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Detection of the eaeA Gene in *Escherichia coli* Isolated from Broiler Chickens by Polymerase Chain Reaction

Short Communication

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ABSTRACT

The aim of this study was to isolate *Escherichia coli* from chickens and to determine the presence of the eaeA gene, a virulence factor detected in *Escherichia coli*, in the isolates by polymerase chain reaction (PCR). Different chicken organs (lung, liver and spleen) were inoculated onto blood agar and biochemical tests were performed on the suspicious isolates. *Escherichia coli* was isolated from 67.5% (81/120) of the samples. DNA was extracted from these isolates and was amplified by PCR, using a pair of primers derived from the eaeA (virulence) gene. In the agarose gel examination of PCR products, 48.1% (39/81) of the isolates were determined to have this virulence factor. Correct amplification with a molecular length of 384 bp was obtained in the analysis of all the isolates by species-specific PCR, which confirmed the results of the detection of the eaeA gene in *Escherichia coli*. The eaeA gene, which is mainly responsible for the virulence of *Escherichia coli*, is commonly present in *Escherichia coli* strains isolated from this region and the significance of this situation for animal and public health was discussed.

KEY WORDS broiler chicken, eaeA gene, *Escherichia coli*, PCR.

INTRODUCTION

Colibacillosis is one of the most important bacterial diseases in poultry which is caused by *Escherichia coli* (Zakeri and Kashefi, 2012). In poultry, pathogenic *E. coli* isolates usually produce extraintestinal infections such as respiratory tract infection, septicemia, swollen head syndrome or a 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 (Zahraei *et al.* 2007). It was showed that *E. coli* that caused disease in chickens are collectively known as avian pathogenic *E.a coli* (APEC) (Zakeri and Kashefi, 2012). The production of cytotoxins in *E. coli* isolates has been extensively studied among isolates from humans and several animal species (Zakeri and Kashefi, 2012). 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. Same studies 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 cells.

Interestingly, a distinct labile toxin (LT) which was found to be active on vero cells only was produced among 11% and 22.5% of turkeys and chickens, respectively. It 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 (Zahraei *et al.* 2007).

The possible role of this cytotoxin in the development of SHS (Swollen head syndrome) was not investigated. Some studies reported that 12.5% of the healthy pigeons whose feces were cultured had Shiga toxin-producing *E. coli* (STEC) that carried a variant stx2 gene. The eae and E-hlyA genes were not detected among stx-positive isolates when tested by polymerase chain reaction (PCR) (Zakeri and Kashefi, 2012).

The attaching and effacing (A/E) lesion that is characteristic of avian pathogenic (APEC) strains is reported to be associated with strains isolated from animals. These bacteria colonize the small intestine, where they attach tightly to the epithelial cells of the villus and cause typical A/E lesions. The genes encoding the proteins responsible for A/E lesions map to a chromosomal "pathogenicity island" termed the "locus of enterocyte effacement" (LEE). Intimin, an outer membrane protein, encoded by eaeA, is a bacterial adhesion molecule that mediates the intimate bacterium host cell interaction characteristic of A / E lesions. The objective this study was to detect the presence of the eaeA gene responsible for A/E lesions in chickens by PCR.

MATERIALS AND METHODS

In all, 120 *E. coli* strains were isolated from visceral organs of poultry (lung, liver and spleen) that died from colibacillosis. The samples were immediately transferred to the laboratory where they were processed.

Isolation and identification

Bacteria from diseased animals were isolated from necropsy specimens and cultured on 5% sheep blood and Mac Conkey agar. *E. coli* strains were stored in tryptone soy broth (oxoid, Hampshire, UK) with 15% glycerol at -70 ˚C. All of isolated and identified *E. coli* strains were positive for lactose adonitol, methyl-red, indol and were negative for H_2S and urease.

Amplification of eaeA gene sequences by PCR

The presence of the eaeA gene, which encodes intimin, was verified by PCR analysis. DNA samples for these analyses were obtained from suspicious cultures. A few colonies from the suspicious cultures were transferred into an eppendorf tube containing 300 µL of distilled water and the suspension was incubated at 56 ˚C for 30 min. The samples were treated with 300 µL of K buffer (20 m*M* Tris at pH 8.0), 150 m*M* NaCl, 10 m*M* EDTA and 0.2% SDS) and proteinase K (200 µL/mL). Following 30 min of boiling, the same amount of phenol (saturated with HCl) was added to the suspension. The suspension was shaken vigorously by hand for 5 min and then centrifuged at 11600 g for 10 min. The upper phase was carefully transferred into another eppendorf tube and sodium acetate (0.1 volumes) and ethanol (2.5 volumes) were added to the suspension, which was left overnight at -20 ˚C to precipitate the DNA. The pellet, obtained following the centrifugation at high speed for 10 min, was washed twice with 95% and 70% ethanol, respectively, each step followed by 5-min centrifugation. Finally, the pellet was dried and resuspended in 50 µL of distilled water.

PCR

PCR was performed with a touchdown thermocycler (Hybaid, Middlesex, UK) in a total reaction volume of 50 µL, containing 5 µL of 10X PCR buffer (10 m*M* Tris HCl at pH 9.0), 50 m*M* KCl, 0.1% Triton X-100, 5 µL of 25 m*M* $MgCl_2$, 250 m*M* deoxynucleotide triphosphate, 2 unit of Taq DNA polymerase (MBI, Fermentas), 1 µ*M* of each primer and 5 µL of template DNA. Amplification was obtained with 35 cycles following an initial denaturating step at 95 ˚C for 1 min. Each cycle involved denaturating at 95 ˚C for 1 min, annealing at 65 ˚C for 2 min, and synthesis at 72 ˚C for 1.5 min. The amplified products were visualized by ethidium bromide (0.5 µg/mL) staining after electrophoresis at 70 volts for 1 h in 1.5% agarose gels. PCR products with a molecular size of 384 bp were considered eaeA-positive *E. coli*.

Primers

The primers were chosen to flank the eaeA gene. The sequence of primer pairs used as follows (Zahraei *et al.* 2007):

eaeA 1, GAC CCG GCA CAA GCA TAA GC
eaeA 2, CCA CCT GCA GCA ACA AGA GG

RESULTS AND DISCUSSION

Culture findings

E. coli was isolated and identified by biochemical tests from 67.5% (81/120) of the samples collected from the organs of chickens. All *E. coli* isolates were catalase, indole and methyl-red positive, and oxidase, citrate, H_2S and VP negative.

PCR findings

PCR product with 384 bp was obtained in the analysis of all the isolates by species-specific PCR, which confirmed the results of the detection of the eaeA gene in *E. coli* from chickens by PCR. With PCR, the eaeA gene was determined to be present in 48.1% of the isolates (Figure 1).

E. coli is present in the normal microflora of the intestinal tract and environment of poultry, but certain strains designated as avian pathogenic *E. coli* (APEC) possess specific virulence factors and are able to cause avian colibacillosis. This disease is a serious problem for the poultry in-

dustry, since it causes great economic loss. The most severe manifestation of avian colibacillosis is septicemia, which is characterized by air sacculitis, pericarditis, perihepatitis and salpingitis (Gannon *et al.* 1993). Although there are a wide range of different virulence factors that may play a role in the pathogenesis of *E. coli*, we investigated the presence of only one virulence gene's encoding putative accessory virulence factor, intimin (encoded by eaeA). In this study, the presence of the eaeA gene, which encodes intimin, was verified by PCR analysis. Molecular tests have been designed for the detection of many virulence genes and are often the most sensitive methods for detecting them; however, using these techniques for the screening of more than one gene is labor intensive and costly. In one study, Debroy and Maddox (2001) claimed that the most commonly observed virulence factor in bovine isolates was the presence of eaeA genes, which occur in about 30% of the isolates. The same researchers reported a 13.2% isolation rate for eaeA from chickens. Isolation rates of eaeA genes from different *E. coli* strains have been reported in epidemiological studies from various locations worldwide. For instance, the eaeA gene was detected in 60% of strains by Bi *et al.* (1999). Another researcher detected the eaeA gene in 60.9% of *E. coli* strains in chickens in Kenya (Kariuki *et al.* 2002). In our study, the eaeA gene isolation rate was 48%. This is in agreement with the finding published by Kariuki *et al.* (2002), but different from what Debroy and Maddox (2001). The eaeA 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 (Zahraei *et al.* 2007).

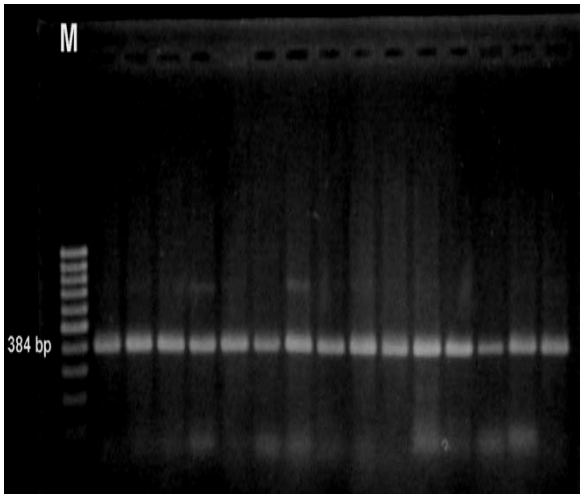

Figure 1 Agarose gel stained with ethidium bromide with polymerase chain reaction (PCR) products of *E. coli* isolates (M: 100 bp DNA ladder)

The carriage of eaeA gene sequence has been detected in *E. coli* isolates from avian sources in recent studies. It detected eaeA gene in 40% of gulls, in 7% of pigeons and in 57% of the broiler flocks, which were contaminated (Zahraei *et al.* 2007). 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 eaeA gene of *E. coli* in Finland. Our samples were taken from different chicken flocks and this may have contributed to the differences in the results. In addition, this study showed that the eaeA gene was widespread among the chicken population in Tabriz, Iran. Distinct eaeA specific primers have been described by Gannon *et al.* (1993), but in the present study the primer combinations eaeA 16S-F1 and eaeA 16S-R1 were very specific in amplifying a 384 bp fragment of the eaeA gene of *E. coli*. Pathogenic *E. coli* strains regularly cause disease in people exposed to contaminated food (Kariuki *et al.* 2002). *E. coli* infection is a common cause of diarrhea in infants in developing countries, and can manifest as haemorrhagic colitis and hemolytic uremic syndrome (O'Brien and Holmes, 1987). These diseases are a result of virulence factors.

CONCLUSION

The results of this experiment indicated that the eaeA gene, which is mainly responsible for the virulence of *E. coli*, is commonly present in *E. coli* strains isolated from this region and the significance of this situation for animal and public health was discussed.

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