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Analysis of main virulence factor in haemolytic *Escherichia coli* isolated from diarrheal specimens of growing and finishing pigs

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ABSTRACT

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A total of 70 haemolytic *Escherichia coli* strains isolated from 310 growing and finishing pigs with diarrhea in 27 porcine farms were screened for the analysis of main virulence factor. Porcine enterotoxigenic *E. coli* strains typically express K88 or F18 fimbria and heat-labile (LT) and/or heat-stable (STa, STb) enterotoxins. We examined the virulence factors of haemolytic *E. coli* strains for K88, F18, F41, 987P and K99 fimbrial genes; LT, STa, STb and Stx2e toxic genes isolated by a multiplex polymerase chain reaction (PCR). The haemolytic *E. coli* strains were found in 18 (66.7%) farms and 14.5% (45 of 70 haemolytic isolates) of isolates in 12 (44.4%) farms possessed at least one of virulence genes detected. Of 70 haemolytic *E. coli* isolates, 28 (40.0%) strains harbored fimbrial genes: K88 (32.1%), F18 (67.9%), F41 (0), 987P (0), F41 (0); toxic genes: LT (39.3%), STb(39.3%/2.4%), STa (39.3%/2.4%), Stx2e (57.1%/38.1%) in fimbrial and non-fimbrial isolates respectively. A total of 42 (60.0%) isolates carried no fimbrial genes, including 25 (59.5%) isolates that did not have any of the virulence genes. Interestingly, *E. coli* harboring Stx2e gene were prominant

(30 of 45 virulent *E. coli*) and among those, 46.7% (14 of 30 Stx2e+) of isolates possessed with F18 fimbrial gene, whereas 53.3% (16 of 30 Stx2e+) were not harboring any fimbrial genes tested. These results suggest a diverse range of virulence genes associated with diarrhea in growing and finishing pigs.

Key Words: *Escherichia coli*, Prevalence, Virulence genes, Porcine diarrhea, PCR

CONTENTS

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhea and death in young pigs. The key virulence factor of ETEC in diarrhea are fimbrial adhesins and enterotoxins [1, 2, 25, 36, 43]. The fimbriae enable the ETEC to colonize the small intestine of piglets by mediating adhesion to the microvilli of epithelial cells [23]. Two classes of enterotoxins are produced by ETEC, heat-labile toxin (LT) and heat-stable toxin (ST). Each stimulates secretion of electrolytes and fluids by intestinal epithelial cells, resulting in diarrhea and dehydration. Whereas LTs are homologous with regard to their mode of action and molecualr mass [7, 18], there is considerbly heterology among STs [6]. The 2 main types of ST produced by ETEC isolated from pigs are STa (ST I or ST mouse) and STb (ST II or ST pig) [6]. Some *E. coli* isolated recovered from pigs with diarrhea may also secrete LT or ST I or ST II enterotoxins [27, 33, 40]. ETEC strains that produce K88 (F4) or F18 fimbria are the most commonly associated with diarrhea in young pigs [8, 11, 13, 14, 33, 35, 39]. In addition, F18 fimbria is present on *E. coli* strains isolated from postweaning diarrhea [10, 24, 40]. There are also other fimbrial structures, such as K99 (F5), 987P (F6), or F41 (F7), which are associated with porcine ETEC strains. These strains have become much less frequently or even rarely isolated [13]. The K88 fimbrial strains usually produce both LT and STb enterotoxins with or without STa. The F18 fimbrial ETEC strains typically produce STa and STb heat-stable toxins, sometimes with Shiga toxin two variant (Stx2e) [3, 13, 21, 35]. The Stx2e toxic gene are isolated from pigs with edema disease [19], but there are also some reports showing the production of this toxin by diarrhaegenic *E. coli* isolates, suggesting its pathogenic role

in postweaning diarrhea [7, 16, 41].

There is a differentiation in the prevalence of virulent *E. coli* depending on animal ages, regions, and country, but a few reports have been found in Korea and they are not including the data from Jeju-do, the partially isolating province in the country.

The purpose of this study was to analysis the virulence factor of fimbrial adhesins and enterotoxins among *E. coli* strains recently isolated from growing and finishing pigs with diarrhea in Jeju-do and Jeollanam-do.

MATERIALS AND METHODS

Samples

The samples tested in this study were taken from 230 fecal samples of 22 porcine farms in Jeju-do and from 80 ones of 5 farms in Jeollanam-do during the period from March to November 2007. All specimens were submitted to the laboratory under chilling on ice within 48 hours.

Bacterial isolation and characterization

The collected specimens from each pigs were cultured in sheep blood agar plates and MacConkey agar (Difco, Detroit, MI, USA). Plates were incubated at 37℃ aerobically overnight, and apparently pure or nearly pure cultured colonies were included for the future test. Both blood agar and MacConkey agar cultures were subcultured on the blood agar to verify the haemolytic capability. The beta-haemolysis was defined as a zone of complete erythrocyte lysis surrounding a bacterial colony observed in an incubated blood agar plate. Final *E. coli* colonies were visually examined for cultural characteristics, including colony appearance and color, glucose and lactose fermentation and the API20E kit. All these *E. coli* isolates were frozen at -70℃ after the addition of 20% glycerol (aMRESCO, Ohio, USA).

PCR primers

Primer sets for multiplex PCR were used a previously described method

[5, 44]. Base sequences and predicted sizes of amplified products for the specific oligonucleotide primers used in this study are shown in Table 1.

Table 1. PCR primers used to amplify virulence genes of *E. coli* isolates in this study

Primers	Forward/Reverse primers(5′–3′)	Size of products (bp)
K88-F	tga atg acc tga cca atg gtg gaa cc	584
K88-R	gcg ttt act ctt tga atc tgt ccg ag	
F18-F	tgg cac tgt agg aga tac cat tca gc	334
F18-R	ggt ttg acc acc ttt cag ttg agc ag	
F41-F	tta gca gcg aag atg agt gat ggg	515
F41-R	gta cta cct gca gaa aca cca gat cc	
987P-F	gcc agt cta tgc caa gtg gat act tc	390
987P-R	gtt tgt atc agg att ccc tgt ggt gg	
K99-F	gcg act acc aat gct tct gcg aat ac	230
K99-R	gaa cca gac cag tca ata cga gca	
LT-F	acg gcg tta cta tcc tgt cta tgt gc	275
LT-R	ttg gtc tcg gtc aga tat gtg att ct	
STa-F	gtc agt caa ctg aat cac ttg act ct	156
STa-R	cat gga gca cag gca gga tta caa ca	
STb-F	gct aca aat gcc tat gca tct aca ca	125
STb-R	cat gct cca gca gta cca tct cta ac	
Stx2e-F2	ecgg tat cct att ccc agg agt tta cg	598
Stx2e-R2	gtc ttc cgg cgt cat cgt ata aac ag	

DNA extraction and PCR reaction

A total of 70 isolates were examined for genes of fimbriae and enterotoxins. A commercial PCR kit, QIAGEN Multiplex PCR kit (QIAGEN, CA, USA), was used to amplify the genes for K88, F18, F41, 987P, K99, LT, STa, STb and Stx2e by mixing PCR primers in a multiplex PCR reaction by following the manufacture instruction. The multiplex PCR was complete by an initial heat activation of 15 min at 95℃ followed by 25 cycles of 30 sec at 94℃, 90 sec at 63℃, and 90 sec at 72℃ and an extension of 10 min at 72℃.

The amplified product was visualized by standard gel electrophoresis of 10 ㎕ of the final reaction mixture in a 3% agarose gel(BMA, Rockland, USA). Amplified DNA fragments of specific sizes were located by ultraviolet fluorescence after staining ethidium bromide. DNA Ladder 100 bp plus (Bioneer, Korea) was used as molecular size marker.

RESULTS

Prevalence of pathogenic *E. coli*

The beta-haemolytic properties of *E. coli* strains were determined on sheep blood agar plates. The haemolytic *E. coli* were isolated from 18 (66.7%) out of 27 farms collected and 12 (44.4%) farms had *E. coli* positive for the detection of virulence gene. Of 70 (22.6%) haemolytic *E. coli* isolates recovered from 310 fecal samples, 45 isolates (64.3%) possessed fimbrial adhesin genes and/or toxic genes (Table 2). Both prevalence of haemolytic and virulence gene possessing *E. coli* were higher in Jeollanam-do than in Jeju-do.

Table 2. Prevalence of pathogenic *E. coli* isolated from fecal specimens of pigs

	Jeju -do	Jeollanam -do	Total
No. of farms tested	22(100)	5(100)	27(100)
No. of farms positive for haemolytic *E. coli*	14(63.6)	4(80.0)	18(66.7)
No. of farms positive for *E. coli* harboring virulence genes	9(40.9)	3(60.0)	12(44.4)
No. of samples tested	230(100)	80(100)	310(100)
No. of samples positive for haemolytic *E. coli*	48(20.9)	22(27.5)	70(22.6)
No. of samples positive for *E. coli* harboring virulence genes	27(11.7)	18(22.5)	45(14.5)

Prevalence of virulence genes

According to multiplex PCR test for 70 haemolytic *E. coli,* 28 (40.0%)

isolates possessed one of five fimbrial genes. Among the fimbrial isolates, F18+ (67.9%) isolates were more prominent than K88+ (32.1%) isolates. And there was no *E. coli* isolates harboring F41, 987P and K99 fimbrial gene. Among 42 (60.0%) non-fimbrial isolates, 17 isolates exhibited at least one toxic gene and most of these (38.1%) harbored genes for Stx2e. The gene for LT enterotoxin was found only in fimbrial isolates, and the Stx2e toxic gene was detected in 57.1% of the fimbrial isolates (Table 3).

Table 3. Prevalence of individual virulence genes in haemolytic *E. coli* isolated from pigs with diarrhea

Fimbrial isolates(28 isolates, 40.0%)								
K88	F18	F41	987P	K99	LT	STb	STa	Stx2e
9	19	0	0	0	11	11	11	16
32.1%	67.9%	0%	0%	0%	39.3%	39.3%	39.3%	57.1%
Non-fimbrial isolates(42 isolates, 60.0%)								
No virulence gene					LT	STb	STa	Stx2e
25					0	1	1	16
59.5%					0%	2.4%	2.4%	38.1%

Patterns of virulence genes in haemolytic *E. coli* isolates

The patterns of detected virulence genes are presented in Fig 1 and Table 4. A total of 8 different patterns were observed in the basis of the virulence gene exhibitions. The patterns A (F18/Stx2e/LT/STa) and E (K88/LT/STa/STb) had 4 different genes and were observed from 2 isolates in 1 farm and 6 isolates in 2 farms, respectively. The patterns C (F18/STa/STb) and F (K88/LT/STb) having 3 different genes were found

in 2 and 3 isolates in 1 and 3 farms, respectively. The patterns B (F18/Stx2e) and D (F18/STa) having 2 different genes were found in 14 and 1 isolates of 7 and 1 farms, respectively. In special, *E. coli* harboring Stx2e gene were prominant (30 of 45 virulent *E. coli*) and among those, 46.7% (14 of 30 Stx2e+) of isolates possessed with F18 fimbrial gene, whereas 53.3% (16 of 30 Stx2e+) were not harboring any fimbrial genes tested, and there is one isolates harboring only STa/STb. .

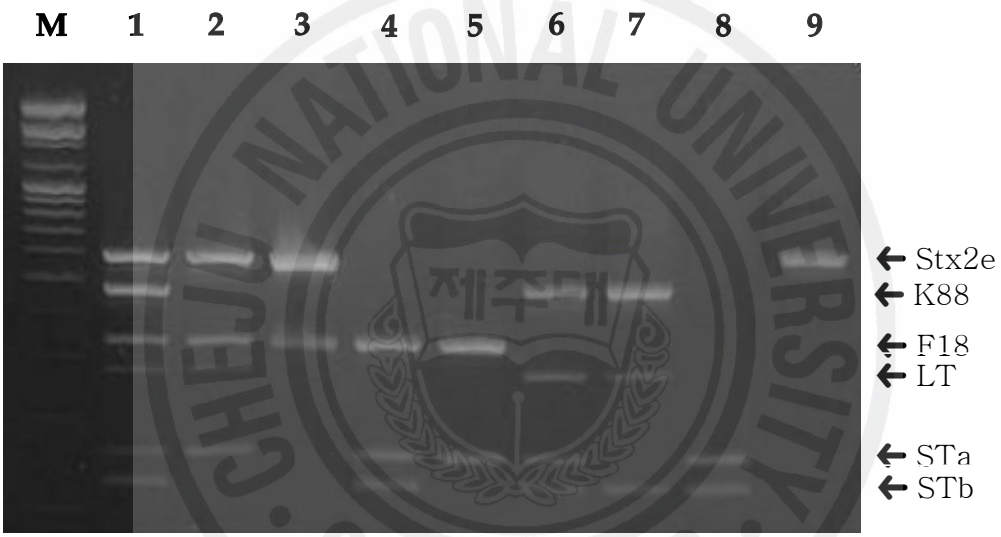

Fig. 1. Results from the multiplex PCR. Lane M:100bp plus DNA ladder (Bioneer); lane 1: pool of samples of 2, 5 and 6; lane 2: Stx2e/F18/LT/STa; lane 3: Stx2e/F18; lane 4: F18/STa/STb; lane 5: F18/STa; lane 6: K88/LT/STa/STb; lane 7: K88/LT/STb; lane 8: STa/STb; lane 9: Stx2e.

Table 4. Prevalence of virulence genes in haemolytic *E. coli* isolated from fecal specimens of pigs

Patterns	Detected virulence genes	No. of farms(%)	No. of isolates(%)
A	F18/Stx2e/LT/STa	1(8.3)	2(4.4)
B	F18/Stx2e	7(58.3)	14(31.1)
C	F18/STa/STb	1(8.3)	2(4.4)
D	F18/STa	1(8.3)	1(2.2)
E	K88/LT/STa/STb	2(16.7)	6(13.3)
F	K88/LT/STb	3(25.0)	3(6.7)
G	STa/STb	1(8.3)	1(2.2)
H	Stx2e	8(66.7)	16(35.6)
	Total	12(100)	45(100)

Distributions of virulence gene patterns in pig farms

Among 12 pig farms, isolated *E. coli* had at least one of virulence genes. Only 5 farms showed only one pattern (pattern B or H) of virulence gene, while the other 7 exhibited more than one virulence gene pattern. Surprisingly, 8 *E. coli* strains possessed 6 different virulence gene patterns were found in one pig farm (Table 5).

Table 5. Distribution of haemolytic *E. coli* harboring virulence gene in swine farms

Patterns of virulence genes harboring	No. of farms	No. of isolates	Pig farms*
H	3	6	P3, P11, P13
B	2	3	P2, P9
AH	1	3	P8
BF	1	2	P6
BH	2	12	P26, P27
EF	1	6	P23
EH	1	5	P14
BCDFGH	1	8	P12
Total	12	45	

* Indicates the pig farms: P1-P22 in Jeju-do; P23-P27: Jeollanam-do

DISCUSSION

In South Korea, 9,382,000 pigs in 11,300 porcine farms have been bred until December 2006 and they are including 396,925 pigs in 173 farms of Jeju-do, the biggest island in Korea, which is partially isolated from the main land. In this province it has not been yet reported on occurrence of some foreign swine diseases, such as classical swine fever (just once 5 years ago), foot and mouth disease, and Aujeszky's disease, in contrast with in the main land. For this reason, pigs and pig products can never enter from other regions to Jeju province, even from the main land of South Korea. This province policy has provided a clean environment for these diseases in swine herds, but instead of that, has maintained or increased many endemic diseases, including Porcine Circovirus infection and porcine reproductive and respiratory syndrome. Therefore, this study was designed to investigate the characterization of *Escherichia coli* strains isolated from growing and finishing pigs with diarrhea in Jeju-do. and to compare with the results in Jeollanam-do in South Korea.

The haemolytic characteristic of *E. coli* is known as a virulence marker in pathogenic *E. coli* [14, 29]. The haemolytic *E. coli* were isolated only 70 (22.6%) out of 310 fecal samples and 45 (64.5%) of 70 haemolytic *E. coli* isolates possessed at least one virulence gene in this study. It implied that haemolysin in *E. coli* is not necessary for the pathogenecity of postweaning diarrhea (PWD) as the previous published data [4, 22, 36], and also the majority of fecal specimens tested in this study were taken from pig with diarrhea caused by other pathogens such as *Lawsonia intracellularis, Salmonella* spp., *Brachyspira hyodysentaeriae* and/or *B. pilosicoli* (data not shown). It is difficult to compare the prevalence of haemolytic *E. coli* and virulent gene-positive *E. coli* in

sampling regions because of the different sampling sizes. However, total prevalence in Jeollanam-do were higher than in Jeju-do.

This study confirmed that only F4+ isolates and F18+ isolates were the fimbrial haemolytic *E. coli* strains associated with growing and finishing pigs with diarrhea. Previous studies indicated that the F4+ ETEC represented nearly a half of the strains isolated from diarrhegic pigs [13, 14]. And Zhang *et al.* (2007) revealed a slight higher prevalence for F4 fimbrial gene. Roughly 65% of the fimbrial *E. coli* isolates carry the F4 fimbrial gene. Similarly, Wilson and Francis (1986) reported the presence of fimbriae among 223 *E. coli* strains originated from pigs with PWD and detected 72% as F4+. Nagy *et al.* (1990) tested 205 *E. coli* isolates from pigs with PWD and showed that 61% were F4+. In contrast, in this study the F4 fimbrial gene shows lower prevalence 12.8% (9/70). Several studies, Ojeniyi *et al.* (1994) found 25.8% of F4+ isolates originated from pigs with PWD. Nakazawa *et al.* (1987) found that only 3% of the PWD strains from Japan were K88+, Choi and Chae (1999) found that 5.4% of ETEC strains were F4+ in Korea. Chen *et al.* (2004) found a low prevalence of F4+ *E. coli* strains isolated from pigs with PWD in eastern China. Twenty-one (9.8%) strains carried the F4 fimbrial gene. This may partially be due to that vaccination programs containing F4-antigen could be changing the fimbriae prevalence in ETEC or STEC as were reported by others, and this “immune pressure" might cause lower the prevalence of other fimbriae, such as F6 fimbrial in *E. coli* isolates caused PWD in China [17, 37]. ETEC strains tend to produce multiple enterotoxin for causing diarrhea in pigs [31], recent studies showed that K88ac strains that express only LT or STb enterotoxin are sufficiently virulent to cause diarrhea in gnotobiotic pigs [2, 43].

Kwon *et al.* (1999) revealed the F41 fimbrial adhesin gene in Korean ETEC strains isolated from preweaning pigs. In another study, some

ETEC strains produced F41 only[26]. Chen *et al.* (2004) found that 1.9% (4/215) of F41+, 6.0% (13/215) of F6+ and 2.3% (5/215) of F5+ isolates from pigs with PWD. Whereas none the F41, F6, and F5 fimbrial genes detected in our *E. coli* isolates.

This study revealed the Stx2e toxic gene was commonly detect in *E. coli* strains isolated from growing and finishing pigs with diarrhea. The Stx2e genes were present in 57.1% (16/28) of fimbrial isolates and all with only F18 fimbrial gene, but in 38.1% (16/42) of non-fimbrial isolates, including 3 strains from Jeollanam-do. Nagy et al. (1999) found that 3 strains of 19 F18-positive *E. coli* isolates possessed only Stx2e but our result is out of accordance with those of the previous studies. Frydendahl (2002) found that 35 strains (40.7%) of 86 F18-positive *E. coli* possessed Stx2e and/or other toxic genes whereas only 1 strain (3.1%) of 32 non-fimbrial isolates was positive for Stx2e gene. Zhang *et al.* (2007) detected that the Stx2e toxic gene was found almost in F18+ isolates only, 17.1% (30/175) in fimbrial isolates from young pigs with diarrhea in the US. Chen *et al.* (2004) found that only a small number (6.1%) of strains were Stx2e gene-positive and F18 positive isolates were consistently unique stx2e gene-negative.

The enterotoxin-producing pattern of *E. coli* is variable in accordance with countries and regions. STa-producing *E. coli* strains were more prevalent than LT-producing strains in the United States and Denmark, whereas LT-producing strains were more prevalent than STa-producing strains in England and Japan [12, 30, 32, 42]. In this study the presence of the STa and LT producing strains was equal in fimbrial isolates. And none LT-producing strains detected in non-fimbrial isolates. A total of 8 different virulence gene patterns exhibited in *E. coli* isolated in this study. More than one pattern was found from 7 (58.3%) of 12 farms possessing any *E. coli* expressed at least one virulence gene and in

special, one farm possessed the various haemolytic *E. coli* strains showing 6 different patterns. Although these differentiations depend on the sampling sizes, regions, animal ages and seasons, it indicates that the potential pathogenic *E. coli* is widely spreaded in swine farms and the strain character may change continually even in the same swine farm due to the possibilities of gene transfer and of bacterial contamination from farm to farm, from animal to animal and/or other transfer methods.

In this study, there are no many *E. coli* strains tested due to selecting only haemolytic isolates but it revealed that various *E. coli* strains are associated with porcine diarrhea, even in the same pig farm, and not only most prominent *E. coli* strains are F18+ and Stx2e+ but also the strains with only Stx2e+ are comparably found in Jeju swine herds. This finding is not unique, but it is different from the previous reports. Further studies are needed to ascertain whether the *E. coli* strains with only Stx2e gene are adapted to Jeju area partially isolated from the mainland of Korea or find also highly in other regions in Korea, and if so, to analyse their pathogenicity and to determine any fimbria associated with bacterial attachment to pig intestinal epithelium.

CONCLUSION

We examined the prevalence of virulence genes in haemolytic *E. coli* strains isolated from growing and finishing pigs with diarrhea in Jeju-do and Jeollanam-do. We detected that pigs with diarrhea are carriers of haemolytic *E. coli* bearing virulence genes. We characterized 70 isolates from growing and finishing pigs on 27 swine farms, and 45 isolates possessed at least one virulence gene. The virulence genes included F18 or K88 fimbrial genes, STa, STb, and Stx2e toxic genes. F18+ isolates prominent than K88+ isolates. Stx2e gene was found in 57.1%/38.1% of fimbrial isolates/non-fimbrial isolates. The potential pathogenic *E. coli* with various virulence genes is associated with pig diarrhea within the same farm.

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