Detection of *eaeA*, *hlyA*, *stx1* and *stx2* genes in pathogenic *Escherichia coli* isolated from broilers affected with colibacillosis

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Abstract:

BACKGROUND: Foodborne outbreaks associated with shiga toxin-producing *Escherichia coli* (STEC) have been well documented worldwide. STECs are major causative agents of gastroenteritis in humans that may be complicated by hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenia purpura (TTP). **OBJECTIVES:** The aim of this study was to investigate the presence of virulence-associated genes including eaeA, hlyA, stx1 and stx2 in *Escherichia coli* strains isolated from broiler's Colibacillosis in the northeast of Iran. **METHODS:** From a total of 78 *E.coli* strains isolated from yolk sac infection, hepatitis and pericarditis, that were referred to educational veterinary clinic during 2011-2014, subculturing of the isolates was performed using selective media and a typical colony from each sample was subjected to multiplex PCR assay for identification of the presence of STEC important virulence-associated genes (eaeA, hlyA,stx1 and stx2) causing shiga toxin-mediated diseases. **RESULTS:** Of 78 *E.coli* isolates, one isolate was positive for both eaeA and hlyA genes while negative for stx1 and stx2 genes. **CONCLUSIONS:** The results showed low prevalence of STEC virulence genes associated with human infections in avian pathogenic *E.coli* (APEC) strains isolated from different flocks of broilers affected with colibacillosis.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) or verotoxin-producing *Escherichia coli* remains a major cause of foodborne-related gastrointestinal diseases in humans (Wani et al., 2004), particularly since these infections may result in life-threatening sequel such as hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenia purpura (TTP) (Best et al., 2005; Dhanashree and ShrikarMallya., 2008; Feng, 2013; Mohammadi et al., 2013; Paton and Paton., 1998; Paton and Paton., 2002; Paton and Paton., 2005; Wani et al., 2004). Ruminants are considered to be the main reservoir of STECs (Paton and Paton., 1998). Other domestic animals, such as pigs, poultry, cats and dogs can also harbor STECs (Dhanashree and Shrikar-Mallya, 2008; Kobayashi et al., 2002; Wani et al., 2004). The pathogenicity of these bacteria is mainly mediated by shiga toxins(Stx1,

Stx2 and their variants) encoded by stx1 and stx2 genes. Within human disease-associated strains, those producing Shiga toxin type 2 appear to be more commonly responsible for serious complications such as HUS than those producing only Shiga toxin type 1 (Feng, 2013; Fode-Vaughan et al., 2003; Paton and Paton., 1998). In addition, a subset of STEC strains considered to be highly virulent for humans has the capacity to produce attaching and effacing lesions on intestinal mucosa, a property encoded on a pathogenicity island termed the locus for enterocyte effacement (LEE). LEE encodes a type III secretion system and *E. coli* secreted proteins, which deliver effector molecules to the host cell and disrupt the host cytoskeleton. LEE also carries eaeA, which encodes an outer membrane protein (intimin) required for intimate attachment to epithelial cells. The eaeA gene has been used as a convenient diagnostic marker for LEE-positive STECs (Mohammadi et al., 2013; Paton and Paton., 1998; Paton and Paton., 2002; Paton and Paton., 2005; Wani et al., 2004). Another putative accessory virulence factor produced by STEC strains (both LEE positive and LEE negative) is plasmid-encoded enterohemolysin (hlyA) (Paton and Paton., 1998; Paton and Paton., 2002; Wani et al., 2004). This haemolysin seems to have an important role in complex diseases (HC and HUS) in humans. (Beutin et al., 1994). Accordingly, the capacity to rapidly determine the STECs virulence profile associated with the mentioned serious diseases is important to ensure the public safety. As chicken products are suspected to be a source of foodborne pathogenic *Escherichia coli* infections in humans (Heuvelink et al., 1999; Mellata, 2013; Tabatabaei et al., 2011), we have described using multiplex PCR assays for detection of the important marker genes associated with major STEC-outbreaks, eaeA and hlyA in the first step, then stx1 and stx2 in isolates harboring eaeA and/or hlyA. *Escherichia coli* strains isolated from chicken visceral organs suffering from clinical colibacillosis were screened to analyze their potential hazard for public health.

Materials and Methods

Sampling and bacterial culture: A total of 78 *E.coli* isolates were obtained from broiler chickens belonging to different flocks which were diagnosed as affected with systemic Colibacillosis. These isolates of *E.coli* were swabbed from affected organs of birds harboring lesions of Colibacillosis such as hepatitis and pericarditis, each isolate represented one flock. They were sampled during the years of 2011 to 2014 in the northeast of Iran. The strains were isolated from liver, blood (heart) and egg yolk sac. The respective specimens were plated on MacConkey agar (Merck, Germany) and suspected colonies were confirmed as *E.coli* using biochemical tests, afterwards a loopful of typical colonies on overnight culture were inoculated on Brain Heart Infusion (BHI) broth (Hi-Media, India) to transport to the food microbiology laboratory. The samples were incubated at 37°C for 24h and were then cultured on Sorbitol-MacConkey agar(Hi-Media, India) supplemented with cefexime 0.05mg/l and potassium tellurite 2.5mg/l. After incubating overnight at 37°C, both sorbitol fermenting and non-fermenting colonies were chosen separately for DNA extraction.

DNA extraction: Crude DNA extracts were prepared by using boiling method, in brief, bacterial colonies were suspended in 200µl of sterile distilled water, then microtubes were vortexed and the bacterial suspensions were lysed by boiling in a water bath at 100°C for 10 min. The lysates were centrifuged at 15000 rpm for 15 min. The supernatants were transferred to sterile nuclease free microtubes and frozen at -18 °C until used as templates for PCR assay.

PCR analysis: Extracted DNA was subjected to multiplex polymerase chain reaction

(m-PCR) for detection of eaeA and hlyA genes. Strains that were positive for eaeA and/or hlyA were further tested in another m-PCRassay for the presence of the genes encoding shiga toxins (Stx1 and Stx2). Amplifications were carried out in single-tubes, 25µl reaction mixture in an automated thermal cycler (TC-512, Techne, England). The PCR cocktail consisted of 3µl DNA template, 11.5µl of 2x Taq premix (consists of ParstousTaq DNA polymerase, reaction buffer, dNTPs mixture, protein stabilizer and 2x solution of loading dye) and 1.25µl of each primer(10 pm/µl, AccuPower PCR PreMix, Bioneer). Deionized distilled water was added to make a final volume of 25 µl. Details of the primers nucleotide sequences, the specific gene region amplified and the predicted size of PCR products are listed in Table1, according to Paton and Paton (Paton and Paton., 1998). Amplification reactions were performed in duplicate for 35 cycles. For all reactions the mixture was heated at 94°c for 5 min prior to thermocycling for initial denaturation. Each cycle consisted of 1 min for denaturation at 94°c, 1min of annealing at 54°c and 1 min of extension at 72°c. This was followed by final extension of 10 min at 72°c. Both duplex PCRs had the same temperatures and conditions (Hosseini et al., 2013). In addition, the *E.coli* O157:H7 reference strain (ATCC35150) was used as positive control for detection of virulence genes and sterile distilled water as negative control in all PCR runs.

The amplified products were visualized by standard gel electrophoresis using 3 µl of the final reaction mixture on 1.5% agarose gel in TAE buffer (89 mMTris, 89 mM glacial Acetic acid, 0.5 M EDTA) for 45 min at 100 V. A 100 bp DNA ladder molecular weight marker (Fermentas, UK.) was included in each electrophoretic run to allow identification of the amplified products. The agarose gel was stained with DNA Green Viewer and photographed using a gel documentation system (Stratagene EE 2, Germany).

Results

Among 78 isolates of *E. coli* isolated from chicken colibacillosis, only one strain (1.28%) harbored eaeA and hlyA genes simultaneously. This strain was screened for the presence of stx1 and stx2 genes by m-PCR and was found to be negative for these genes.

Discussion

Despite the fact that most avian pathogenic Escheichia coli (APEC) infections are extra intestinal, some APECs contain traits associated with the intestinal *E.coli* pathotypes, including enteropathogenic *E.coli* (EPEC), enterotoxigenic *E.coli* (ETEC), enteroinvasive *E.coli* (EIEC) and enterohemorragic *E.coli* (EHEC) (Barnes et al., 2008). However, information on *E.coli* isolates genotypes in poultry as a food source is of considerable importance in the food safety or public health's viewpoint. In this investigation the possible pathogenicity of APECs in producing human shiga toxin-mediated disease including HC and HUS was addressed by screening the important virulence associated genes.For the rapid and sensitive detection, PCR has proven to be of great diagnostic value (Mohammadi et al., 2013, Paton et al., 1996). Cultivation in liquid medium (BHI broth in this study) and then plating on CT-SMAC may increase the number of bacteria and therefore assist in the detection of STECs which are present in low numbers or in a physiologically stressed state (Mohammadi et al., 2013). It should be noted that STEC include both sorbitol fermenting and non-fermenting strains (Harrigan., 1998), therefore all colonies (sorbitol-positives and negatives) were picked from SMAC for DNA extraction.

Target virulence factors in the present study were as follow: 1-chromosomal eaeA gene (Gerrish et al., 2007), 2-plasmid hlyA gene (Dhanashree and Shrikar Mallya, 2008,

Table 1. Sequence of used primers and the size of amplified products.

Primer	Sequence(5´-3´)	Specificity	Amplicon size (bp)
stx1F	ATAAATCGCCATTCGTT GACTAC	nt 454–633 of A subunit coding region of stx1	180
stx1R	AGAACGCCCACTGAGATCATC		
stx2F	GGCACTGTCTGAAACTGCTCC	nt 603–857 of A subunit coding region of stx2	255
stx2R	TCGCCAGTTATCTGACATTCTG	(including stx2 variants)	
eaeAF	GACCCGGCACAAGCATAAGC	nt 27–410 of eaeA (this region is conserved be-	384
eaeAR	CCACCTGCAGCAACAAGAGG	tween EPEC and STEC)	
hlyAF	GCATCATCAAGCGTACGTTCC	nt 70–603 of EHEC hlyA	534
hlyAR	AATGAGCCAAGCTGGTTAAGCT		

Gerrish et al., 2007, Paton and Paton, 2002) 3-phage stx1 and stx2genes (Dhanashree and ShrikarMallya, 2008, Gerrish et al., 2007). Testing for eaeA and hlyA genes confirms the presence of the LEE pathogenicity island and the large virulence plasmid, respectively, and detection of either stx1 or stx2 genes confirms the presence of STECs (Boerlin et al., 1999; Paton and Paton., 2002). Upon transit through the stomach, STEC must adhere to the luminal surface of the large intestine in order to effectively colonize the host and compete with normal microbiota. The best-characterized adhesion and an absolutely essential virulence factor, is the ~94-kDa outer-membrane protein intimin, encoded by the eaeA gene (Vanaja et al., 2013) as many of the STEC strains that do not produce putative accessory virulence factors such as intimin and enterohemolysin have a low degree of virulence in humans (Paton and Paton., 1998). It should be noted that though a number of sporadic cases of HUS were caused by eaeA-negative STECs (Beutin et al., 1999, Mohammadi et al., 2013, Paton et al., 1999), harboring the hlyA gene is a significant contributor to shiga toxin-mediated disease, as there is increasing evidence of hemolysin gene being the marker for STECs (Dhanashree and ShrikarMallya., 2008, Schroeder et al., 2002). For this reason we have assayed both eaeA and hlyA in all isolates simultaneously. In the following, isolates harboring eaeA and/or hlyA were tested for the presence of other principal virulence factors for human infections, stx1 and stx2.

Results obtained in our study revealed none of the 78 *E.coli* strains can be regarded as important carriers of zoonotic *E.coli*. However, one (1.28%) eaeA and hlyA positive isolate was found which did not produce shiga toxins (stx1 or stx2). The absence of stx genes in this isolate could be due to the fact that stx gene is bacteriophage coded and the isolate would have lost the same during preservation (Dhanashree and Shrikar Mallya, 2008, Schroeder et al., 2002).

Our results are in agreement with the earlier findings of Wani et al. (2004) who reported none of 426 *E.coli* isolates from fecal samples originating from chickens and pigeons in India was positive for stx1 and stx2 and the percentage rate of eaeA and hlyA was 2.74% and 1.74%, respectively (Wani et al., 2004). Janben et al. (2001) also did not find any STEC virulence factors (stx1, stx2, eaeA and hlyA genes) in 80 *E.coli* strains of poultry internal organs having died from colibacillosis (Janben et al., 2001). Other research findings indicating prevalence level similar to our results are as follow: Krause et al. (2005) who have reported 2.3% eaeA positive while all their screened isolates were negative for stx gene (Krause et al., 2005); Mellata et al. (2001) in Algeria who observed none of the 50 studied intestinal avian *E.coli* strains harbored eaeAand hlyAgenes(Mellataet al., 2001) and the study of Schroeder et al. (2003) that also did not reveal any STEC in retail chicken and turkey obtained from Washington, DC, USA (Schroeder et al., 2003). In contrast, Kobayashi et al.

(2002) detected a high percentage (57%) of fecal samples of contaminated chicken flocks in Finland bearing eaeA gene, while they lacked stx and hlyA genes (Kobayashi et al., 2002).

Low prevalence rate of STEC virulence genes associated with human infections in this research corroborate the fact that most APECs isolated from poultry are specific clonal types that are pathogenic only for birds and represent a low risk of disease for people or other animals (Barnes et al., 2008; Cayaet al., 1999; Ron., 2006). APECs also are much less toxigenic than pathogenic *E.coli* in mammals and human beings (Barnes et al., 2008; Blanco et al., 1997; Janben et al., 2001; Mellataet al., 2001). Moreover, infection with STEC in chicken requires flagella but not intimin, the surface adhesion responsible for attachment of the organism to epithelial cells in mammals (Barnes et al., 2008; Best et al., 2005; La Ragioneet al., 2005). In summary, the data presented in this study show a low presence of infective STECs occurrence in colibacillosis cases of chicken flocks in the northeast of Iran. The results are in contrast with Tabatabaei et al. (2011) who have isolated STEC from 4% chicken fecal samples in Iran (Tabatabaei et al., 2011). Therefore, further investigations are required to evaluate the role of poultry as a putative vehicle of infective STEC to human. Furthermore, knowledge in distribution of the virulence-associated genes may be a useful instrument to design comprehensive epidemiological studies.

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جستجوی فاکتورهای حدت سویه‌های وروتوکسیژنیک اشریشیا کولای جدا شده از نیمچه‌های گوشتی شمال شرق ایران

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چکیده

زمینه مطالعه: شیوع بیماریهای غذا زاد ناشی از اشریشیا کولای تولید کننده شیگا توکسین در سراسر جهان کاملاً شناخته شده است. این توکسینها عوامل عمده گاسترو انتریتهای جوامع انسانی می‌باشند که گاها با کولیت خونریزی دهنده، سندرم اورمیک-همولیتیک و پورپورا ترمبو سایتو پنی پیچیده تر نیز می‌گردد. **هدف:** هدف این مطالعه بررسی حضورفاکتورهای حدت شامل eaeA، hlyA، stx۱ و stx۲در سویه‌های اشریشیا کولای جداشده از نیمچه‌های گوشتی مبتلا به کلی باسیلوزدر شمال شرق ایران بود. **روش‌کار:** تعداد ۷۸ سویه اشریشیا کولای از موارد هپاتیت و پریکاردیت نیمچه‌های گوشتی ارجائی به کلینیک آموزش دانشکده در طی سال‌های ۱۳۹۰-۱۳۹۳ جداسازی گردید. این سویه‌ها با استفاده از محیط‌های انتخابی کشت مجدد گردیده و یک کلنی تیپیک از هر نمونه به روش PCR چند گانه از نظر حضور ژن‌های حدت STEC (eaeA، hlyA، stx۱ و stx۲) مؤثر در بیماریهای ناشی از شیگا توکسین مورد بررسی قرار گرفت. **نتایج:** از تعداد ۷۸ سویه جداشده اشریشیا کولای، تنها یک ایزوله واجد ژن‌های eaeA و hlyA بود که فاقد ژن‌های stx۱ و stx۲ بود. **نتیجه گیری نهایی:** نتایج این بررسی نشان دهنده شیوع پایین ژن‌های حدت سویه‌های ورو توکسیژنیک در اشریشیا کولای جدا شده از نیمچه‌های گوشتی مبتلا به کلی باسیلوزیس می‌باشد.

واژه های کلیدی: کلی باسیلوز، اشریشیا کولای، شیگا توکسین

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