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Original article

Prevalence of pathogenicity island ETT2 in Escherichia coli isolated from piglets with diarrhea in northeast of China

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Abstract

Our aim was to investigate the prevalence of the pathogenicity island ETT2 and to examine the relationship between the ETT2 locus and other virulence factors in *Escherichia coli (E. coli)* isolated from piglets with diarrhea. A total of 354 *E. coli* strains isolated from scouring piglets were tested using PCR for the presence of the ETT2 locus. The *E. coli* strains were also analyzed for enterotoxins, fimbriae, non-fimbrial adhesin, Shiga toxins, pathogenicity islands, α-haemolysin (hlyA), afa8 gene cluster and autotransporter protease (sepA) genes. The results showed that 215 (60.7%) of the isolates possessed the ETT2 island. In 215 ETT2-positive *E. coli* strains, the virulence genes found were EAST1 (27.0%), irp2 (18.6%), paa (15.4%), STb (7.9%), LT (6.5%), ler (4.7%), hlyA (3.7%), AIDA-I (3.7%), K88 (3.7%), eae (3.3%), STa (2.8%), afaD (1.4%), afaE (1.4%), K99 (0.9%) and sepA (0.47%), *respectively*, and the isolates could be assigned into 25 different virulence factor patterns. In 139 ETT2-negative *E. coli* strains, the virulence genes detected were EAST1 (38.9%), paa (14.4%), STb (11.5%), AIDA-I (10.1%), irp2 (7.9%), sepA (2.16%), LT (0.7%), STa (0.7%), eae (0.7%), ler (0.7%), hlyA (0.7%) and K88 (0.7%), *respectively*, and the isolates could be classified into 13 different virulence factor patterns. Moreover, the occurrence of LT gene of ETT2-positive *E. coli* strains was far more than that of ETT2-negative *E. coli* strains.

Key words: *Escherichia coli*, pathogenicity island, ETT2, piglets, diarrhea

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Introduction

Escherichia coli (*E. coli*) is an important cause of intestinal and extraintestinal diseases in humans and animals (Tan et al. 2012). On the basis of genetic and clinical criteria, strains of *E. coli* can be classified into several different categories (Veilleux and Dubreuil 2006). Of these categories, certain pathogenic *E. coli* strains are the major causes of diarrhea in piglets (Kwon et al. 2002, Vu et al. 2006, Wang et al. 2010) and this disease poses negative economic implications for the pork industry due to high morbidity and mortality worldwide (Madoroba et al. 2009). The pathogenic *E. coli* causes disorders mainly attributed to virulence factors such as enterotoxins, Shiga toxins and adhesions (Zhang et al. 2007, Toledo et al. 2012).

Although toxins and adhesins are the predominant virulence factors, pathogenic *E. coli* of animal origin always harbors several mobile genetic elements such as plasmids, pathogenicity islands (PAI) and bacteriophages encoding a network of various virulence traits (Choi et al. 2001, Cheng et al. 2012). PAIs represent a distinctive subset of genomic islands (GEIs) that are acquired by horizontal gene transfer via transduction, conjugation and transformation (Gal-Mor et al. 2006). These islands always code for several bacterial virulence factors, e.g. adhesins, iron-scavenging siderophores, capsules, endotoxins and exotoxins, type III and type IV secretion systems (Dobrindt et al. 2004, Benedek et al. 2007).

Of these, the *E. coli* type III secretion system 2 (ETT2) island has been found to be highly prevalent in isolates from colibacillosis in pigs and mastitis in cows (Prager et al. 2004, Cheng et al. 2012). ETT2 island was first discovered through the analysis of genome sequences of enterohemorrhagic *E. coli* O157:H7 (Ren et al. 2004). This novel pathogenicity island was subsequently identified as a 29.9 kb element integrated at the tRNA locus *glyU* (Hacker et al. 2003). The ETT2 locus consists of more than 35 genes, including *yqe, yge, etr, epr, epa, eiv,* etc., which is homologous to the *Salmonella* type III secretion system located on *Salmonella* pathogenicity island 1 (SPI-1) (Ideses et al. 2005, Cheng et al. 2012). Of the ETT2 genes, etrA, which has been identified between the *apr* and *epa* genes as an ETT2 regulator, had been used as a special marker for the detection of ETT2 locus previously (Osawa et al. 2006). Moreover, mutational inhibition of two regulatory genes from the ETT2 cluster in pathogenic *E. coli* leads to greatly increased secretion of proteins encoded by the LEE PAI and to increased adhesin of *E. coli* to human intestinal cells (Zhang et al. 2004). This finding suggests that ETT2 island plays a role in the pathogenesis of *E. coli* infection. However, respective effector proteins encoded by the ETT2 island have not been identified as yet (Wang et al. 2016).

Although ETT2 locus is commonly present in pathogenic *E. coli* strains isolated from livestock, so far, little is known concerning the virulence profiles of ETT2-positive *E. coli* strains isolated from scouring piglets. Therefore, the purpose of this study was to investigate the prevalence of the ETT2 locus in *E. coli* strains from scouring piglets. In addition, the association of the ETT2 locus with other virulence factors, such as enterotoxin (LT, STa, STb, EAST1), fimbriae (K88, K99, 987P, F41, F18, F17), non-fimbrial adhesin (AIDA-I, paa, eae), Shiga toxin (Stx1, Stx2, Stx2e), pathogenicity islands (HPI, LEE), α-haemolysin (hlyA), afa8 gene cluster (afaD, afaE) and sepA, was also investigated. This investigation may provide significant epidemiologic information of porcine pathogenic *E. coli* so as to develop effective vaccines and diagnostic reagents that may prevent diarrhea among piglets.

Materials and Methods

Bacterial isolates

A total of 354 *E. coli* field isolates were derived from fecal samples of 1-25 days old piglets with diarrhea in Liaoning province, Heilongjiang province and Jilin province of China from 2014 to 2016, and each fecal sample was collected from an individual piglet. Fecal samples of the diarrheic piglets were collected using sterile swabs that were placed into Eppendorf tubes and immediately transported to the laboratory in ice-cooled containers, and then fecal samples were streaked on MacConkey agar plates and incubated at 37°C aerobically overnight. Three lactose positive colonies from each MacConkey agar plate were subcultured on MacConkey agar plates and incubated overnight at 37°C. One fermenting colony from each subcultured MacConkey agar plate was confirmed to be *E. coli* by standard biochemical tests (Mast group Ltd, Merseyside, UK). After isolation, the confirmed *E. coli* strains were stored in Luria-Bertani (LB) broth containing 20% glycerol at -70°C until examination.

Reference strains

E. coli reference strains were used as positive controls and *E. coli* strain JM109 was used as negative control for this study. DN0402 (LEE, sepA), DN89B (Stx1, hlyA, LEE, HPI, EAST1), DN84B (EAST1, HPI, F17), DN68A (HPI, F17, afa8), S1 (AIDA-I) and E118A (ETT2, paa) were stored in our labora-

Table 1. PCR primers for detection of pathogenicity island ETT2 and other virulence factors of *E. coli* isolates in this study.

Virulence factor	Forward Primer	Reverse Primer	Size of product (bp)	Reference
ETT2	CTTCTTCCTAACGAAACATCATTAC	TGACATATCAACTTTCTCTTACGC	913	Osawa et al. (2006)
LT-I	TAGAGACCGGTATTACAGAAATCTGA	TCATCCCGAATTCTGTTATATATGTC	282	Cheng et al. (2006)
STa	GGGTTGGCAATTTTTATTTCTGTA	ATTACAACAAAGTTCACAGCAGTA	183	Cheng et al. (2006)
STb	ATGTAAATACCTACAACGGGTGAT	TATTTGGGCGCCAAAGCATGCTCC	360	Cheng et al. (2006)
EAST1	ATGCCATCAACACAGTATATC	TCAGGTCGCGAGTGACGG	117	Zhang et al. (2007)
K88	GATGAAAAAGACTCTGATTGCA	GATTGCTACGTTCAGCGGAGCG	841	Cheng et al. (2006)
K99	CTGAAAAAAACACTGCTAGCTATT	CATATAAGTGACTAAGAAGGATGC	543	Cheng et al. (2006)
987P	GTTACTGCCAGTCTATGCCAAGTG	TCGGTGTACCTGCTGAACGAATAG	463	Cheng et al. (2006)
F41	GATGAAAAAGACTCTGATTGCA	TCTGAGGTCATCCCAATTGTGG	682	Cheng et al. (2006)
F18	ATGAAAAGACTAGTGTTTATTTCTT	TTACTTGTAAGTAACCGCGTAAGCC	520	Cheng et al. (2006)
F17	GGGCTGACAGAGGAGGTGGGGC	CCCGGCGACAACTTCATCACCGG	411	Vu-Khac et al. (2006)
AIDA-I	ACAGTATCATATGGAGCCA	TGTGCGCCAGAACTATTA	586	Zhang et al. (2007)
paa	CCATAAAGACAGCTTCAGTGAAAA	GTATTACTGGTACCACCACCATCA	162	Zhang et al. (2007)
afaD	GTTGAACTGAGTCTTAATACCAGTG	TGAGCATTCTCCGCTAACTGATAAT	354	Lalioui et al. (1999)
afaE	CTAACTTGCCATGCTGTGACAGTA	TTATCCCCTGCGTAGTTGTGAATC	302	Lalioui et al. (1999)
eae	ATATCCGTTTTAATGGCTATCT	AATCTTCTGCGTACTGTGTTCA	425	Cheng et al. (2006)
ler	AACAAGCCCATACATTCAGC	GCCATCATCAGGCACATTAG	169	This study
irp2	AAGGATTCGCTGTTACCGGAC	TCGTCGGGCAGCGTTTCTTCT	287	Cheng et al. (2006)
Stx1	ATTCGCTGAATGTCATTCGCT	ACGCTTCCCAGAATTGCATTA	664	Cheng et al. (2006)
Stx2	GAATGAAGAAGATGTTTATAGCGG	GGTTATGCCTCAGTCATTATTAA	281	Cheng et al. (2006)
Stx2e	GAATGAAGAAGATGTTTATAGCGG	TTTTATGGAACGTAGGTATTACC	454	Cheng et al. (2006)
hlyA	GCATCATCAAGCGTACGTTCC	AATGAGCCAAGCTGGTTAAGCT	533	Paton et al. (1998)
sepA	TAAAACCCGCCGCCTGAGTA	TGCCGGTGAACAGGAGGTTT	611	Boerlin et al. (2005)

tory. C83903 (K88, LT, STb, EAST1), C83920 (K99, F41, STa), C83529 (K99, STa), C83915 (987P, STa), C83684 (F18ab, Stx2e), O157ŁvH7 (Stx1, Stx2, LEE) and CMCC44498 (F18ab, Stx2e) were purchased from China Institute of Veterinary Drug Control. 2134P (F18ac) was kindly provided by Prof. Eric Cox (Laboratory of Immunology, Faculty of Veterinary Medicine, Ghent University, Belgium).

PCR primers

Primers used for amplification of ETT2 island were slightly modified from previous study (Osawa et al. 2006). *E. coli* isolates were also examined for enterotoxins (LT, STa, STb, EAST1), fimbriae (K88, K99, 987P, F41, F18, F17), non-fimbrial adhesins (AIDA-I, paa, eae), Shiga toxins (Stx1, Stx2, Stx2e), pathogenicity islands (HPI, LEE), α-haemolysin (hlyA), afa8 gene cluster (afaD, afaE) and sepA genes. These virulence genes were detected by PCR as described previously (Lalioui et al. 1999, Paton et al. 2002, Boerlin et al. 2005, Cheng et al. 2006, Vu et al. 2006, Zhang et al. 2007). New primers for ler gene were designed with an annealing temperature of 52°C and a product size of 169bp based upon the gene sequence of LEE PAI (GeneBank: FM201464.1) using Primer 5.0 software. The nucleotide sequences and the predicted sizes of the amplified products for the specific PCR primers used are shown in Table 1.

DNA extraction and PCR reaction

DNA templates were obtained by suspending a colony of *E. coli* grown overnight on LB agar plates in 50 µL of LB medium and boiled for 10 min. The 50 µL PCR mixtures contained the following: 1× EasyTaq buffer (Mg^{2+} plus), 5 U of EasyTaq polymerase (Beijing TransGen Biotech, China), 0.25 mM of each dNTP (Beijing TransGen Biotech, China), 0.4 µM of each primer, 2 µL of DNA template, and deionized water to a final volume of 50 µL. The PCR reactions were completed in microcentrifuge tubes for the GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, USA).

To amplify gene for ETT2, PCR amplification was conducted under a program consisting of 5 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 49°C, and 1 min at 72°C; and final extension of 7 min at 72°C. To amplify genes for LT, STa, STb, F18, F17, eae, ler, irp2 and sepA, PCR amplification was conducted under a program consisting of 5 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 57°C, and 40 s at 72°C; and final extension of 7 min at 72°C. To amplify genes for K88, K99, 987P, F41, Stx1, Stx2, Stx2e and hlyA, PCR amplification was conducted under a program consisting of 5 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 54°C, and 1 min at 72°C; and final extension of 7 min at 72°C. To amplify genes for AIDA-I, paa, afaD, afaE and EAST1, PCR amplification was conducted under a program consisting of 5 min at 95°C; 30 cycles

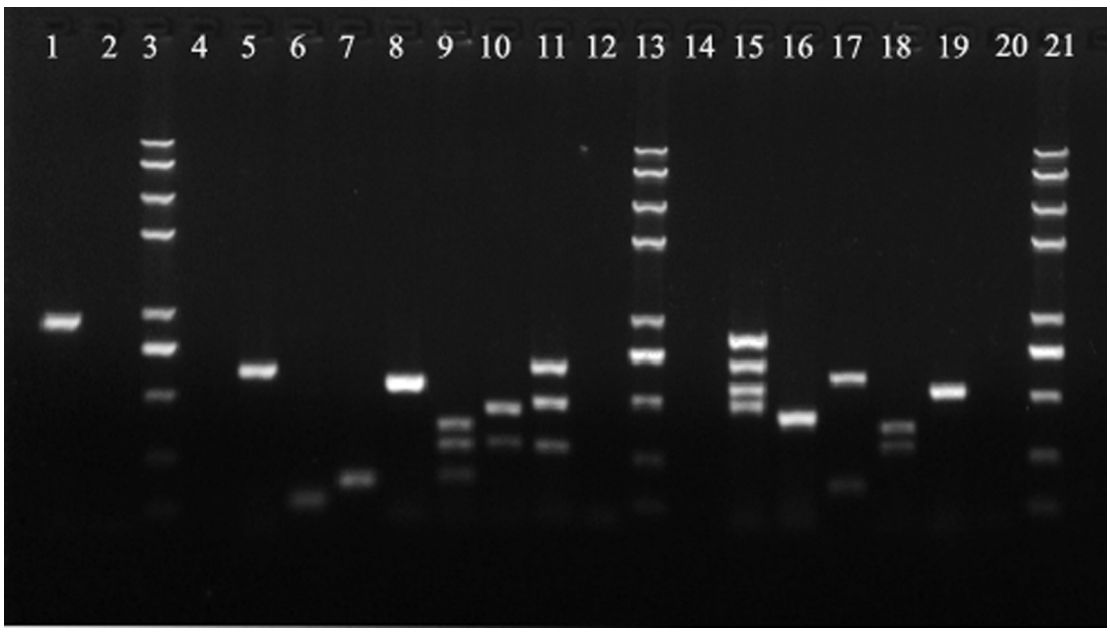

Fig. 1. Results from the PCR amplification with primers as listed in Table 1: Lane 1: reference strain positive for ETT2 island; Lane 2, 4, 12, 14, 20: negative control; Lane 3, 13, 21: Tran2K Plus II DNA marker; Lane 5: reference strain positive for sepA gene; Lane 6: reference strain positive for EAST1 gene; Lane 7: reference strain positive for ler gene; Lane 8: reference strain positive for hlyA gene; Lane 9: reference strains positive for STb, LT and STa genes, respectively; Lane 10: reference strains positive for LEE and HPI islands, respectively; Lane 11: reference strains positive for stx1, stx2e and stx2 genes, respectively; Lane 15: reference strains positive for K88, F41, K99 and 987P genes, respectively; Lane 16: reference strain positive for F17 gene; Lane 17: reference strains positive for AIDA-I and paa genes, respectively; Lane 18: reference strain positive for afaD and afaE genes, respectively; Lane 19: reference strain positive for F18 gene.

Table 2. Prevalence of ETT2 island among pathogenic *E. coli* strains isolated from diarrheic piglets[a].

	ETT2 positive *E. coli* strains	ETT2 negative *E. coli* strains
ETT2 only	100 (46.5)	–
Toxins only	68 (31.6)	45 (32.4)
Adhesins only	13 (6.0)	9 (6.5)
Both toxins and adhesins	34 (15.8)	19 (13.7)
None[b]	–	66 (47.5)
Total	215	139

[a] Values given in parenthesis are in percentage.
[b] *E. coli* isolates negative for both toxins and adhesins.

of 30 s at 94°C, 30 s at 51°C, and 40 s at 72°C; and final extension of 7 min at 72°C.

After amplification, all PCR products were separated by 1% agarose gel electrophoresis of 10 μL of the final reaction mixture. Reference positive and negative control strains and Tran2K Plus II DNA marker (Beijing TransGen Biotech, China) were used to identify amplified products. Amplified products were visualised with ethidium bromide under an UV illuminator and images were recorded using AlphaImager 2200 software (Alpha Innotech, USA).

Statistical analysis

Statistical analysis was performed using SPSS version 16.0. The χ^2 test was used to analyse the data with p values <0.05 considered to be statistically significant.

Ethics Statement

The samples were collected under the permission of the owner of the piggery. The present study did not involve endangered or protected species. The animal

Table 3. Prevalence of other virulence factors from 215 ETT2-positive *E. coli* and 139 ETT2-negative *E. coli* isolates from diarrheic piglets.

Virulence factor	ETT2 postive *E. coli* strains (n=215)	ETT2 negative *E. coli* strains (n=139)	p-value
Toxin genes			
LT	14 (6.5)	1 (0.7)	p<0.01
STa	6 (2.8)	1 (0.7)	ns
STb	17 (7.9)	16 (11.5)	ns
EAST1	58 (27.0)	54 (38.9)	p<0.05
irp2	40 (18.6)	11 (7.9)	p<0.01
eae	7 (3.3)	1 (0.7)	ns
Ler	10 (4.7)	1 (0.7)	p<0.05
sepA	1 (0.5)	3 (2.2)	ns
hlyA	8 (3.7)	1 (0.7)	ns
afaD	3 (1.4)	0 (0)	ns
Fimbrial and non-fimbrial adhesin genes			
AIDA-I	8 (3.8)	14 (10.1)	p<0.05
Paa	33 (15.4)	20 (14.4)	ns
K88	8 (3.7)	1 (0.7)	ns
K99	2 (0.9)	0 (0)	ns
afaE	3 (1.4)	0 (0)	ns

study complied with the Animal welfare Act by following the NIH guidelines (NIH Pub. No. 85-23, revised 1996), and protocols were approved and supervised by Animal Care and Use Committee of Northeastern University.

Results

After PCR reaction was performed to detect the ETT2 island and other virulence genes, the specific PCR products of expected sizes were revealed by agarose gel electrophoresis in the reference strains, *respectively* (Fig. 1). PCR amplification showed that 60.7% (215/354) of the 354 *E. coli* field isolates were positive for ETT2 locus. Of the 215 ETT2-positive *E. coli* strains, 46.5% (100/215) carried the ETT2 locus only, and 53.5% (115/215) carried other virulence factors in addition to ETT2 locus. Among the 215 isolates known to carry genes for other virulence factors, 6.0% (13/215) carried genes for the adhesins only, 31.6% (68/215) carried genes for the toxins only, and 15.8% (34/215) carried genes for both adhesins and toxins. Of the 139 ETT2-negative *E. coli* strains, 6.5% (9/139) carried genes for the adhesins only, 32.4% (45/139) carried genes for the toxins only, 13.7% (19/139) carried genes for both adhesins and toxins, and 47.5% (66/139) carried genes for neither adhesins nor toxins (Table 2).

Of the 215 ETT2-positive *E. coli* strains, EAST1 (26.98%) and irp2 (18.60%) were the most prevalent toxins, but only irp2 was significantly more frequent than in ETT2-negative strains (p<0.01). The percentage of LT (6.5%, p<0.01) and Ler (4.7%, p<0.05) of ETT2-positive *E. coli* strains was far more than that of ETT2-negative *E. coli* strains. More than half of the 215 ETT2-positive *E. coli* isolates were lacking any of the fimbrial or non-fimbrial adhesin genes, and only 47 isolates carried one or more of the adhesin genes. Similarly, low percentage of adhesin genes were detected in the 139-ETT2 negative *E. coli* strains, and only 28 *E. coli* isolates carried at least one adhesin genes. Among the isolates, ADIA-I showed statistically significant difference (p<0.05) in comporison to ETT2-positive strains. (Table 3).

The distribution of virulence factors among 215-ETT2 positive and 139 negative *E. coli* isolates are summarized in Table 4. The results indicated that isolates without adhesin genes showed a greater variability in toxin-associated gene profiles and were found in 11 different combinations of which ETT2/EAST1 (29 isolates, 13.5%) was the most common combination. Considering all virulence factors together, ETT2/irp2 (22 isolates, 10.2%) showed statistically significant difference (p<0.05) in comporison to ETT2-negative strains. Similarly, of the 139 ETT2--negative *E. coli* strains, adhesin negative strains also showed multiplex genotypes of which EAST1

Table 4. Distribution of other virulence factors among 215 ETT2-positive *E. coli* isolates from diarrheic piglets[a].

Virulence factor		ETT2 postive *E. coli* strains (n=215)	ETT2 negative *E. coli* strains (n=139)	p-value of a statistical test
Colonization factor plus	Toxin-associated gene			
AIDA-I	irp2	2 (0.9)	0 (0)	ns
	STb/EAST1	3 (1.4)	8 (5.8)	p<0.05
	LT/STb/EAST1	3 (1.4)	0 (0)	ns
paa	EAST1	3 (1.4)	2 (1.4)	ns
	irp2	4 (1.9)	1 (0.7)	ns
	STb	0 (0)	1 (0.7)	ns
	irp2/EAST1	1 (0.5)	0 (0)	ns
	ler/hlyA	1 (0.5)	0 (0)	ns
	eae/ler	2 (0.9)	0 (0)	ns
	eae/ler/hlyA	5 (2.3)	1 (0.7)	ns
afaE	afaD	1 (0.5)	0 (0)	ns
K88	LT/STb/EAST1	2 (0.9)	0 (0)	ns
	irp2/STa/sepA/hlyA	1 (0.5)	0 (0)	ns
AIDA-I/paa	STb/EAST1	0 (0)	6 (4.3)	p<0.05
Paa/K88	LT/STa/STb/EAST1/sepA	3 (1.4)	1 (0.7)	ns
Paa/K99	STa	2 (0.9)	0 (0)	ns
afaE/K88	LT/afaD	2 (0.9)	0 (0)	ns
None[b]	STb	1 (0.5)	0 (0)	ns
	EAST1	29 (13.5)	31 (22.3)	p<0.05
	irp2	22 (10.2)	5 (3.6)	P<0.05
	sepA	0 (0)	1 (0.7)	ns
	ler	0 (0)	1 (0.7)	ns
	STb/EAST1	1 (0.5)	0 (0)	ns
	irp2/EAST1	9 (4.2)	6 (4.3)	ns
	irp2/ler	1 (0.5)	0 (0)	ns
	Stx2e/sepA	0 (0)	1 (0.7)	ns
	eae/ler/hlyA	1 (0.5)	0 (0)	ns
	LT/STb/EAST1	4 (1.9)	0 (0)	ns
	None[c]	100 (46.5)	66 (47.5)	ns

[a] Values given in parenthesis are in percentage.
[b] *E. coli* isolates negative for fimbrial and non-fimbrial adhesin genes.
[c] *E. coli* isolates negative for toxin-associated gene.

(31 isolates, 22.3%) was the most common one. The 7 major genotypes of the 139 ETT2-negative *E. coli* strains, EAST1 (31 isolates, 22.3%), AIDA-I/STb/ /EAST1 (8 isolates, 5.8%), AIDA-I/paa/STb/EAST1 (6 isolates, 4.3%) showed statistically significant difference (p<0.05) in comporison to ETT2 positive strains.

Discussion

Following the detection of the first PAI in uropathogenic *E. coli* in 1990, PAIs have also been identified in other *E. coli* pathotypes as well as in different Gram-positive and Gram-negative bacteria (Hacker et al. 2003). Of the PAIs, the *E. coli* type III secretion system is responsible for secretion and injection of virulence-associated factors into the cytosol of host cells, causing diarrheal disease (Yao et al. 2009). ETT2 is an additional type III secretion system and is shown to be present in the majority of pathogenic *E. coli* strains, but the role of ETT2 in the pathogenesis of *E. coli* infection remains unclear (Yao et al. 2009, Cheng et al. 2012). In the present study, to better understand the pathogenic implication of ETT2, we investigated the prevalence of the ETT2 locus and examined the relationship between the ETT2 locus and other virulence factors in *E. coli* strains isolated from piglets with diarrhea. The results revealed that ETT2 locus (215 isolates, 60.7%) was commonly present in 354 *E. coli* field strains isolated from diarrheal piglets. This result is in accordance with Osawa et al. (2006) and Cheng et al. (2012), who found that 65% (54/83) and 85.9% (79/92) of the diarrheagenic *E. coli* isolates carried the ETT2 locus, *respectively*. In addition, in the previous study,

Cheng et al. (2012) and Prager et al. (2004) showed that ETT2 locus was highly associated with Stx2e, while 76.1% (70/92) and 100% (124/124) of the ETT2 positive *E. coli* strains carried Stx2e, *respectively*. On the contrary, in the present study, no isolates carried the Stx2e gene, and ETT2 locus mainly associated with EAST1 and HPI among the 215 ETT2-positive *E. coli* strains, It is possible that such difference could be due to different age (suckling piglet vs. weaned piglet) and different region (northeast of China vs. east of China and Germany) for bacterial isolation, because the distribution and frequencies of virulence factors in *E. coli* can vary considerably from region to region (Kim et al. 2004, Zhang et al. 2007, Toledo et al. 2012) and vary at different age (Kwon et al. 2002, Toledo et al. 2012).

ETT2 represents an additional member of genomic islands or pathogenicity islands of *E. coli* which may contribute to virulence traits of *E. coli* pathovars (Prager et al. 2004). Previous epidemiological study concerning ETT2 island had focused on several different regions located at the whole ETT2 locus by PCR, and the results showed that ETT2 had undergone mutational attrition in almost all strains (Hacker et al. 2003, Liu et al. 2004, Prager et al. 2004, Ren et al. 2004, Cheng et al. 2012). However, it showed that although the ETT2 secretion system may be inhibited, the ETT2 pathogenicity island is still capable of exerting powerful phenotypic effects through gene regulation, e.g. regulators from the ETT2 locus influence expression and secretion of proteins by the LEE secretion system even if the ETT2 gene cluster was present in deletion types of *E. coli* strains (Zhang et al. 2004). A previous study also showed the presence of ETT2 in septicemic *E. coli*, where it contributed to virulence in a 1-day-old chick model, although it had several premature stop codons and a 5 kb deletion (Ideses et al. 2005). These findings indicated that the ETT2 locus is important as a virulence factor in pathogenic *E. coli* strains no matter intact form or deletion types. Therefore, the present study only detected the etrA gene, which was located in almost all deletion types of the ETT2 islands (Hacker et al. 2003, Prager et al. 2004, Cheng et al. 2012), and this gene had been regarded as a special marker for the detection of ETT2 island previously.

In addition, most of the 215 ETT2-positive *E. coli* strains and the 139 ETT2-negative *E. coli* strains carried multiple toxin genes. E.g., of the 215 ETT2-positive *E. coli* strains, 4.7% (10/215) contained genes for both EAST1 and irp2, 4.2% (9/215) contained genes for LT, STb and EAST1, 2.8% (6/215) contained genes for eae, ler and hlyA, and 1.7% (4/215) contained genes for both STb and EAST1. Of the 139 ETT2-negative *E. coli* strains, 10.1% (14/139) contained genes for both STb and EAST1, and 4.3% (6/139) contained genes for both irp2 and EAST1. This means that pathogenic *E. coli* causes diarrhea in piglets not only due on single toxin factor, but also due to synergy of multiplex virulence factors that have a synergistic effect in enhancing pathogenicity, making it challenging to control diarrhea of piglets.

Of the 215 ETT2-positive *E. coli* strains, 78.1% (168/215) are lacking any of the fimbrial or non-fimbrial adhesin genes, and only 21.9% (47/215) carried one or more of the adhesin genes. Similar results were also obtained from 139 ETT2-negative *E. coli* strains. This phenomenon is similar to the previous studie (Nakazawa et al. 1997, Soderlind et al. 1998), while a small quantity of fimbriae among pathogenic *E. coli* strains isolated from piglets. A possible explanation of this phenomenon could be that frequent use of vaccines focused on fimbrial antigen had apparently changed the pattern of diarrheagenic *E. coli* (Do et al. 2006). Moreover, it has been demonstrated that experimental pigs would not develop the disease even if being inoculated with a diarrheal *E. coli* strains if pathogenic *E. coli* cannot attach to epithelial cells by adhesions. Therefore, this phenomenon mentioned above probably due to the presence of unknown adhesins on the surface of the adhesion-negative *E. coli* strains, and this claim remains to be verified by further investigation.

In conclusion, the results of the present study indicate that ETT2 locus is widely distributed among *E. coli* strains isolated from piglets with diarrhea. The results also showed that virulence profiles between ETT2-positive *E. coli* strains and ETT2-negative *E. coli* strains show no significant difference.

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