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Original Article

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Abstract

Enterobacter infection can cause diseases such as bacteremia, lower respiratory tract infections, skin and soft-tissue infections, urinary tract infections, etc. Enterobacter could be isolated from natural environments. Enterobacter infections can require prolonged hospitalization and powerful and expensive antimicrobial agents. Increase in the number of resistant and multi-resistant strains of bacteria is a major concern to health officials worldwide. Therefore, the objective of the study was to evaluate the prevalence of antibiotic-resistant *Enterobacter* sp. recovered from wild birds collected in Taif province, Saudi Arabia. Fifty isolates were recovered from cloacal samples of resident wild birds and 32 isolates from cloacal samples of migratory wild birds. Sixteen percent of the isolates from resident birds were multi-resistant to 3-4 groups of antimicrobials, and 84% of the isolates were multi-resistant to 5-10 groups of antimicrobials. Six percent of the isolates from migratory birds were multi-resistant to four groups of antimicrobials and 94% of the isolates were multi-resistant to 5-10 groups of antimicrobials. Sixty-five percent of the most potent multidrug-resistant (MDR) bacterial isolates (> 7 antibiotics) belonged to *Enterobacter cancerogenus*. Aminoglycoside (*strA* and *strB*), b-lactam (bla_{TEM}), tetracycline (*tetA* and *tetB*) and gentamycin (*Gmr*) resistance genes were detected in all MDR isolates. Multiple plasmids of high molecular weights were found in the same MDR host strain. This suggests that there is a potential for multidrug-resistant bacterial transmission between birds. This study highlights the role of wild birds in the dissemination of multidrug-resistant bacteria and antimicrobial resistance genes around the world.

Keywords: antibiotic-resistant bacteria, *Enterobacter cancerogenus*, wild birds, antibiotic resistance genes, plasmids, 16S-rRNA

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Introduction

Emergence of resistance to multiple antimicrobial agents in pathogenic bacteria has become a significant public health threat as there are fewer, or even sometimes no, effective antimicrobial agents available for infections caused by these bacteria. Gram-positive and Gram-negative bacteria are both affected by the emergence and rise of antimicrobial resistance. The problem of increasing antimicrobial resistance is even more threatening when considering the very limited number of new antimicrobial agents that are in development (ECDC/EMEA, 2009; Boucher et al., 2009). Pathogenic bacteria in humans and animals resistant to commonly used antibiotics have become a major global healthcare problem in the 21st century. Nowadays, antibiotic-resistant bacteria can be found in all different ecological niches. Selective pressure in favour of bacteria possessing these genes has emerged from the abusive use of antibiotics mainly in hospitals, agriculture and animal farming (Kummerer, 2004).

Enterobacter species are common causes of nosocomial infections in humans. Apart from *E. hormaechei*, *E. asburiae* and *E. aerogenes*, which represent the most frequently encountered Enterobacter species in clinical specimens, there are several further *Enterobacter* taxa associated with human disease. The most common is *E. sakazakii*, a yellow-pigmented and biochemically heterogeneous species with an unusual thermotolerance (Nazarowec-White and Farber, 1997). *Enterobacter sakazakii*, first described in 1980 (Farmer et al., 1980), is an important agent of life-threatening neonatal sepsis and meningitis, with mortality rates of 40-80% (Grimont and Grimont, 1992). In some cases these diseases are complicated by the development of brain abscesses and other secondary disorders (Burdette and Santos, 2000). *Enterobacter sakazakii* was also shown to cause an outbreak of severe necrotizing enterocolitis in a neonatal intensive care unit in Belgium (Van Acker et al., 2001). Although the guts of insects were shown to contain the species (Kuzina et al., 2001) and dried infant formula milk has been implicated in both outbreaks and sporadic cases of *E. sakazakii* meningitis, its natural habitat remains obscure (Nazarowec-White and Farber, 1997). Izard et al. (1981) described Enterobacter strains, previously called *Enterobacter* H3, as *E. amnigenus*. These bacteria have been isolated from water and from several clinical specimens, e.g. respiratory tract, wound, blood and feces (Farmer et al., 1985a), and were shown to cause sepsis and other infections in man (Capdevila et al., 1998). Strains of *E. cancerogenus* have been isolated predominantly from human blood and spinal fluid (Farmer et al., 1985b) and were found to be etiological agents of wound and urinary tract infections, sepsis and osteomyelitis (Abbott and Janda, 1997). *Enterobacter taylorae*, described in 1985 by Farmer et al. (1985b), was shown to be a junior synonym of *E. cancerogenus* (Grimont and Ageron, 1989). A further, widely distributed *Enterobacter* species is *E. gergoviae*. *Enterobacter* species have been isolated from environmental samples and clinical specimens and is likely to cause sepsis and urinary tract infections (Grimont and Grimont, 1992). Although there is some information on antimicrobial susceptibility patterns of recently established *Enterobacter* species, in most cases only a small number of strains/species and/or a few antibiotics have been examined. Data on natural sensitivity or resistance are exceptional.

Wild birds have been reported as a possible reservoir and dispenser for multidrug-resistant bacteria (Radhouani et al., 2012; Simões et al., 20012; Shobrak and Abo-Amer, 2014). The presence of bacteria of potential zoonotic importance among wild birds has public health significance. Water contact and acquirement by food seem to be the main factors of transmission of resistant bacteria from human or veterinary origin to wild animals (Kozak et al., 2009). Wild birds or general wild animals could, therefore, serve as reservoirs of resistant bacteria and genetic factors of antimicrobial resistance (Dolejska et al., 2009; Shobrak and Abo-Amer, 2014).

Antimicrobial resistance has been known as an emerging worldwide problem in both human and veterinary medicine, and antimicrobial use is considered the most important factor for the emergence, selection, and distribution of antimicrobial-resistant bacteria (Winokur et al., 2001).

The problem of antimicrobial resistance worldwide is one of the foremost issues we will face in the coming decades. We strongly believe that there is an urgent requirement for research on how to comprehensively address the problems of antimicrobial resistance. The problem of resistance as a public health threat has increased significantly over the last decade and local solutions are needed. Because the wild birds other than other animals might play an important role in the transmission of multidrug-resistant bacteria across the world because of their migration and there was no reports stating the relationship between the transmission of MDR bacteria and wild birds in the Middle East region, especially in Saudi Arabia, the aim of this work was proposed. The objective of this study was to obtain detailed understanding of possible wild birds as carriers and spread of drug-resistant *Enterobacter* sp. in the environment which could form a potential hazard to human and animal health by transmission of antimicrobial-resistant strains to waterways and environmental sources through faecal deposits. Therefore, this work was approached to isolate, molecularly characterize and verify the prevalence of antimicrobial-resistant *Enterobacter* sp. in wild birds from different areas with an attention on the detection of multidrug-resistant (MDR) bacteria.

Materials and Methods

Sampling sites and birds capture: Different types of resident and migrating wild birds were captured during 2013-2014, using misnets and clap-nets, from different areas in Taif province, Saudi Arabia. Birds were captured within a vegetated area covering 500 m radius. Resident birds were Philby's Partridge (*Alectoris philbyi*), Arabian Serin (*Crithagra rothschildi*), Lappet-faced Vulture (*Torgos tracheliotos*), Arabian Babbler (*Turdoides squamiceps*), Sand Partridge (*Ammoperdix heyi*), White-spectacled Bulbul (*Pycnonotus xanthopygos*), Black Scrub Robin (*Cercotrichas podobe*) and Rüppell's Weaver (*Ploceus*

galbula). Migrating birds were Sand Martin (*Riparia riparia*), Isabelline Shrike (*Lanius isabellinus*), Barn Swallow (*Hirundo rustica*), Willow Warbler (*Phylloscopus trochilus*) and Tawny Pipit (*Anthus campestris*). These wild birds specially, migrating birds, were in contact with birds in chicken farms and consequently to human being. The birds were characterized and identified according to Porter and Aspinall (2010).

Sampling from cloacal swabs: Sterile cotton swabs wetted with sterile normal saline water were inserted in the cloacae of the resident and migrating resident wild birds, and placed in sterile vials. After collection of cloacal swabs, the birds were then freed. The samples were transported immediately to the laboratory in an ice box for microbiological studies.

Isolation and identification of Enterobacter sp.: Standard methods were used for the enrichment, isolation, identification, and biochemical confirmation of *Enterobacter* sp. isolates (Clesceri et al., 1998). All samples were processed within 4 h. The swab samples were enriched in buffered peptone water at 37°C for 24 h. Subsequently, the cultures were streaked on MacConkey agar and incubated at 37°C overnight. The plates were investigated for lactose fermenting bacteria (red colonies) and different single colonies were then collected and purified.

All isolates were morphologically characterized by Gram stain. For biochemical characterization, the isolates were inoculated in tryptic soy broth (TSB), and incubated for approximately 4 h until the cultures were turbid. Identification of the isolates was performed following a series of biochemical tests including oxidase, methyl red, Voges-Proskauer reactions, indole, citrate, catalase, urea hydrolysis, gelatin hydrolysis, lactose fermentation, nitrate reduction, casein hydrolysis and sugar fermentation. MDR Coliform bacteria were isolated on MacConkey agar plates and identified by standard biochemical methods (Barrow and Feltham, 1993).

Identification by API 20E: The identification of the strains was confirmed with a commercial identification system for Enterobacteriaceae (API 20E strips, Bio Merieux). This identification system contains biochemical key reactions for most clinically significant Enterobacteriaceae species. The inoculum for the identification tests was a suspension from an overnight culture on nutrient agar (Oxoid) in physiological saline solution at 10^6 colony-forming units (CFU)/mL; incubation times were 24 h at 37°C.

Antimicrobial susceptibility test: The bacterial isolates were screened for antimicrobial agent sensitivity testing. The antimicrobial sensitivity phenotypes of *Enterobacter* spp. isolates were determined using a Kirby-Bauer disk diffusion assay according to the standards and interpretive criteria described by CLSI (2012).

A total of 15 antimicrobial discs with 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) were used.

Plates of Mueller-Hinton medium were swabbed with TSB broth inoculated with the bacterial isolates and incubated to a turbidity of 0.5 McFarland standard. The commercially prepared antimicrobial disks (4x50, BIO-RAD) were placed on the inoculated plates. The plates were incubated at 35°C for 20 hours. Diameters (millimeters) of the clear zones of growth inhibition around the antimicrobial agent disks, including the 6-mm disk diameter, were measured by using precision calipers. The zone diameter for individual antimicrobial agents was then explained into sensitive, intermediate and resistant categories according to the interpretation table of the CLSI (2012). Multi-drug resistance (MDR) refers to resistance to 3 or more antimicrobials.

Molecular identification of the most MDR bacteria

Preparation of genomic DNA: Bacterial colonies were picked up with a sterilized toothpick and suspended in 0.5 ml of sterilized saline in a 1.5 ml centrifuge tube. Centrifugation was performed at 10,000 rpm for 10 min. After removal of supernatant, the pellet was suspended in 0.5 ml of InstaGene Matrix (Bio-Rad, USA) and incubated 56°C for 30 min and then heated at 100 °C for 10 min. After heating, supernatant was used for PCR (Abo-Amer, 2011).

PCR of 16S rRNA gene: 1 µl of template DNA was added in 20 µl of PCR reaction solution. 27F (5´-AGA GTT TGA TCM TGG CTC AG-3´) and 1492R (5´-TAC GGY TAC CTT GTT ACG ACT T-3´) primers were used. Thirty-five amplification cycles were performed at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec. DNA fragments amplified were ~ 1,400 bp.
Unincorporated PCR primers and dNTPs were removed from the PCR products by using Montage PCR Clean up kit (Millipore).

16S rRNA gene sequencing: The purified PCR products of approximately 1,400 bp were sequenced by using two primers which were 518F (5´-CCA GCA GCC GCG GTA ATA CG-3´) and 800R (5´-TAC CAG GGT ATC TAA TCC-3´). Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

Selected sequences of other microorganisms with the greatest similarity to the 16S rRNA sequences of bacterial isolates were extracted from the nucleotide sequence databases and aligned using CLUSTAL W (1.81) Multiple Sequence Alignment generating phylogenetic tree. The 16S rRNA gene sequences of the bacterial isolates reported in this paper were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with accession numbers: AB776820 (Enterobacter *cancerogenus* NMB2-1), AB776821 (Enterobacter *cancerogenus* NMB2-2), AB776822 (Enterobacter *cancerogenus* NMB7-1), AB776823 (Enterobacter *cancerogenus* NMB8-1), AB776824

(Enterobacter *cancerogenus* NMB8-2), AB776825
(Enterobacter *cancerogenus* NMB10-2), AB776826
(Enterobacter *cancerogenus* MB18-1), AB776827
(Enterobacter *cancerogenus* MB18-2), AB776828
(Enterobacter *cancerogenus* MB20-1), AB776829
(Enterobacter *cancerogenus* MB20-2), LC005844
(*Enterobacter cancerogenus* NMB7-2), LC005847
(*Enterobacter cancerogenus* MB14-1), LC005848
(*Enterobacter cancerogenus* MB14-2), LC005853
(*Enterobacter cancerogenus* MB21-1) and LC005854
(*Enterobacter cancerogenus* MB21-2.

Plasmid isolation: Plasmid DNA of MDR bacterial isolates was extracted by the alkaline lysis method (Anderson and McKa, 1983), analysed by electrophoresis through 0.8% agarose gel, visualised under UV transilluminator, and photographed and recorded using the Gel Documentation system (Model G:BOX, Syngene). To estimate the molecular weight of plasmids, *Escherichia coli* V517 (MTCC 131) was used as a source of standard plasmid marker, which harbored eight different plasmids with known molecular weight.

PCR screening for antimicrobial resistance genes: All the MDR *Enterobacter* spp. isolates were examined for the presence of antimicrobial resistance genes. Several colonies of each MDR *Enterobacter* isolate cultivated on Nutrient Agar (Oxoid) were resuspended in 300 µl sterile distilled water. The cells were lysed by boiling in an aqueous bath at 95°C for 10 min. The mixture was then centrifuged at 16000 *g* for two min. The supernatant was used as template DNA for PCR. Multiplex-PCRs (mPCR) were optimized to enable simultaneous amplification of multiple resistance genes. The conditions for mPCR were an initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min, with a single final extension at 72°C for 7 min. All mPCRs were performed in a 25-µl reaction mixture containing 1×PCR buffer, 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 60 pmol of each primer, 1 U of Taq DNA polymerase, and 100 ng of template DNA. The primers used for PCR are listed in Table 1. The PCR products were analyzed by electrophoresis through a 1.5% agarose gel, staining with ethidium bromide (0.5µg/ml), and then visualization using the GelDoc (BioRad). A DNA ladder of 100 bp (Promega) was used as the molecular weight marker.

Statistical analysis: All analyses were carried out according to one-way analysis of variance (ANOVA) and assessed 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 experiments were performed in triplicate.

Table 1 PCR primers used in identification of resistance genes

Gene	Primer sequence (5´→-3´)[a]	Product size (bp)	Reference or source
bla$_{TEM}$	F, GCA CGA GTG GGT TAC ATC GA R, GG□ CCT CCG ATC GTT GTC AG	310	Carlson et al., 1999
strA	F, CTT GGT GAT AAC GGC AAT TC R, CCA ATC GCA GAT AGA AGG C	548	AF273682
strB	F, ATC GTC AAG GGA TTG AAA CC R, GGA TCG TAG AAC ATA TTG GC	509	AF024602
tetA	F, GCT ACA TCC TGC TTG CCT TC R, CAT AGA TCG CCG TGA AGA GG	210	X61367
tetB	F, TTG GTT AGG GGC AAG TTT TG R, GTA ATG GGC CAA TAA CAC CG	659	Lai-King et al., 1999
Gmr	F, AAG CGC ACG AAG CGC GGG CTG R, AAG GCG GGC CTC AAG GAG GTC	414	Frana et al., 2001

a F, forward; R, reverse

Results

Isolation of Enterobacter spp. from birds: Eighty-two bacterial isolates were recovered from the cloacal swabs of resident and migrating wild birds on MacConkey agar plates. Fifty isolates were recovered from the resident wild birds and 32 isolates from the migratory wild birds. These were lactose fermenting bacteria (red colonies) and Gram-negative bacteria.

Antibiotic Susceptibility test: The fifty bacterial isolates recovered from resident wild birds were screened for antimicrobial agent sensitivity testing. One hundred percent and 88 % of the bacterial isolates recovered from resident birds were resistant to Oxacillin and Chloramphenicol, respectively. Moreover, 88% and 84% of the bacterial isolates recovered from resident birds were resistant to Lincomycin and Oxytetracycline, respectively. Ninety-six percent of the bacterial isolates recovered from resident birds were multidrug-resistant (≥ 3 antibiotics) to all 15 antibiotics tested. Ninety-six percent of the bacterial isolates recovered from migrating birds were sensitive to Enrofloxacin.

The thirty-two bacterial isolates recovered from migrating wild birds were screened for antimicrobial agent sensitivity testing. One hundred percent of the bacterial isolates recovered from migrating birds were resistant to Oxacillin, Chloramphenicol, Oxytetracycline and Lincomycin. Moreover, 87.5% and 81% of the bacterial isolates recovered from migrating birds were resistant to Ciprofloxacin and Ampicillin, respectively. All bacterial isolates recovered from migrating birds were sensitive to Norfloxacin, Gentamycin and Enrofloxacin, and 87.5 % of the bacterial isolates recovered from migrating birds were sensitive to Piperacillin.

Multidrug-resistant (MDR) bacteria: Multidrug resistance profiles of the 50 bacterial isolates from resident birds are shown in Table 2. Eight percent of the isolates were multi-resistant to three groups of antimicrobials, and 8% of the isolates were multi-resistant to four antimicrobial groups. The rest were multi-resistant (84%) to 5-10 groups of antimicrobials. Multidrug resistance of the 32 bacterial isolates from migratory birds is presented in Table 3. Six percent of the isolates were multi-resistant to four groups of antimicrobials. The other isolates (94%) were multi-resistant to 5-10 groups of antimicrobials.

Table 2 Antibiotic resistance profiles of bacterial isolates from resident birds

Isolates	No. of isolates	No. of antibiotics	Antibiotic resistance profiles
NMB 1-1	2	6	OX[1],C,N,CIP,T,L
NMB 1-2	2	6	OX,C,N,CIP,T,L
NMB 2-1	2	10	CEC, OX,AM,C,CL,N,CIP,T,L,AX
NMB 2-2	2	9	CEC, OX,AM,C,CL,CIP,T,L,AX
NMB 3-1	2	8	OX,AM,N,CIP,T,L,AX,PRL
NMB 3-2	2	9	OX,AM,C,N,CIP,T,L,AX,PRL
NMB 4-1	2	5	OX,N,CIP,T,L
NMB 4-2	2	6	OX,C,N,CIP,T,L
NMB 5-1	2	5	OX,C,N,T,L
NMB 5-2	2	6	OX,C,N,CIP,T,L
NMB 6-1	2	4	OX,C,T,L
NMB 6-2	2	4	OX,C,T,L
NMB 7-1	2	7	OX,C,CL,CT,CIP,T,L
NMB 7-2	2	10	CEC,OX,AM,C,CL,CIP,T,L,AX,PRL
NMB 8-1	2	8	CEC,OX,AM,C,CL,T,L,AX
NMB 8-2	2	8	CEC,OX,AM,C,CL,T,L,AX
NMB 9-1	2	3	OX,T,L
NMB 9-2	2	3	OX,T,L
NMB 10-1	2	8	CEC,OX,AM,C,CL,N,CIP,L
NMB 10-2	2	8	CEC,OX,AM,C,CL,N,CIP,L
NMB 11-1	2	5	OX,C,CL,CT,CIP
NMB 11-2	2	5	OX,C,CL,CT,CIP
NMB 12-2	2	12	OX,AM,C,CL,N,CT,CIP,T,NOR,L,CN,ENR
NMB 13-1	2	6	OX,C,N,CT,CIP,L
NMB 13-2	2	6	OX,C,N,CT,CIP,L

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

Table 3 **Antibiotic resistance profiles of bacterial isolates from migrating birds**

Isolates	No. of isolates	No. of antibiotics	Antibiotic resistance profiles
MB 14-1	2	8	CEC[1],OX,AM,C,CL,CIP,T,L
MB 14-2	2	8	CEC,OX,AM,C,CL,CIP,T,L
MB 15-1	2	10	CEC,OX,AM,C,CL,CT,CIP,T,L,AX
MB 15-2	2	8	CEC,OX,C,CL,CT,T,L,AX
MB 16-1	2	6	OX,AM,C,CIP,T,L
MB 16-2	2	4	OX,C,T,L
MB 17-1	2	9	OX,AM,C,CT,CIP,T,L,AX,PRL
MB 17-2	2	9	OX,AM,C,CT,CIP,T,L,AX,PRL
MB 18-1	2	10	CEC,OX,AM,C,CL,CT,CIP,T,L,AX
MB 18-2	2	10	CEC,OX,AM,C,CL,CT,CIP,T,L,AX
MB 20-1	2	9	CEC,OX,AM,C,CL,CIP,T,L,AX
MB 20-2	2	8	CEC,OX,AM,C,CL,CIP,T,L
MB 21-1	2	9	CEC,OX,AM,C,CL,CIP,T,L,AX
MB 21-2	2	8	CEC,OX,AM,C,CL,CIP,T,L
MB 22-1	2	5	OX,C,CIP,T,L
MB 22-2	2	5	OX,C,CIP,T,L

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

*R, resistant; I, Intermediate; S, Sensitive

Identification of MDR bacteria: Forty-six multidrug bacterial isolates (> 7 antibiotics) were identified following a series of biochemical tests (Table 4). Results indicated that 65% of the bacterial isolates belonged to *Enterobacter* sp. The identification of the strains was confirmed with a commercial identification system for Enterobacteriaceae (API 20E strips, Bio Merieux). The MRD *Enterobacter* sp. isolates and their bird origin are shown in Table 5.

Molecular characterization of MDR isolates

***16S rRNA analysis*:** For further characterization, 16S rRNA encoding genes of MDR bacterial isolates 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 Figure 1. Results showed highest matching of isolates NMB2-1, NMB2-2, NMB7-1, NMB8-1, NMB8-2, NMB10-2, MB18-1, MB18-2, MB20-1, MB20-2, NMB7-2, MB14-1, MB14-2, MB21-1 and MB21-2 to members of the Enterobacter group. As demonstrated, the 16S rRNA sequences of the Enterobacter isolates are greatest closely associated to Enterobacter *cancerogenus*. These results are compatible with the conclusions of the morphological, biochemical, API 20E and serological characterization. These findings recommend that the MDR bacterial isolates are new strains.

Resistance genes: The simultaneous detection of different antimicrobial resistance genes such as *bla*$_{TEM}$, *strA*, *strB*, *tetA*, *tetB*, and *Gmr* was achieved using multiplex- PCRs (Table 6). Fifteen MDR isolates (~ 65%) were demonstrated to harbor *bla*$_{TEM}$, *strA*, *strB*, *tetA*, *tetB*, and *Gmr* with sizes of 10 bp, 548 bp, 509 bp, 210 bp, 658 bp and 414 bp, respectively. Twenty-three MDR isolates (100%) were shown to carry *bla*$_{TEM}$, *tetA*, *tetB* and *Gmr*.

Plasmid Analysis: Twelve MDR isolates (52%) were shown to carry three plasmids (~ 115, 93, 75 Kb) (Table 6). Seven MDR isolates (30%) were demonstrated to harbor 2 plasmids (~ 93, 75 Kb) and 2 MDR isolates (9%) harbored 2 plasmids (~ 115, 75 Kb). Moreover, 2 MDR isolates (9%) carried 2 plasmids (~ 48 Kb, 65 Kb).

Table 4 Morphological and biochemical characteristics of multi-resistant isolates (> 7 antibiotics)

Bacterial isolates	No. of isolates	Characteristic tests															identification
		Gram staining	Catalase	Oxidase	Indole	Methyle red	Voges-Proskauer	Citrate	Lactose	Glucose fermentation	Gelatin hydrolysis	Casein hydrolysis	Nitrate reduction	Urea hydrolysis	H_2S on TSI	Motility	
NMB 2-1	2	G-	+	-	-	-	+	+	+	-	-	-	+	-	-	+	*Enterobacter* sp.
NMB 2-2	2	G-	+	-	-	-	+	+	+	-	-	-	+	-	-	+	*Enterobacter* sp.
NMB 7-1	2	G-	+	-	-	-	+	+	+	-	-	-	+	-	-	+	*Enterobacter* sp.
NMB 7-2	2	G-	+	-	-	-	+	+	+	-	-	-	+	-	-	+	*Enterobacter* sp.
NMB 8-1	2	G-	+	-	-	-	+	+	+	-	-	-	+	-	-	+	*Enterobacter* sp.
NMB 8-2	2	G-	+	-	-	-	+	+	+	-	-	-	+	-	-	+	*Enterobacter* sp.
NMB 10-2	2	G-	+	-	-	-	+	+	+	-	-	-	+	-	-	+	*Enterobacter* sp.
MB 14-1	2	G-	+	-	-	-	+	+	+	-	-	-	+	-	-	+	*Enterobacter* sp.
MB 14-2	2	G-	+	-	-	-	+	+	+	-	-	-	+	-	-	+	*Enterobacter* sp.
MB 18-1	2	G-	+	-	-	-	+	+	+	-	-	-	+	-	-	+	*Enterobacter* sp.
MB 18-2	2	G-	+	-	-	-	+	+	+	-	-	-	+	-	-	+	*Enterobacter* sp.
MB 20-1	2	G-	+	-	-	-	+	+	+	-	-	-	+	-	-	+	*Enterobacter* sp.
MB 20-2	2	G-	+	-	-	-	+	+	+	-	-	-	+	-	-	+	*Enterobacter* sp.
MB 21-1	2	G-	+	-	-	-	+	+	+	-	-	-	+	-	-	+	*Enterobacter* sp
MB 21-2	2	G-	+	-	-	-	+	+	+	-	-	-	+	-	-	+	*Enterobacter* sp

G -, gram negative; +, positive reaction; −, negative reaction

Table 5 **Bacterial isolates, their identification and their bird origin**

Isolates	Identification	No. of isolates	Bird type
NMB 2-1	*Enterobacter* sp.	2	Arabian Babbler (*Turdoides squamiceps*), resident bird
NMB 2-2	*Enterobacter* sp.	2	Arabian Babbler (*Turdoides squamiceps*), resident bird
NMB 7-1	*Enterobacter* sp.	2	Rüppell's Weaver (*Ploceus galbula*), resident bird
NMB 7-2	*Enterobacter* sp.	2	Rüppell's Weaver (*Ploceus galbula*), resident bird
NMB 8-1	*Enterobacter* sp.	2	Black Scrub Robin (*Cercotrichas podobe*), resident bird
NMB 8-2	*Enterobacter* sp.	2	Black Scrub Robin (*Cercotrichas podobe*), resident bird
NMB 10-2	*Enterobacter* sp.	2	Arabian Serin (*Crithagra rothschildi*), resident bird
MB 14-1	*Enterobacter* sp.	2	Isabelline Shrike (*Lanius isabellinus*), migrating bird
MB 14-2	*Enterobacter* sp.	2	Isabelline Shrike (*Lanius isabellinus*), migrating bird
MB 18-1	*Enterobacter* sp.	2	Tawny Pipit (*Anthus campestris*), migrating bird
MB 18-2	*Enterobacter* sp.	2	Tawny Pipit (*Anthus campestris*), migrating bird
MB 20-1	*Enterobacter* sp.	2	Sand Martin (*Riparia riparia*), migrating bird
MB 20-2	*Enterobacter* sp.	2	Sand Martin (*Riparia riparia*), migrating bird
MB 21-1	*Enterobacter* sp.	2	Isabelline Shrike (*Lanius isabellinus*), migrating bird
MB 21-2	*Enterobacter* sp.	2	Isabelline Shrike (*Lanius isabellinus*), migrating bird

Table 6 Antibiotic resistance genes and plasmids of multi-resistant isolates (> 7 antibiotics)

Isolates	Resistance genes	plasmids
Enterobacter cancerogenus NMB 2-1	bla_{TEM}, *strA*, *strB*, *tetA*, *tetB*, *Gmr*	~ 115, 93, 75 Kb
Enterobacter cancerogenus NMB 2-2	bla_{TEM}, *strA*, *strB*, *tetA*, *tetB*, *Gmr*	~ 115, 93, 75 Kb
Enterobacter cancerogenus NMB 7-1	bla_{TEM}, *tetA*, *tetB*, *Gmr*	~93, 75 Kb
Enterobacter cancerogenus NMB 7-2	bla_{TEM}, *strA*, *strB*, *tetA*, *tetB*, *Gmr*	~ 115, 93, 75 Kb
Enterobacter cancerogenus NMB 8-1	bla_{TEM}, *strA*, *strB*, *tetA*, *tetB*, *Gmr*	~ 115, 93, 75 Kb
Enterobacter cancerogenus NMB 8-2	bla_{TEM}, *strA*, *strB*, *tetA*, *tetB*, *Gmr*	~ 115, 93, 75 Kb
Enterobacter cancerogenus NMB 10-2	bla_{TEM}, *tetA*, *tetB*, *Gmr*	~ 93, 75 Kb
Enterobacter cancerogenus MB 14-1	bla_{TEM}, *tetA*, *tetB*, *Gmr*	~ 93, 75 Kb
Enterobacter cancerogenus MB 14-2	bla_{TEM}, *tetA*, *tetB*, *Gmr*	~ 93, 75 Kb
Enterobacter cancerogenus MB 18-1	bla_{TEM}, *strA*, *strB*, *tetA*, *tetB*, *Gmr*	~ 115, 93, 75 Kb
Enterobacter cancerogenus MB 18-2	bla_{TEM}, *strA*, *strB*, *tetA*, *tetB*, *Gmr*	~ 115, 93, 75 Kb
Enterobacter cancerogenus MB 20-1	bla_{TEM}, *strA*, *strB*, *tetA*, *tetB*, *Gmr*	~ 115, 93, 75 Kb
Enterobacter cancerogenus MB 20-2	bla_{TEM}, *tetA*, *tetB*, *Gmr*	~ 93, 75 Kb
Enterobacter cancerogenus MB 21-1	bla_{TEM}, *strA*, *strB*, *tetA*, *tetB*, *Gmr*	~ 115, 93, 75 Kb
Enterobacter cancerogenus MB 21-2	bla_{TEM}, *tetA*, *tetB*, *Gmr*	~ 93, 75 Kb

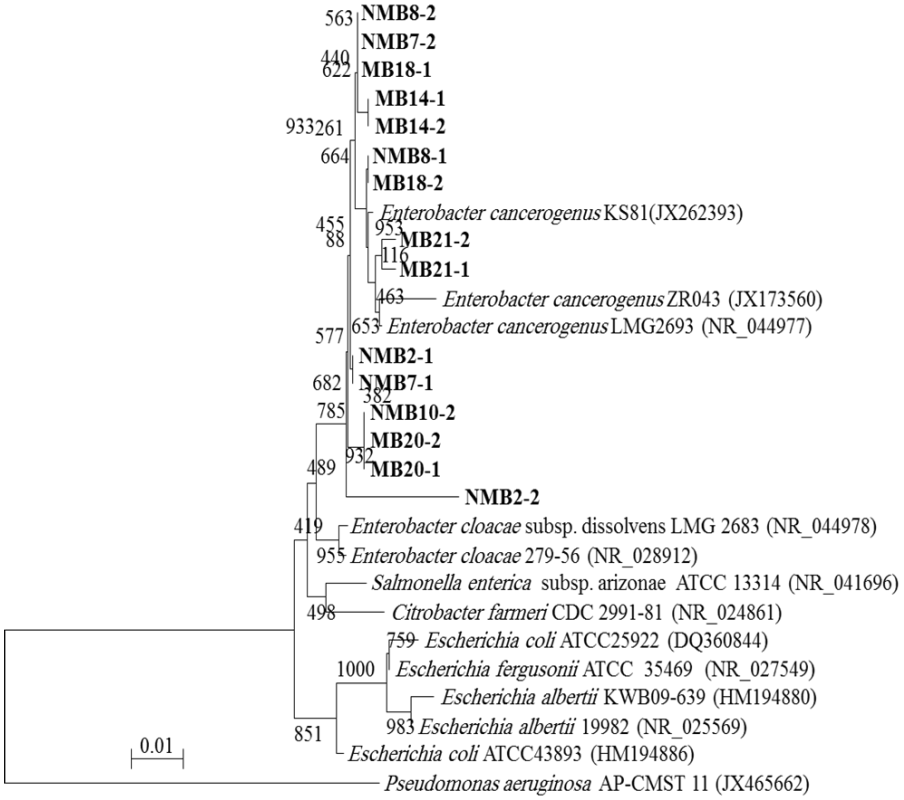

Figure 1 A phylogenetic tree of multidrug-resistant bacterial isolates based on the nucleotide sequences of 16S rRNA genes was constructed by neighbor-joining method. The scale bar shows the genetic distance. The number presented next to each node shows the percentage bootstrap value of 1000 replicates.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

Discussion

The overall aim was to study the current environmental contamination of antibiotic resistance among bacteria, and in *Enterobacter* sp. in particular, due to the use/overuse of antimicrobials in veterinary and human medicine and the lack of infection control. Wild birds were used as indicators of the level of antibiotic resistance contamination in the environment. Therefore, this work was aimed to determine the prevalence and diversity of antibiotic resistance in *Enterobacter* sp. from different wild birds and to determine the prevalence and genotypes of extended spectrum beta-lactamases (ESBLs) in birds.

Sixteen percent of the isolates from resident birds were multi-resistant to 3-4 groups of antimicrobials, and 84% of the isolates were multi-resistant to 5-10 groups of antimicrobials. Six percent of the isolates from migratory birds were multi-resistant to four groups of antimicrobials and 94% of the isolates were multi-resistant to 5-10 groups of antimicrobials. Forty-six multidrug bacterial isolates (> 7 antibiotics) were identified as follows: 65% of the bacterial isolates belonged to *Enterobacter cancerogenus*.

These results indicate a low percentage of isolates of Gram-negative bacteria, among which *Enterobacter* sp. were the most frequent. Similar results showed a low percentage of isolates of Gram-negative bacteria clinically healthy psittacines, among which *E. coli* and *Enterobacter* spp. were the most frequent (Bowman and Jacobson, 1980; Hidasi et al., 2013). A high frequency of *Enterobacter* sp. isolates was noted in this study (30.12%) (Hidasi et al., 2013). While this genus is commonly isolated in samples from cloaca, feces, and eyes of psittacines, it may also be detected as a secondary agent in systemic, respiratory, and intestinal infections (Rosskopf and Woerpel, 1996).

A range of potentially pathogenic bacteria were isolated from the cloacae of resident and migrating wild birds in Taif province, Saudi Arabia. The most prevalent bacterial species isolated from both birds in this study, *Enterobacter* sp. and *Escherichia coli*, are common residents of the intestinal tract of numerous vertebrate species (Janda and Abbott, 1998). Many of the same bacterial species were isolated from both types of birds indicating there is a potential for transmission of bacteria between birds. Wildlife such as wild birds is normally not exposed to clinically used antimicrobial agents, but can acquire antimicrobial-resistant bacteria through contact with humans, domesticated animals and the environment, where water polluted with feces seems to be the most important vector. Increased introduction of antimicrobial agents into the environment via medical therapy, agriculture and animal husbandry has resulted in selective pressures on bacterial populations.

A multiplex-PCR was carried out for accurate detection of antibiotic resistance genes. The present study showed that the antibiotic resistance genes bla_{TEM}, *strA*, *strB*, *tetA*, *tetB* and *Gmr* were found in 65% of the isolates. Moreover, the antibiotic resistance genes bla_{TEM}, *tetA*, *tetB* and *Gmr* were present in 35% of the isolates. Previous results showed that the predominant tetracycline resistance gene *tetA* was present in both human and zoonotic strains, followed by *tetB* and *tetC* genes (Benacer et al., 2010). The *strA* gene was the most frequently found in all streptomycin-resistant strains and has been reported to be associated with plasmids (Randall et al., 2004). Both *strA* and *aadA* genes were present in 11 strains, which most probably carried plasmids containing the *strA* gene and the class 1 integron harboring the *aadA* gene (Benacer et al., 2010).

Plasmid is one of the known most important mediators in facilitating the fast spreading of antibiotic resistance among bacteria (Dale and Park, 2004). Our result revealed that 52% of the isolates harbored three large plasmids (~ 115, 93, 75 Kb), 30% carried 2 plasmids (~ 93, 75 Kb), 2% harbored 2 plasmids (~ 115, 75 Kb) and 2% carried 2 plasmids (~ 48 Kb, 65 Kb). Recent results have reported that large plasmid sizes of ~ 115, 93, and 75 Kb were observed in MDR *Escherichia* isolates from wild birds (Shobrak and Abo-Amer, 2014). Various large plasmids (~ 52 to 100 Kb) were found in isolates of MDR Enterobacteriaceae (Huang et al., 2012). The present study reported that multiple plasmids carrying multiple antibiotic resistance genes were found in the same host strain as reported by Huang et al. (2012). The high molecular weight plasmids, carrying resistance genes and transfer-associated genes, might be potential elements for regional and universal spread of antibiotic resistance. This is the first report showing the relationship between the transmission of MDR *Enterobacter cancerogenus* and wild birds in the Middle East region especially in Saudi Arabia.

Based on these results, the transmission of MDR *Enterobacter cancerogenus* between birds may occur, although evidence suggests it may be frequent. It is advisable to continue monitoring both birds for MDR pathogenic bacteria. Determining the virulence of these bacterial species would also be valuable.

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บทคัดย่อ

การดื้อยาปฏิชีวนะและการจำแนกลักษณะทางอณูชีวโมเลกุลของแบคทีเรีย *Enterobacter cancerogenus* ที่แยกได้จากนกป่าในจักหวัด Taif ประเทศซาอุดิอาระเบีย

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การติดเชื้อ *Enterobacter* เป็นสาเหตุของโรค เช่น มีเชื้อแบคทีเรียในกระแสเลือด การติดเชื้อในทางเดินหายใจส่วนล่าง การติดเชื้อที่ผิวหนังและเนื้อเยื่ออ่อน การติดเชื้อในทางเดินขับถ่ายปัสสาวะ เป็นต้น *Enterobacter* สามารถถูกแยกได้จากสิ่งแวดล้อมในธรรมชาติ การติดเชื้อ *Enterobacter* จำเป็นต้องมีการอยู่พักรักษาตัวในโรงพยาบาลระยะยาว และใช้ยาปฏิชีวนะที่มีฤทธิ์แรงและราคาสูง การเพิ่มขึ้นของจำนวนสายพันธุ์ที่ดื้อยาและดื้อต่อยาหลายชนิดของเชื้อแบคทีเรียเป็นเรื่องใหญ่ที่น่ากังวลสำหรับบุคลากรทางการแพทย์ทั่วโลก ดังนั้น วัตถุประสงค์ของการศึกษาจึงมีเพื่อประเมินความชุกของ *Enterobacter* sp. ที่ดื้อต่อยาปฏิชีวนะ โดยเก็บจากนกป่าในจังหวัด Taif ประเทศซาอุดิอาระเบีย มีเชื้อที่แยกได้ 50 ชนิดจากตัวอย่าง cloaca ของนกป่าประจำถิ่น และ 32 ชนิดที่แยกได้จาก cloaca ของนกป่าอพยพ ร้อยละ 16 ของเชื้อที่แยกได้จากนกประจำถิ่นดื้อต่อยาหลายชนิดจำนวน 3-4 กลุ่มของยาปฏิชีวนะ และร้อยละ 84 ของเชื้อที่แยกได้ดื้อต่อยาปฏิชีวนะหลายชนิด 5-10 กลุ่มของยาปฏิชีวนะ ร้อยละ 6 ของเชื้อที่แยกได้จากนกอพยพมีความสามารถดื้อยาหลายชนิดจำนวน 4 กลุ่มของยาปฏิชีวนะ และร้อยละ 94 ของเชื้อที่แยกได้ต้านต่อยาหลายชนิดจำนวน 5-10 กลุ่มของยาปฏิชีวนะ ร้อยละ 65 ของเชื้อที่แยกได้และมีฤทธิ์สูงที่สุดในการต้านต่อยาหลายชนิด (MDR) (ยาปฏิชีวนะมากกว่า 7 ชนิด) เป็นเชื้อ *Enterobacter cancerogenus* พบยีนที่ดื้อต่อยา Aminoglycoside (*StrA* และ *strB*), b-lactam (bla_{TEM}), tetracycline (*tetA* and *tetB*) and gentamycin (*Gmr*) จากเชื้อ MDR ที่แยกได้ทั้งหมด พบ plasmid หลายอันที่มีน้ำหนักโมเลกุลสูงจาก MDR ของสายพันธุ์โฮสต์ที่เหมือนกัน ข้อมูลนี้แนะว่ามีความเป็นไปได้ในการถ่ายทอดเชื้อแบคทีเรียที่ดื้อต่อยาหลายชนิดระหว่างนก การศึกษานี้ได้เน้นบทบาทของนกป่าในการแพร่กระจายของแบคทีเรียที่ดื้อต่อยาหลายชนิด และยีนที่ดื้อต่อยาปฏิชีวนะทั่วโลก

คำสำคัญ: แบคทีเรียที่ดื้อต่อยาปฏิชีวนะ *Enterobacter cancerogenus* นกป่า ยีนที่ดื้อต่อยาปฏิชีวนะ พลาสมิด 16S-rRNA

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