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 lincospectine 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 occurrence 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 broiler 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 sterile 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 sterile deionized water, then incubated at 100°C for 10 min to release the DNA, and afterwards 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 (CinnaGen 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 $MgCl_2$, 1 µL of 10 mM deoxynucleoside triphosphate (CinnaGen Inc, Iran), 1 µL of each primer, 0.5 µL of *Taq* DNA polymerase (CinnaGen Inc, Iran), 5 µL of the template DNA and 7 µL (13 µL in the second protocol) of sterile dionised

Table 1: Primers sequences used in multiplex PCR

Gene	Primer	Oligonucleotid sequence (5-3)	Number of nucleotide	Fragment size (bp)	Ref.
stx1	*vt1*	CGC TGA ATG TCA TTC GCT CTG C	22	302	Rey *et al*. (2003)
	vt2	CGT GGT ATA GCT ACT GTC ACC	21		
stx2	*vt1*	CTT CGG TAT CCT ATT CCC GG	20	516	Rey *et al*. (2003)
	vt2	CTG CTG TGA CAG TGA CAA AAC GC	23		
eae	*eae1*	GAG AAT GAA ATA GAA GTC GT	20	775	Rey *et al*. (2003)
	eae2	GCG GTA TCT TTC GCG TAA TCG CC	23		
espB	*espB1*	GGC GTT TTT GAG AGC CA	17	260	Cid *et al*. (2001)
	espB2	GAT GCC TCC TCT GCG A	16		
alr	*E.c1*	CGT GAA GAG GCT AGC CTG GAC GAG	24	366	Yokoigawa *et al*. (1999)
	E.c2	AAA ATC GGC ACC GGT GGA GCG ATC	24		

water. Using a thermal cycler (Techne 512, UK), the multiplex PCR condition was as follows: 94°C for 10 min for initial denaturation of DNA followed by 30 cycles of 94°C for 1 min, 84°C for 1 min (64°C in the second protocol) and 72°C for 1 min.

The amplified products were visualized by gel electrophoresis using 10 µL of the final reaction mixture on a 1.2% agarose gel in TBE buffer. The samples were electrophoresed for 1 h at 100 V. Each gel contained molecular size markers (Gene ruler 100 bp DNA ladder plus, Fermentas).

Antibiogram

Antibiotic sensitivity testing of *E. coli* isolates was assayed by Kirby-Bauer method and the following antibiotic discs were used: Enrofloxacin (NFX), Sulfamethoxasol + Trimethoprim (SXT), Tetracycline (T), Chloramphenicol (C), Lincomycin + Spectimomycin (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 antisera.

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 2: Occurrence of virulence factors among different serotypes of *E. coli* isolated from water

Sample	Serotyping	*alr*	*espB*	*stx1*	*stx2*	*eae*
1	N	+	+	+	-	+
2	O2	+	+	+	-	+
5	N	+	-	+	+	-
18	O2	+	-	+	-	-
26	O2	+	+	+	-	+
32	N	+	+	-	+	-
36	N	+	+	+	-	-
37	O2	+	-	+	-	-
40	O2	+	+	+	+	+
49	O2	+	-	+	-	+
53	O2	+	+	+	-	-
57	O2	+	-	+	+	-
61	N	+	-	+	-	+

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

Sample	Serotyping	*alr*	*espB*	*stx1*	*stx2*	*eae*
1	O2	+	-	+	-	-
2	O2	+	-	+	-	-
5	O2	+	-	-	-	-
18	O2	+	-	-	-	-
26	N	+	-	-	+	-
32	N	+	-	-	-	-
36	O2	+	-	-	-	-
37	O157	+	+	+	+	+
40	N	+	+	-	+	+
49	N	+	+	+	+	-
53	N	+	-	-	+	-
57	N	+	+	+	+	-
61	N	+	-	-	+	-

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

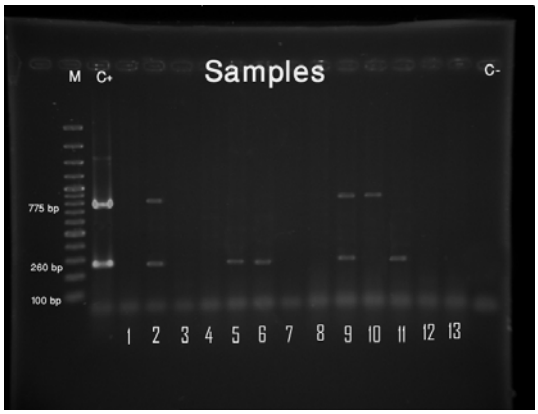

Fig. 1: Multiplex PCR of isolated *E. coli* from water, using primer set *espB*: 260 bp, *eae*: 775 bp. Lane M: 100 bp Marker (Fermantas). Lane 2, 5, 6, 9, 11 are positive *espB* (2, 26, 32, 40, 53 samples), Lane 2, 9, 10 are positive *eae* (2, 40, 49 samples), Lane C+: *E. coli* O157 (Strain No. 84-4, Tarbiat Modarres University) as positive control, and Lane C-: negative control (Water)

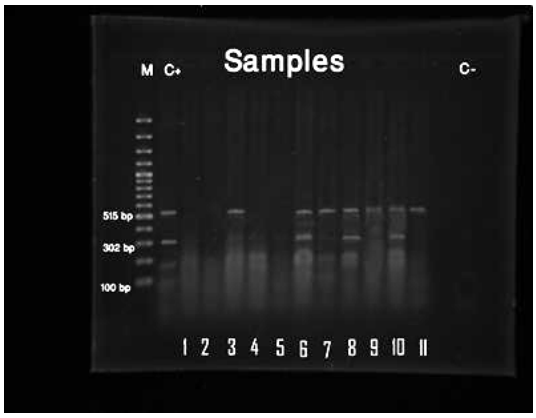

Fig. 2: Multiplex PCR of isolated *E. coli* from carcasses, using primer set *stx1*: 302 bp, *stx2*: 516 bp. Lane M: 100 bp Marker (Fermantas). Lane 6, 8, 10 are *stx1* positive (37, 49, 57 samples), Lane 3, 6, 7, 8, 9, 10, 11 are *stx2* positive (26, 32, 40, 49, 53, 57, 61 samples), Lane C+: *E. coli* O157 (Strain No. 84-4, Tarbiat Modarres University) as positive control, and Lane C-: negative control (Water)

Two isolates from water and carcasses were serotyped as O2. On the basis of molecular characteristics, a sample of water and one of carcass had the *espB*, *stx2* and *eae* genes in common, although five samples of both water and carcass shared the *stx1* gene as well.

Escherichia coli isolates showed variable percentages of sensitivity and resistance to the different antibiotics. A high level of resistance against Tetracycline in *E. coli* isolates from water and carcasses was 100% and 85%, respectively.

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 non-typeable 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 lincospectin, 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.

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