Detection of *E. coli* isolated from cheese by using virulence factors by PCR technique in AL-diwaniyia city

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Abstract

The study was conduct to detection of Enterohemorrhagic *Escherichia coli* (EHEC) by important virulence factors genes such as hemolysin toxin genes (*hlyA* and *hlyB*) by using PCR technique from cattle milk product (cheese). *Escherichia coli* is an important bacterium that can cause food borne disease, the raw milk, milk products, and undercooked ground meat products are the primary sources of outbreaks. A total 50 chees sample was collected from local market in al-diwaniyia city the study was done in period between August and October 2014. The PCR primers for *hlyA and hlyB* gene were designed by this study from NCBI-Genbank published sequence. The PCR results was show (38) positive isolates out of 50 isolates at (76%), (23) isolates producing *hlyA* gene and (15) isolates producing *hlyB* gene whereas (9) of positive isolates were producing both hemolysin toxin genes. In conclusion, the PCR was appeared very sensitive and highly specific assay serve as suitable molecular diagnostic tool for detection Enterohemorrhagic *Escherichia coli* producing hemolysin toxins.
Key words: *Escherichia coli*, PCR, virulence factors, cheese.

تشخيص الايشيريشيا كولاي المعزولة من الجبن باستخدام جينات الضراوة بطريقة بلمرة الحامض النووي في محافظة الديوانية

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الخلاصة

هدفت الدراسة الى الكشف عن جرثومة الايشيريشيا كولاي التي هي من اهم أنوع البكتيريا التي يمكن أن تسبب الأمراض التي تنتقل عن طريق الأغذية ، كالحليب الخام ومنتجات الألبان ، ومنتجات اللحوم غير المطبوخة جيدا و هي تعد المصدر الرئيسي لتفشي المرض. تم استخدام تقنية تفاعل البلمرة سلسلة (PCR) في الكشف عن أهمية الضراوة لعوامل الجينات مثل الجينات (*hlyA*) (*hlyB*) المنتجة من الايشريشيا كولاي المعوية Enterohemorrhagic (EHEC) المعزولة من منتجات الألبان للماشية (الجبن) وقد صممت البرايمرات للجينات PCR لـ *hlyB hlyA* في هذه الدراسة من NCBI-بنك الجينات. تم جمع 50 عينة من الاجبان من الاسواق المحلية لمركز مدينة الديوانية. وكانت نتائج PCR (38) عزلة إيجابية وبنسبة (76٪) ، (23) عزلة أنتجت جين *hlyA* و(15) عزلة أنتجت جين *hlyB* في حين (9) عزلات انتجت كلا جيني الذيفان الحال للدم. نستنتج ان الجينات (*hlyA*) و (*hlyB*) للذيفان الحال للدم هي من عوامل الضراوة الهامة المنتجة بواسطة الإيشيريشيا القولونية Enterohemorrhagic في الإنسان وان استخدام PCR وقد أظهر حساسية عالية جدا ومحددة للغاية في الفحص باستخدام تشخيص الحامض النووي الجزيئي للكشف عن الايشيرشيا القولونية المنتجة للذيفانات الحالة للدم.
الكلمات المفتاحية: الابشيريشيا القولونية ، تقنية بلمرة الحامض النووي ، عوامل الضراوة ، الاجبان.

Introduction

Raw milk is usually colonized by a variety of many zoonotic pathogens such as *Campylobacter, enterohaemorragic Escherichia coli, Salmonella spp., Listeria monocytogenes, Staphylococcus aureus and Yersinia enterocolitica* therefore; they represent an important source of foodborne pathogens. These pathogens in milk have been linked to the environment in the farm, mixing clean milk with mastitis milk and

from livestock (1). Enterohemorrhagic *Escherichia coli* (EHEC) comprise an important group of zoonotic enteric pathogens. In humans, some EHEC infections causes in bloody or non-bloody diarrhea, this may be complicated by hemorrhagic (2). EHEC are transmitted by the fecal–oral route. They can be spread between animals by direct contact or via water troughs, shared feed, contaminated pastures or other environmental sources. Ruminants, particularly cattle and sheep, are the most important reservoir hosts for EHEC which is mainly transmitted to humans by the consumption of contaminated food and water, or by contact with animals, feces and contaminated soil (3). Person-to-person transmission can contribute to disease spread during outbreaks; however, humans do not appear to be maintenance hosts for this organism (4, 5). The production of active extracellular α-hemolysin requires the products of the four linked genes *hlyC, hlyA, hlyB,* and *hlyD.* α-Hemolysin is synthesized as an inactive polypeptide and converted in its active form by the addition of a fatty acid group catalyzed by the *HlyC* protein. The secretion of α-hemolysin is signal peptide independent and mediated by a specific membrane trans locator system encoded by *hlyB* and *hlyD* (6). hemolysin toxin gene (*hlyA*) and (*hlyB*) gene is important virulence factors producing by *Escherichia coli* infected human and production of α-hemolysin were described to be 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, 8). 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 factors *hlyA* and *hlyB* gene.

Materials and methods

Samples collection: Fifty (50) cattle cheese samples were collected from different markets in Diwanyia province. The samples placed in sterile container that transferred into microbiology laboratory, College of Veterinary Medicine and store in refrigerator until bacterial isolation.

Bacterial isolation: *Escherichia coli* was isolated from cheeses samples by inoculation on Brain Heart Infusion (BHI) broth media at 37°C overnight for primary enrichment culture and then the bacterial growth were inoculated on Eosin methylene blue agar and sheep blood agar at 37°C overnight for selective isolation of pure culture *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 micro centrifuge tubes and then transferred in centrifuge at 10000 rpm for 1 minute. The supernatant was discarded and the bacterial cells pellets were used in genomic DNA extraction and the extraction was done according to company instruction. The extracted gDNA was checked by Nano drop spectrophotometer, then store in -20C at refrigerator until perform PCR assay.

Polymerase chain reaction (PCR): PCR assay was performed by using specific primers for detection hemolysin toxin genes (*hlyA*) and *(hlyB).* These primers were designed from NCBI-Gen Bank published sequence *E. coli hlyA* and *hlyB gene* (Genbank code: AF037577.1, M10133.1) by using primer3 plus design online. *hlyA*-F primer and *hlyA*-R primer, hlyB-F primer and hlyB-R primer were provided by (Bioneer company. Korea) (Table 1). 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 1µl of 10pmole of forward primer and 1µl 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 thermo cycler (Mygene Bioneer. Korea) by set up the following thermo cycler conditions; initial denaturation temperature of 95 °C for 5 min; followed by 30 cycles at denaturation 95 °C for 30 s, annealing 58 °C for 30 s, and extension 72 °C for 1min and then final extension at 72 °C for 5 min. The PCR products were examined by electrophoresis in a 2% agarose gel, stained with ethidium bromide, and visualized under UV trans illuminator.

Table (1): Specific primers for detection of hemolysin toxin genes (*hlyA*) and *(hlyB)* by PCR technique.

Primer	Sequence	PCR product size
hlyA	(AGAATGGCACGGCGATTACT) F (CAGCACTGCCGCCTAATTTA) R	150 bp
hlyB	(CAGTTACCCGCCTTGGTGAT) F (CTTGCAATTGCGATGCGTTG) R	535 bp

Results

The results of isolation from cheese sample was show (38) positive isolates out of 50 of *Escherichia coli* isolates at (76%), (23) isolates producing *hlyA* gene and (15) isolates producing *hlyB* gene whereas, the results of isolate confirm by PCR technique PCR amplification of *hlyA* and *(hlyB*) genes in positive samples was shown clear PCR product bands when examined by electrophoresis in a 2% agarose gel, stained with ethidium bromide, and visualized under UV trans illuminator at 150 bp *hlyA* PCR product (Fig.1), and 535bp *hlyB* PCR product (Fig. 2).

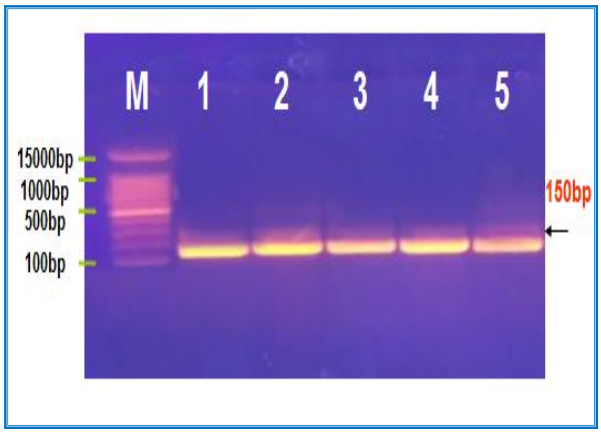

Fig. (1): Agarose gel electrophoresis of PCR assay show the positive results of hemolysin toxin *hlyA* gene in *Escherichia coli*. Where, Lane (M) DNA marker (100bp), Lane (1-5) some positive samples for hemolysin toxin *hlyA* at 150bp PCR product.

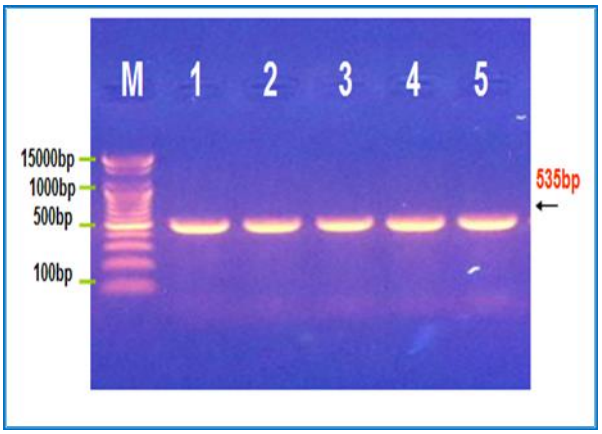

Fig. (2): Agarose gel electrophoresis of PCR assay show the positive results of hemolysin toxin *hlyB* gene in *Escherichia coli*. Where, Lane (M) DNA marker (100bp), Lane (1-5) some positive samples for hemolysin toxin *hlyB* at 535bp PCR product.

Discussion

The polymerase chain reaction PCR was appeared as sensitive and specific assay that used in detection of hemolysin toxin genes (*hlyA*) and (*hlyB*) producing Enterohemorrh-

agic *Escherichia coli* (EHEC) isolated from cattle cheese samples. Where, PCR assay results were shown (38) positive samples out of 50 samples at (76%). (23) isolates producing *hlyA* gene and (15) isolates producing *hlyB* gene whereas, (9) isolates that producing both hemolysin toxin genes. The ability to detect of hemolysin toxin gene (*hlyA*) *and* (*hlyB*) producing Enterohemorrhagic *E. coli* (EHEC) was increased many times when a validated PCR assay was used. PCR is increasingly accepted to be the most sensitive means of determining whether indirect method (Pure culture *E. coli*) or a fecal specimen contains EHEC (9). The multiplex PCR assay also used by (10) who develop multiplex PCR assay for the rapid detection of virulence factors genes in Enterohemorrhagic *E. coli* (EHEC) in fecal samples of 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 (11). *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 (12). Even though a-haemolysin is often expressed among haemolytic *E. coli* isolates, it seems to be unusual among isolates of EHEC (13). 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. (14)Similar finding were demonstrated in the samples collected from healthy sheep and cattle. A recent study (15,16) who described the presence of virulence factor genes in feces of cattle, (17) sheep and pigs in Queensland, Australia was identified 19 of 105 (18%), 70 of 101 (69%), and 27 of 129 (21%) bovine, ovine, and porcine fecal isolate respectively. In conclusion, a PCR approach is advantageous in rapidly detecting hemolysin toxin gene (*hlyA*) and (*hlyB*) are important virulence factor producing by Enterohemorrhagic *Escherichia coli* infected cattle and used multiplex PCR was appeared sensitive and specific assay serve as suitable molecular diagnostic tool for detection pathogen.

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