academicJournals

Vol. 7(31), pp. 4001-4005, 2 August, 2013
DOI: 10.5897/AJMR2013.5748
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Development of an enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) to detect and genotype enterotoxigenic *Escherichia coli* of calf origin

Xiu-Yan LANG, Yang-Long ZHANG, Hui-Ting JIANG, Jiao LIU and Hong-Bo NI*

College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang Province 163319, P.R. China.

Accepted 3 June, 2013

We develop an enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) DNA fingerprinting technique for genotyping enterotoxigenic *Escherichia coli* (ETEC) in calves. Using the ERIC-PCR, we detected 66 ETEC strains in calves isolated from 11 farms. The ETEC strains could be grouped into 17 types, with type D predominant. The ERIC-PCR system is an efficient method for identification, typing and tracking of ETEC. Serotyping using a specific O antigen revealed 15 serotypes, with serotype O111 most prevalent (21.2%). We were unable to serotype 21.5% of the ETEC strains we had previously identified by ERIC-PCR. Our results indicate that some ETEC strains had identical genotypes, however their serotype might differ and vice versa. The ERIC-PCR method is rapid, sensitive, repeatable and reliable, and we believe it should be applied for the genotyping of ETEC in calves.

Key Words: Calf, enterotoxigenic *Escherichia coli*, ERIC-PCR, genotyping, serotyping.

INTRODUCTION

Colibacillosis in calves is caused by enterotoxigenic *Escherichia coli* (ETEC), and usually occurs in animals younger than 1-month-old. Serotyping is the traditional identification methods. Previous reports indicate that differences in serotype have an impact upon the protective effect of immunization. Additionally, the O antigen of some ETECs were found to strongly cross-react among different serotypes, as demonstrated by slide agglutination (Chowdhury et al., 2010). Because there are so many E. coli serotypes, it can be extremely difficult to specifically diagnose E. coli pathogens and prevent any resulting disease (Dudal et al., 2011). With the development of molecular biology techniques, We believe that Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) methods could resolve the genotypes of the large number of O antigen serotypes, thereby enabling identification of an appropriate immunogenic strain (Guay, 2009; Flores and Okhuysen, 2009; Indrawattana et al., 2011). These researchers determined the sequence distribution and copy numbers on chromosomes for ERICs and showed interspecies specificity. This technique can be used in strain typing of ETECs based on their unique numbers and sizes of pattern (Prabhu et al., 2010; Bialek et al., 2009). This would lay the foundation for further epidemiological and etiological investigations of ETEC. The aim of our study was to establish an ERIC-PCR method for genotyping of ETEC strains in calves.

*Corresponding author. E-mail: nihongbo@sina.com. Tel: +86-459-6819197. Fax: +86-459-6819191.

Table 1. Prevalence of E. coli isolated from calves.

Site of origin	Number of strains
BW	13
WD	9
FD	5
DQ	3
GN	6
HE	4
MD	2

MATERIALS AND METHODS

Bacterial strains and main reagent

A total of 66 fecal samples from calves positive for the presence of ETEC were collected from 11 farms in Heilongjiang, China (Table 1). Taq DNA polymerase was purchased from Promega Biotech (Madison, WI, USA). A 100 bp DNA ladder marker was purchased from MBI Biotech (Lansing, MI, USA). Nutrient broth, nutrient agar and MacConkey medium were all purchased from Hangzhou Microbial Chemical Reagent Company (Hangzhou, China). Agarose was purchased from Biowest (Shanghai, China).

Primer synthesis

ERIC-PCR specific primers (ERIC-1, 5'-ATG TAA GCT CCT GGG GAT TCA C-3'; ERIC-2, 5'-AAG TAA GTG ACT GGG GTG AGC G-3') based on a report by Versalovic et al. (1991) were synthesized by Harbin Boshi Biotechnology.

Serotyping

Serotyping of E. coli clinical samples was performed by slide agglutination with antiserum following conventional methods. After the biochemical identification of strains of *E. coli* densely streaked with inoculating loop on ordinary agar medium. Cultures were incubated at 37°C for 24 h, washed in 2 mL of 0.5% carbolic acid saline and centrifuged at 295 ×g for 5 min. Supernatants were harvested by centrifugation at 4,722 ×*g* for 10 min, and the bacterial cell pellet incubated at 121°C for 2 h to destroy the K antigen. Samples were stored at 4°C until required for determination of O antigen titer. Slide agglutination was conducted by mixing a small drop of antiserum on a glass slide and mixing with O antigen. The slide is tilted to mix and then observed after 3 to 5 min; however, a positive agglutination reaction can be seen within 30 s.

DNA preparation

Genomic DNA was extracted from E. coli clinical samples by the phenol-chloroform method (Mühling et al., 2008). The quality and purity of DNA samples were identified by agarose gel electrophoresis; DNA concentration was determined with a spectrophotometer.

ERIC-PCR

For the ERIC-PCR, each reaction comprised 2.5 µL of 10× buffer (Mg^{2+}-free), 2.5 µL of each primer, 2 µL of Mg^{2+}, 0.5 µL of Taq DNA polymerase, 150 ng/µL template, 10 µL of double-distilled H_2O. The thermal cycling protocol we used involved an initial denaturation step at 95°C for 5 min, followed by 30 cycles at 94°C for 1 min, 51°C for 5 min, 68°C for 8 min, and a final extension step at 65°C for 16 min after the 30th cycle. Amplicons were subjected to 1.5% (w/v) agarose gel electrophoresis and visualized using ethidium bromide staining and a UV gel imager.

Clinical samples tested and data analysis

We genotyped 66 E. coli isolated samples from calves using our developed ERIC-PCR method. We analyzed the banding patterns for each isolate following electrophoresis. Bands at the same position among all isolates were recorded as 1; any other bands were labeled 0. All data were input into the NTSYS-pc 2.10 software suite, and analyzed by the unweighted pair group method with arithmetic mean (UPGMA) to produce a dendrogram.

RESULTS AND DISCUSSION

The ERIC-PCR generates distinct amplification products that can be differentiated by agarose gel electrophoresis. Amplicons ranged between 100 and 3,000 bp, with 2 to 10 bands produced (Figure 1). ERIC-PCR genotyping method results showed that the size, number, characteristic bands compared to other research with the same DNA fingerprint. Cluster analysis of ETEC strains showed they should be classified into five major groups and had more than 75% similarity. We identified some genetically distant strains with a large number of differences at the gene level.

The 66 strains of ETEC genotyped were grouped into 17 different serotypes. The major genotype was type D, accounting for 21.2% (14/66) of samples and eight ETEC serotypes. The next most prevalent genotypes were types F and L (13.6% each type; 9/66), accounting for eight and five serotypes, respectively. The type I genotype accounted for 10.6% (7/66) of samples and three serotypes. The type C genotype was prevalent in 9.1% of samples (6/66) and was made up of five serotypes. The remaining were prevalent at 1.5 to 6.1% for the remaining samples. In our study, all strains were grouped into 15 serotypes, with serotype O111 the most prevalent (21.2%). However, 21.5% of all samples could not be ascribed a serotype.

Although serotyping is the most widely used method in epidemiological studies, it may not be the most appropriate for ETEC strain typing. The serotyping method is simple, with intuitive features, but it has the disadvantages that it can only identify limited phenotypic characteristics, is susceptible to external environmental factors, and is subjective (Shringi et al., 2012). In recent years, many molecular biological techniques, such as ERIC-PCR, pulsed field gel electrophoresis, amplified fragment length polymorphism, restriction fragment length polymorphism, random primers amplified polymorphic DNA typing (Wijetunge et al., 2012), and repetitive

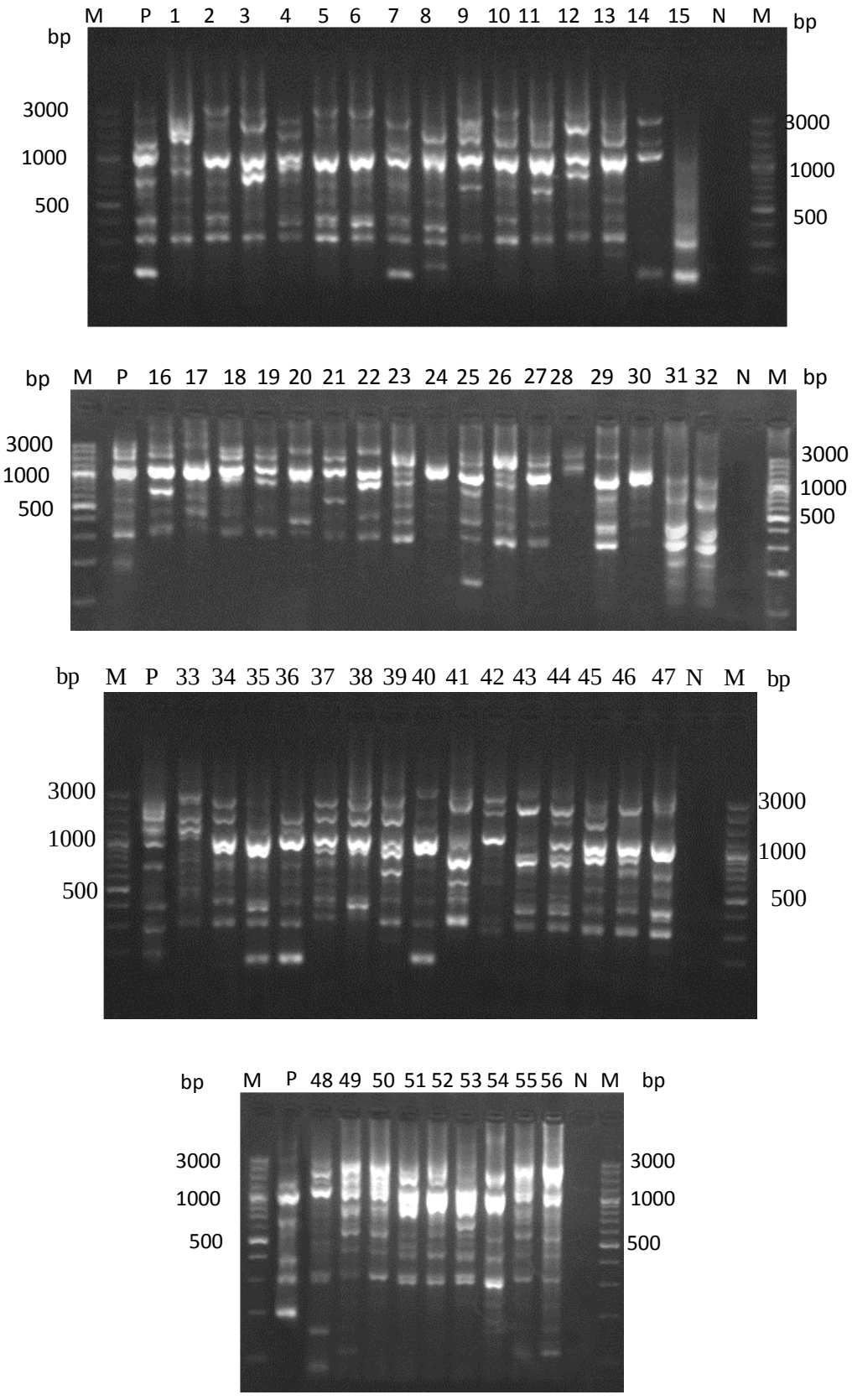

Figure 1. The Electrophoresis result by the ERIC-PCR reaction system. M, 100 bp DNA ladder marker; 1-66, ERIC-PCR amplification results; P, positive controls; N, negative controls.

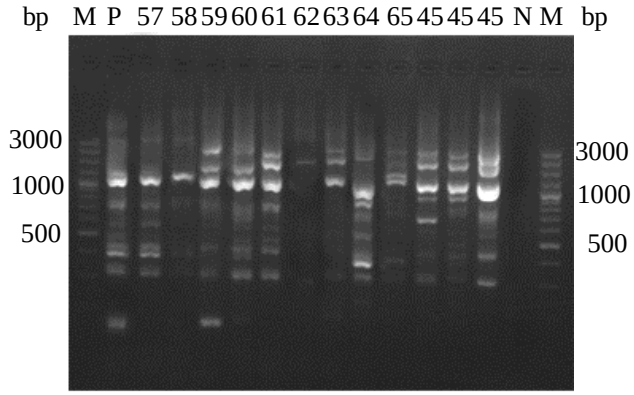

Figure 1. Contd

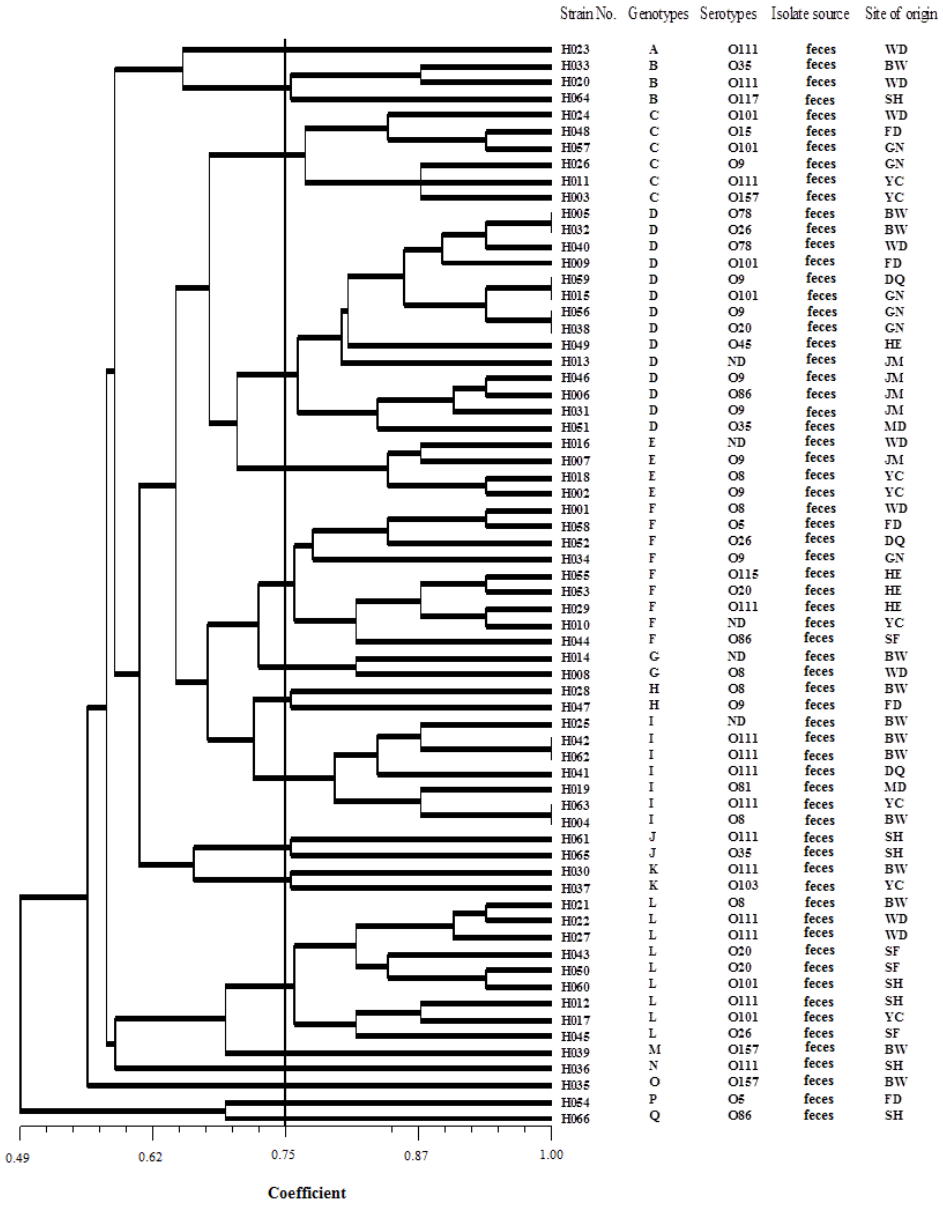

Figure 2. Dendrogram of E.*coli* fingerprint by cluster analysis.

bacterial DNA elements PCR (Pérez-Moreno et al., 2011) have been developed for genotyping. These methods allow for the analysis of microbial genomic DNA and determination of their evolution; this in turn can lead to their molecular classification and identification. These molecular biological technologies overcome the limitations of traditional methods. The ERIC-PCR method is rapid, sensitive, repeatable and reliable; therefore, we believe it could be broadly applied in the molecular classification of microbes especially ETEC strains (Guimarãesa et al., 2011). ERIC-PCR genotyping method and serotyping method were used in this study, and the results showed that different genotypes of isolates were with the same serotypes, and with no serotyping isolates also can genotyping. Our results show that the five strains that were not serotyped were grouped among five different genotypes, indicating that epidemic ETEC strains belong to a variety of genotypes. Comparison of serotyping method, ERIC-PCR genotyping method is a fast and reliable method.

The ERIC-PCR method is currently being used for molecular epidemiological analyses of human intestinal flora, Salmonella, Listeria, *Haemophilus parasuis* and *Vibrio cholerae* (Ahmed et al., 2012). Molecular epidemiological analysis leads to the discovery of new strains and results in a better understanding of known strains. It plays an extremely important role in the investigation of disease epidemiology, disease prevention and outbreak control. We use the ERIC-PCR typing technique to analyze the source of pathogenic ETEC in cattle. ERIC-PCR clustering analysis could be seen on the genetic relationship of dendrogram from different isolates. The GN and JM cattle farm were detected D-type, D-type was the most predominant flora, follow by F-type detected in the HE cattle farm, and SF cattle farm detected belonging to L-type. The numbers of genotypes were from two types to nine types of isolates from the other cattle farm, suggesting that there was considerable genotypic variability of ETEC. The ERIC-PCR can help determine the source of outbreaks by tracing the source of infection and possible transmission route(s), clearing a strain that might be associated with clinical manifest-tations or disease, and enhance understanding infectious disease epidemiology. Therefore, ERIC-PCR method may provide important reference for ETEC of calves in Heilongjiang province of China in epidemiological investigation and preliminary etiological analysis.

ACKNOWLEDGEMENTS

Our work was financially supported by grants from the Technological Innovation Team Building Program of the University of Heilongjiang Province (No. 2010td05), the Doctoral Initiating Project of Heilongjiang Bayi Agricultural University (No. 2031011018), and the Education Department of Heilongjiang Province (1251G044).

REFERENCES

Ahmed MU, Dunn L, Ivanova EP (2012). Evaluation of current molecular approaches for genotyping of Campylobacter jejuni strains. Foodborne Pathog. Dis. 9(5):375-385.

Bialek WA, Wojtasik A, Majchrzak M, Sosnowski M, Parniewski P (2009). $(CGG)_4$-based PCR as a novel tool for discrimination of uropathogenic *Escherichia coli* strains: comparison with enterobacterial repetitive intergenic consensus-PCR. J. Clin. Microbiol. 47(12):3937-3944.

Chowdhury F, Begum YA, Alam MM, Khan AI, Ahmed T, Bhuiyan MS, Harris JB, LaRocque RC, Faruque ASG, Endtz H, Ryan ET, Cravioto A, Svennerholm AM, Calderwood SB, Qadri F (2010). Concomitant Enterotoxigenic *Escherichia coli* Infection Induces Increased Immune Responses to *Vibrio cholerae* O1 antigens in tatients with cholera in Bangladesh. J. Infect. Immun. 78(5):2117-2124.

Dudal KA, Lindner B, Brade H, Leimbach A, Brzuszkiewicz E, Dobrindt U, Holst O (2011). The lipopolysaccharide of the mastitis isolate *Escherichia coli* strain 1303 comprises a novel O-antigen and the rare K-12 core type. Microbiology 157(6):1750-1760.

Flores J, Okhuysen PC (2009). Enteroaggregative *Escherichia coli* infection. J. Curr. Opin. Gastroenterol. 25(1):8-11.

Guay DRP (2009). Cranberry and Urinary Tract Infections. J. Drugs 69(7):775-807.

Guimarãesa AS, Dornelesa EMS, Andrade GI, Lage AP, Miyoshib A, Azevedob V, Gouveia AMG, Heinemann MB (2011). Molecular characterization of Corynebacterium pseudotuberculosis isolates using ERIC-PCR. Vet. Microbiol. 153(3):299-306.

Indrawattana N, Nibaddhasobon T, Sookrung N, Chongsanguan M, Tungtrongchitr A, Makino S, Tungyong W, Chaicumpa W (2011). Prevalence of Listeria monocytogenes in Raw Meats Marketed in Bangkok and Characterization of the Isolates by Phenotypic and Molecular Methods. J. Health Popul. Nutr. 29(1):26-38.

Mühling M, Woolven-Allen J, Murrell JC, Joint I (2008). Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. ISME J. 2(4):379-392.

Pérez-Moreno MO, Centelles-Serrano MJ, Cortell-Ortolá M, Fort-Gallifa I, Ruiz J, Llovet-Lombarte MI, Jardí-Baiges AM (2011). Molecular epidemiology and resistance mechanisms involved in reduced susceptibility to amoxicillin/clavulanic acid in Klebsiella pneumoniae isolates from a chronic care centre. Int. J. Antimicrob. Agents 37(5):462-466.

Prabhu V, Isloor S, Balu M, Suryanarayana VVS, Rathnamma D (2010). Genotyping by ERIC-PCR of *Escherichia coli* isolated from bovine mastitis cases. Indian J. Biotechnol. 9(3):298-301.

Shringi S, Garcíab A, Lahmersa KK, Potter KA, Muthupalani S, Swennes AG, Bessera TE (2012). Differential Virulence of Clinical and bovine-biased Enterohemorrhagic *Escherichia coli* O157:H7 Genotypes in Piglet and Dutch belted rabbit models. Infect. Immun. 80(1):369-380.

Wijetunge DS, Dunn P, Wallner-Pendleton E, Lintner, V, Lu H, Kariyawasam S (2012). Fingerprinting of poultry isolates of Enterococcus cecorum using three molecular typing methods. J. Vet. Diagn. Invest. 24(6):1166-1171.