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RESEARCH ARTICLE

Detection of hemolysin genes producing Enterohemorrhagic Escherichia coli isolated from sheep by Multiplex PCR technique

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Abstract

Enterohemorrhagic Escherichia coli (EHEC) is a bacterium that can cause severe food borne disease. Primary sources of outbreaks are raw or undercooked ground meat products, raw milk and fecal contamination of vegetables. It can cause severe gastrointestinal disease, including fatal infections, and is being detected more frequently worldwide. In this study we used Multiplex Polymerase chain reaction technique (PCR) for detection hemolysin toxin genes (hlyA) and (hlyB) as virulence factors producing by Enterohemorrhagic Escherichia coli (EHEC) isolated from diarrheic sheep. The PCR primers for hlyA and hlyB gene were designed by this study from NCBI-Genbank published sequence (Genbank: AF037579.1, AM690761.1). The Multiplex PCR results was show (34) positive isolates out of 50 isolates at (68%), (20) isolates producing hlyA gene and (14) isolates producing hlyB gene whereas, (16) isolates not producing hemolysin toxin gene. In conclusion, hemolysin toxin gene (hlyA) and (hlyB) gene is important virulence factors producing by Enterohemorrhagic Escherichia coli infected sheep and used Multiplex PCR was appeared very sensitive and highly specific assay serve as suitable molecular diagnostic tool for detection Enterohemorrhagic Escherichia coli producing hemolysin toxins.

INTRODUCTION

Enterohemorrhagic Escherichia coli (EHEC) 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 hemorrhagic (1). 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 (2, 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 translocator system encoded by hlyB and held (6). 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 cytoskeleton 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 Multiplex PCR procedure by targeting the virulence factors hlyA and hlyB gene.

Materials and Methods

Sample collection: 50 fecal samples were collected from sheep infected with diarrhea from different field in Diwanyia province. The samples placed in sterile 25 ml container that transferred into microbiology Laboratory College of veterinary medicine and store in refrigerator until 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 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 (Presto TM Mini gDNA Bacteria Kit. Geneaid, USA). 1ml of overnight bacterial growth on BHI broth was placed in 1.5ml microcentrifuge tubes and then 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 -20C at refrigerator until perform PCR assay.

Multiplex Polymerase chain reaction (PCR): PCR assay was performed by using specific primers for detection hemolysin toxin genes (hlyA) and (hlyB). These primes were designed from NCBI-GenBank published sequence E. coli EHEC-hlyA and hlyB gene (Genbank code: AF037579.1, AM690761.1) 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 (GTATTCGGCACAGCAGAGAAA) and hlyA-R primer (TTAATGCTG GCAGCTGTGTC), hlyB-F primer (TTACTGGCGCTACCGATCTC) and hlyB-R primer (CGAATAACCGGTGCAACAAT) 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 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 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 55 °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 1.5% agarose gel, stained with ethidium bromide, and visualized under UV transilluminator.

Results and Discussion

The Multiplex Polymerase chain reaction PCR was appeared as sensitive and specific assay that used in detection of hemolysin toxin genes (hlyA) and producing (hlyB) Enterohemorrhagic Escherichia coli (EHEC) isolated from fecal samples of sheep. Where, PCR assay results were shown (34) positive samples out of 50 samples at (68%). Twenty isolates producing hlyA gene and fourteen isolates producing hlyB gene whereas, sixteen isolates not producing hemolysin toxin gene. PCR amplification of hlyA and (hlyB) genes in positive samples was shown clear PCR product bands on agarose gel electrophoresis at 323bp hlyA and 575bp hlyB PCR products (figure 1).

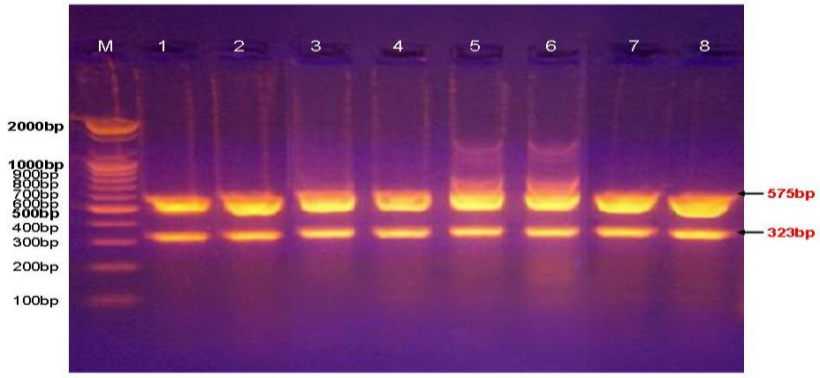

Figure (1): Agarose gel electrophoresis of PCR assay showing the positive results of hemolysin toxin hlyA and hlyB gene in Escherichia coli. Where, Lane (M) DNA marker (100bp), Lane (1-8) positive samples for hemolysin toxin hlyA at 323bp and hlyB 575bp PCR product.

The ability to detect of hemolysin toxin gene (hlyA) and (hlyB) producing Enterohemorrhagic Escherichia 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 Escherichia 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. Similar finding were demonstrated in the samples collected from healthy sheep and cattle. Previous study (14) who described the presence of virulence factor genes in feces of cattle, 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 samples, 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 sheep and used multiplex PCR was appeared sensitive and specific assay serve as suitable molecular diagnostic tool for detection pathogen.

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