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Research Article

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

Ayşe KILIÇ[1], Hasan Basri ERTAŞ[2], Adile MUZ[2], Gökben ÖZBEY[2], Hakan KALENDER[1]

[1]Veterinary Control and Research Institute, 23200, Elazığ - TURKEY

[2]Department of Microbiology, Faculty of Veterinary Medicine, Fırat University, 23119, Elazığ - TURKEY

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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 *E. coli*, in the isolates by polymerase chain reaction (PCR). Different chicken organs were inoculated onto blood agar and biochemical tests were performed on the suspicious isolates. *E. coli* was isolated from 48% (48/100) 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% (48/100) of the isolates were determined to have this virulence factor.

Key Words: *Escherichia coli,* PCR, chicken, *eaeA* gene

Tavuklardan İzole Edilen *Escherichia coli*'de *eaeA* Geninin Polimeraz Zincir Reaksiyonu ile Tespiti

Özet: Bu çalışmada tavuklardan *Escherichia coli* izolasyonu ve izolatlarda virulens faktörlerinden *eaeA* geninin Polimeraz Zincir Reaksiyonu ile saptanması amaçlandı. Tavukların iç organlarından kanlı agara ekim yapılarak şüpheli kolonilere uygulanan bazı biyokimyasal testler neticesinde numunelerin % 48 (48/100)'inde *E. coli* spp. izolasyonu yapıldı. İzolatlardan ekstrakte edilen DNA'lar *E. coli*'de tespit edilen *eaeA* geni (virulence geni)'nden türetilen bir çift primer kullanılarak PZR'de amplifikasyona tabii tutuldu. PZR ürünlerinin agaroz jelde değerlendirilmesi neticesinde % 48 (48/100)'inde bu virulens geninin mevcut olduğu belirlendi.

Anahtar Sözcükler: *Escherichia coli,* PCR, tavuk, *eaeA* geni

Introduction

Escherichia coli is a major component of the normal intestinal flora of animals. These bacteria belong to many different serotypes and can be isolated from the feces of both healthy and diseased animals. Whereas most of the bacteria are not pathogenic, some have acquired genes that can impart virulence. Pathogenic *E. coli* strains have been associated with gastroenteric diseases, such as diarrhea and hemorrhagic colitis. Pathogenic *E. coli* strains cause significant loss of neonatal animals. In the last few decades, understanding of the genetic basis and molecular mechanisms of the bacterial virulence of *E. coli* has shed light on the mechanisms of pathogenesis associated with this diverse group of bacteria (1,2).

E. coli strains have been identified by several methods, including animal models, tissue culture assays, immunoassays, and by using DNA probes that detect specific virulence factors (2). All these approaches require isolation and biochemical identification of single *E. coli* colonies, making detection of the virulence factors time consuming and expensive.

PCR is a powerful molecular biology technique that was introduced to facilitate the detection of these virulence factors (2); however, its direct application to fecal specimens is impeded by the presence of inhibitors in such crude materials (3). For this reason, most studies using this technique worked with isolated colonies and/or extraction and partial DNA purification (4-6).

*E-mail: aykilic23@hotmail.com

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 (7). 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, 100 *E. coli* strains were isolated from visceral organs of poultry that died from colibacillosis (lung, liver, and spleen), in order to determine the presence of virulence-associated genes at a local poultry abattoir in eastern Turkey. 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 MacConkey agar. *E. coli* strains were stored in tryptone soy broth (Oxoid, Hampshire, UK) with 15% glycerol at –70 °C.

Of these isolated and identified *E. coli* strains, 100% were positive for lactose adonitol, methyl-red, and indol, and 100% 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 ºC for 30 min. The samples were treated with 300 µl of K buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 10 mM EDTA, and 0.2% SDS) and Proteinase K (200 µg/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 11,600 ×g for 10 min. The upper phase was carefully transferred into another Eppendorf tube, and sodium acetate (0.1 volume) and ethanol (2.5 volume) were added to the suspension, which was left overnight at –20 ºC 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 mM Tris HCl (pH 9.0), 50 mM KCl, and 0.1% Triton X-100), 5 µl of 25 mM $MgCl_2$, 250 mM deoxynucleotide triphosphate, 2U of Taq DNA polymerase (MBI, Fermentas), 1 µM of each primer, and 5 µl of template DNA. Amplification was obtained with 35 cycles following an initial denaturating step at 95 ºC for 1 min. Each cycle involved denaturating at 95 ºC for 1 min, annealing at 65 ºC for 2 min, and synthesis at 72 ºC 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 were as follows:

eaeA 1: GAC CCG GCA CAA GCA TAA GC;

eaeA 2:CCA CCT GCA GCA ACA AGA GG (8).

Results

Culture findings

E. coli was isolated and identified by biochemical tests from 48% (48/100) 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

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

biochemical tests (Figure). With PCR, the *eaeA* gene was determined to be present in 48% of the isolates.

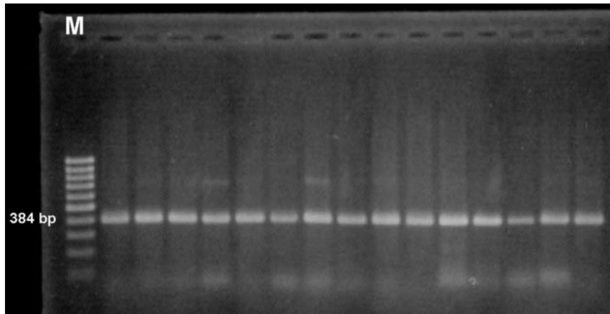

Figure. Agarose gel stained with ethidium bromide with polymerase chain reaction (PCR) products of *E. coli* isolates (M: 100-bp DNA ladder).

Discussion

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 industry, 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 (9).

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 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 (10). 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. (11). Another researcher detected the *eaeA* gene in 60.9% of *E. coli* strains in chickens in Kenya (12). In our study, the *eaeA* gene isolation rate was 48%. This is in agreement with the finding published by Kariuki et al. (12), but different from what Debroy and Maddox reported (10). 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 Elazığ, Turkey. Distinct *eaeA*-specific primers have been described by Gannon et al. (9), 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.* In this study, *E. coli* was identified in 48% of the chicken samples and 48% of the *E. coli* isolates were amplified by PCR.

Pathogenic *E. coli* strains regularly cause disease in people exposed to contaminated food (12). *E. coli* infection is a common cause of diarrhea in infants in developing countries, and can manifest as haemorrhagic colitis and hemolytic uremic syndrome (13). These diseases are a result of virulence factors.

We found 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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