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*Full Length Research Paper*

# Rapid diagnosis of ETEC and HPI-harboring *Escherichia coli* infection in newborn piglets with diarrhea

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**Diarrhea is a common and important disease in industrial pig farms and the pathogenic *Escherichia coli* infection is the main cause of morbidity and mortality in newborn piglets. The preliminary diagnoses on this disease are mainly depending on clinical symptom and detailed body dissection. To further shorten the diagnosis time and increase the determination efficiency for newborn piglet diarrhea caused by *E. coli*, a rapid method was established based on the fast bacterial culturing followed by the PCR examining for the virulence factor genes, such as enterotoxin ST1, ST2, LT1 and high pathogenicity island (HPI). A total of 151 rectal swab samples were obtained from live diarrheic piglets from Jiangsu province, China. Following the first cultivation in LB broth at 37°C for 6 h, all the samples were detected by the PCR methods, and the data show that 95 cases (62.91%) were infected with HPI-harboring *E. coli*, 24 cases (15.89%) were infected with Enterotoxigenic *E. coli* (ETEC) and 14 cases (9.27%) were infected with ETEC and HPI-harboring *E. coli*. In addition, 2660 bacteria isolates were picked from all the 133 bacterial cultures which contained HPI-harboring *E. coli* and/or ETEC and the data of PCR examination determined that only 57 isolates were HPI-harboring *E. coli*, 20 were ETEC and 3 were both ETEC and HPI-harboring *E. coli*. This research not only revealed that HPI-harboring *E. coli* and ETEC are the prevalent pathogen of newborn piglet diarrhea, but also suggested that the method used in this study is specific, easier and more rapid to perform in the diagnosis of the infection of diarrheagenic *E. coli* with high accurate rate than the bacterial isolation and identification.**

**Key words:** Piglet, diarrhea, *Escherichia coli*, rapid detection, virulence factor, determination.

## INTRODUCTION

The newborn piglet diarrhea is frequently due to infection by one or the other pathogens, such as bacteria, viruses and parasites. After the first few days of life, increasing interaction with other animals and the environment, including introduction of artificial feeding, the risk of exposure to enteropathogens was increased (Carpenter et al., 2005; Oli et al., 1998; Stevenson, 1999). As we all know, pathogenic *Escherichia coli*, especially Enterotoxigenic *E. coli* (ETEC), are a frequent, important and global cause of severe, watery diarrhea in the newborn (suckling) pigs.

ETEC is defined as a pathogen containing *E. coli* isolates that elaborate at least one member of two defined groups of enterotoxins, namely: heat-labile (LT) and heat-stable enterotoxin (ST) (Nair et al., 1998; Smith et al., 1970;). Enterotoxins are extracellular proteins or peptides, which are able to alter the functions of enterocytes by increasing secretion and reducing absorption, and therefore they are the more efficient virulence attributes of ETEC (Blanco et al., 1991; Nagy et al., 2005, 1999). In addition, most ETEC isolated from

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diarrhoeic pigs can produce one or more of the following fimbriae: F4 (K88), F5 (K99), F6 (987P), F17, F18 and F41 (Blanco et al., 1991; Garabal et al., 1997; Nagy et al., 1999; Ojeniyi et al., 1994; Wilson et al., 1986), which allow the bacteria adhering to the small intestinal epithelium but without inducing significant morphological changes (Nataro et al., 1998).

Furthermore, the old topic of horizontal gene transfer (HGT) has become fashionable (Syvanen et al., 1999; Jain et al., 1999). From the overwhelming surge of genome sequence information, more and more candidates for horizontally transferred genes are being identified (Coton et al., 2009; Cruz et al., 2009). It is clear that genes have flowed through the biosphere, as in a global organism. There are a number of well documented examples in which bacterial adaptation has been influenced by HGT, such as the high-pathogenicity island (HPI) of pathogenic *Yersinia* in *E. coli* (Buchrieser et al., 1998; Carniel et al., 1992, 1998; Fetherston et al., 1994; Hacker et al., 2000; Perry et al., 1990; Petermann et al., 2008; Schubert et al., 1998), or the new "plasmid-associated" pathogenicity island PAI2173 which encodes the tetB gene conferring tetracycline resistance (Fekete et al., 2003). Detection of HGT can provide an optimistic control strategy on the selected disease, as well as providing the foundation for considerations of a ubiquitous role of HGT in shaping modern eukaryotic species.

The aims of the research were to establish a rapid method to further shorten the diagnosis time and increase the determination efficiency for newborn piglet diarrhea caused by HPI-harboring *E. coli* and/or ETEC, determine the relationship between the newborn piglet diarrhea and the pathogenic *E. coli* infection, as well as investigate the virulence factor genes of the diarrheagenic *E. coli* isolates.

## MATERIALS AND METHODS

### Collection of diseased samples

From March to September, 2008, a total of 151 rectal swab samples were collected from live diarrheic piglets from 51 swine industrial farms in Jiangsu province, China. All the diseased piglets with a mean age of 5.2 ± 3.6 days all suffered from watery diarrhea with a 1 - 3-day latency period and peaks around one week after birth, and the samples were collected within 36 h after diarrhea. Bringing each test sample to a final volume of 500 µl with LB broth (10 g tryptone, 10 g NaCl, 5 g yeast extract, $H_2O$ to 1 L, pH 7.4), they were store at -70°C.

### Primers

The different sets of primers (Table 1) used for PCR amplification were described previously (Cheng et al., 2005, 2006; Schubert et al., 1998) and synthesized by Sangon Biological Engineering Technology and Service Co. Ltd. (Shanghai, P. R. China). The primers that target the genes of STa, STb and LTa were mixed together (named enterotoxin primers set, each 50 mmol/l) to detect ETEC, and the primers that target the genes of HMWP2 were mixed together (named HPI primers set, each 50 mmol/l) to detect HPI-harboring *E. coli* in the samples. The primers that target the genes of F4, F5, F6 and F41 were mixed together (named fimbria primers set I, each 50 mmol/l) to identify adhesion factor genes of the bacteria isolates, and the primers that target the F18 genes were mixed together (named fimbria primers set II, each 50 mmol/l) to identify and distinguish F18ab$^+$ and F18ac$^+$ *E. coli*.

### Rapid diagnosis of pathogenic *E. coli* infection in newborn piglets

100 µl dilution of each rectal swab sample were transferred into separate tubes containing 2 ml of LB broth and grow the liquid cultures with vigorous agitation at 37°C for 6 h. 0.5 ml of the liquid cultures was transferred to labelled microcentrifuge tubes, and centrifuge at 10,000 rpm for 5 min. The supernatants were discarded and each pellet resuspended in 200 µl of distilled water. After boiling for 10 min, the suspensions were chilled on ice for 5 min, centrifuged at 10,000 rpm, at 4°C for an additional 5 min and the supernatants were used directly or stored at 4°C before PCR detection.

To detect ETEC and/or HPI-harboring *E. coli* in the liquid cultures of rectal swab 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 ($Mg^{2+}$ plus), 5 IU of *Taq* polymerase, 4 µl of dNTP mixture (each 2.5 mmol/l), 1 µl of enterotoxin primers set or HPI primers set, 2 µl of DNA template, and deionized water to a final volume of 50 µl. Cycling parameters for each primer set are outlined in Table 2. Each sample underwent 32 cycles of PCR amplification. PCR products were separated by 1% agarose gel electrophoresis along with DL2000 DNA markers and visualized after staining with ethidium bromide on a UV transilluminator.

### Determination of the virulence factor genes of the bacteria isolates

Following rapid diagnosis of ETEC and/or HPI-harboring *E. coli* infection, all the positive samples were submitted to separative cultivation of bacteria by streaking on Mackonkey agar plates. Following incubation at 37°C overnight, twenty colonies were picked out from each sample and cultured on LB agar plates at 37°C for 24 h. All the 2660 bacteria isolates were submitted to PCR detection for the genes of enterotoxins (STa, STb and LTa), HPI and fimbriae (F18, F4, F5, F6 and F41). The extraction of DNA templates and PCR assays were performed as described above, while enterotoxin primers set was used for STa, STb and LTa, HPI primers set for HPI, fimbria primers set I for F4, F5, F6 and F41, and fimbria primers set II for F18ab and F18ac. In addition, all the ETEC and/or HPI-harboring *E. coli* were serotyped by using uni-factor serum of O antigen of *E. coli*.

## RESULTS

### Rapid detection of pathogenic *E. coli* infection in newborn piglets

The specificity of the PCR assays were described previously (Cheng et al., 2006). Among the 151 rectal swab samples from live diarrheic piglets, 95 (62.91%) samples (from 32 farms) only contained HPI-harboring

**Table 1.** Primers used for PCR amplification in this study.

| RR | Primer sequence 5'-3' | Position in open reading frame (accession number in GenBank) | Size of product (bp) | Reference |
|---|---|---|---|---|
| **STa** | | | | |
| F | GGGTTGGCAATTTTTATTTCTGTA | 298-321 (M25607) | 183 | (Cheng et al., 2006) |
| R | ATTACAACAAAGTTCACAGCAGTA | 457-480 (M25607) | | |
| **STb** | | | | |
| F | ATGTAAATACCTACAACGGGTGAT | 1-20 (M35729) | 360 | (Cheng et al., 2006) |
| R | TATTTGGGCGCCAAAGCATGCTCC | 334-357 (M35729) | | |
| **LTa** | | | | |
| F | TAGAGACCGGTATTACAGAAATCTGA | 579-604 (AB011677) | 282 | (Cheng et al., 2006) |
| R | TCATCCCGAATTCTGTTATATATGTC | 835-860 (AB011677) | | |
| **HMWP2** | | | | |
| F | AAGGATTCGCTGTTACCGGAC | 241-261 (L18881) | 280 | |
| R | TCGTCGGGCAGCGTTTCTTCT | 507-527 (L18881) | | |
| **F4** | | | | |
| F | GATGAAAAAGACTCTGATTGCA | 35-56 (M29374, M29376), 407-428 (M25302) | 841 | (Cheng et al., 2006) |
| R | GATTGCTACGTTCAGCGGAGCG | 860-881 (M29374, M29376), 1226-1247 (M25302) | | |
| **F5** | | | | |
| F | CTGAAAAAAACACTGCTAGCTATT | 70-93 (M35282) | 543 | (Cheng, et al. 2006) |
| R | CATATAAGTGACTAAGAAGGATGC | 589-612 (M35282) | | |
| **F6** | | | | |
| F | GTTACTGCCAGTCTATGCCAAGTG | 707-730 (U50547) | 463 | (Cheng et al., 2006) |
| R | TCGGTGTACCTGCTGAACGAATAG | 1146-1169 (U50547) | | |
| **F41** | | | | |
| F | GATGAAAAAGACTCTGATTGCA | 254-275 (X14354) | 682 | (Cheng et al., 2006) |
| R | TCTGAGGTCATCCCAATTGTGG | 914-935 (X14354) | | |
| **F18** | | | | |
| F1 | ATGAAAAGACTAGTGTTTATTTCTT | 160-184 (M61713) | 513 or 516 | (Cheng et al., 2005) |
| F2 | CGTGAACGGTAAAACACAGGG | 504-524 (M61713) | 169 | |
| R | TTACTTGTAAGTAACCGCGTAAGCC | 648-672 (M61713) | | |

*E. coli*, 24 (15.89%) samples (from 8 farms) just contained ETEC, 14 (9.27%) samples (from 5 farms) contained both ETEC and HPI-harboring *E. coli*, while 18 samples (from 6 farms) were not detected with ETEC or HPI-harboring *E. coli* (Table 3). The data suggested that at least, 133 cases (from 45 farms) of the 151 diarrheic piglets were infected with pathogenic *E. coli*. In addition, among the 38 samples which contained ETEC, 34 were LTa-positive, 9 were STa-positive and 25 were STb-positive.

## Determination of the virulence factor genes of the bacteria isolates

Two thousand, six hundred and sixty bacteria isolates were picked from all the 133 bacterial cultures which contained HPI-harboring *E. coli* and/or ETEC, and the data of PCR examination (Table 4) determined that only 57 isolates (from 55 samples) were HPI-harboring *E. coli*, 20 were ETEC (from 16 samples) and 3 were (from 3 samples) both ETEC and HPI-harboring *E. coli*. Among

**Table 2.** PCR cycling conditions in this study.

| Primer set | Time at denature at 94°C | Annealing | Time at extension at 72°C | Total no. of cycles |
|---|---|---|---|---|
| Enterotoxin primers set | 30 s | 60 –56°C for 30s (decreasing 1°C every two cycles) | 1 min | 32 |
| HPI primers set | 30 s | 64–58 °C for 30s (decreasing 1°C every two cycles) | 1 min | 32 |
| Fimbria primers set I | 30 s | 66–62 °C for 30s (decreasing 1°C every two cycles) | 1 min | 32 |
| Fimbria primers set II | 30 s | 66–62 °C for 30s (decreasing 1°C every two cycles) | 1 min | 32 |

**Table 3.** The results of rapid detection of *E. coli* infection in newborn piglets with diarrhea.

| Virulence factor type | HPI | LTa | STa | STb | Numbers of samples |
|---|---|---|---|---|---|
| | + | | | | 95 |
| | + | + | | + | 10 |
| | + | | + | | 1 |
| | + | | + | + | 2 |
| | + | + | + | + | 1 |
| | | + | | | 11 |
| | | + | | + | 8 |
| | | + | + | | 1 |
| | | | + | + | 1 |
| | | + | + | + | 3 |
| Total | 95 | 34 | 9 | 25 | 133 |

the 57 HPI-harboring E. coli isolates tested, one (26.24%) was F4+, 9 (3.75%) were F6+, while 10 were F4+ and F6+. Among the 20 ETEC isolates, 8 (3.75%) were LTa+, STb and F6+, 3 (3.75%) were LTa+, STb, F4+ and F6+, 4 (3.75%) were LTa+ and F6+, 2 (3.75%) were STa+, STband F6+, 2 (3.75%) were STa+, and one was LTa+, STa, STb, F4+ and F6+. All the 3 both ETEC and HPI-harboring E. coli isolates were LTa+, STb and F4+. No single F5+, F41+and F18+ isolate was detected. In addition, all the 80 *E. coli* isolates were O serotyped with the most prevalent serotype been O138 accounting for 62.50% (50/80), followed by O65 (12.50%), O21 (8.75%), O139 (6.25%), O141 (5.00%), O9 (2.50%), O159 (1.25%) and O55 (1.25%).

### Contrast between PCR detection and conventional diagnosis by bacteria isolation and identification

The diagnosis time were first compared and the data show that the effective time of PCR detection method is not exceeding 10 h with 6 h bacterial cultivation, 0.5 h extraction of DNA templates, 2 h PCR examination and 1.5 h electrophoresis of PCR products, while the working time of conventional diagnosis by bacteria isolation and identification usually was 3 to 4 days. Then, the diagnosis efficiency were also analyzed and compared. The results of this research show that the ETEC and/or HPI-harboring *E. coli* were found to exist in 133 (88.08%) of the 151 samples (45 of the 51 investigated farms) by PCR detection method, while only 57 isolates could be detected in 55 samples by bacteria isolation and identification, although in each sample was picked out 20 bacteria isolates. This suggested that the PCR detection methods used in this study is specific, easier and more rapid to perform in the diagnosis of the infection of diarrheagenic *E. coli* with high accurate rate than the conventional diagnosis by bacterial isolation and identification.

## DISCUSSION

Pathogenic *E. coli* is a common porcine enteric pathogen, causing diarrhea in newborn piglets or post-weaning porcine edema disease, and ETEC is considered to be

**Table 4.** Summary of the O serotype and virulence factors of the 80 *E. coli* isolates from diarrheic piglets.

| Virulence factor / O serotype | HPI | LTa | STa | STb | F4 | F6 | Numbers of isolates |
|---|---|---|---|---|---|---|---|
| O138 | | + | | + | | + | 8 |
| O138 | | + | | + | + | + | 3 |
| O138 | | + | | | | + | 4 |
| O138 | | | + | + | | + | 2 |
| O138 | | | + | | | | 2 |
| O138 | | + | + | + | + | + | 1 |
| O138 | + | + | | + | + | | 3 |
| O138 | + | | | | + | + | 5 |
| O138 | + | | | | | + | 4 |
| O138 | + | | | | | | 18 |
| O65 | + | | | | | | 10 |
| O21 | + | | | | | | 7 |
| O139 | + | | | | | + | 5 |
| O141 | + | | | | | | 4 |
| O9 | + | | | | + | | 1 |
| O9 | + | | | | | | 1 |
| O159 | + | | | | | | 1 |
| O55 | + | | | | + | + | 1 |
| Total | 60 | 19 | 5 | 17 | 14 | 33 | 80 |

the main categories of diarrhoeagenic *E. coli*. But moreover, it is worth noting that the high-pathogenicity island has been identified in pathogenic *E. coli* strains causing diarrhea and dysentery in calves, rabbits, piglets and human (Carniel et al., 1992; Fetherston et al., 1994; Paauw et al., 2009). On the other hand, most *E. coli* are the normal inhabitants of the intestinal tracts of animals (Levine, 1987; Martins et al., 2000), and it is very difficult to obtain the pathogenic *E. coli* in the rectal swab samples from live diarrheic piglets by bacteria isolation and identification. Therefore, diagnosis of pathogenic *E. coli* infection has focused increasingly on the rapid detection method, although the conventional diagnosis method by bacteria isolation and identification has occupied a central place in the history.

Among many detection methods, PCR is a major advance in molecular diagnostics of *E. coli* infection for its good sensitivity and specificity (Nataro et al., 1998). As a matter of fact, the clinical diagnosis of *E. coli* infection in piglets, especially in the rectal swab samples from live diarrheic animals, usually just need confirming whether the pathogenic *E. coli* was/were existed in the sample(s), and not always need bacterial isolation and identification anymore while it is so difficult to pick out the pathogenic isolate(s). According to this point of view, a rapid detection method was established in this study based on the fast bacterial culturing followed by the PCR examining for the virulence factor genes, which could extremely shorten the diagnosis time and increase the determination efficiency for newborn piglet diarrhea caused by *E. coli*.

This is perfectly supported by the data of this research fortunately. Such as, in our research, a total of 151 rectal swab samples were obtained from live diarrheic piglets from 51 swine industrial farms, and the data of PCR method could confirmed that 88.08% diarrheic piglets (from 88.24% farms) were infected with ETEC and/or HPI-harboring *E. coli*. But among the 2660 bacteria isolates picked from the 133 bacterial cultures, only 80 isolates were HPI-harboring *E. coli* and/or ETEC, while most isolates may were the important members of the normal microbiologic flora of piglets (Moneoang et al., 2009).

This research not only revealed that ETEC are still the prevalent pathogen of newborn piglet diarrhea, but also found that the HPI-harboring *E. coli* isolates were more frequently detected in the diarrhea samples. Furthermore, most $HPI^+$ isolates (95.0%) were toxin negative which might contribute to the virulence of these pathogenic *E. coli* isolates that causes newborn piglet diarrhea, and this attractive hypothesis has to be verified by comparing the virulence of the parental strain and of the isogenic mutants in a suitable infection mode. By the way, the pathogenic *E. coli* associated with neonatal diarrhea belong to a limited number of serogroups with O138, O65, O21, O139, O141, O9, O159, and O55, while O138 being the most commonly found in ETEC and $HPI^+$ isolates, which were different from the isolates from porcine post weaning diarrhea (PWD) and pig edema disease (ED) (Cheng et al., 2006).

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