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# Frequency of virulence factors in *Escherichia coli* isolated from suckling pigs with diarrhoea in China

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ABSTRACT

*Escherichia coli*-associated diarrhoea is an important disease adversely affecting the pig industry. This study was conducted to investigate the frequency of virulence factors expressed by *E. coli* strains isolated from suckling pigs with diarrhoea in China. A total of 381 *E. coli* strains, obtained from 290 faecal samples from pigs on 38 farms, were tested for fimbriae (K88, K99, 987P, F41, F18, F17), non-fimbrial adhesins (AIDA-I, paa, CS31A, eae, saa), enterotoxin (LT-I, LT-II, STa, STb, EAST1), Shiga toxin (Stx1, Stx2, Stx2e), pathogenicity islands (HPI, LEE), α-haemolysin (hlyA), *afa*8 gene cluster (*afa*D, *afa*E) and *sepA* genes by PCR. Out of the 381 isolates, 206 carried at least one virulence gene. Of the 206 virulence positive isolates, the virulence factor genes detected were EAST1 ($n = 120$), *irp*2 ($n = 59$), *paa* ($n = 50$), STb ($n = 41$), AIDA-I ($n = 34$), LT-I ($n = 23$), *ler* ($n = 11$), *hly*A ($n = 9$), K88 ($n = 8$), *eae* ($n = 8$), STa ($n = 7$), *sep*A ($n = 6$), F18 ($n = 5$), *afa*D ($n = 3$), *afa*E ($n = 3$), K99 ($n = 2$) and Stx2e ($n = 1$), with most isolates carrying multiple virulence genes. These results demonstrate that relatively few isolates from the study population express K88, K99, LT-I or STa, but that EAST1 (58%), *irp*2 (29%), AIDA-I (16.5%), *paa* (24%) and STb (20%) are frequent virulence factors expressed by *E. coli* strains isolated from suckling pigs with diarrhoea in China.



## Introduction

Infection with pathogenic *Escherichia coli* is a common cause of diarrhoea in suckling pigs worldwide (Toledo et al., 2012) and poses adverse economic effects for the pig industry, due to high morbidity, mortality and reduced growth rates (Zhang et al., 2007). Most *E. coli*-induced diarrhoea is caused by enterotoxigenic *E. coli* (ETEC), with strains expressing enterotoxins and/or fimbrial adhesins (Vu-Khac et al., 2007). Fimbriae (K88, K99, 987P, F41, F18 and F17) enable bacteria to colonise the small intestinal epithelium of pigs (Vu-Khac et al., 2007; Zhang et al., 2007; Kim et al., 2010), with both heat-labile (LT-I, LT-II) and heat-stable (STa, STb) enterotoxins stimulating intestinal epithelial cells to secrete electrolytes and fluids, resulting in diarrhoea and dehydration (Kwon et al., 2002; Zhang et al., 2007). In addition, enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) is widely expressed in pathogenic *E. coli* strains, isolated from humans and animals affected with diarrhoea (Vu-Khac et al., 2007; Zhang et al., 2007; Toledo et al., 2012).

In addition to enterotoxins and fimbrial adhesins, pathogenic *E. coli* from pigs can express several other virulence factors, such as non-fimbrial adhesins, pathogenicity islands (PAIs), Shiga toxigenic *E. coli* (STEC)-associated virulence factors and the autotransporter protease, SepA. Non-fimbrial adhesins, such as adhesin involved in diffuse adherence (AIDA-I), porcine attaching and effacing-associated (paa) factor, CS31A and *afa*8 gene cluster, are widely distributed in diarrhoea-causing *E. coli* isolates from young pigs (Contrepois et al., 1989; Zhang et al., 2007). These adhesins are responsible for bacterial attachment to receptors on host epithelial cells, but are devoid of any fimbrial structures (Ngeleka et al., 2003). The *afa*8 gene cluster encodes proteins involved in adhesion to (controlled by *afa*E) and internalisation into (controlled by *afa*D) epithelial cells (Jouve et al., 1997; Lalioui et al., 1999).

PAIs are regions on the bacterial chromosome where virulence genes have accumulated (Benedek and Schubert, 2007). The two most important PAIs, locus of enterocyte effacement (LEE) and high-pathogenicity island (HPI), have been identified in pathogenic *E. coli* strains causing diarrhoea in pigs (Cheng et al., 2006). The LEE island encodes attaching and effacing factor (eae) and a transcriptional regulator, ler (Jores et al., 2004; Barba et al., 2005). Among the HPI-located genes, the *irp*2 gene can be used as a specific marker for the detection of HPI (Rakin et al., 1999).

Shiga toxigenic *E. coli* comprise a serologically diverse group of bacteria that cause diarrhoea in young pigs (Barman et al., 2008), expressing virulence factors, which include Shiga toxins (Stx1, Stx2 and their variants), LEE PAI, α-haemolysin (hlyA), and the STEC autoagglutinating adhesin (saa) (Gyles, 2007; Toledo et al., 2012). Previous studies have indicated that SepA increases the

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adherence of pathogenic *E. coli* to intestinal epithelial cells, causing atrophy of intestinal villi and inflammation of the intestinal mucosa (Goswami et al., 2008).

*E. coli* isolates from animals with diarrhoea generally harbour multiple virulence factors that have a synergistic effect in enhancing pathogenicity, posing a challenge for control of diarrhoea in young pigs. Additionally, the distribution and frequency of virulence factors can vary considerably from region to region (Zhang et al., 2007; Kim et al., 2010; Toledo et al., 2012) and over time in a specific region (Wani et al., 2004; Farooq et al., 2009). The present study was designed to acquire more information and increase the understanding of the epidemiology of pathogenic *E. coli* strains currently in circulation in China. The frequency of 25 virulence factors was investigated in bacterial isolates from suckling pigs, obtained from farms in different geographical locations.

## Materials and methods

### *Bacterial isolates and their characterisation*

From 2010 to 2012, a total of 290 faecal samples were collected from suckling pigs with diarrhoea on 38 farms in 14 regions in the northeast of China. Faecal samples were collected from individual pigs using a sterile swab that was placed into an Eppendorf tube and transported to the laboratory within 12 h. Samples from each pig were streaked onto MacConkey agar plates and incubated aerobically at 37 °C overnight. Three fermenting colonies with the appearance of *E. coli* were randomly selected from each plate and re-cultured on MacConkey agar plates at 37 °C for a further 24 h. From each re-cultured plate, one lactose-positive colony was picked and identified by standard biochemical procedures to establish its identity as an *E. coli* strain. All confirmed *E. coli* isolates were stored in Luria–Bertani (LB) broth, containing 20% glycerol at −70 °C for further studies. A panel of *E. coli* reference strains was used as positive controls for PCR, with *E. coli* strain JM109 used as a negative control (see Appendix A: Supplementary Table 1).

### *Preparation of bacterial DNA*

All *E. coli* isolates and reference strains were grown on LB agar plates and incubated aerobically at 37 °C overnight. A colony from each plate was inoculated into 3 mL LB medium and incubated at 37 °C for 24 h with 200 rpm shaking on a HZQ-X100 orbital shaker (Harbin Dongming Medical Instrument Factory). A 500 μL volume of an overnight culture was centrifuged at 10,000 *g* for 5 min and the bacterial pellet washed twice in phosphate-buffered saline (PBS, pH 7.4). The bacterial pellet was suspended in 50 μL deionised water and boiled for 10 min, followed by chilling on ice for 5 min and centrifugation at 10,000 *g* for 5 min. Supernatants were used as DNA templates for PCR amplification.

### *PCR for virulence genes*

PCR (single or multiplex) was used to detect genes encoding fimbriae (K88, K99, 987P, F41, F18, F17), non-fimbrial adhesins (AIDA-I, *paa*, CS31A, *eae*, *saa*), enterotoxins (LT-I, LT-II, STa, STb, EAST1), Shiga toxins (Stx1, Stx2, Stx2e), HPI, α-haemolysin (*hly*A), *afa*8 gene cluster (*afa*D, *afa*E) and *sep*A, as described previously (Bertin et al., 1998; Paton and Paton, 1998, 2002; Lalioui et al., 1999; Boerlin et al., 2005; Cheng et al., 2006; Vu Khac et al., 2006; Zhang et al., 2007; Osman et al., 2012). To amplify the *ler* gene, primers were designed, based on the gene sequence of LEE (FM201464.1) obtained from GenBank using Primer Premier 5.0 software (Premier Biosoft). Nucleotide sequences and predicted sizes of the amplified products for the specific oligonucleotide primers used are shown in Supplementary Table 2 (see Appendix A). The PCR reactions were performed in microcentrifuge tubes for the GeneAmp PCR System 9700 thermocycler (Applied Biosystems). Each PCR mixture contained 5 μL 10× EasyTaq buffer ($Mg^{2+}$ plus), 5 U EasyTaq polymerase (Beijing TransGen Biotech), 0.25 mM each deoxynucleotide triphosphate (Beijing TransGen Biotech), 0.4 μM each oligonucleotide primer, 2 μL DNA template and deionised water to a final volume of 50 μL. An initial denaturation step at 95 °C for 5 min was performed prior to 30 cycles of PCR, followed by a final extension step at 72 °C for 7 min.

Amplicons were visualised by 1% agarose gel electrophoresis of 10 μL of the final reaction mixture. DNA amplicons of specific sizes were identified by ultraviolet fluorescence after staining with ethidium bromide and their size was estimated by comparison with Tran2K Plus II DNA marker (Beijing TransGen Biotech). All amplicons were sequenced to confirm the specificity of the primers.

### *Statistical analysis*

Statistical analysis was performed using SPSS version 12.0. The $\chi^2$ test was used to analyse the data with $P$ values <0.05 considered to be statistically significant.

## Results

### *Isolation of virulence gene positive strains*

Amplicons of expected sizes were visualised by agarose gel electrophoresis in the reference strains (see Appendix A: Supplementary Fig. 1). After biochemical identification, a total of 381 *E. coli* strains were isolated from 290 faecal samples. Of these isolates, PCR analysis demonstrated that 206/381 (54%) carried one or more virulence genes. Virulence gene positive *E. coli* strains isolated from the same faecal samples harbouring the same virulence genes were regarded as being only one strain (Table 1).

### *Enterotoxin-associated genes*

Of the 206 isolates expressing one or more virulence genes, 182/206 (88%) were positive for at least one toxin-associated gene. For the enterotoxin genes, EAST1 (120/206, 58.3%) was the most frequent, followed by STb (41/206, 19.9%), LT-I (23/206, 11.2%) and STa (7/206, 3.4%). No LT-II positive strains were detected. For PAI genes, *irp*2 was the most frequent (59/206, 28.6%). In contrast, *eae* and *ler* were less common and were detected in only 8/206 (3.9%) and 11/206 (5.3%) isolates, respectively. For STEC-associated toxin genes, 9/206 (4.4%) isolates were positive for the *hly*A gene and 1/206 (0.5%) isolates carried the Stx2e gene. No isolates carried the Stx1 or Stx2 genes. In addition, *afa*D and *sep*A were detected in 3/206 (1.5%) and 6/206 (2.9%) isolates, respectively.

**Table 1**
Total number of faecal samples collected from young pigs on different farms in China.

| Farm location | Number of farms | Number of faecal samples | Number of virulence positive *E. coli* isolates | Total number of *E. coli* isolates | Year |
|---|---|---|---|---|---|
| Shuangcheng | 4 | 22 | 19 | 24 | 2010 |
| Anda | 5 | 16 | 9 | 18 | 2011 |
| Mingshui | 3 | 20 | 9 | 28 | 2012 |
| Qinggang | 5 | 40 | 29 | 49 | 2012 |
| Wangkui | 4 | 29 | 15 | 35 | 2012 |
| Lanxi | 2 | 14 | 12 | 23 | 2012 |
| Zhaodong | 4 | 33 | 17 | 38 | 2012 |
| Beian | 1 | 11 | 10 | 19 | 2012 |
| Mudanjiang | 1 | 4 | 6 | 9 | 2012 |
| Hailin | 1 | 7 | 13 | 15 | 2012 |
| Ningan | 3 | 35 | 30 | 42 | 2012 |
| Siping | 1 | 15 | 8 | 23 | 2012 |
| Daqing | 3 | 30 | 18 | 39 | 2012 |
| Haebin | 1 | 14 | 11 | 19 | 2012 |
| Total | 38 | 290 | 206 | 382 | 2010–2012 |