Detection of hemolysin virulence factor gene of Enterohemorrhagic *Escherichia coli* (EHEC) isolated from feces of infected sheep by using Polymerase Chain Reaction Technique

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(Received 22 April 2014, Accepted 25 September 2014)

Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) are the most important recently emerged group of food-borne pathogens. It can cause severe gastrointestinal disease, including fatal infections, and is being detected more frequently worldwide. In this study the Polymerase chain reaction technique (PCR) were used for detection of hemolysin toxin gene (hlyA) as virulence factor producing by Enterohemorrhagic *Escherichia coli* (EHEC) isolated from diarrheic sheep. The PCR primers for hlyA gene were designed according to a program from NCBI-Genbank published sequence (Genbank code: X94129.1). Fifty fecal samples were collected from sheep suffering from diarrhea, (32) isolates (64%) of *Escherichia coli* were detected and diagnosed by routine laboratory tests. The results were shown only (13) positive isolates in PCR technique for detection of hemolysin toxin gene at (40.62%) which present as Enterohemorrhagic *Escherichia coli* out of (32) *E. coli*. In conclusion, hemolysin toxin gene (hlyA) is important virulence factor of Enterohemorrhagic *Escherichia coli* infected sheep and using PCR was appeared very sensitive and highly specific assay serve as suitable molecular diagnostic tool for detection Enterohemorrhagic *Escherichia coli* producing hemolysin toxin.
Key words: hemolysin, virulence factor gene, EHEC, diarrhea, PCR technique.

تشخيص جين عامل الفوعة الهيمولايسين لبكتيريا الايشيريشيا القولونية النزفية المعزولة من براز الاغنام المصابة باستخدام تقنية تفاعل سلسلة البلمرة

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الخلاصة

تعد بكتيريا الايشيريشيا القولونية النزفية المعوية (EHEC) في الوقت الحاضر من اهم المجاميع البكتيرية الممرضة التي تنقل بواسطة الاغذية، وتكون سببا لأمراض الجهاز الهضمي الحادة وقد تكون سببا للوفاة، وتم تشخيصها بكثرة في جميع انحاء العالم. في هذه الدراسة استخدمنا تقنية تفاعل سلسلة البلمرة للكشف عن وجود جين ذيفان الهيمولايسين (hlyA) كعامل ضراوة ينتج من قبل بكتيريا (EHEC) المعزولة من حالات اسهال من الاغنام. تم تصميم البادئات لجين (hlyA) حسب برنامج من قبل بنك الجينات NCBI (Genbank code: X94129.1). جمعت (50) عينة من اغنام تعاني من حالات اسهال ، وجدت (32) عزلة من بكتيريا الايشيريشيا القولونية (64%) وتم تشخيصها بطرق التشخيص الروتينية بالمختبر. اظهرت نتائج فحص تفاعل سلسلة البلمرة وجود (13) عزلة موجبة حاوية على جين ذيفان الهيمولايسين بنسبة (40.62%) من مجموع (32) عزلة من بكتيريا الايشيريشيا القولونية النزفية المعوية (EHEC). استنتجت الدراسة ان الجين المسؤول عن انتاج ذيفان الهيمولايسين هو عامل ضراوة مهم لبكتيريا الايشيريشيا القولونية النزفية المعوية (EHEC) التي تصيب الاغنام وان فحص تقنية تفاعل سلسلة البلمرة هو فحص حساس وذو خصوصية عالية لتشخيص بكتيريا الايشيريشيا المعوية (EHEC) الفارزة لذيفان الهيمولايسين.
الكلمات المفتاحية: جين الهيمولايسين ، الايشيريشيا القولونية النزفية ، الإسهال ، الاغنام ، تقنية تفاعل سلسلة البلمرة.

Introduction

Enterobacteriaceae family is the most major bacterial etiologic causes of diarrhea in sheep and goats especially in young ages because the normal flora have not been configured (1). *Escherichia coli* (EHEC) a member of Enterobacteriaceae comprise an important group of zoonotic enteric pathogens, in humans, some EHEC infections result in bloody or non-bloody diarrhea, which may be complicated by hemorrhage (2). The transmission of EHEC is by the fecal–oral route. Cattle and small ruminants are the major natural reservoir of these foodborne pathogens, they can be spread between animals by direct contact or via water troughs, shared feed, contaminated pastures or other environmental sources and mainly transmitted to humans by the consumption of contaminated food and water, or by contact with animals (3, 4). In different mammalian species the strains of *E.coli* which cause the extraintestinal disease often have the ability to lyse erythrocyte (5). The production of active extracellular α-hemolysin needs the products of the four linked genes hlyC, hlyA, hlyB, and hlyD. α-Hemolysin is formed as an inactive polypeptide and converted in its active form by the addition of a fatty acid group catalyzed by the hlyC protein. The production of α-hemolysin is signal peptide independent and mediated by a specific membrane translocator system encoded by hlyB and hlyD (6). Hemolysins are consider as an important virulence factors of bacteria causing extra intestinal diseases, tissue damage facilitating bacterial dissemination, releasing of host nutrients, and may also modulate host signaling pathways affecting several processes, including inflammatory responses, host cell survival, and cytoskeletal dynamics (7). Recently, traditional microbiological culturing techniques are being replaced by polymerase chain reaction (PCR) based techniques for the identification and detection of *E.coli* as it is less laborious and saves significant amount of time. PCR assays are proven specific and sensitive in detecting the major virulence genes of *E.coli*, therefore the purpose of this study was to detect pathogenic *E. coli* (EHEC) using PCR procedure by targeting the virulence factor hlyA gene.

Materials and methods

Sample collection: 50 fecal samples were collected from sheep suffered from diarrhea from different fields in Diwanyia province. The samples placed in sterile 25 ml container that transferred into microbiology laboratory, College of Veterinary Medicine for bacterial isolation.

Bacterial isolation: *Escherichia coli* was isolated from fecal samples by inoculation on Brain Heart Infusion Broth media at 37°C overnight for primary enrichment culture and then the bacterial growth were streaked on MacConkey agar and Eosin methylene blue agar overnight for selective isolation of pure culture *Escherichia coli* isolates. Biochemical identification tests (IMViC and TSI) were also used for more detection of *Escherichia coli* isolates.

Bacterial genomic DNA extraction: Bacterial genomic DNA was extracted from *E. coli* isolates by using (PrestoTM Mini gDNA Bacteria Kit. Geneaid. USA). 1ml of overnight bacterial growth on BHI broth was placed in 1.5ml microcentrifuge tubes and than transferred in centrifuge at 10000 rpm for 1 minute. After that, the supernatant was discarded and the bacterial cells pellets were used in genomic DNA extraction and the extraction was done according to company instruction. After that, the extracted gDNA was checked by Nanodrop spectrophotometer, then store in -20°C at deep freezer until perform PCR assay.

Polymerase chain reaction (PCR): PCR assay was performed by using specific primer for detection hemolysin toxin gene (hlyA). These primers were designed from NCBI-GenBank published sequence *E. coli* EHEC-hlyA gene (Genbank code: X94129.1) by using primer3 plus design online. The primers were used to amplify a 696bp fragment of highly conserved regions of hlyA gene in Enterohemorrhagic *Escherichia coli* (EHEC). hlyA-F primer (AGCGTACGTTCCGCTGGCAA) and hlyA-F primer (ACCCGCTGCAG

CTTTTGTTCCT) were provided by (Bioneer company. Korea). Then PCR master mix was prepared by using (AccuPower® PCR PreMix kit. Bioneer. Korea). The PCR premix tube contains freeze-dried pellet of (Taq DNA polymerase 1U, dNTPs 250µM, Tris-HCl (pH 9.0) 10mM, KCl 30mM, MgCl2 1.5mM, stabilizer, and tracking dye) and the PCR master mix reaction was prepared according to kit instructions in 20µl total volume by added 5µl of purified genomic DNA and 1.5µl of 10pmole of forward primer and 1.5µl of 10pmole of reverse primer, then complete the PCR premix tube by deionizer PCR water into 20µl and briefly mixed by Exispin vortex centrifuge (Bioneer. Korea). The reaction was performed in a thermocycler (Mygene Bioneer. Korea) by set up the following thermocycler conditions; initial denaturation temperature of 95 °C for 5 min; followed by 30 cycles at denaturation 95 °C for 30 s, annealing 60 °C for 30 s, and extension 72 °C for 1min and then final extension at 72 °C for 10 min. The PCR products were examined by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV transilluminator.

Results

From 50 fecal samples collected from sheep suffering from diarrhea, 32 isolates (64%) of *Escherichia coli* were detected and diagnosed in laboratory (table 1).

Table (1): Number and percentage of *E. coli* isolates from the fecal samples.

Fecal samples		Specimens no.	%
E. coli isolates	Positive	32	64
	Negative	18	36
Total		50	100

Table (2): Results of hemolysin toxin gene (hlyA) producing Enterohemorrhagic *Escherichia coli* (EHEC) isolates by PCR technique.

(hlyA) gene		*E. coli* isolates no.	%
PCR	Positive	13	40.625
	Negative	19	59.375
Total		32	100

PCR technique results were shown 13 positive samples of hemolysin toxin gene (hlyA) producing Enterohemorrhagic *Escherichia coli* (EHEC) out of 32 *E. coli* isolates at (40.62%) (table 2).

PCR assay results were dependent on the extracted bacterial genomic DNA by using (PrestoTM Mini gDNA Bacteria Kit. Geneaid. USA) this kit was used Spin column-based nucleic acid purification its solid phase extraction method to quickly purify nucleic acids. This method relies on the fact that nucleic acid will bind to the solid phase of silica under certain conditions. Therefore this kit was appeared rapid in 1 hours extraction and simple method for purification of *Escherichia coli* bacterial genomic DNA from overnight bacterial growth on enrichment BHI broth media at 37°C and the agarose gel electrophoresis was appeared, there were sharp DNA bands without nucleic acid lysis (fig. 1). PCR amplification of hlyA gene in positive samples was shown clear PCR product bands on agarose gel electrophoresis at 696bp PCR product (fig. 2).

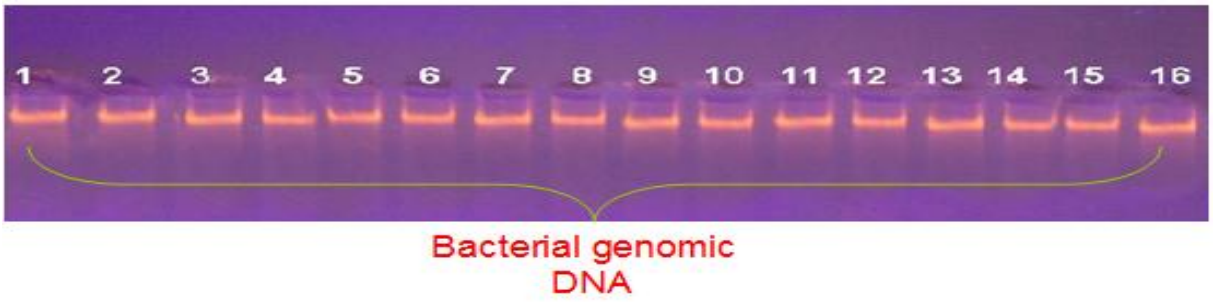

Fig. (1): Agarose gel electrophoresis of bacterial genomic DNA that show clear and sharp genomic DNA bands without DNA lysis in all *Escherichia coli* isolates samples.

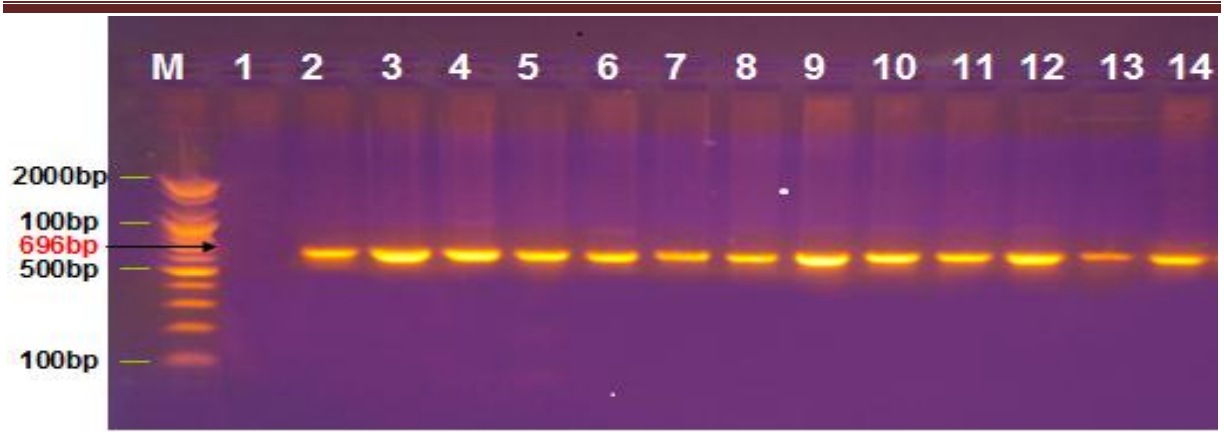

Fig. (2): Agarose gel electrophoresis of PCR assay show the positive results of hemolysin toxin hlyA gene. Where, Lane (M) DNA marker (100bp), lane (1) negative control sample, Lane (2-14) positive samples for hemolysin toxin hlyA at 696bp PCR product.

Discussion

In this study out of (50) fecal samples (32) isolates (64%) of *E.coli* are identified, these results reflect the importance role of *E.coli* as causative agent of diarrhea, also emphasize the role of sheep as major reservoir for pathogenic *E. coli.* Polymerase Chain Reaction is increasingly accepted to be the most sensitive means of determining whether indirect method (Pure culture *E. coli*) or a fecal specimen contains EHEC (8). The PCR assay also used by (9) who develop multiplex PCR assay for the rapid detection of virulence factors genes in Enterohemorrhagic *Escherichia coli* (EHEC) in fecal samples derived from healthy and clinically affected cattle, sheep, pigs, and goats. Haemolysin is considered to be the main factor responsible for cell detachment in vitro (10). *E. coli* produces several other haemolysins. Enterohaemolysin of enterohaemorrhagic *E. coli* (EHEC), designated Ehx or hly EHEC, is very similar to HlyA with regard to its genetic organization and calcium ion dependency although it is cell-bound (11). Even though a-haemolysin is often expressed among haemolytic *E. coli* isolates, it seems to be unusual among isolates of EHEC (12). PCR for detection of virulence factor producing by EHEC in sheep is very important finding to demonstrate predominate of *E. coli* serotype that mainly effect the sheep and causes severe diarrhea. Similar finding are demonstrated in the samples collected from healthy sheep and cattle. A study (13) described the presence of virulence factor genes in feces of cattle, sheep and pigs in Queensland-Australia which identified 19 of 105 (18%), 70 of 101 (69%), and 27 of 129 (21%) bovine, ovine, and porcine fecal samples respectively. While (14) found the virulence factors genes in 102 (49.8%) of the *E.coli* strains in feces of cattle in Brazil. In conclusion, PCR approach is advantageous in rapidly detecting hemolysin toxin gene (hlyA) is important virulence factor of Enterohemorrhagic *Escherichia coli* isolated from infected sheep. PCR is appeared sensitive and specific assay serve as suitable molecular diagnostic tool for detection pathogen.

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