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Research Paper

Role of wild birds as carriers of multi-drug resistant *Escherichia coli* and *Escherichia vulneris*

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Abstract

Emergence and distribution of multi-drug resistant (MDR) bacteria in environments pose a risk to human and animal health. A total of 82 isolates of *Escherichia* spp. were recovered from cloacal swabs of migrating and non-migrating wild birds. All bacterial isolates were identified and characterized morphologically and biochemically. 72% and 50% of isolates recovered from non-migrating and migrating birds, respectively, showed positive congo red dye binding (a virulence factor). Also, hemolysin production (a virulence factor) was showed in 8% of isolates recovered from non-migrating birds and 75% of isolates recovered from migrating birds. All isolates recovered from non-migrating birds were found resistant to Oxacillin while all isolates recovered from migrating birds demonstrated resistance to Oxacillin, Chloramphenicol, Oxytetracycline and Lincomycin. Some bacterial isolates recovered from non-migrating birds and migrating birds exhibited MDR phenotype. The MDR isolates were further characterized by API 20E and 16S rRNA as *E. coli* and *E. vulneris*. MDR *Escherichia* isolates contain ~1-5 plasmids of high-molecular weights. Accordingly, wild birds could create a potential threat to human and animal health by transmitting MDR bacteria to water streams and other environmental sources through their faecal residues, and to remote regions by migration.

Key words: *E. coli*, *E. vulneris*, multi-drug resistance (MDR), migrating and non-migrating birds, congo red binding, hemolysin, API 20E, 16S rRNA, plasmid profile.

Introduction

During the time of World War II, antimicrobial agents were first widely utilized to treat bacterial infections. Since that time, antimicrobial agent use has gradually increased. In addition to huge use in the treatment of human illnesses, antibiotics are commonly used in veterinary medicine and usually added to the nourish of food producing animals. Random use of antimicrobial agents may cause existing populations of microorganisms that are antimicrobial resistant or multi-drug resistant (MDR). These microorganisms may be shed in faeces with following contamination of soil, food, and aquatic environments. While antimicrobial agents have confirmed a successful way against bacterial contamination and infection, their widespread use has produced a reservoir of antimicrobial agents and MDR microorganisms. The occurrence and persistence of antimicrobial resistant bacteria in such environments as sediment (Timoney *et al.*, 1978), soil (Trevors, 1987), surface water (Bell *et al.*, 1980; Wnorowski, 1993), municipal drinking water (Armstrong *et al.*, 1981; Calomiris *et al.*, 1984; Moffie and Mouton, 1988) and sewage (Altherr and Kasweck, 1982; Middleton and Ambrose, 2005; Murray *et al.*, 1984; Walter and Vennes, 1985), is a growing public health concern.

The appearance of MDR bacteria of animals, birds and human (Grobbel *et al.*, 2007) is accompanied by co-contamination of the environment leading to a great health

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Table 1 - Types, names, location and status of collected migrating and non-migrating wild birds.

No. and types of birds	English names	Scientific names	Location	Status
Non-migrating birds 1	Sand Partridge	*Ammoperdix heyi*	Jabra	Resident
2	Arabian Babbler	*Turdoides squamiceps*	Meisan	Resident
3	White-spectacled Bulbul	*Pycnonotus xanthopygos*	Samnan Wa Samneen	Resident
4	Rüppell's Weaver	*Ploceus galbula*	Samnan Wa Samneen	Resident
5	Black Scrub Robin	*Cercotrichas podobe*	Samnan Wa Samneen	Resident (regionally endemic)
6	Arabian Serin	*Crithagra rothschildi*	Jabra	Resident (Endemic to the Arabian Peninsula)
7	Rüppell's Weaver	*Ploceus galbula*	Jabra	Common Resident
8	Philby's Partridge	*Alectoris philbyi*	Samnan	Resident (Endemic to the Arabian Peninsula)
9	Lppet-faced Vulture	*Torgos tracheliotos*	Mahazat as-Sayd Protected area	Resident
Migrating birds1	Isabelline Shrike	*Lanius isabellinus*	National Wildlife Research Center	Passage migrant and winter visitor
2	Barn Swallow	*Hirundo rustica*	Wadi Al Arj	Passage migrant and winter visitor
3	Tawny Pipit	*Anthus campestris*	Wadi Al Arj	Passage migrant and winter visitor
4	Willow Warbler	*Phylloscopus trochilus*	NWRC	Passage migrant and winter visitor
5	Sand Martin	*Riparia riparia*	Wadi Al Arj	Passage migrant and winter visitor
6	Isabelline Shrike	*Lanius isabellinus*	Wadi Al Arj	Passage migrant and winter visitor

alarm (Martinez, 2009). In addition to the present detection of MDR bacteria in high human density sites (Cole *et al.*, 2005), their incidence in more distant regions like high mountains or the arctic is more concern (Caprioli *et al.*, 1991; Sjolund *et al.*, 2008). The presence of bacteria of potential zoonotic importance among migrating and non-migrating wild birds has public health significance. Different of pathogenic bacterial species have been isolated from wild birds. Migrating and non-migrating wild birds can also act as reservoirs of coliform bacteria, such as *E. coli*, carrying antimicrobial-resistance genes. 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 (Cole *et al.*, 2005; Kozak *et al.*, 2009). Migrating and non-migrating wild birds or general wild animals could therefore serve as reservoirs of resistant bacteria and genetic factors of antimicrobial resistance (Dolejska *et al.*, 2009).

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). Enteropathogenic strains, such as the vero cytotoxin-producing *E. coli* O157:H7 strain, are the agent of colibacillosis and have been isolated from healthy or diseased wild birds, including migrants such as *Ardea cinerea, B. canadensis, Cygnus columbianus, U. aalge,* and *Columba palumbus* (Hubalek, 1994). These migrating birds can become carriers of *E. coli* strains resistant to antimicrobial agents and can be responsible for the spread of R plasmids over the world (Kanai *et al.*, 1981). Migratory waterfowl may serve as reservoirs of antimicrobial resistance of thermotolerant faecal indicator organisms (Middleton and Ambrose, 2005). *E. coli* isolates originating from Arctic birds carry antimicrobial resistance determinants.

The problem of antimicrobial resistance worldwide is one of the foremost issues that we 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. The aim of this study was to obtain detailed understanding of possible migrating and non-migrating wild birds carry and spread of drug-resistant *Escherichia* spp. 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, characterize and verify the prevalence of antimicrobial resistant *Escherichia* spp. in migrating and non-migrating wild birds with an attention on the detection of MDR bacteria.

Materials and Methods

Sampling sites and birds capture

Fifteen different types of migrating and non-migrating wild birds (Three of each species) were captured (Table 1), using misnets and clap-nets, from different areas (Jabra; Meisan, Samnan Wa Samneen, National Wildlife Research Center (near Besel village, Wadi Jaleel and Wadi

Al Arj) at Taif province, Saudi Arabia. Birds were captured within a vegetated area covering 500 m radius. Birds were characterized and identified according to Porter and Aspinall (Porter and Aspinall, 2010). Capturing the wild birds was approved by the Saudi Wildlife Authority, Taif province.

Sampling from cloacal swabs

Sterile cotton swabs wetted with sterile normal saline water were inserted in the cloacae of the migrating and non-migrating 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.

Isolation and identification of *Escherichia* spp.

Standard methods were used for the enrichment, isolation, identification, and biochemical confirmation of *Escherichia* spp. isolates (Clesceri *et al.*, 1998). All samples were processed within 4 h. 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) that precipitated bile and had a dark red center. Different single colonies were then collected and purified.

All isolates were morphologically characterized by Gram stain. For biochemical characterization, isolates were inoculated in tryptic soy broth (TSB), and incubated for approximately 4 h until the cultures were turbid. Identification was done according to Buchanan and Gibbons (1974) following a series of biochemical tests included oxidase, methyl red, Voges-Proskauer reactions, indole, citrate, catalase, urea hydrolysis, gelatin hydrolysis, lactose fermentation, nitrate reduction, casein hydrolysis and sugar fermentation. Moreover, identification of *Escherichia* spp. isolates was further confirmed by analytical profile index API 20E strips (Bio Merieux).

Congo red binding

To estimate the Congo red dye binding (Styles and Flammer, 1991), *Escherichia* spp. isolates were grown at 37 °C for 24 h on tryptic soy agar supplemented with 0.02% Congo red (Sigma) and 0.15% bile salt (Difco). Positive colonies were red, while negative colonies were appeared pale. Based on the intensity of red color, the binding was recorded as +, ++, and +++.

Hemolysis

To investigate hemolysis (Hacker *et al.*, 1983), overnight cultures of *Escherichia* spp. isolates were streaked on sheep blood agar and incubated at 37 °C for 24 h. The appearance of a zone of erythrocyte lysis around or under bacterial colonies indicated hemolysis.

Antimicrobial susceptibility test

Only the bacterial isolates that confirmed to be *Escherichia* spp. based on the results of the biochemical tests were selected for antimicrobial agent sensitivity testing. The antimicrobial sensitivity phenotypes of *Escherichia* spp. isolates were determined using a Kirby-Bauer disk diffusion assay according to the standards and interpretive criteria described by CLSI (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 (30 μg), Norfloxacin (10 μg), Lincomycin (2 μg), Gentamycin (10 μg), Amoxicillin (25 μg), Enrofloxacin (5 μg) and Piperacillin (100 μg) were used. This antimicrobial panel was chosen to include antibiotics with potential efficiency against *Escherichia* spp. isolates. These antimicrobial agents were chosen on the basis of their importance in treating human or animal *E. coli* infections and their use as feed additives to promote growth in animals and on the basis of their ability to provide diversity for representation of different antimicrobial classes (Krumperman, 1983).

Plates of Mueller-Hinton medium were swabbed with TSB broth inoculated with *Escherichia* spp. 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 h. The 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 translated into sensitive, intermediate and resistant categories according to the interpretation table of the CLSI (CLSI, 2012). Multi-

Table 2 - Distribution of *Escherichia* spp. in different types of birds.

Sample source	No. of samples tested	No. of samples positive for *Escherichia* spp. detection	% of positive samples	No. of *Escherichia* spp. isolates
Non-migrating birds	27	25	92%	50
Migrating birds	18	17	94%	32
Total	45	42	93%	82

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 sterilizes 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 100 °C for 10 min. After heating, supernatant was used for PCR.

PCR of 16S rRNA gene

One microliter of template DNA was added in 20 μL of PCR reaction solution. 27F (AgA gTT TgA TCM TGG CTC Ag) and 1492R (TAC ggY TAC CTT gTT ACg ACT T) primers were used. 35 amplification cycles were performed at 94 °C for 45 s, 55 °C for 60 s, and 72 °C for 60 s. DNA fragments were amplified ~ 1,400 bp.

Unincorporated PCR primers and dNTPs were removed from PCR products by using Montage PCR Clean up kit (Millipore).

Sequencing

The purified PCR products of approximately 1,400 bp were sequenced by using two primers which were 518F (CCA gCA gCC gCg gTA ATA Cg) and 800R (TAC CAg ggT ATC TAA TCC). 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 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 the accession numbers: AB758349 (*E. coli* WB3-1), AB758350 (*E. coli* WB3-2), AB758351 (*E. coli* MB17-1), AB758352 (*E. coli* MB17-2), AB758353 (*E. vulneris* WB7-2), AB758354 (*E. vulneris* MB14-1), AB758355 (*E. vulneris* MB14-2), AB758356 (*E. vulneris* MB21-1) and AB758357 (*E. vulneris* MB21-2).

Plasmid analysis

Plasmid extraction of MDR isolates was carried out by alkaline lysis technique (Anderson and McKa, 1983). The isolated plasmid was then separated using a horizontal 1% agarose gel electrophoresis technique. *Shigella flexneri* containing plasmids of known molecular weights was used as a standard. The log of the molecular weights (in base pairs) *vs.* the distances (cm) traveled for the standard plasmids was plotted and then the sizes of unknown plasmids were estimated.

Curing of plasmid

Plasmid curing was achieved for MDR isolates by physical method by incubating bacterial cells at 45 °C as reported by Fortina and Silva (1996). The bacterial isolates were incubated in TSB at 45 °C overnight for plasmid curing. Other flasks were incubated at 37 °C as a control. The sodium dodecyl sulphate (1% SDS) treatment method of Tomoeda *et al.* (1968) was also performed for plasmid curing. Loss of plasmid and antibiotic susceptibility testing of antibiotics to which bacteria were resistant were used to confirm the curing.

Statistic 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.

Results

Isolation and identification of *Escherichia* spp

Six types of migrating wild birds and nine types of non-migrating birds were captured from Taif area for isolation of *Escherichia* spp. Captured birds were characterized and identified (Table 1). Pure colonies of bacteria were isolated from birds on MacConkey agar plates from cloacae samples and preliminary identification as *Escherichia* spp.

The distribution pattern of *Escherichia* spp. isolates is summarized in Table 2. The present results revealed that 92% of total samples collected from birds were found *Escherichia* spp. positive. The prevalence range of all two types of sample sources found positive was from 92% in non-migrating birds to 94% in migrating birds. Present study showed significantly a high percentage of migrating bird samples contained *Escherichia* spp. ($p < 0.05$).

Among bacterial strains isolated from birds, a total of 82 (50 isolates from non-migrating birds and 32 isolates from migrating birds) (Table 2) were selected and subjected to morphological and biochemical tests. The biochemical tests which were used for identification of *Escherichia* sp. isolates from birds are summarized in Table 3. The biochemical reactions revealed that all bacterial isolates belonged to two species of the genus *Escherichia* which were E. coli and *E. vulneris*. Also, API 20E identification confirmed the results obtained by the biochemical tests.

Table 3 - Characteristic tests of *Escherichia* spp. isolates.

Characteristic tests	*E. coli* reaction	% of *E. coli* isolates	*E. vulneris* reaction	% of *E. vulneris* isolates
Gram Staining	G-, short bacilli	100	G-, short bacilli	100
MacConkey agar	Pink colony	100	Pink colony	97
Catalase Test	+	100	+	95
Oxidase Test	-	100	-	98
Indole Test	+	80	-	92
Methyle Red Test	+	80	+	88
Voges-Proskauer Test	-	70	-	89
Citrate Test	-	90	-	87
Lactose Test	+	100	+	99
Sugar Fermentation	+	95	+	98
Gelatin Hydrolysis Test	+	80	-	84
Casein Hydrolysis test	+	75	-	87
Nitrate Reduction Test	+	90	+	95
Urea Hydrolysis test	+	85	-	92
H_2S on TSI	+	88	-	90
Motility	+	90	+	88

G -= gram negative; + = positive reaction; - = negative reaction.

Virulence markers of *Escherichia* spp

To investigate the pathogencity of *Escherichia* spp. isolates, congo red binding tests were carried out. 8% of isolates obtained from non-migrating birds were strongly positive congo red binding whereas 25% of isolates from migrating birds were strongly positive (Table 4). These results indicated that positive congo red binding of isolates was significantly higher in migrating birds and lower in non-migrating birds ($p < 0.05$). Congo red binding has been used as a potential virulence marker, which indicated that these isolates were pathogens.

Moreover, in order to investigate hemolysin production prevalence among the bacterial isolates, hemolysis tests were achieved. 8% bacterial isolates obtained from non-migrating birds caused hemolysis in sheep blood agar plates while 75% of isolates from migrating birds caused hemolysis (Table 4). These results indicated that hemolysis of isolates was significantly higher in migrating birds and lower in non-migrating birds ($p < 0.05$).

Antimicrobial susceptibility of *Escherichia* sp isolates

Antimicrobial susceptibility profiles of 50 *Escherichia* spp. isolates from samples of non-migrating bird sources have been demonstrated in Table 5. Resistance spectrum of *Escherichia* spp. isolates for 15 antimicrobial agents tested in descending order was respectively Oxacillin, Lincomycin, Oxytetracycline, Amoxicillin, Cephalexin, Neomycin, Ampicillin, Cefaclor, Colistin, Piperacillin, Chloramphenicol, Ciprofloxacin, Norfloxacin, Gentamycin and Enrofloxacin. All isolates were found resistant to Oxacillin. 88%, 84% and 72% of isolates were found resistant to Lincomycin, Oxytetracycline and Amoxicillin, respectively. Moreover, 4% strains were found intermediate resistant to Cefaclor, Chloramphenicol, Gentamycin, Amoxicillin and Enrofloxacin, and 8% isolates were found intermediate resistant to Colistin, Oxytetracycline and Piperacillin. 32% and 48% isolates were found intermediate resistant to Neomycin and Cephalexin, respectively. 60-100% of isolates demonstrated sensitive to Cefaclor, Ampicillin, Chloramphenicol, Colistin, Ciprofloxacin, Norfloxacin, Gentamycin, Enrofloxacin and Piperacillin. Sensitive to Cefaclor, Ampicillin, Chloramphenicol, Colistin, Ciprofloxacin, Norfloxacin, Gentamycin, Enrofloxacin and Piperacillin was significantly high in the isolates obtained from non-migrating birds ($p < .05$). 72-100% of isolates demonstrated resistance to Amoxicillin, Oxytetracycline, Lincomycin and Oxacillin.

Table 4 - Virulence characteristics of *Escherichia* spp. isolates.

Sample source	No. and percentage of *Escherichia* pp. isolates	
	Congo red binding	Hemolysis
Non-migrating birds	+++ (4, 8%), ++ (10, 20%) and + (22, 44%)	4, 8%
Migrating birds	+++ (8, 25%) and ++ (8, 25%)	24, 75%

Table 5 - Antibiotic susceptibility profiles of 50 selected strains of *Escherichia* spp. isolated from non-migrating birds. Antimicrobial susceptibility was performed according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2012).

Antibiotic discs, code and concentration	No. of *Escherichia* spp. isolates (%)		
	Resistant	Intermediate	Sensitive
Cefaclor, CEC (30 µg)	14(28)	2(4)	34(68)
Oxacillin, OX (1 µg)	50(100)	0(0)	0(0)
Ampicillin, AM (10 µg)	20(40)	0(0)	30(60)
Chloramphenicol, C (30 µg)	6(12)	2(4)	42(84)
Cephalexin, CL (30 µg)	24(48)	24(48)	2(4)
Neomycin, N (30 µg)	24(48)	16(32)	10(20)
Colistin, CT (10 µg)	12(24)	4(8)	34(68)
Ciprofloxacin, CIP (5 µg)	4(8)	0(0)	46(92)
Oxytetracycline, T (30 µg)	42(84)	4(8)	4(8)
Norfloxacin, NOR (10 µg)	2(4)	0(0)	48(96)
Lincomycin, L (2 µg)	44(88)	0(0)	6(12)
Gentamycin, CN (10 µg)	2(4)	2(4)	46(92)
Amoxicillin, AX (25 µg)	36(72)	2(4)	12(24)
Enrofloxacin, ENR (5 µg)	2(4)	2(4)	46(92)
Piperacillin, PRL (100 µg)	6(12)	4(8)	40(80)

Resistance to Amoxicillin, Oxytetracycline, Lincomycin and Oxacillin was significantly high in the isolates from non-migrating birds ($p < 0.05$).

Antimicrobial susceptibility profiles of 32 bacterial isolates of *Escherichia* spp. from samples of migrating birds have been presented in Table 6. Resistance spectrum of *Escherichia* spp. isolates for 15 antimicrobial agents tested in descending order was respectively Oxacillin, Chloramphenicol, Oxytetracycline, Lincomycin, Ciprofloxacin, Ampicillin, Cefaclor, Cephalexin, Amoxicillin, Colistin and Piperacillin. 100% of isolates were found resistant to Oxacillin Chloramphenicol, Oxytetracycline and Lincomycin. 87.5%, 75%, 50%, 37.5% and 12.5% of isolates were found resistant to Ciprofloxacin, Ampicillin,

Table 6 - Antibiotic susceptibility profiles of 32 selected strains of *Escherichia* spp. isolated from migrating birds. Antimicrobial susceptibility was performed according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2012).

Antibiotic discs, code and concentration	No. of *Escherichia* spp. isolates (%)		
	Resistant	Intermediate	Sensitive
Cefaclor, CEC (30 µg)	20(62.5)	0(0)	12(37.5)
Oxacillin, OX (1 µg)	32(100)	0(0)	0(0)
Ampicillin, AM (10 µg)	24(75)	2(6)	6 (18)
Chloramphenicol, C (30 µg)	32(100)	0(0)	0(0)
Cephalexin, CL (30 µg)	20 (62.5)	0(0)	12(37.5)
Neomycin, N (30 µg)	0 (0)	30(94)	2(6)
Colistin, CT (10 µg)	12(37.5)	0(0)	20(62.5)
Ciprofloxacin, CIP (5 µg)	28(87.5)	4(12.5)	0(0)
Oxytetracycline, T (30 µg)	32(100)	0(0)	0(0)
Norfloxacin, NOR (10 µg)	0(0)	2(6)	30(94)
Lincomycin, L (2 µg)	32(100)	0(0)	0(0)
Gentamycin, CN (10 µg)	0 (0)	0 (0)	32(100)
Amoxicillin, AX (25 µg)	16(50)	4(12.5)	12(37.5)
Enrofloxacin, ENR (5 µg)	0 (0)	0(0)	32 (100)
Piperacillin, PRL (100 µg)	4(12.5)	0(0)	28(87.5)

Amoxicillin, Colistin and Piperacillin, respectively. 62.5% of isolates were found resistant to Cefaclor and Cephalexin. Moreover, 6% and 94% strains were found intermediate resistant to Ampicillin and Neomycin, respectively, and 12.5% isolates were found intermediate resistant to Ciprofloxacin and Amoxicillin. 62.5-100% of isolates found sensitive to Colistin, Norfloxacin, Gentamycin, Enrofloxacin and Piperacillin indicative sensitive to these antimicrobial agents was significantly high in isolates of migrating birds ($p < 0.05$). 50-100% isolates demonstrated resistance to Oxacillin, Chloramphenicol, Oxytetracycline, Lincomycin, Ciprofloxacin, Ampicillin, Cefaclor, Cephalexin and Amoxicillin. These results indicated that resistance to Oxacillin, Chloramphenicol, Oxytetracycline, Lincomycin, Ciprofloxacin, Ampicillin, Cefaclor, Cephalexin and Amoxicillin was significantly high in isolates from migrating birds ($p < 0.05$).

MDR phenotype of *Escherichia* spp isolates

MDR patterns of *Escherichia* spp. isolates are shown in Table 7. Resistance to Oxacillin, Ampicillin, Neomycin, Ciprofloxacin, Oxytetracycline, Lincomycin, Amoxicillin and Piperacillin (OX,AM,N,CIP,T,L,AX,PRL) was significantly higher in the isolates from non-migrating bird, Arabian Babbler (Turdoides squamiceps), than in those from other non-migrating birds ($p < 0.05$). Isolates from non-migrating bird, Ruppell's Weaver (Ploceus galbula), were significantly more resistant to Cefaclor, Oxacillin, Ampicillin, Chloramphenicol, Cephalexin, Ciprofloxacin, Oxytetracycline, Lincomycin, Amoxicillin and Piperacillin (CEC,OX,AM,C,CL,CIP,T,L,AX,PRL) than those from other non-migrating birds ($p < 0.05$). Resistance to Cefaclor, Oxacillin, Ampicillin, Chloramphenicol, Cephalexin, Ciprofloxacin, Oxytetracycline, Lincomycin, Amoxicillin (CEC,OX,AM,C,CL,CIP,T,L,AX) was significantly higher in isolates from migrating bird, Isabelline Shrike (Lanius isabellinus) than in isolates from other migrating birds ($p < 0.05$).

Molecular characterization of most MDR *Escherichia* spp isolates

The bacterial isolates (WB3-1, WB3-2, MB17-1, MB17-2, WB7-2, MB14-1, MB14-2, MB21-1 and MB21-2) showed MDR phenotype were further identified by 16S rRNA encoding gene analysis. Part of the 16S rRNA encoding gene of these isolates was PCR-amplified and sequenced. The resulting nucleotide sequences were compared to available sequences in the databases. A phylogenetic tree illustrating the results of 16S rRNA analysis is demonstrated in Figure 1. As demonstrated, the 16S rRNA sequences of the isolates WB3-1, WB3-2, MB17-1 and MB17-2 are most closely related to *E. coli* (~98%) while as , the 16S rRNA sequences of the isolates WB7-2, MB14-1, MB14-2, MB21-1 and MB21-2 are most closely related to *E. vulneris* (~98%).

Plasmid profile

Agarose-gel electrophoresis of plasmid DNA from 9 isolates of MDR *Escherichia* (E. coli WB3-1, *E. coli* WB3-2, *E. vulneris* WB7-2, *E. vulneris* MB14-1, *E. vulneris* MB14-2, *E. coli* MB17-1, *E. coli* MB17-2, *E. vulneris* MB21-1 and *E. vulneris* MB21-2) is shown in Table 7 and Figure 2. Isolates E. coli WB3-1 and *E. coli* WB3-2 contain one large plasmid of ~115 kb whereas isolates *E. coli* MB17-1 and *E. coli* MB17-2 contain 5 plasmids of ~115 kb, 75 kb, 27 kb, 24 kb and 18 kb. Isolate *E. vulneris* WB7-2 contain two plasmid of ~115 kb and 93 kb. Isolates *E. vulneris* MB14-1 and *E. vulneris* MB14-2contain one plasmid of ~39 kb while as isolates *E. vulneris* MB21-1 and *E. vulneris* MB21-2 contain one plasmid of ~75 kb. The results showed that all of MDR *Escherichia* isolates contain a high-molecular weight plasmid DNA.

Plasmid curing studies of MDR *Escherichia* isolates were performed to detect if the drug resistance observed in this study was plasmid-mediated. No plasmid bands were detected after the electrophoretic separation of crude DNA

Table 7 - Antibiotic resistance and plasmid profiles of *Escherichia* spp. isolates and their origins.

Organisms	No. of isolates	Bird origin	Antibiotic resistance profiles	Plasmid profiles
E. coli WB3-1	2	*Turdoides squamiceps*	*OX AM N CIP T L AX PRL	115 kb
E. coli WB3-2	3	*Turdoides squamiceps*	OX AM N CIP T L AX PRL	115 kb
E. coli MB17-1	2	*Anthus campestris*	OX AM CT CIP T L AX PRL	115, 75, 27, 24, 19 kb
E. coli MB17-2	4	*Anthus campestris*	OX AM CT CIP T L AX PRL	115, 75, 27, 24, 19 kb
E. vulneris WB7-2	3	*Ploceus galbula*	CEC OX AM C CL CIP T L AX PRL	115, 93 kb
E. vulneris MB14-1	3	*Lanius isabellinus*	CEC OX AM C CL CIP T L	93 kb
E. vulneris MB14-2	2	*Lanius isabellinus*	CEC OX AM C CL CIP T L	93 kb
E. vulneris MB21-1	3	*Lanius isabellinus*	CEC OX AM C CL CIP T L AX	75 kb
E. vulneris MB21-2	2	*Lanius isabellinus*	CEC OX AM C CL CIP T L	75 kb

*Oxacillin, OX; Ampicillin, AM; Neomycin, N; Ciprofloxacin, CIP; Oxytetracycline, T; Lincomycin, L; Amoxicillin, AX; Piperacillin, PRL; Colistin, CT; Cefaclor, CEC; Chloramphenicol, C.

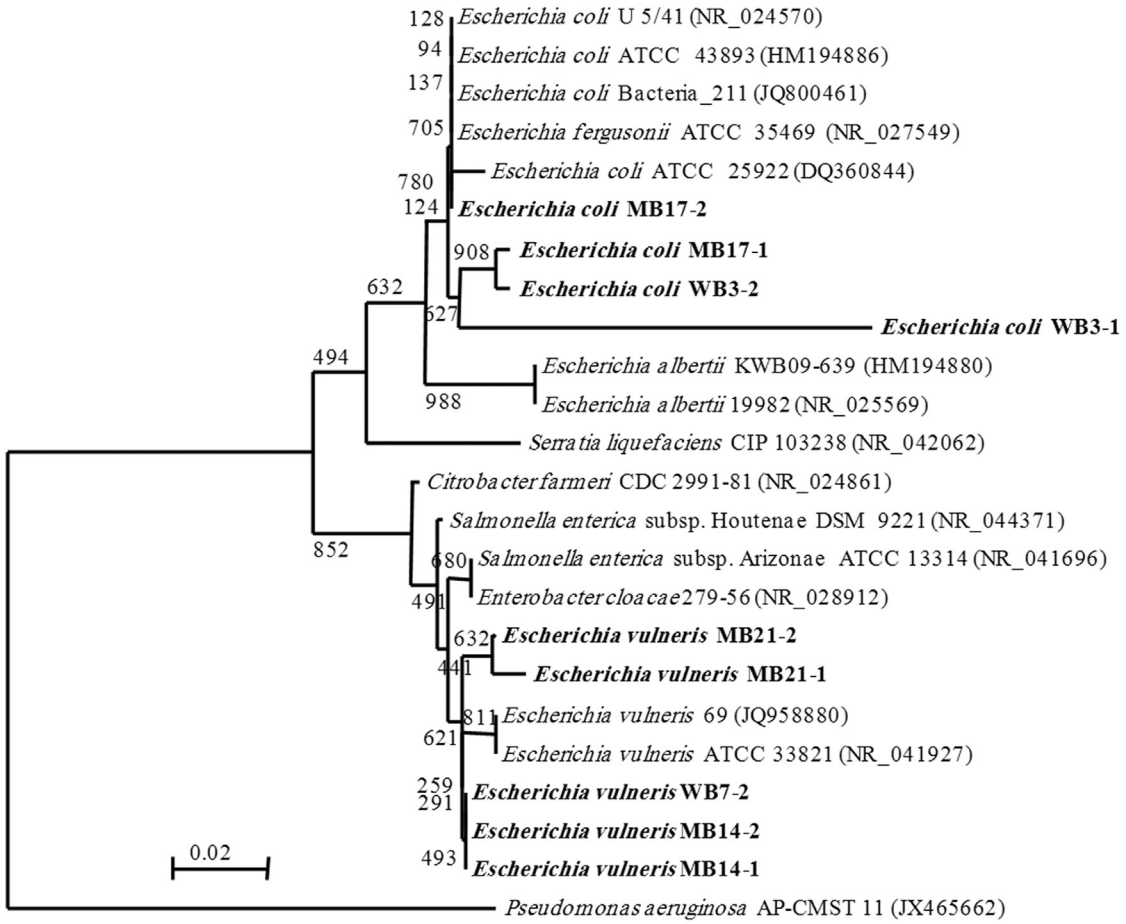

Figure 1 - A phylogenetic tree of *Escherichia* isolates (bold font) based on the nucleotide sequences of the partial 16S rRNA genes. The tree was constructed by using the neighbor-joining method, and genetics distances were computed by the Jukes-Cantor model. The scale bar indicates the genetic distance. The number shown next to each node indicates the percentage bootstrap value of 1000 replicates. The GenBank accession numbers of the bacteria are shown in parentheses. The sequence from *Pseudomonas aeruginosa* was treated as the out-group.

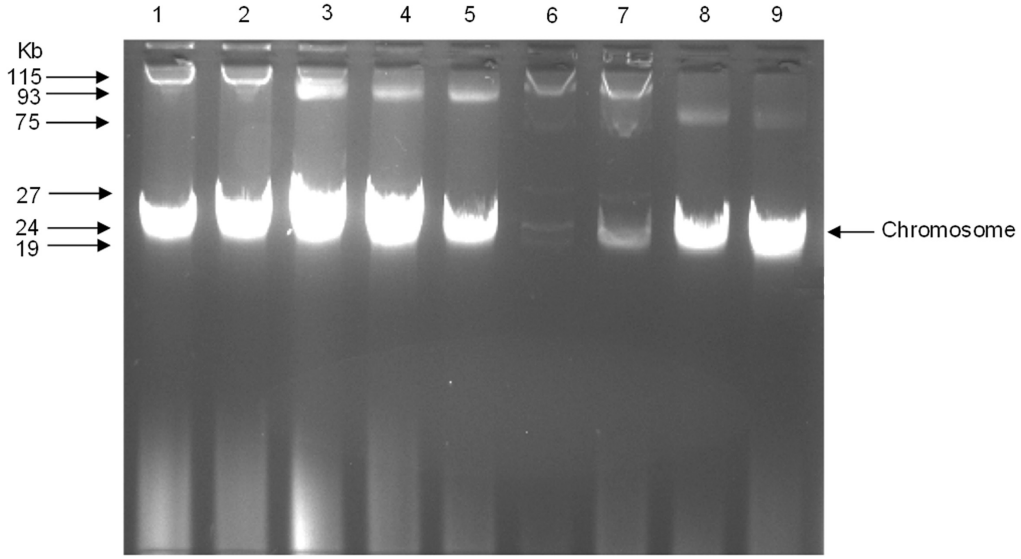

Figure 2 - Agarose (1%) gel electrophoresis of plasmid DNA from *Escherichia* isolates:1, *E. coli* WB3-1; 2, *E. coli* WB3-2; 3, *E. vulneris* WB7-2; 4, *E. vulneris* MB14-1; 5, *E. vulneris* MB14-2; 6, *E. coli* MB17-1; 7, *E. coli* MB17-2; 8, *E. vulneris* MB21-1; and 9, *E. vulneris* MB21-2. Arrows indicated the interest bands of plasmids with their molecular weights.

extracts from cured isolates. Loss of plasmids correlated with loss of resistance to antibiotics in cured strains. Loss of resistance to multiple antibiotics was also demonstrated.

Discussion

Fifty isolates of *Escherichia* spp. recovered from non-migrating birds and 32 isolates from migrating birds were identified as two species of E. coli and *E. vulneris*. The prevalence range of samples found *Escherichia* spp. positive was from 92% in non-migrating birds to 94% in migrating birds. These results indicated significantly a high percentage of migrating bird samples contained *Escherichia* spp. Binding of congo red dye by E. coli is associated with the pathogenicity of the organism (Styles and Flammer, 1991). The present results indicated that positive congo red binding of *Escherichia* spp. isolates was significantly higher in migrating birds and lower in non-migrating birds. Congo red binding has been used as a potential virulence marker, which indicated that these isolates were pathogens. Moreover, hemolysin production is presumed to be a virulence factor in extraintestinal, *e.g.* urinary tract, infections caused by *E. coli* (Hacker, 1983). The results indicated that hemolysin production of isolates was significantly higher in migrating birds and lower in non-migrating birds. These results indicated that these isolates showed one to two virulence factors (congo red binding and hemolysis). *E. coli* is one of the common microbial flora of gastrointestinal tract of birds, animals and human but may become pathogenic to both (Levine, 1987). The present results indicated that migrating birds act as long-distance vectors for a wide range of *Escherichia* spp. that might be infectious to humans. This generates the potential for establishment of novel foci of emerging or re-emerging communicable diseases along bird migration routes.

On other hand, 100%, 88%, 84% and 72% of *Escherichia* spp. isolates from non-migrating birds were exhibited resistant to Oxacillin, Lincomycin, Oxytetracycline and Amoxicillin, respectively. Resistance to Amoxicillin, Oxytetracycline, Lincomycin and Oxacillin was significantly high in *Escherichia* spp. isolates from non-migrating birds. In addition, 50-100% of *Escherichia* spp. isolates recovered from migrating birds showed resistance to Oxacillin, Chloramphenicol, Oxytetracycline, Lincomycin, Ciprofloxacin, Ampicillin, Cefaclor, Cephalexin and Amoxicillin. Our results indicated that resistance to Oxacillin, Chloramphenicol, Oxytetracycline, Lincomycin, Ciprofloxacin, Ampicillin, Cefaclor, Cephalexin and Amoxicillin was significantly high in isolates from migrating birds. Previous results reported that 72% and 21% of *E. coli* isolates recovered from the faeces of wild Canada geese (*Branta canadensis*) and choughs (*Pyrrhocorax pyrrhocorax*) that were feeding and/or living in close to livestock waste were found resistant to amoxicillin-clavulanic acid of (Blanco *et al.*, 2009; Cole *et al.*, 2005). The wild birds in our study might have come into contact with antimicrobial residues and resistant bacteria from human waste in waterways. Moreover, because some antimicrobials utilized by human and veterinary medicine do not completely destroy, they may distribute through the environment in wastewater and soil (Sarmah *et al.*, 2006). The difference in the prevalence of antimicrobial resistance in wildlife living in natural habitats in different geographic sites may reflect different levels of general pollution in the local environment (Osterblad *et al.*, 2001). Wildlife, such as the black-headed gull (*Larus ridibundus*) or the Russian rook (*Corvus frugilegus*), might transport resistant bacteria from other areas to natural environments and then act as reservoirs, maintaining antimicrobial resistance within natural ecosystems (Blanco *et al.*, 2009; Cole *et al.*, 2005; Dolejska *et al.*, 2007). A range of extended-spectrum β-lactamase-producing *E. coli* isolates recovered from seagull feces from Porto, Portugal, beaches exhibited a high rate of cefotaximase-15 resistance (Simoes *et al.*, 2010).

All isolates from migrating and non-migrating birds examined in this study exhibited MDR phenotype to approximately 3-10 antimicrobial agents. Resistance to OX,AM,N,CIP,T,L,AX,PRL was significantly high in *Escherichia* spp. isolates recovered from non-migrating bird, Arabian Babbler (Turdoides squamiceps). *Escherichia* spp. Isolates recovered from non-migrating bird, Ruppell's Weaver (Ploceus galbula), were significantly more resistant to CEC,OX,AM,C,CL,CIP,T,L,AX,PRL. Resistance to CEC,OX,AM,C,CL,CIP,T,L,AX was significantly high in *Escherichia* spp. isolates recovered from migrating bird, Isabelline Shrike (Lanius isabellinus). MDR were significantly higher in the isolates from migrating birds than those from non-migrating birds. These results indicated that the isolates from non-migrating birds might gain the antimicrobial-resistance from isolates of migrating birds. Moreover, no significant differences were observed between the patterns of resistance among the swab sample isolates from either non-migrating birds or migrating birds indicative cross-transfer of antimicrobial-resistance. A high frequency of *E. coli* isolates from migrating Canada geese sampled on the eastern shore of Maryland in the United States were resistant to penicillin G, ampicillin, cephalothin, and sulfathiazole (Middleton and Ambrose, 2005). Due to indiscriminate exploitation of antimicrobial agents, such high prevalence of multi-drug resistance may apparently be occurred which may ultimately replace the drug sensitive microorganisms from antimicrobial saturated environment (Van de Boogard and Stobberingh, 2000). The present finding indicated that spreading of drug-resistant bacteria by migrating birds is worldwide. Wildlife is normally not clinically exposed to antimicrobial agents but can get antimicrobial resistant bacteria through contact with humans, domestic animals and the environment, and the water contaminated with faeces seems to be the most important vector.

Escherichia spp. isolates exhibited MDR phenotypes were further identified by 16S rRNA analysis. The results indicated greatest similarity to members of the *Escherichia* group, which matches the conclusions of the morphological, biochemical and API 20E analysis. The 16S rRNA gene of isolates WB3-1, WB3-2, MB17-1 and MB17-2 shares ~98% similarity with that of uropathogenic *E. coli* ATCC 25922 (American Type Culture Collection, Rockville, MD, USA) indicative that these bacteria are new isolates of the bacterium *E. coli*. The 16S rRNA gene of isolates WB7-2, MB14-1, MB14-2, MB21-1 and MB21-2 shares ~98% similarity with that of *E. vulneris* ATCC 33821. These results suggest that these bacteria are new isolates of the bacterium *E. vulneris*. Also, this is the first finding reported that *E. coli* and *E. vulneris* isolates originating from these birds carry MDA determinants. *E. vulneris* was recognized as a new species of the family Enterobacteriaceae only in 1982 and associated with human wounds (Brenner *et al.*, 1982). *E. vulneris* has been isolated from animals, human, the environment, and potable water. *E. vulneris* can colonize the respiratory tract, female genital tract, urinary tract, and stool in human. The existence of several resistance to multiple classes of antimicrobial agents indicates that infection caused these isolates would be difficult to treat using antimicrobials currently available. The diversity of the cloacal microbial community in migrating and non-migrating birds, caught at Taif region, was assessed by cultivation and 16S rRNA analysis provide a better understanding of the bird' potential to harbor and disperse MDR pathogens.

Large plasmids of sizes ~ 115, 93, 75 kb were observed in MDR *Escherichia* isolates. These results indicated that plasmids with high molecular weights were found to be responsible for resistance to multiple antimicrobial compounds. Similar findings reported that large plasmids with sizes equal to or greater than 50 kb were observed in MDR *E. coli* isolates from animals (Karczmarczyk *et al.*, 2011). Our results indicated that all of MDR *Escherichia* isolates contain a high-molecular weight plasmid DNA. High molecular weight plasmid found in MDR bacteria was reported by Lay *et al.* (2012). To detect the potential relation of multidrug resistance with plasmid in the bacterial isolates, plasmid curing was performed for the MDR *Escherichia* isolates. Curing of plasmid had resulted in loss plasmids. The plasmid cured cells became sensitive to all resistant antibiotics, indicative antibiotic resistance marker genes were located in plasmid. Our study conclude that multiple resistant *Escherichia* isolates and plasmid containing multidrug resistant genes are present in wild birds may act as a possible source of transfer of these highly resistant bacteria and their genes to human.

In conclusion, wild birds and especially migrating species can become long-distance vectors for a wide range of antimicrobial resistant microorganisms. Also, these results confirm the significant role of *Escherichia* spp. as reservoirs and carriers of multidrug-resistant plasmids. Therefore, more concerns are recommended for staff hygiene in processing and handling of birds and misuse of antimicrobial agents should be decreased or stopped by careful use of antimicrobial agents for the safety of public health. On the way to most successfully reduce the threat of diseases associated with wild birds, the public health and animal health sectors must work together in developing approaches to decline human contact to pathogens carried by non-migrating and migrating birds. A successful public educational operation could also put in viewpoint and clarify tradition and realities about the risk of obtaining infections associated with wild birds.

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