The relation of water contamination and Colibacillosis occurrence in poultry farms in Qom province of Iran

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Summary

Seventy poultry farms' drinking water was tested for Escherichia coli contamination in Qom province in Iran. The cases of colibacillosis from positive farms were also collected and tested. The isolates were examined for serotype, detection of virulence genes by multiplex PCR and antibiotic resistance. Thirty poultry farm water samples were E. coli positive (18.57%), although 13 E. coli isolates were recovered from carcasses of related farms. The isolates belonged to O2 serogroup and one O157, with approximately 29% of the strains being non-typeable. Two isolates from water and carcasses were serotyped O2 and one sample serotyped O157, which needs to be further studied. The PCR method was on the basis of showing virulence genes of espB, stx1, stx2 and eae. One sample from water and one from a carcass were shared espB, stx2 and eae genes. Stx1 and stx2 genes were common in a sample from both water and carcass, although five samples from both water and carcass shared a stx1 gene as well. All isolates showed maximum sensitivity and resistance to lincompectine and tetracycline, respectively.

Key words: Colibacillosis, Drinking water, PCR, Poultry farms, Qom province

Introduction

Avian colibacillosis is considered to be the major bacterial disease in the poultry industry worldwide. It has been found to be a major infectious disease in birds of all ages. This disease has an important economic impact on poultry production worldwide. Avian colibacillosis is an infectious disease of birds caused by Escherichia coli, which is considered as one of the principal causes of morbidity and mortality, associated with heavy economic losses to the poultry industry by its association with various disease conditions, either as the primary pathogen or as a secondary pathogen. The risk for colibacillosis increases with increasing infection pressure in the environment. A good housing hygiene and avoiding any possible contamination conditions are very important.

Drinking water as an essential nutrient in metabolism for birds is of concern to poultry performance due to its potential for E. coli contamination. Although water does not provide ideal conditions for pathogenic microorganisms to multiply, they will generally survive long enough to allow waterborne transmission. Escherichia coli is one of the main poultry pathogens responsible for water contamination. Contaminated water with faecal coliform severely affects the performance of broilers, and Escherichia coli strains cause systemic disease in poultry.

Colibacillosis, which is caused by E. coli, causes considerable economic and welfare problems in broilers, due to its frequent occurrence and its adverse effects on growth and health. There are many reports about water contamination with E. coli in poultry farms. Amaral et al. (1999, 2001) reported that the samples from water
sources and reservoirs were contaminated by 
*E. coli* in 10 broiler and laying hen farms 
evidencing faecal pollution of the samples. 
Also, Amaral (2004) reported colibacillosis 
ocurrence by underground water origin 
contaminated by *E. coli*. Goan *et al.* (1992) 
assessed water samples from 105 wells of 65 
flocks in the United States, and reported that 
faecal coliforms were present in 43% of the 
samples. Therefore, the present study was 
conducted to show the possibility of 
colibacillosis with water origin in some 
broader farms.

Materials and Methods

**Samples**

Drinking water (100 ml) of 70 broiler 
farms in Qom province was collected from 
pipe water. The samples were taken after 
flaming the outlet and allowing the water to 
run for 5 min. The samples were placed in 
sterile glass jars and stored in a cool box to 
transport to the lab and were tested 4-5 h 
after sampling. One ml of each sample was 
added to lactose broth with a durham tube in 
a five-tube MPN dilution series and 
incubated for 24 h at 35.5°C. For 
confirmation positive tubes were inoculated 
into brilliant green broth with a durham tube and 
incubated for 48 h at 35.5°C. To detect 
faecal coliforms, the positive tubes were 
inoculated into brilliant green broth with a 
durum tube and incubated for 24 h at 45°C. 
The IMViC test was carried out as a 
supplementary test. Positive *E. coli* tubes 
were streaked onto eosin methylene blue 
agar and incubated for 24 h at 37°C. Typical 
colonies with a dark center or a metallic 
sheen were selected, gram stained and 
inoculated into *E. coli* broth for 24 h at 37°C 
and *E. coli* was confirmed. The cases of 
colibacillosis from positive farms were 
collected and samples were taken from 
pericardium in an aseptic condition. The 
samples were streaked onto McConkey agar 
and then EMB agar and incubated for 24 h at 
37°C (Vanderzant and Splittstoesser, 1992).

**Serotyping**

For the O antigen group determination a 
dense suspension of the organism was taken 
as 3-5 match head size amounts of the 
organism from an agar plate or slope and 
placed in 3 ml of 0.85% saline. The 
suspension was heated to 100°C for 60 min 
and centrifuged at 900 × g for 20 min. The 
supernatant was removed and 0.5 ml of 
0.85% saline was added to resuspend the 
precipitate. A homogenized suspension was 
used as the antigenic suspension for O-
antigen grouping. A drop of polyvalent 
antiserum (O1, O26, O86a, O111, O119, 
O127a, O128) and monovalent antisera (O2, 
O78, O114, O115, O124, O157) (Mast 
Diagnostics Company, Amiens, France) was 
placed on the clean slide. A drop of the 
antigen suspension was placed next to the 
antiserum drop on the slide and mixed well 
in order to observe the agglutination.

Detection of virulence gene by 
multiplex PCR

The reference *E. coli* strain used as 
positive control was *E. coli* O157 (strain No. 
84-4, Tarbiat Modarres University) and 
stere deionized water was used as a 
negative control. In multiplex PCR 
amplification, 26 *E. coli* isolates of water 
and carcass origin and positive control strain 
were cultured in LB agar for 24 h at 37°C. 
To extract the bacterial DNA, 6 to 8 colonies 
were picked and suspended in 100 µL of 
stere deionized water, then incubated at 
100°C for 10 min to release the DNA, and 
afterswards centrifuged at 6000 × g for 5 min. 
The supernatant was used in the PCR 
reaction as the template DNA. The base 
sequence and predicted size of the amplified 
product for each of the oligonucleotide 
primers (Cinna Gen Inc, Iran) used in this 
study are shown in Table 1. Primers were 
used in two different protocols. In the first 
protocol, *E. coli* (EC) primers which were 
the same as in the second protocol including 
stx1, stx2, eae and espB primers. EC primers 
confirmed the isolates as *E. coli* (for alanine 
racemase (*alr*) gene). Amplification 
reactions were performed in a 25 µL volume 
containing 2.5 µL of 10 × PCR buffer, 1 µL 
of 50 mM MgCl₂, 1 µL of 10 mM 
deoxynucleoside triphosphate (CinnaGen 
Inc, Iran), 1 µL of each primer, 0.5 µL of 
*Tag* DNA polymerase (CinnaGen Inc, Iran), 
5 µL of the template DNA and 7 µL (13 µL 
in the second protocol) of sterile dionised
Isolated (GM).
Spectinomycin (LI + SP) and Gentamycin
Chloramphenicol (C), Lincomycin +
Trimethoprim (SXT), Tetracycline (T),
Enrofloxacin (NFX), Sulfamethoxasol +
and the following antibiotic discs were used:

isolates was assayed by Kirby-Bauer method
that eight isolates were O2 positive, whereas
of 13 confirmed the isolates as
were positive with EC primer which
amplification (Tables 2 and 3). All isolates
virulence genes by multiplex PCR
have been used to determine the different
ruler 100 bp DNA ladder plus, Fermentas).
contained molecular size markers (Gene
electrophoresed for 1 h at 100 V. Each gel
in TBE buffer. The samples were
final reaction mixture on a 1.2% agarose gel
by gel electrophoresis using 10 µL of the

Antibiogram
Antibiotic sensitivity testing of E. coli
isolates was assayed by Kirby-Bauer method
and the following antibiotic discs were used:
Enrofloxacin (NFX), Sulfamethoxasol +
Trimetoprim (SXT), Tetracycline (T),
Chloramphenicol (C), Lincomycin +
Spectinomycin (LI + SP) and Gentamycin
(GM).

Results
The results showed that 13 samples of
water were E. coli positive (18.57%).
Isolated E. coli from water and carcasses
have been used to determine the different
virulence genes by multiplex PCR
amplification (Tables 2 and 3). All isolates
were positive with EC primer which
confirmed the isolates as E. coli. Serotyping
of 13 E. coli isolates of water origin showed
that eight isolates were O2 positive, whereas
the others could not be typed. Among the 13
E. coli isolates of carcass origin, just one
isolate was O157 positive, whereas 5
isolates were O2 positive and the others
could not be serotyped with the available antiserum.

Among the 13 isolates of water, 7
isolates (53.84%) showed espB gene (Fig.
1), 12 isolates (92.30%) showed stx1, 4
isolates (30.76%) carried stx2 and 6 isolates
(46.15%) possessed eae (Fig. 1)(Table 2).
Among the 13 isolates of carcasses, 4
isolates (30.76%) had espB gene, 5 isolates
(38.46%) carried stx1 (Fig. 2), 7 isolates
(53.84%) had stx2 (Fig. 2) and 2 isolates
(15.38%) possessed eae (Table 3). None of
the isolates of water and carcasses had
Haemolysis on blood agar.

Table 1: Primers sequences used in multiplex PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Oligonucleotide sequence (5-3)</th>
<th>Number of nucleotides</th>
<th>Fragment size (bp)</th>
<th>Ref.</th>
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<td>stx1</td>
<td>vt1</td>
<td>CGG TGA AGT AGT TGG CTC GTC</td>
<td>22</td>
<td>302</td>
<td>Rey et al. (2003)</td>
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<td>CGT GGG ATA GCT ACT GTC ACC</td>
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<td>stx2</td>
<td>vt1</td>
<td>CGG TGA AGT AGT TGG CTC GTC</td>
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</tr>
<tr>
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<td>CGT TGC TAA GAG TCA AA AAC GC</td>
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<td></td>
</tr>
<tr>
<td>eae</td>
<td>eae1</td>
<td>GAG AAT GAA ATA GGA GTC GT</td>
<td>20</td>
<td>775</td>
<td>Rey et al. (2003)</td>
</tr>
<tr>
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<td>eae2</td>
<td>GGG GTA ATC TCT TCC GCG TAA</td>
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</tr>
<tr>
<td>espB</td>
<td>espB1</td>
<td>GGC GTC TTT GAG AGC CA</td>
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<td>260</td>
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</tr>
<tr>
<td></td>
<td>espB2</td>
<td>GAT GCC TCC TCT TCG A</td>
<td>16</td>
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</tr>
<tr>
<td>alr</td>
<td>E.c1</td>
<td>CGT GAA GAG GCT ACC CTG GAC GAG</td>
<td>24</td>
<td>366</td>
<td>Yokoiyawa et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>E.c2</td>
<td>AAA ATC GGC ACC GGT GGA GCC ATC</td>
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<td></td>
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Table 2: Occurrence of virulence factors among different serotypes of E. coli isolated from water

<table>
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<tr>
<th>Sample</th>
<th>Serotyping</th>
<th>alr</th>
<th>espB</th>
<th>stx1</th>
<th>stx2</th>
<th>eae</th>
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<td>-</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>2</td>
<td>O2</td>
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<td>-</td>
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<tr>
<td>5</td>
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</tbody>
</table>

N = Non serotyped, + = Presence of gene, and - = Absence of gene

Table 3: Occurrence of virulence factors among different serotypes of E. coli isolated from carcasses

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<tr>
<th>Sample</th>
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<th>espB</th>
<th>stx1</th>
<th>stx2</th>
<th>eae</th>
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<tbody>
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<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>O2</td>
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</tr>
<tr>
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<tr>
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<td>O2</td>
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<td>-</td>
<td>-</td>
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</table>

N = Non serotyped, + = Presence of gene, and - = Absence of gene
**Discussion**

Avian colibacillosis is a common systemic disease and is responsible for a significant proportion of the mortality found in poultry flocks. In our study, faecal coliform was detected in 43% of the samples from the water sources, indicating the occurrence of faecal pollution that could be due to free access of wild and domestic animals to the superficial water sources, disposal of animal excreta and dead carcasses, and even the drainage of human sewage from the rural villages. There are many reports about water contamination with *E. coli* in poultry farms. Amaral (2004) reported that colibacillosis occurs due to contaminated underground water. He et al. (2007) showed that 90% of the water samples from wells and 100% of the samples originating from springs had bacteria indicative of faecal pollution. In the present study, 55% of the wells were contaminated with coliforms. Out of these, 35% were identified as *E. coli*. Also, 100% of the springs were contaminated with coliforms. Out of these, 50% were identified as *E. coli*.

Verotoxins or shigatoxins are cytotoxins produced by some enteropathogenic *E. coli* (EHEC or STEC). VT1 (*Stx1*) and VT2 (*Stx2*) are two major types of Verotoxins that have been recognized. The *eae* gene encodes a protein named intimin which is responsible for intimate attachment of *E. coli* to the enterocytes causing attaching and effacing (A/E) lesions in the intestinal mucosa (Agin and Wolf, 1997). The *espB* protein, encoded by the *espB* gene, also helps bacteria to attach to the enterocytes (McDaniel and Kaper, 1997). Both *eae* and *espB* genes are part of a pathogenicity island termed as the locus for enterocyte effacement (LEE). There are several studies about virulence genes in *E. coli*, especially by using multiplex PCR (Pass et al., 2000; Sami et al., 2007). Fantinatti et al. (1994) found that 3 of 17 *E. coli* isolates (11%)
from septicemia in chickens produced Verotoxin, and these three isolates demonstrated the highest level of pathogenicity, indicating a correlation between toxin production and pathogenicity. Zahraei Salehi et al. (2007) detected stx2 gene in 75% of 12 avian isolates using multiplex PCR. One isolate possessed stx1, two isolates carried eae sequence and three isolates presented espB. In this study, 7 E. coli isolates of carcasses had stx2 gene, 5 isolates were positive for stx1, 2 isolates had eae and 4 isolates showed espB. This study is in agreement with the finding of Zahraei Salehi et al. (2007).

Escherichia coli isolates pathogenic for poultry commonly belong to certain serogroups, particularly the serogroups O78, O2 and O1, but other O serogroups and nontypeable ones are also capable of producing the disease. In our study, in the majority of the flocks, most of the isolated E. coli, 8 isolates from water and 5 isolates from carcasses, belonged to the O2 serogroup. A study in Iran showed that the most common serogroups belonged to O78, O128, O2, O111 and O124 (Zahraei Salehi and Yahya Raeyat, 2001). Ike et al. (1990) recorded a high incidence of serogroups O2 and O78 in cases of colibacillosis in Japan. Sri Purnomo et al. (1992) indicated that O1, O2 and O78 serogroups are the most commonly associated serogroups with colibacillosis in Indonesia. Veere Gowda et al. (1996) showed that the most prevalent serogroups in India are O2 and O78.

In the present study, all isolates showed maximum resistance and sensitivity to tetracycline and lincomycin, respectively. Reinthaler et al. (2003) found that most of the E. coli strains from sewage exhibited the most resistance to TE. Tetracycline resistance was also observed among E. coli isolates and has been frequently reported in poultry products (Sackey et al., 2001). Tabatabaei and Nasirian (2003) reported that 94% of E. coli isolates from chickens in poultry farms of Tehran were resistant to TE. Hofacre (2002) reported that 90% of E. coli poultry isolates were resistant to TE. Our results are similar to their findings. The significant increase in the incidence of resistance against antibiotics in the E. coli strains isolated from broiler chickens is probably due to the increased use of antibiotics as feed additives for growth promotion and prevention of diseases, use of inappropriate antibiotics for treatment of diseases, resistance transfer among different bacteria and possible cross-resistance between antibiotics used in poultry. In the present study, the findings demonstrate that colibacillosis with water origin in Qom province is more likely. So, the usage of hygienic drinkers or continuous water chlorination and regular washing of the pipe system and waterers with disinfectants are recommended.

Acknowledgements

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