenteriae B4-1	~4 kb
Salmonella Typhimurium B5-1	~14 kb
Salmonella Typhimurium B5-2	~14 kb
Salmonella Typhimurium B6-1	~14 kb
Salmonella Typhimurium B6-2	~14 kb
Shigella dysenteriae B8-1	~3 kb
Shigella dysenteriae B8-2	~3 kb
Shigella dysenteriae B10-1	~92 kb
Shigella dysenteriae B11-1	~92 kb
Shigella dysenteriae B12-1	~3 kb
Shigella dysenteriae B12-2	~3 kb
Shigella dysenteriae B18-1	~92 kb
Shigella dysenteriae B18-2	~92 kb

Results

Isolation and identification of multidrug-resistant bacteria: One hundred and sixty six bacterial isolates were selected for antibiotic resistance, morphological, biochemical and further investigations. These bacterial isolates were screened for antimicrobial sensitivity profiles. Antibiotic susceptibility pattern of the bacterial isolates is summarized in Table 3. Resistance range of the bacterial isolates for 15 antibiotics examined in downward order was separately Lincomycin, Oxacillin, Oxytetracycline, Chloramphenicol, Ampicillin, Ciprofloxacin, Amoxicillin, Neomycin, Piperacillin, Gentamycin, Cephalexin, Cefaclor, Enrofloxacin, Norfloxacin and Colistin. No strain was found sensitive to Lincomycin, Oxacillin, Oxytetracycline and Chloramphenicol. Moreover, 1%-35% isolates showed intermediate resistant to 12 antibiotics out of total 15 investigated. Fifty to one hundred percent of bacterial isolates

demonstrated resistance to Lincomycin, Oxacillin, Oxytetracycline, Chloramphenicol, Ampicillin and Ciprofloxacin. All isolates were found resistant to Lincomycin.

Multi-resistant bacterial isolates (Table 4) were identified morphologically and biochemically (Table 5) and further confirmed by API 20E strips. Out of the 42 cloacal samples evaluated in this study only 20 (48%) were positive for *Salmonella, Shigella* and *Proteus*. Only 5 cloacal samples were positive for *Salmonella* from Chicken-5-2, Chicken-6-1, Chicken-6-2, Chicken-9-2 and Chicken-5-1 with an incidence of 9.6% *Salmonella* isolates in this study. A total of 30 *Shigella* isolates (18%) were isolated from 11 cloacal samples from Pigeon (Zajel)-3-1, Pigeon (Zajel)-4-1, Chicken-11-1, Farmyard pigeon-13-2, Farmyard pigeon-14-2, Quail-17-2, Quail-8-1, Quail-8-2, Farmyard pigeon-12-1, Farmyard pigeon-12-2 and Chicken-18-2. Only 4 cloacal samples were positive for *Proteus* from Chicken-9-1, Chicken-10-1, Quail-17-1 and Chicken-18-1 with an incidence of 4.8% *Proteus* isolates. The serological tests revealed that *Salmonella* isolates included 2 serotypes; *Salmonella* Typhimurium and *Salmonella* Typhi. *Shigella* and *Proteus* isolates were identified as *Shigella dysenteriae* and *Proteus vulgaris*, respectively.

Some bacterial isolates of the current investigation showed resistance to more than 3 antibiotics. One hundred percent of the *Salonella*, *Shigella* and *Proteus* isolates tested in this investigation exhibited resistance to 3-13 antibiotics from the 15 tested antibiotics (Table 4). All *Salmonella* isolates showed resistance to 5-11 antibiotics while all *Proteus* isolates exhibited resistance to 8-13 antibiotics. Moreover, all *Shigella* isolates demonstrated resistance to 3-10 antibiotics. The most found MDR pattern of *Salmonella* Typhimurium was CEC, OX, AM, CL, N, CIP, T, L, CN, AX, PRL; while it was OX, AM, C, N, CIP, T, L, CN, AX, PRL for *Shigella dysenteriae*. Moreover, the MDR pattern of *Proteus vulgaris* was OX, AM, C, CL, N, CIP, T, NOR, L, CN, AX, ENR, PRL.

Amplification of invA and mdh genes: The PCR amplification of nucleotide fragment inside the *Salmonella* invasion gene, *invA* gene, of the bacterial isolates was investigated as a method of differentiating *Salmonella* from other bacteria. A group of MDR bacterial isolates was examined. The PCR product of *invA* gene (~284 bp DNA fragment) in the *Salmonella* isolates were identified (Table 6). On the other hand, none of the non-*Salmonella* isolates produced specific PCR product. The non-*Salmonella* isolates produced a non-specific PCR product that was clearly different in size from the specific 284 bp product. Because malic acid dehydrogenase, an enzyme of the citric acid cycle, is common to organisms, the membrane-associated and cytoplasmic malate dehydrogenase gene (*mdh*) coding for this enzyme was also investigated. The molecular weight of the *mdh* PCR product was 261 bp as expected. All *Salmonella*, *Shigella* and *Proteus* isolates generated positive results (Table 6).

Plasmid profiles: Table 7 shows the resistance plasmids in multidrug-resistant isolates. *Salmonella* Typhi X6-1 and *Salmonella* Typhi X6-2 harboured ~120 kb plasmids. All strain of *Salmonella* Typhimurium harboured ~14 kb plasmids except *Salmonella* Typhimurium X9-2 harboured ~140 kb and 14 kb plasmids. All *Shigella dysenteriae* strains carried ~3 kb plasmids except *Shigella dysenteriae* X11-1, *Shigella dysenteriae* B10-1, *Shigella dysenteriae* B11-1, *Shigella dysenteriae* B18-1 and *Shigella dysenteriae* B18-2 harboured ~92 kb plasmids; *Shigella dysenteriae* B4-1, *Shigella dysenteriae* X13-2 and *Shigella dysenteriae* X14-2 carried ~4 kb plasmids; and *Shigella dysenteriae* X17-2 harboured ~7 kb plasmids. All *Proteus vulgaris* strains harboured ~4 kb and 5 kb plasmids except *Proteus vulgaris* X10-1 harboured ~5 kb plasmids.

16S rRNA gene analysis: For further characterization, 16S rRNA encoding genes of MDR bacterial isolates (*Shigella dysenteriae* X11-1, *Shigella dysenteriae* X17-2, *Shigella dysenteriae* B10-1, *Shigella dysenteriae* B11-1, *Shigella dysenteriae* B18-1, *Shigella dysenteriae* B18-2, *Salmonella* Typhimurium X9-2, *Salmonella* Typhimurium B5-1, *Salmonella* Typhimurium B6-1, *Salmonella* Typhimurium B6-2, *Salmonella* Typhi X6-1, *Salmonella* Typhi X6-2, *Proteus vulgaris* X6-2, *Proteus vulgaris* X10-1, *Proteus vulgaris* X17-1 and *Proteus vulgaris* X18-2) were PCR-amplified and sequenced. The nucleotide sequences of MDR bacterial isolates were compared to existing sequences in the databases. A dendrogram demonstrating the results of 16S rRNA analysis is shown in Fig 1. The results show highest matching of the isolates X11-1, X17-2, B10-1, B11-1, B18-1 and B18-2 to members of the *Shigella* group. As demonstrated, the 16S rRNA sequences of the *Shigella* isolates are most closely associated to *Shigella dysenteriae*. Furthermore, the results indicate greatest similarity of the isolates X9-2, B5-1, B6-1 and B6-2 to members of the *Salmonella* group. As demonstrated, the 16S rRNA sequences of the *Salmonella* isolates are most closely associated to *Salmonella* Typhimurium. In addition, the results indicate greatest similarity of the isolates X6-1 and X6-2 to members of the *Salmonella* group. As shown, the 16S rRNA sequences of the *Salmonella* isolates are most closely associated to *Salmonella* Typhi. Moreover, the results indicate greatest similarity of the isolates X6-2, X10-1, X17-1 and X18-2 to members of the *Proteus* group. As explained, the 16S rRNA sequences of the *Salmonella* isolates are most closely associated to *Proteus vulgaris*. These results are compatible with the conclusions of the morphological, biochemical, API 20E and serological characterization. These findings confirm that the MDR bacterial isolates are new strains.

Discussion

Salmonella, *Shigella* and *Proteus* are significant zoonotic pathogens and their incidence in animals creates a steady threat to human being (Muragkar et al., 2005). In the current work, *Salmonella* Typhi, *Salmonella* Typhimurium, *Shigella dysenteriae* and *Proteus vulgaris* were isolated from cloacal swab samples of domestic birds. The overall isolation rate of 9.6% of *Salmonella*, 18% of *Shigella* and 4.8% of *Proteus* indicates that there is at least a moderate prevalence of infection in the backyard chickens, posing a risk to

industrial chicken farms, and public health. Consequently, the courtyard chickens should be taken into account when any prophylactic program is planned at controlling *Salmonella* infections. Gast and Beard (1990) reported that cloacal swabs were utilized to supply indication of strong intestinal occupation by salmonellae in individual birds. The isolation of *S.* Typhimurium from the cloacal swabs of Italian pigeons was reported (Cena, 1999).

Approximately 50-100% of the bacterial isolates showed resistance to Lincomycin, Oxacillin, Oxytetracycline, Chloramphenicol, Ampicillin and Ciprofloxacin. All isolates were found resistant to Lincomycin. The practice of antimicrobials in veterinary medication as food animal growth promoting agent and in humans through the earlier decade has resulted in massive stress for supporting antimicrobial resistance amongst bacterial pathogens worldwide (Hakanen et al., 2001). Presently, there is an increasing worry about the expansion of multidrug-resistant bacteria causing zoonosis and taking a vital animal tank, for example *Salmonella* strains (Kariuki et al., 2006).

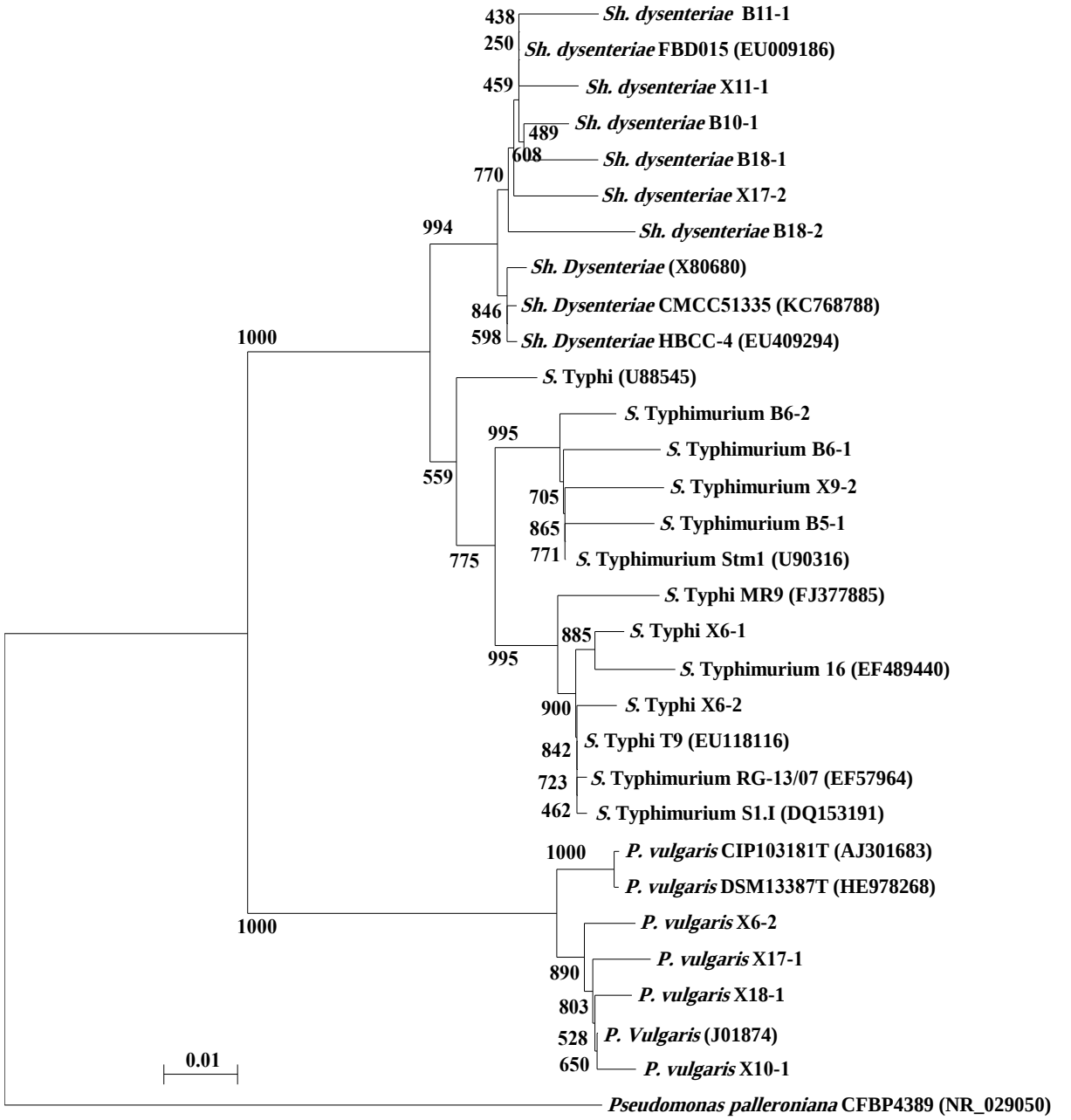

Figure 1 A phylogenetic tree of multidrug-resistant isolates based on the nucleotide sequences of 16S rRNA genes was constructed by neighbor-joining method. The scale bar shows the genetic distance. The number presented next to each node shows the percentage bootstrap value of 1000 replicates. The GenBank accession numbers of the bacteria are presented in parentheses. The *Pseudomonas palleroniana* was treated as the out-group.

The most found multidrug-resistant (MDR) pattern of *Salmonella* Typhimurium was CEC, OX, AM, CL, N, CIP, T, L, CN, AX, PRL; while it was OX, AM, C, N, CIP, T, L, CN, AX, PRL for *Shigella dysenteriae*. Moreover, the MDR pattern of *Proteus vulgaris* was OX, AM, C, CL, N, CIP, T, NOR, L, CN, AX, ENR, PRL. Ampicillin resistance was noticed in all *Salmonella* and *Proteus* isolates, and in some *Shigella* isolates, matching the results obtained by Suresh et al. (2006). The resistance to tetracycline was similarly observed in 100% of the bacterial isolates, higher than described reports (Bada-Alambedji et al., 2006). One of the most frequently used growth promoters is Tetracycline. Consequently, resistance of bacterial isolates to tetracycline might be predicted to be members of this class (chlortetracycline and oxytetracycline) that are usually utilized as antibiotic growth supporters (Jones and Ricke, 2003). The high resistance of bacterial isolates might be described by the potential distribution of the *tetA* resistance gene, as detected in Italy (Pezzella et al., 2004). The significantly high resistance rates to usually used antimicrobial substances amongst the *Salmonella*, *Shigella* and *Proteus* isolates examined create an important threat issue for the handling of foodborne *Salmonella*, *Shigella* and *Proteus* infections in individuals.

This study revealed the emergence of multiple drug-resistant *Salmonella, Shigella and Proteus* from poultry. Therefore, carefully reducing the use of antibiotics in livestock farms and medical practices must be requested. Because of haphazard application of antimicrobial compounds, such high frequency of multidrug resistance of bacteria might obviously happen which could finally substitute the drug-sensitive bacteria from antibiotic flooded ecosystem (Van de Boogard and Stobberingh, 2000).

Darwin and Miller (1999) reported that an important protein present in inner membrane of *Salmonella*, encoded by *invA* gene, was essential for attacking epithelial cells in humans and animals. The PCR product of *invA* gene (284 bp DNA fragment) in only the *Salmonella* isolates were detected. Previous results reported that *invA* gene had sequences unique to *Salmonella* (Rahn et al., 1992; Shanmugasamy et al., 2011). Previous results also reported that the incidence of *Salmonella* was distinguished in 192 samples of fowl carcasses from fowl farms in Shiraz province (Iran), and all isolates were examined for *invA* gene and were confirmed as *Salmonella* positive by the expected PCR product of 284-bp (Zahraei Salehi et al., 2005). The *mdh* PCR product (261 bp) was also detected in all bacterial isolates. The molecular weight of the *mdh* PCR product was 261 bp of *Salmonella* Typhimurium isolated from stool and food samples (Lin and Tsen, 1999).

In the present study, *Salmonella* Typhi harboured ~120 kb plasmids. All strain of *Salmonella* Typhimurium harboured ~14 and 140 kb plasmids. All *Shigella dysenteriae* strains carried ~3, 4, 7 and 92 kb plasmids. All *Proteus vulgaris* strains harboured ~4 kb and 5 kb plasmids. A previous study demonstrated that a significant number of *Salmonella* and *Shigella* isolates, found to harbour the plasmid-encoded multi-drug resistance, were resistant to Chloramphenicol, Ampicillin, Ceftriaxone, Trimethoprim-sulfamethoxazole, Cefuroxime and to a slighter degree to Ciprofloxacin and Ofloxacin (Suh Yah, 2010). The MDR bacterial isolates of *Salmonella* Typhi harbored plasmids of 120 kb and 14 kb (Karmaker et al., 1991). The resistance of gastroenteric *Salmonella and Shigella* strains to antimicrobial agents has increased due to the production of extended-spectrum β-lactamases (ESBLs) encoded on plasmids (Yujuan and Ling, 2006). Therefore, the current results suggest that the MDR bacteria might rise from sensitive bacteria by the taking of multidrug-resistant plasmid from antibiotic-resistant enteric bacteria.

This study revealed that the prevalence rate of MDR *Salmonella*, *Shigella* and *Proteus* in domestic birds at Taif province were 9.6%, 18% and 4.8%, respectively. A significant number of the plasmid-encoded MDR *Salmonella*, *Shigella* and *Proteus* isolates were exhibited to harbour transferable plasmid genes resistant to antibiotics. The presence of these plasmid-encoded MDR suggests that these strains could increase public health problems if distributed in general human population. The high level of antimicrobial resistance of pathogenic bacteria isolated from poultry might cause a possible hazard to public health. Procedures must be applied to decrease the hazard of infection of poultry and to prevent infection by consumption of avian products.

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บทคัดย่อ

การแยกและจำแนกลักษณะทางอณูชีวโมเลกุลของแบคทีเรีย *Salmonella Shigella* และ *Proteus* ที่ดื้อต่อยาหลายชนิดในสัตว์ปีก

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การเพิ่มขึ้นของแบคทีเรียในทางเดินอาหารที่ต้านยาหลายชนิด โดยเฉพาะ *Salmonella*, *Shigella* และ *Proteus* ในสัตว์ปีกได้กลายเป็นภาวะคุกคามทางสาธารณสุขที่สำคัญ ดังนั้น งานนี้จึงมีวัตถุประสงค์เพื่อค้นหาว่านกเลี้ยงมีบทบาทในการปรากฏและการถ่ายทอดแบคทีเรียที่ต้านต่อยาหลายชนิด (MDR) ในจังหวัด Taif ประเทศซาอุดิอารเบีย เชื้อ *Salmonella*, *Shigella* และ *Proteus* ได้ถูกแยกจากนกเลี้ยงหลากหลายชนิดที่มีสุขภาพดี (n=42) จากหลายแหล่ง แบคทีเรียที่แยกได้ถูกเก็บจากการป้าย cloaca ของนก และใช้เทคนิค non-selective และ selective pre-enrichment แบคทีเรียจำนวน 166 isolate ได้ถูกคัดกรองสำหรับความไวต่อยาปฏิชีวนะ เช่น Cefaclor, Oxacillin, Ampicillin, Chloramphenicol, Cephalexin, Neomycin, Colistin, Ciprofloxacin, Oxytetracycline, Norfloxacin, Lincomycin, Gentamycin, Amoxicillin, Enrofloxacin and Piperacillin แบคทีเรีย isolate ที่เป็น MDR ได้ถูกระบุชนิดโดยใช้การจำแนกลักษณะทางสัณฐานวิทยา ทางชีวเคมี และยืนยันด้วย API 20E strip ทำการทดสอบทางซีรั่มวิทยา และการวิเคราะห์ 16S rRNA นกที่เก็บตัวอย่างได้ร้อยละ 48 ให้ผลบวกต่อการเป็นแบคทีเรียที่ก่อโรคและดื้อยา ได้แก่ *Salmonella*, *Shigella* และ *Proteus* การจำแนกชนิดทาง phenotype ได้รับการพิสูจน์โดยการเพิ่มจำนวนของยีน *invA* and *mdh* ด้วยวิธี PCR มีการพบว่าแบคทีเรีย isolate ที่เป็น MDR เป็นแหล่งของการดื้อยาหลายชนิดที่สร้างมาจาก plasmid ผลการศึกษาเหล่านี้ได้แสดงว่าสัตว์ปีกอาจเป็นแหล่งรังโรคของ *Salmonella*, *Shigella* และ *Proteus* ที่ต้านต่อยาหลายชนิด และอาจเป็นวิธีการที่เป็นไปได้ในการแพร่กระจายเชื้อโรคเหล่านี้สู่คนได้

คำสำคัญ: เชื้อแบคทีเรียดื้อยา นกเลี้ยง ยีน *invA* ยีน *mdh* *Proteus* *Salmonella* *Shigella*

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