ANTIBIOTIC SUSCEPTIBILITY, SEROTYPING AND PATHOGENICITY EVALUATION OF AVIAN *ESCHERICHIA COLI* ISOLATED FROM BROILERS IN NORTHERN IRAN

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Summary

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The aim of this study was to determine the main serotypes of avian-associated *E. coli* and their susceptibilities to common antimicrobial agents used in Iranian poultry industry. Furthermore the invasion characteristics of the poultry-associated isolates were evaluated. Eighty *Escherichia coli* (*E. coli*) strains were isolated from 63 broiler farms with colibacillosis clinical signs in Mazandaran province, northern of Iran. Antibacterial susceptibility testing of the isolates with ten selected antibiotics was conducted according to the standard methods and avian pathogen serotypes, O1, O2, O18 and O78:K80, were determined using the specific antisera. In addition, invasion properties of the isolates were examined based on Congo red dye agar test. According to the results, 73.75%, 71.25% and 65% of isolates were resistant to oxytetracycline, tetracycline and erythromycin, respectively, while resistance rates against enrofloxacin, florfenicol and norfloxacin were 7.5%, 7.5% and 2.5% respectively. The O78 and O1 were the predominant serotypes among *E. coli* isolates. Nine resistance patterns were observed in the *E. coli* isolates with predominant patterns being distributed widely across broilers indicating a striking diversity of resistance patterns in the areas. Cross-resistance between animal and human antimicrobial agents may be possible, thus, their proper usage in veterinary medicine is necessary.

Key words: broilers, *Escherichia coli*, Iran, multi-drug resistance, serotyping

Colibacillosis in chickens refers to any localised or systemic infection caused entirely or partly by *Escherichia coli* (Barnes *et al.*, 2003), enteric Gram-negative, flagellated bacterium which is part of the normal flora in the digestive

tract of chickens (Nakamura *et al.*, 1992). Although the microorganism has low pathogenicity for chickens, pathogenic avian strains of *E. coli* (APEC) are identified, which belong to a small range of serotypes including O78:K80, O1:K1 and

O2:K1 (Barnes *et al.*, 2003). Several pathological signs due to infection with an *E. coli* strain can be distinguished in poultry: septicemia, granuloma, inflammation of air sacs, cellulitis, swollen head syndrome, peritonitis, salpingitis, osteomyelitis, panophthalmitis and omphalitis/yolk sac infection. In broilers, colibacillosis mainly results in respiratory infections (airsacculitis) and peritonitis/pericarditis (Pourbakhsh *et al.*, 1997). Colibacillosis is mainly treated with antibiotics, but the use of these drugs is costly and the period in which broilers can be treated is limited because of the withdrawal time. Also, significant increase in appearance of antibiotic-resistant strains of *E. coli* isolated from poultry has complicated the problem (Khoshkhoo & Peighambari, 2005; Johnson *et al.*, 2007). Serotyping of the antigens is a very useful method for detecting pathogenic *E. coli* strains in clinical specimens, foods, and environmental samples and for understanding the epidemiology of the pathogen (Wang *et al.*, 2010). In this study, colibacillosis related *E. coli* strains isolated from poultry farms were analysed to determine the main serotypes of avian-associated *E. coli* and their susceptibilities to common antimicrobial agents used in Iranian poultry industry. Furthermore, the invasion characteristics of the poultry-associated isolates were evaluated.

The study was conducted in Mazandaran province (Northern Iran) during May and December 2012. Liver samples were collected aseptically from chickens with severe clinically signs after necropsy from 63 colibacillosis-affected broiler farms (20 liver samples per flock in four batches) and were placed into sterile Whirl-pak bags. Clinical signs observed in depressed birds were sneezing and coughing, and the most significant gross lesion

was airsacculitis. No antibiotic treatment was used prior to sampling time and the specimens were submitted to the laboratory on ice pieces in less than 6 h.

Homogenised samples were inoculated onto MacConkey's agar (Merck, Germany) plates and incubated at 37 °C for 24 h. Suspected *Escherichia coli* colonies were subsequently inoculated on Eosin Methylene Blue (EMB) agar (HiMedia, India) and incubated as above. Isolates were Gram-stained, tested for catalase and oxidase, sulfide-indole-motility (SIM), methyl red, Voges-Proskauer, citrate (IMVIC), triple sugar iron (TSI), and then stored on nutrient agar until used for antimicrobial susceptibility tests. Antimicrobial drug susceptibility was determined by a disc-diffusion method on Mueller-Hinton (MH) agar plates (Merck, Germany), according to the antibiogram standard methods (Bauer *et al.*, 1966). The following antibiotic discs were applied: ampicillin (10 µg), amoxicillin (25 µg), co-amoxiclav (20 µg), tetracycline (30 µg), oxytetracycline (30 µg), erythromycin (15 µg), florfenicol (30 µg), flumequine (30 µg), enrofloxacin (5 µg), and norfloxacin (10 µg). All antibiotic discs obtained from Padtan Teb, Iran.

Avian pathogenic strains were determined by agglutination test with specific antiserum raised against O1, O2, O18, and O78 antigens (Sifin, Bioconnections, UK) according to the protocol previously described (Allan *et al.*, 1993). Isolates pathogenicity test was carried out based on Congo red dye agar test previously explained by Berkhoff & Vinal (1986). Briefly, the colonies were streaked on Congo red agar (HiMedia, India) and incubated for 72 h at 25 °C. The reaction was detected at 18, 24, 48 and 72 h. Appearance of red colonies within 72 h was recorded as a positive reaction (invasive

strains). Negative colonies did not bind the dye and remained white or grey even after 72 h and were declared negative.

A total of 80 *E. coli* isolates were recovered from 63 clinically affected broiler farms in Mazandaran province, Iran.

Regarding the individual antimicrobials, *E. coli* resistance was observed for all 10 antimicrobial agents. The isolates showed high levels of resistance (>50%) to tetracycline (71.25%), oxytetracycline (73.75%), erythromycin (65%), and ampicillin (62.5%). The antimicrobials detected at low levels of resistance (<10%) were enrofloxacin (7.5%), norfloxacin (2.5%) and florfenicol (7.5%) (Table 1). Antimicrobial resistance levels of the broiler-originated *E. coli* isolates to the tetracycline group (71.25%–73.75%) dominated over other groups while their antimicrobial susceptibility level to the fluoroquinolone group was still high (resistance levels were less than 10%). With antimicrobials from the β -lactam groups, levels of resistance of *E. coli* isolates were higher to the older (ampicillin and amoxicillin) than to newer antimicrobials (co-amoxiclav). Each of the eighty *E. coli*

isolates showed resistance to at least two antibiotics. Table 2 shows the distribution of isolates into different groups of resistance patterns and the percentage of occurrence in relation to total isolates.

Among the 80 *E. coli* strains 86.25% belonged to the four serogroups with 17 to O1, 14 to O2, 8 to O18 and 30 to O78 (Table 3). Out of the eighty *E. coli* isolates subjected to Congo red binding assay, 71 (88.75%) isolates were positive and 9 (11.25%) isolates were negative.

Antimicrobial resistance among bacteria isolated from food animals is a matter of concern to public health and animals as well. Investigations on *E. coli* isolates from poultry have shown increased resistance to antimicrobials (Khoshkhoo & Peighambari, 2005; Li *et al.*, 2010). Johnson *et al.* (2007) demonstrated a close similarity between resistant human and poultry *E. coli* isolates.

In this study, fifty (62.5%) of all isolates were resistant to ampicillin. High percentages (89.6% and 96.7%) of resistance to ampicillin were reported by Gundogan *et al.* (2006) and Khoshkhoo & Peighambari (2005), respectively.

Table 1. Antibiotic resistance patterns of 80 *E. coli* isolated from broilers with colibacillosis symptoms

Antibiotic	Number (%) of sensitive isolates	Number (%) of intermediate isolates	Number (%) of resistant isolates
A (10 µg)	18 (22.50)	12 (15.00)	50 (62.50)
AMX (25 µg)	46 (57.50)	8 (10.00)	26 (32.50)
AMC (20 µg)	59 (73.75)	7 (8.75)	14 (17.50)
TE (30 µg)	3 (3.75)	10 (12.50)	57 (71.25)
OTC (30 µg)	7 (8.75)	14 (17.50)	59 (73.75)
E (15 µg)	14 (17.50)	14 (17.50)	52 (65.00)
FF (30 µg)	58 (72.50)	16 (20.00)	6 (7.50)
FM (30 µg)	33 (41.25)	8 (10.00)	39 (48.75)
NFX (5 µg)	66 (82.50)	8 (10.00)	6 (7.50)
NX (10 µg)	71 (88.75)	7 (8.75)	2 (2.50)

A, ampicillin; AMX, amoxicillin; AMC, co-amoxiclav; TE, tetracycline; OTC, oxytetracycline; E, erythromycin; FF, florfenicol; FM, flumequine; NFX, enrofloxacin; NX, norfloxacin.

Table 2. Multiresistance patterns of *E. coli* isolates. The left column present the number of tested drugs against which the isolates are resistant, the right one – drug combinations. The number of isolates resistant against each combination is given in brackets.

Number of drugs	Drug combinations (number of isolates)
2	OTC+TE (1)
3	A+TE+OTC (2); A+OTC+AMX (1); TE+OTC+E (3)
4	A+TE+OTC+ E (10); TE+OTC+ E+FM (2)
5	A+TE+OTC+ E+FM (12)
6	A+TE+OTC+AMX+E+FM (11)
7	AMC+A+TE+OTC+AMX+E+FM (5); AMC+A+TE+OTC+AMX+E+FM (1)
8	AMC+A+TE+OTC+AMX+E+FM+FF(2); AMC+A+TE+OTC+AMX+E+FM+NFX (2)
9	AMC+A+TE+OTC+AMX+E+FM+FF+NFX (2)
10	All drugs tested (2)

A, ampicillin; AMX, amoxicillin; AMC, co-amoxiclav; TE, tetracycline; OTC, oxytetracycline; E, erythromycin; FF, florfenicol; FM, flumequine; NFX, enrofloxacin; NX, norfloxacin.

Table 3. Prevalence of different serotypes of *E. coli* isolated from broilers with colibacillosis symptoms

<i>E. coli</i> serotypes	Number of isolates (%)	Number of invasive isolates (%)
O1	17 (21.25)	16 (94.1)
O2	14 (17.5)	12 (85.7)
O18	8 (10)	6 (75)
O78: (K80)	30 (37.5)	30 (100)
Other serotypes	11 (13.75)	7 (63.6)
Total	80 (100)	71 (88.7)

Two (2.5%) out of 80 *Escherichia coli* isolates were resistant to norfloxacin, variable results were obtained by different authors. Khoshkhoo & Peighambari (2005) and Alimehr *et al.* (1999) reported 52.7% and 20.4% resistance to norfloxacin among *Escherichia coli* isolates from broilers, respectively. On the other hand, 0% resistance to norfloxacin was mentioned by Ali Akond *et al.* (2009). Such

differences may well be related to the source, frequency and type of *E. coli* isolates encountered in different geographical areas.

The results revealed that 6 (7.5%) out a total of 80 *Escherichia coli* isolates were resistant to enrofloxacin. Similar results were obtained by different authors, 4% and 9.3% of avian *Escherichia coli* were found resistant to enrofloxacin by Zakeri

& Kashefi (2012), and Hanson *et al.* (2002). In the current study, 6 (7.5%) out of all isolates were resistant to florfenicol. Similar percentage of florfenicol resistant *Escherichia coli* (10%) was recorded among avian *Escherichia coli* isolates by Gregova *et al.* (2012).

The results showed that the least sensitivity was related to tetracycline (3.75%) and oxytetracycline (8.75%). These results are in line with the results of Zakeri & Kashefi (2012) and Hanson *et al.* (2002). High resistance to these antibiotics may be explained by the fact that both are frequently used by poultry industry in Iran.

Multi-drug resistance (MDR) appeared as a real problem as the majority of strains were resistant to at least two antibiotics. Increased MDR has been reported in *E. coli* isolates in many countries including Iran (Khoshkhoo & Peighambari, 2005; Li *et al.*, 2010). A similar finding on MDR of *E. coli* strains has been reported from other parts of the world (Khan *et al.*, 2002; Guerra *et al.*, 2003; Rahman *et al.*, 2008). Antimicrobial susceptibility test is very important to choose efficient antimicrobial treatment against avian *E. coli*. Results showed that among MDR types, numbers of antimicrobial agents were different – between 2 to 12. This is in line with the results of another study on poultry *E. coli* (Khoshkhoo & Peighambari, 2005). Direct contacts and animal-origin foods can spread MDR bacterial isolates into human population (Soulsby, 2008). After colonisation of resistant bacteria in the human intestinal tract, the antibiotic resistance gene can be transferred to microflora or pathogenic bacteria. Shedding of resistant bacteria in the environment can infect animals and then come back to humans through the food chain (Hawkey, 2008).

Similar to previous studies (Cavalieri *et al.*, 1984; Gross, 1994), present results showed that O78, O1 and O2 were the dominant serotypes of *E. coli* in colibacillosis infected poultry. Also, Allan *et al.* (1993) showed that 61% of 44 isolates were typeable, with O1, O2 and O78 being the most frequent serotypes.

The result of the Congo red binding assay indicated that the majority (88.75%) of cases of colibacillosis produced Congo red positive colonies. *In vitro* Congo red pathogenicity test could be clearly correlated with invasion ability and pathogenicity. This finding is in agreement with a previous study (Berkhoff & Vinal, 1986).

In conclusion, this study shows that multidrug resistant *E. coli* isolates are prevalent among broilers in Iran. It is recommended that the use of antimicrobial agents in food animals should follow prudent use guidelines to minimise the selection and spread of resistant bacteria. Also, utilisation of some antibiotics such as tetracycline, oxytetracycline and erythromycin in poultry farms should be revised. Moreover, it seems that antimicrobial resistance test former to therapy at the farm can be helpful for better antibiotic treatments.

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