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Prevalence of ETT2-Harboring *Escherichia coli* Infection in Piglets with Edema and/or Diarrhea

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To investigate into the prevalence of ETT2-harboring *Escherichia coli* (ETT2$^+$ *E. coli*) infection in piglets with edema and/or diarrhea disease, a total of 90 intestinal mucosa samples were collected from 40 cases of porcine postweaning diarrhea, 32 cases of edema disease and 18 cases of diarrhea concurrent edema disease from the Jiangsu Province, China. Following first cultivating in LB broth at 37 °C for 6 h, all the samples were detected by the PCR methods, and the data show that 13 cases (32.50%) of porcine postweaning diarrhea were infected with ETT2^ÿ *E. coli*, while 35 cases (92.11%) and 11 cases (91.67%) were positive in edema disease and diarrhea concurrent edema disease respectively.

Key words: ETT2, *Escherichia coli*, Prevalence, Piglet.

Porcine postweaning diarrhea (PWD) is commonly associated with enterotoxigenic *Escherichia coli* (ETEC), while pig edema disease (ED) is more frequently associated with shigatoxin-producing *E. coli* (STEC) (Nagy *et al*., 1999; Chen *et al*., 2004). The predominant virulence factors of ETEC and STEC are toxin(s) and adhesion(s), e.g. ETEC strains were characterised by heat-labile enterotoxin (LT), heat-sensitive enterotoxin (ST) as well as adhesive fimbriae F4, F5, F6, F41 and F18 (Bertschinger *et al*., 1990; Choi *et al*., 2001; Kennan *et al*., 1995; Moon *et al*., 1986; Salajka *et al*.,1992), while STEC strains were characterised by shigatoxin subtype 2e (stx2e) and fimbria F18 (Fekete *et al.*, 2002; Frydendahl, 2002; Imberechts *et al*., 1992; Pollard *et al*., 1990; Rippinger *et al*., 1995).

Furthermore, pathogenic *E. coli* of animal origin always contain several mobile genetic elements such as plasmids, bacteriophages or pathogenicity islands (PAI) encoding a network of various virulence traits (Oelschlaeger *et al*., 2004). The locus of enterocyte effacement (LEE) and high-pathogenicity island (HPI) have been identified among ETEC and STEC strains isolated from piglets (Cheng *et al*., 2005 and 2006). It is also worth notice that the *E. coli* type three secretion system 2 (ETT2) island, approximately 29.9 kb in size and localised adjacent to the tRNA locus *glyU*, was discovered through the analysis of genome sequences of enterohemorrhagic *E. coli* (Perna *et al*., 2001; Hayashi *et al*., 2001). There are at least 35 genes coding by ETT2 locus, including *epr*, *epa*, and *eiv* (Fig. 1), which were similar to the

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sequence of several Salmonella pathogenicity islands (Spi-1, Spi-2, Spi-3, etc.) (Blanc-Potard *et al*., 1999; Hansen-Wester *et al*., 2001).

Since ETT2 has been designated as pathogenicity island, it is necessary to investigate the prevalence of ETT2^{ÿ} *E. coli* infection in piglets with edema and/or diarrhea. In this study, PCR based on *ECs3703* and *ECs3737* genes were developed to detect the presence of ETT2^{ÿ} *E. coli* in 90 intestinal mucosa samples were collected from porcine postweaning diarrhea and edema disease cases

MATERIAL AND METHODS

Primer design

Two sets primers (Table 1) used for PCR amplification were designed and analyzed with DNAStar software based on the published *ECs3703* and *ECs3737* genes of ETT2 island deposited in GenBank, and synthesized by Sangon Biological Engineering Technology and Service Co. Ltd. (Shanghai, P. R. China). The primers that target the *ECs3703* gene were mixed together (named *ECs3703* primers set, each 50mmol/l) and the primers that target the *ECs3737* gene were also mixed together (named *ECs3737* primers set, each 50mmol/l)

Collection of samples

From Mar. to May., 2010, a total of 90 intestinal mucosa samples were collected from piglets with a mean age of 37.6±3.6-day old, including 40 cases of porcine postweaning diarrhea, 32 cases of edema disease and 18 cases of diarrhea concurrent edema disease from 45 large-scale and individual pig farms in the Jiangsu Province, China. Bring each test sample to a final volume of 500 µl with LB broth (10g tryptone, 10g NaCl, 5g yeast extract, H_2O to 1L, pH 7.4) and store at -70 °C.

Bacterial pre-culturing and extraction of DNA templates

Transfer 100 µl dilution of each intestinal mucosa sample into separate tubes containing 2 ml of LB broth and grow the liquid cultures with vigorous agitation at 37°C for 6 h. Take 0.5 ml of the liquid cultures to labeled microcentrifuge tubes, and centrifuge at 12 000 g for 5 min. Discard the supernatants and resuspend each pellet in 200 µl of distilled water. After boiling for 10 min, the suspensions were chilled on ice for 5 min, and then centrifuged at 12 000 g at 4°C for an additional 5 min. Transfer each supernatant to fresh tube and store at 4°C.

PCR detection of ETT2-harbouring *E. coli*

To detect ETT2^{ÿ} *E. coli* in the liquid cultures of intestinal mucosa samples, PCR assays were performed in microcentrifuge tubes for the Applied Biosystems 2720 Thermal Cycler (America). All the reagents were purchased from *Takara* Biotechnology (Dalian) Co., Ltd. The PCR mixture contained 5 µl of 10× PCR buffer (Mg^{2+} Free), 5 µl of $MgCl_2$ (25mmol/L), 5 IU of *Taq* polymerase, 4 µl of dNTP mixture (each 2.5 mmol/l), 1 µl of *ECs3703* or *ECs3737* primers set primers set, 2 µl of DNA template, and deionized water to a final volume of 50 µl. After denaturation at 94 °C for 3 min, 30 cycles of the PCR was performed using the following program: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s (10 min for the final cycle). PCR products were separated by size by 1% agarose gel electrophoresis along with DL2000 DNA markers and visualized after staining with ethidium bromide on a UV transilluminator.

Before the 90 clinic samples were submitted to PCR detection, four ETT2^{ÿ} bacterial strains (S443025, S451524, S452021 and S462234) and one ETT *E. coli* (TG1) were tested as positive and negative controls respectively. Followed by the PCR products of *ECs3703* and *ECs3737* genes from ETT^{+} *E. coli* strain S443025 were cloned into pGEM®-T vector (Promega) and transformed into *E. coli* DH5α, the fragments of *ECs3703* and *ECs3737* genes in the recombinant plasmids were sequenced and compared with the published sequences.

RESULTS

Specificity of the PCR for identification of ETT2 locus

To confirm their specificities, the PCR methods were performed first using the DNA templates from the reference *E. coli* strains. After PCR was performed to detect the *ECs3703* gene, the specific PCR products were revealed by agarose gel electrophoresis in the all four ETT2^{ÿ}bacterial strains respectively (Fig.2), while no specific band was detected in the ETT^{+} *E. coli*.

When PCR was performed to detect *ECs3737* gene, the expected band were detected in all four ETT2^{ÿ} *E. coli*, and no expected band was detected in the ETT^{+} *E. coli*. (Fig.3). To further evaluate the efficacy of the PCR methods, the DNA fragments of *ECs3703* and *ECs3737* genes of strain S443025 were sequenced and compared with the published sequences, and the data showed that the PCR region of *ECs3703* gene was 581 bp and by 99.5%, and the DNA fragment of *ECs3737* gene was 442 bp and by 98.6% identical to the reference sequence (NC_002695) respectively.

Table 1. The PCR primers used in this study

primer	Sequence	Position (gi accession number)	Size of product (bp)
ECs3703F	ATTGCCAAATAATGCCAGAAGAGTCACC	3709989-3710016 (NC_002695)	581
R	TCAATGTTGGACCGAATGTGAACGAATA	3710542-3710569 (NC_002695)	
ECs3737 F	TACTAATGCCATATAGCCCCATAA	3736673-3736696 (NC_002695)	442
R	CTACGCTTTTAACAAACGATTGAT	3737091-3737114 (NC_002695)	

Detection of ETT2^{+} *E.coli* from diseased animals

All the samples were detected by the PCR methods as described above, and the data shown that the ETT2^{+} *E.coli* infection rates of the edema disease and diarrhea concurrent edema disease cases were higher than that of the porcine postweaning diarrhea cases. Among the 90 cases, 13 of the 40 samples (32.50%) of porcine postweaning diarrhea (from 20 farms) were infected with ETT2-harboring *E. coli*, while 35 of the 38 (92.11%) edema disease cases (from 19 farms) and 11 of the 12 (91.67%) diarrhea concurrent edema disease cases (from 6 farms) were ETT2^{+} *E.coli*-positive (Fig.4).

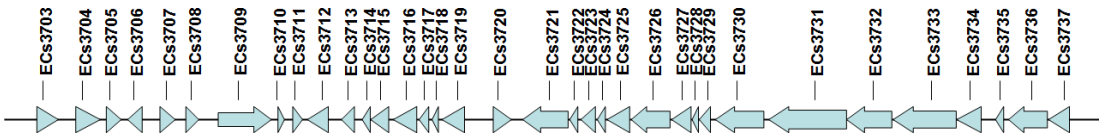
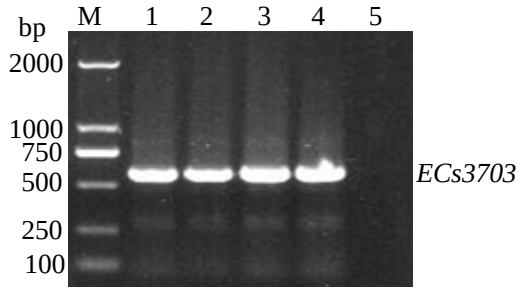
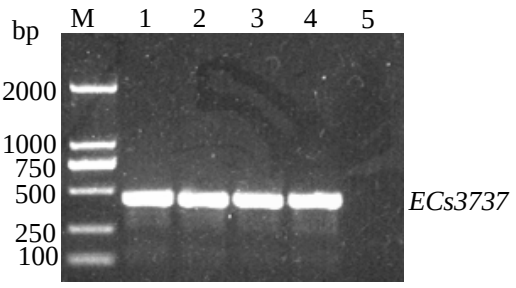


Fig. 1. Genomic position of genes of the pathogenicity island ETT2 derived from the genome sequence of *E. coli* O157:H7 str. Sakai (GenBank accession no. NC 002695)



M. DNA Marker DL2000; 1. S443025; 2. S451524; 3. S452021; 4. S462234; 5.negative control

Fig. 2. Specificity of PCR to detect *ECs3703* gene



M. DNA Marker DL2000; 1. S443025; 2. S451524; 3. S452021; 4. S462234; 5.negative control

Fig. 3. Specificity of PCR to detect *ECs3737* gene

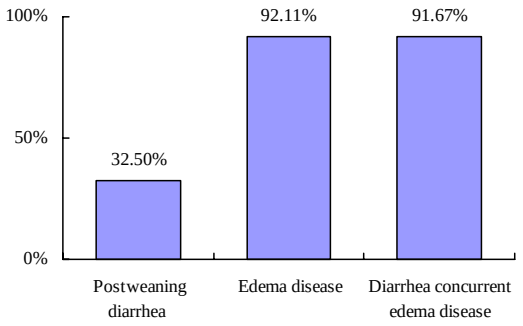


Fig. 4. Prevalence of ETT2-harboring *E. coli* in piglets

DISCUSSIONS

It is well known that piglet pathogenic *E. coli* are the etiological agents of diarrhea as well as edema disease, and virulence factors of pathogenic bacteria may be encoded by particular regions of the prokaryotic genome termed pathogenicity islands. Pathogenicity islands were first described in human pathogens of the species *E. coli*, but have recently been found in the genomes of various pathogens of humans, animals, and plants (Hacker *et al*., 2000).

ETT2 is a second cryptic type III secretion system in *E. coli* which was first discovered through the analysis of genome sequences of enterohemorrhagic *E. coli* O157:H7. ETT2-associated genes, including regulators and chaperones, were found at the same chromosomal location in the majority of genome-sequenced strains, including the laboratory strain K-12 (Hayashi *et al*., 2001). To give an insight into the current profile of pathogenic *E. coli* and a true reflection of the problem herds, it is necessary to investigate into the prevalence of the ETT2$^+$ *E.coli* in diseased piglets.

The ETT2 gene cluster was found to be present in whole or in part in the *E. coli* strains (Prager *et al*., 2004), but *ECs3703* and *ECs3737* genes were existed in ETT2 island whether the entire type or deletion variants, which were based on the analysis of all the published ETT2 genes deposited in GenBank. Therefore, PCR based on *ECs3703* and *ECs3737* genes were developed to detect the presence of ETT2$^+$ *E. coli* in this research. The tests by using reference bacterial strains revealed the efficacy of the developed PCR methods, and the sequencing of the PCR products of *ECs3703* and *ECs3737* genes confirmed the specificity for detecting ETT2$^+$ *E. coli*.

In this research, the data revealed that 32.50% diarrheic piglets were infected with ETT2$^{\ddot{y}}$ *E. coli*, while 92.11% and 91.67% were positive in edema disease and diarrhea concurrent edema disease cases respectively. One of the most worthy of attention is the ETT2$^+$ *E.coli* infection rates of the edema disease and diarrhea concurrent edema disease cases were higher than that of the porcine postweaning diarrhea cases. Therefore, we are suspicious of whether ETT2 could contribute to the virulence of *E. coli* isolates that causing edema disease or diarrhea. The function of ETT2 remains a mystery, as do the niche and signals that might trigger its expression and the identity and nature of its effectors. This attractive suspicion has to be revealed by comparing the virulence of the parental strain and that of the isogenic mutants in a suitable infection mode.

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