Detection of *stx*1, *stx*2, *eae*, *esp*B and *hly* genes in avian pathogenic *Escherichia coli* by multiplex polymerase chain reaction

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Abstract: The purpose of this study was to determine the presence of selected virulence genes in Avian Pathogenic *Escherichia coli* (APEC). We examined 12 APEC isolates which belonged to the most common serotypes in Iran. All 12 isolates were tested for the presence of *stx1, stx2, eae, esp*B and *hly* genes by multiplex polymerase chain reaction in two protocols. In the first protocol the isolates were tested with *EC* and with *hly* primers, and in the second protocol the isolates were examined with *eae, stx1, stx2* and *esp*B primers. Seventy five percent (9) of the isolates carried only *stx2* gene sequence and just one isolate had both *stx1* and *stx2* genes. Furthermore, 2 isolates (16.66 %) possessed *eae* sequence and 3 isolates carried *esp*B (25%). The *hly* gene was not detected in any of the isolates. The findings of this study indicated that the *Stx2* may be widespread among APEC in Iran. *J.Vet.Res. 62,2:37-42,2007.*

Key words: virulence, *Escherichia coli*, multiplex PCR, *stx*1, *stx*2, *eae*, *esp*B, *hly*.

Introduction

Colibacillosis is one of the most important bacterial diseases in poultry which is caused by *Escherichia coli* (2). In poultry, pathogenic *E. coli* isolates usually produce extraintestinal infections such as respiratory tract infection, septicemia, swollen head syndrome, or combination of these manifestations. Respiratory infections due to *E. coli* occur primarily in young broilers whose respiratory tract previously was damaged by some infectious agents or environmental factors (2).

The production of cytotoxins in *E. coli* isolates has been extensively studied among isolates from humans and several animal species (4). Little work has been done to investigate the production of cytotoxin by avian *E. coli* isolated from poultry. Recent studies have demonstrated the presence of a number of verotoxins in some of the APEC isolates. In 1992, Emery *et al.,* found that 5.7% and 7.5% of *E. coli* associated with septicemia in turkeys and chickens, respectively, produced a heat-labile toxin (LT) which was cytotoxic for both Vero and Y^{-1} cells. Interestingly, a distinct LT which was found to be active on Vero cells only was produced among 11% and 22.5% of turkeys and chickens, respectively. Fantinatti *et al.,* (1994) detected cytotoxic activity on Vero cells among 3 out of 17 avian septicemic *E. coli* isolates. These three isolates were among those isolates that demonstrated a high level of pathogenicity when injected to two-day old chicks. Parreira and Yano (1998) showed that 72% of *E. coli* isolates from SHS produced a cytotoxin that was active on Vero and HeLa cells. The cytotoxin was designated as VT2y. The possible role of this cytotoxin in the development of SHS was not investigated. In 2000, Schmidt *et al.,* reported that 12.5% of the healthy pigeons whose feces were

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Table1. Primer sequences used in multiplex PCR

Gene	Primer	Oligonucleotide Sequence (5-3)	Number of nocleotid	Fragment size(bp)	Ref.
*stx*1	vt1	CGC TGA ATG TCA TTC GCT CTG C	22	302	Rey (2002)
	vt2	CGT GGT ATA GCT ACT GTC ACC	21		
*stx*2	vt1	CCT CGG TAT CCT ATT CCC GG	20	516	Rey (2002)
	vt2	CTG CTG TGA CAG TGA CAA AAC GC	23		
eae	eae1	GAG AAT GAA ATA GAA GTC GT	20	775	Rey (2002)
	eae2	GCG GTA TCT TTC GCG TAA TCG CC	23		
*esp*B	espB1	GGC GTT TTT GAG AGC CA	17	260	Cid (2001)
	aspB2	GAT GCC TCC TCT GCG A	16		
hly	hly1	AAC AAG GAT AAG CAC TGT TCT GGC T	25	1177	Yamamoto (1995)
	hly2	ACC ATA TAA GCG GTC ATT CCC GTC A	25		
alr	EC1	CGT GAA GAG GCT AGC CTG GAC GAG	24	366	Yokoigawa (1999)
	EC2	AAA ATC GGC ACC GGT GGA GCG ATC	24		

cultured had Shiga toxin-producing *E. coli* (STEC) that carried a variant *stx2* gene. Parreira and Gyles (2002) used polymerase chain reaction (PCR) amplification and hybridization technique for the detection of stx genes among a number of APEC isolates, and reported that 52 of the 97 APEC isolates carried stx gene sequence. The *eae* and *E-hlyA* genes were not detected among stx-positive isolates when tested by polymerase chain reaction (PCR).

The purpose of this study was to detect the stx (*stx1*, *stx2*), *eae*, *espB*, and *hl*y genes among recently isolated *Escherichia coli* from chickens associated with colibacillosis by multiplex PCR.

Materials and Methods

Bacterial Strains: Among 156 *Escherichia coli* isolated from chickens in Iran during 2000-2001, twelve isolates each belonging to one serotype were selected and tested for the presence of the selected genes. All isolates which had been lyophilized and stored at -20□C, were recultured on nutrient agar, then subcultured on blood, MacConkey, and EMB agar, and incubated for 24 hours at 37□C. Positive control strains were: an *Escherichia coli* O157 strain (designated as 84-4) carrying gene sequences for *stx1*, *stx2*, *eae*, *sep*B provided from microbial collection of Tarbiat Modarres University, an *E. coli* ATCC 35218 carrying gene sequence for *hly*, and an *E. coli* NTCC 11954 carrying gene sequence for alr (alanine racemase) gene (a chromosomal gene which is used to confirm the *E. coli* species).

Multiplex PCR: For multiplex PCR amplification, 12 avian *Escherichia coli* isolates and positive control strains were cultured on LB agar for 24 h at 37□C. To extract bacterial DNA, 6 to 8 colonies of each culture were picked and suspended in 100 □l of sterile deionized water, incubated at 100□C for 10 minutes to release the DNA, and centrifuged at 6000□g for 5 minutes. The supernatant was used in the PCR reaction as the template DNA. Sterile dionized water was used instead of template DNA in negative control tubes. Base sequence and predicted size of amplified product for each oligonucleotide primer (Sinagene, Iran) used in this study were shown in Table 1. Primers were used in two different protocols. In the fist protocol, EC and *hly* primers, and in the second protocol, *stx1*, *stx2*, *eae*, and *esp*B primers were included. *EC* primers confirmed the isolates as *E. coli*. Amplification reactions were performed in a 25□l volume containing 2.5□l of 10X PCR buffer, 1□l of 50 mM $MgCl_2$, 1.5□l of 10 mM deoxynucleoside triphosphate (Sinagene, Iran), 1□l

Table2. Occurrence of virulence genes among different serotypes of avian pathogenic *Escherichia coli*
P: positive, N: negative

Serotype	*EC(alr)*	*esp*B	*stx*1	*stx*2	*eae*	*hly*
O2:K12	P	N	N	P	N	N
O2:K1	P	N	N	P	N	N
O78:K80	P	N	N	P	N	N
O78:K10	P	N	N	P	N	N
O86:K 61	P	N	N	N	P	N
O111:B4	P	N	P	P	N	N
O114:K90	P	P	N	P	N	N
O119:B14	P	N	N	P	N	N
O124:K72	P	N	N	N	N	N
O125:K70	P	N	N	N	P	N
O126:K71	P	P	N	P	N	N
O128:K67	P	P	N	P	N	N

of each primer, 0.5 l of *Taq* DNA polymerase (Sinagene, Iran), 2 l of the template DNA, and 13.5 l (9.5 l in the second protocol) of sterile dionized water. Using a thermal cycler (Techne, UK), the condition for the multiplex PCR was programmed as follows: 94 C for 10 min for initial denaturation of DNA followed by 30 cycles of 94 C for 1 min, 48 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 l of the final reaction mixture on a 1.2 % agarose gel in TBE buffer. The samples were electrophoresed for 1 h at 100 V. Amplified DNA fragments of specific sizes was located by UV fluorescence after staining with ethidium bromide. Molecular size markers (Gene ruler 100 bp DNA ladder plus, Fermentas) were included in each gel.

Results

To determine the prevalence of selected virulence genes among avian *Escherichia coli*, we examined 12 common serotypes in Iran by multiplex PCR amplification (Table 2). All isolates were positive with EC primer that confirmed the isolates as *E. coli*. Nine isolates (75 %) carried only *stx2* gene and just one isolate had both *stx1* and *stx2* (8.33%). Three isolates were positive for *espB* and 2 isolates possessed *eae* gene sequence. All *espB* positive isolates had *stx2* but the *eae* positive isolates were not positive for any other gene sequences. No isolate was positive for *hly* neither by PCR nor on blood agar plate (Fig.1 and Fig.2).

Discussion

Avian pathogenic *Escherichia coli* (APEC) commonly belongs to certain O serogroups such as O78, O2, O1, but other O serogroups and nontypeble ones are also capable of producing the disease (2). Recent studies in Iran showed that the most common serogroups belonged to O78, O128, O2, O111 and O124 (22).

Verotoxins or shigatoxins are cytotoxins produced by some enteropathogenic *Escherichia coli* and are active on Vero cells. VT1 (*Stx1*) and VT2 (*Stx2*) are two major types of Verotoxins that have been recognized. Several reports have indicated the cytotoxic activity of avian *E. coli* on Vero cells. Emery *et al.,* (1992) reported that, in a collection of *E. coli* associated with septicemia, 18 of 82 chickens isolates (22%) and 48 of 436 turkey isolates (11%) produced a cytotoxin which was active on Vero cells. In 1994, Fantinatti *et al.,* found that 3 of 17 *E. coli* isolates (11%) from septicemia in chickens produced Vero toxin and these three isolates demonstrated the highest level of pathogenicity indicating a correlation between toxin production and pathogenicity. Parreira and Yano (1998) described that culture supernatant from 72% of the 50 *E. coli* isolates from the swollen head syndrome produced a cytotoxin detectable on Vero cells. Later, Parriera and Gyles (2002) reported that among 97 clinical isolates (53%) carried stx genes. All the stx-positive isolates were negative for the *eae* and *hly* genes.

A high percentage (83.33%) of the *E. coli* isolates in this study carried stx genes especially *stx2* type. Unlike findings of Parriera and Gyles (2002), we observed that nine isolates (75%) were *stx2*-positive, one isolate was positive for both *stx1* and *stx2*, and there was no isolate carrying only *stx1* gene sequence.

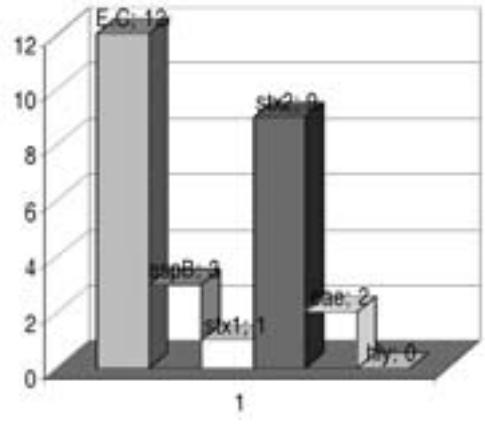

Figure 1. The value of each gene among 12 avian serotypes of *E. coli*

Both isolates of O2 and O78 serogroups, which are among serogroups most commonly associated with colibacillosis, were stx-positive. The serogroup O111 was the isolate that carried both *stx1*, and *stx2* gene sequence. In this study, the presence of *stx2* gene was more prevalent than *stx1*, which has not been reported previously.

The production of different types of hemolysin has been frequently contributed to *E. coli* from intestinal and extraintestinal diseases (16, 20). It causes the release of the Ferro from cells, providing iron for the bacteria. Epidemiological studies have shown that -hemolysin correlates with *E. coli* isolates associated with uropathogenic infection and sepsis (20). However, most studies have reported the avian *E. coli* as non-hemolytic (5, 6,13,15, 22) which corresponds with our findings.

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 (1). The carriage of *eae* gene sequence has been detected in *E. coli* isolates from avian sources in recent studies. Morabito *et al.,* (2001) found that most of the stx positive *Escherichia coli* isolates possessed genes encoding for intimin. Kobayashi *et al.,* (2002) detected *eae E. coli* in 40% of gulls, in 7% of pigeons and in 57% of the broiler flocks, which were contaminated. However, all *eae E. coli* isolates from birds differed from human pathogenic strains due to lack of *EHEC-hlyA* and bfp/EAF as well as distribution of O-serogroups. It was concluded that the birds cannot be regarded as important carriers of zoonotic stx or *eae E. coli* in Finland. The presence of *eae* gene sequence has been indicated in *E. coli* isolates from avian sources in some other reports (7,17). In our study, we were able to detect *eae* gene sequence in only two isolates. None of the stx-positive isolates from avian sources were positive for *eae* (13). These data indicate that domestic animals and birds constitute a natural reservoir of attaching and effacing *Escherichia coli* strains, and some of these are known as human pathogens. Numerous researchers have underlined the strong association between the carriage of *eae* gene and the capacity of stx-positive strains to cause severe human diseases specially Hemolytic Uremic Syndrome (HUS). In this study *eae* gene were not found in stx-positive isolates indicating that most of the avian *E. coli* may not be able to pose a risk to public health.

The *espB* protein, encoded by *espB* gene also helps bacteria to attach to the enterocytes (9). Both *eae* and *espB* genes are part of a pathogenicity island termed as the locus for enterocyte effacement (LEE). The presence of *espB* has been demonstrated in attaching and effacing *Escherichia coli* (AEEC) strains isolated from humans and animals (3,11). In the present study, three isolates from chickens were positive for *espB*, and all *espB* positive isolates were positive for *stx2* too.

Little information is available on the effects of Shiga toxin-producing *Escherichia coli* (STEC) on avian intestine. Sadhu and Gyles (2002) administered STEC orally to 80 day-old chicks and examined the ceca of the chicks 10 days post-challenge by light microscopy, transmission and scanning electron microscopy, and immunohistochemistry. No adherence or tissue abnormality for any isolate could be shown. Available data form previous works confirm that the birds, even the apparently healthy ones, represent a natural reservoir of STEC strains, characterized by the frequent presence of virulence genes associated with disease in humans. Further works are needed to clarify whether these STEC may represent a cause of avian disease or even a potential health hazard for humans. The findings described in our study indicate that stx genes may be widespread

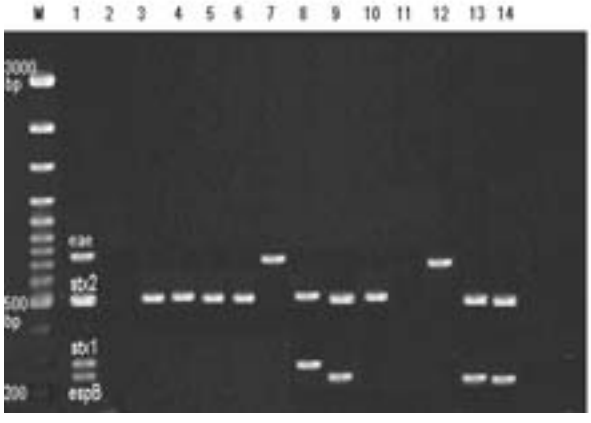

Figure 2: *Escherichia coli* - Multiplex PCR of *Escherichia coli* isolated from poultry, using primer set espB: 260 bp, *stx*1: 302 bp. *stx*2: 515 bp and eae: 775 bp. Lane M: 100 bp Marker (fermentas). Lane 1: *Escherichia coli* O157 (Strain NO. 84-4, Tarbiat Modarres University) as positive control; Lane 2: negative control (Water); Lane 3 to 14: Avian *Escherichia coli* isolate (Lane3: O2:K 12, Lane4: O2:K1, Lane5: O78:K80, Lane6: O78:K10, Lane7: O86:K61, Lane8: O111:B4, Lane9: O114:K90, Lane10: O119:B14, Lane11: O124:K72, Lane12: O125:K70, Lane13: O126:K71, Lane14: O128:K67).

among APEC, but the cytotoxicity should be detected on Vero cell to determine the possible shiga toxins production.

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