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Prevalence and Isoforms of the Pathogenicity Island ETT2 Among *Escherichia coli* Isolates from Colibacillosis in Pigs and Mastitis in Cows

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Abstract To study the prevalence and isoforms of the pathogenicity island ETT2 among pathogenic *Escherichia coli*, as well as to determine the relationship between the ETT2 locus and other virulence factors, PCR amplifications target to the 35 ETT2-associated genes were established and used to investigate the presence of the ETT2 locus in 168 *E. coli* isolates from weaned piglets with edema and/or diarrhea or dairy cows with mastitis. The results showed that the ETT2 locus could be identified in the pathogenic *E. coli* isolates from colibacillosis in pigs and in the ones from mastitis in cows, but the presence of ETT2 among the isolates of porcine origin were significantly higher (85.87%) than that (47.37%) of bovine origin. Furthermore, 11 ETT2 isoforms were found in this research, including an intact form and 10 deletion types. The intact ETT2 was the prevalent form among the pathogenic *E. coli* isolates of porcine origin, and highly associated with the presence of shigatoxin type 2e (Stx2e), while the great majority isolates of bovine origin just carried various deletion types, and no distinct association with other virulence factors, e.g., the presence/absence of LT1, ST2, Cnf2, Tra, HPI, Hly, and F17a fimbriae.

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Introduction

Although toxin(s) and adhesin(s) are the predominant virulence factors [2–4, 6, 11, 16, 17], pathogenic *Escherichia coli* of animal origin always contain several mobile genetic elements such as plasmids, pathogenicity islands (PAI), or bacteriophages encoding a network of various virulence traits [14]. Thereinto, PAIs are a distinct class of genomic islands, which are acquired by horizontal gene transfer. These islands always encode accessory functions such as additional metabolic activities, antibiotic resistance, or properties involved in microbial pathogenesis, fitness, and/or symbiosis [8].

Among the PAIs, the *E. coli* type three secretion system 2 (ETT2) island, approximately 29.9 kb in size and localized adjacent to the tRNA locus *glyU*, was discovered through the analysis of genome sequences of enterohemorrhagic *E. coli* [10, 12–14]. As shown in Fig. 1 [15], there are at least 35 genes coding by the intact ETT2 locus, including *yqe*, *yge*, *etr*, *epr*, *epa*, *eiv*, etc., which were similar to the sequence of several Salmonella pathogenicity islands [1, 7, 9]. Except for the intact form, the ETT2 gene cluster was also found to be present in part in the *E. coli* strains [13, 14]. Compared to the parental *E. coli* strains, the ETT2 or *eivA*-deleted mutants exhibit defects in invasion and intracellular survival, suggesting its involvement in the pathogenesis of *E. coli* infection [18]. However, the ETT2-encoded proteins and their functions in pathogenesis or influenced on the virulence network of *E. coli* infection remain to be detailed defined as yet.

To investigate the prevalence and virulent association of the PAI ETT2 among *E. coli* isolates of different animal

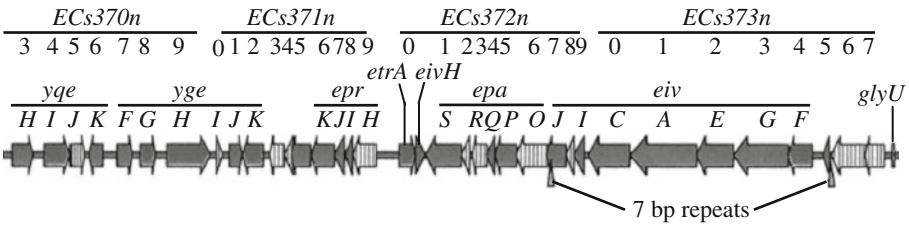

Fig. 1 Genomic position of genes of the pathogenicity island ETT2 derived from the genome sequence of *E. coli* O157:H7 str. Sakai (GenBank accession no. NC_002695)

origins, in this study PCRs targeting 35 ETT2-associated genes were established and used to analyze the prevalence and isoforms of the ETT2 among 168 *E. coli* isolates from piglets with edema and/or diarrhea or dairy cows with mastitis. In addition, the association of the ETT2 with other virulence factors was also investigated.

Materials and Methods

Bacterial Isolates

Ninety-two *E. coli* isolates were isolated from weaned piglets with edema and/or diarrhea, including twenty-two enterotoxigenic *E. coli* (ETEC), forty-six shigatoxin-producing *E. coli* (STEC), and twenty-four STEC/ETEC [2, 3]. Seventy-six pathogenic *E. coli* isolates were from dairy cows with clinical or sub-clinical mastitis, which contain heat-labile toxin type 1 (LT1), heat-stable enterotoxin type 2 (ST2), cytotoxic necrotizing factor type 2 (Cnf2), transfer surface exclusion protein (Tra), hemolysin (Hly), F17a fimbriae, and high-pathogenicity island (HPI) [5]. Four $ETT2^{+}$ (S443025, S451524, S452021, and S462234) and one ETT^{-} (TG1) *E. coli* strains (Yangzhou University) were used as the positive and negative controls, respectively.

Primer Design for ETT2 Locus

Thirty-five sets of PCR primers (Table 1) were designed according to ETT2 *ECs3703–ECs3737* gene sequences in GenBank using the PrimerSelect program in Lasergene software (DNASTAR, Inc), and synthesized by Sangon Biological Engineering Technology and Service Co. Ltd. (Shanghai, China). The two primers of each set were mixed together (each 50 mmol/l) before adding to the PCR mixture, respectively.

Bacterial Genomic DNA Extraction

Both the *E. coli* isolates and reference strains were grown overnight at 37°C on Luria–Bertani agar plates and single colonies were suspended in 50 µl deionized water. After boiling for 10 min, chilling on ice for 5 min and centrifugation at 10,000×*g* for 5 min, the supernatants were used as the DNA templates for PCR amplification.

PCR Detection of ETT2-Associated Genes in Pathogenic *E. coli*

Fifty microliter PCR reactions contained 5 µl of 10× PCR buffer (Mg^{2+} plus), 5 IU of *Taq* polymerase, 4 µl of dNTP mixture (2.5 mmol/l of each, *TaKaRa*), 1 µl of primer set, 2 µl of DNA template, and deionized water 37 µl. The 30-cycle PCRs were performed in 200 µl microcentrifuge tubes on the Applied Biosystems 2720 Thermal Cycler (America) using cycling parameters in Table 2. The PCR products were analyzed by 1% agarose gel electrophoresis along with DL2000 DNA markers.

Before amplification of ETT2-associated genes in 168 pathogenic *E. coli* isolates, the specificity of the PCR was tested using the genomic DNA template from $ETT2^{+}$ (S443025, S451524, S452021, or S462234) or ETT^{-} (TG1) *E. coli* as the positive or negative controls, respectively.

Sequence Analysis

To confirm the creditability of the PCR amplification, the PCR products of 35 ETT2-associated genes from the reference *E. coli* strain S443025 were subcloned into pGEM®-T vector (Promega) and transformed into *E. coli* DH5α, respectively. All the fragments in the recombinant plasmids were sequenced and compared with the published sequences (NC_002695).

Results

PCR Specificities for Amplification of ETT2-Associated Genes

The PCR was first performed using the DNA template from each of five reference *E. coli* strains. Agarose gel electrophoresis showed that 35 PCR products of expected sizes could be amplified from S443025 and S451524 strains, which harbor the intact ETT2 locus. For strain S452021 (with deletion from *ECs3730* to *ECs3735*) or S462234 (with deletion from *ECs3728* to *ECs3735*), 29 or 27 specific PCR products could be amplified. For TG1 strain ($ETT2^{-}$), however, no single PCR product could be detected. To further evaluate the creditability of the PCR methods, the 35 DNA fragments of ETT2-associated genes

Table 1 The PCR primers used in this study

Primer	Sequence	Position (gi accession number)	Size of product (bp)
ECs3703	F ATTGCCAAATAATGCCAGAAGAGTCACC	3709989–3710016 (NC_002695)	581
	R TCAATGTTGGACCGAATGTGAACGAATA	3710542–3710569 (NC_002695)	
ECs3704	F ACATAACGCCGATCTTAACGTCGT	3710965–3710988 (NC_002695)	748
	R TCAACCACGATTAACCTCACGGTA	3711689–3711712 (NC_002695)	
ECs3705	F CACTGTCTATTCTTACACTGCAGA	3711768–3711791 (NC_002695)	418
	R CCTTGCACAATGCATTATTGCTCT	3712162–3712185 (NC_002695)	
ECs3706	F CTTAGCTCGATTATTATTCCTTCG	3712382–3712405 (NC_002695)	354
	R GAAATGGTCTATATGCATGATATT	3712712–3712735 (NC_002695)	
ECs3707	F CCTGAACAGGAGAACAAAAATAAG	3713240–3713263 (NC_002695)	342
	R TTTATTATGTTTGGTGACGAGGGG	3713558–3713581 (NC_002695)	
ECs3708	F GTGATGAATGGGCAAATCAACTAA	3713943–3713966 (NC_002695)	431
	R TAAGAGTTCAATATATGCCTGCGC	3714350–3714373 (NC_002695)	
ECs3709	F CGCTCACGGAAGATGGAATTCTTA	3714780–3714803 (NC_002695)	887
	R ATGAAGTGCCAAAGACATATGGCA	3715643–3715666 (NC_002695)	
ECs3710	F CTAATCCAATCGGTATCAATAATT	3716281–3716304 (NC_002695)	186
	R TAGGATTATCTTCTGAATACATAT	3716443–3716466 (NC_002695)	
ECs3711	F TCGCTCTTGTTGAAAGCAATCTAA	3716716–3716739 (NC_002695)	356
	R AGCCTTACGATTGTCTAGTTCATT	3717048–3717071 (NC_002695)	
ECs3712	F CGATACCACTGTGAACATCTAATTTC	3717146–3717171 (NC_002695)	556
	R CAGCCGTTTATGATTGATGGGATAAT	3717676–3717701 (NC_002695)	
ECs3713	F TTATTCATATCCAAAGAATGTGTT	3717988–3718011 (NC_002695)	387
	R GAAGAGTATATTGTTAAAATCCTC	3718351–3718374 (NC_002695)	
ECs3714	F AGGGTATAAAATTATCCATCACAT	3718434–3718457 (NC_002695)	211
	R AGCTTGATAAAATCTCCTGCAAAT	3718621–3718644 (NC_002695)	
ECs3715	F TTTGCAGGAGATTTTATCAAGCTC	3718622–3718645 (NC_002695)	556
	R CCTAAGAAAGATTATATATGCTCC	3719154–3719177 (NC_002695)	
ECs3716	F ATTAGTTAGAATGGCCCTATACTT	3719228–3719251 (NC_002695)	693
	R TTATTGTTTATCCTGCTGTTGTGC	3719897–3719920 (NC_002695)	
ECs3717	F CAGCGACAATTTTTCTTGCTAATG	3719961–3719984 (NC_002695)	304
	R TCAGTAAGCAATATGCCACCTATT	3720241–3720264 (NC_002695)	
ECs3718	F GGAAGTTTTGTATGATTGCTGAAT	3720292–3720315 (NC_002695)	215
	R TATTATGGACATCAGCAAACAATT	3720483–3720506 (NC_002695)	
ECs3719	F TTATTCCTGAAATGTCCAGTTGCT	3720540–3720563 (NC_002695)	679
	R TAATGATCCGCAGCTGAGACATAT	3721195–3721218 (NC_002695)	
ECs3720	F CTTCACTGTAGGCACTAATGCATT	3722004–3722027 (NC_002695)	467
	R CTCTTACGCAAGATTGGAGTTTAA	3722447–3722470 (NC_002695)	
ECs3721	F TTTATCTTCTGAAGAGAGCTGTTC	3722892–3722915 (NC_002695)	1054
	R AACTGAAAAGCCAACACAAAAGAA	3723922–3723945 (NC_002695)	
ECs3722	F ACGCTATGCTTCACAACTAAA	3724124–3724104 (NC_002695)	147
	R TATAGTTGCAATTGTTCTGGG	3723998–3723978 (NC_002695)	
ECs3723	F TTGGATCCGGCTACAGGT	3724393–3724376 (NC_002695)	109
	R GAGTTTCCAGAATAAAGTTC	3724304–3724285 (NC_002695)	
ECs3724	F GCTAAATTCAGCATTTCGATACCA	3724750–3724773 (NC_002695)	222
	R GAATAGGGCGCTGTATCTGATTT	3724949–3724971 (NC_002695)	
ECs3725	F ATAACATTGTCCAACCGTCCATCG	3725052–3725075 (NC_002695)	617
	R GTCAAATAGCATTTCGCTGATAGC	3725645–3725668 (NC_002695)	
ECs3726	F ATGCCCTGATAACCAAGAATGAAT	3725675–3725698 (NC_002695)	943
	R GATGAAAGTTAATGTTCGGTCTGA	3726594–3726617 (NC_002695)	

Table 1 continued

Primer	Sequence	Position (gi accession number)	Size of product (bp)
ECs3727	F TTCATCTTGCTCAGTCAATACCCA	3726612–3726635 (NC_002695)	111
	R ACTAACGAAAGAAGGTTTGCTGAT	3726699–3726722 (NC_002695)	
ECs3728	F TTATCAATAGACTTCATTTGCCCA	3727264–3727241 (NC_002695)	217
	R TGGACGAAGTCAAGAAAATTGAAC	3727457–3727480 (NC_002695)	
ECs3729	F CCATTATATTTTTCCTCCTGTTCA	3727478–3727501 (NC_002695)	306
	R AAACCTGCAGTCACTGTTAGATAT	3727760–3727783 (NC_002695)	
ECs3730	F ACCAATTTGTGAGTTTAGGCCGTG	3727978–3728001 (NC_002695)	848
	R GTCGTATCAGGAACGTCGAGTTAT	3728802–3728825 (NC_002695)	
ECs3731	F ATTTTCCGTTAGTTCCCCGTACGA	3729261–3729284 (NC_002695)	898
	R TCTTGCTGATAGGATTCGTAGTCA	3730135–3730158 (NC_002695)	
ECs3732	F CATTGAACATCACCTTCTTCCATG	3731289–3731312 (NC_002695)	914
	R TCTGGACGGAGAAGAATATGTCCT	3732179–3732202 (NC_002695)	
ECs3733	F CACGATTCAGGTAAGTTTTTATCC	3732451–3732474 (NC_002695)	944
	R AGAAGATGATGACCTACAAGTAGA	3733371–3733394 (NC_002695)	
ECs3734	F ACATAGTTCTCTCGGTGTTTTTCC	3734146–3734169 (NC_002695)	735
	R ATTGAAGAAGGGCTGTTACTTCCA	3734857–3734880 (NC_002695)	
ECs3735	F TACGAATCATGAGACTTATAATGG	3735240–3735263 (NC_002695)	163
	R AATAAATTACTGTTAGCTTACCTG	3735379–3735402 (NC_002695)	
ECs3736	F CTCATCATTGATAAGGTTGGACAT	3735487–3735510 (NC_002695)	942
	R CTTGATCTCCTTATACAAGCAATC	3736405–3736428 (NC_002695)	
ECs3737	F TACTAATGCCATATAGCCCCATAA	3736673–3736696 (NC_002695)	442
	R CTACGCTTTTAACAAACGATTGAT	3737091–3737114 (NC_002695)	

of strain S443025 were sequenced, and the data showed that all the 35 PCR regions of *ECs3703–ECs3737* genes are absolutely accorded with the expected size (Table 1) and share high homologies with the published sequence (NC_002695), respectively (Table 3).

Prevalence of ETT2 in Pathogenic *E. coli* Isolates

After 30 cycles of PCR amplification, agarose gel electrophoresis showed that the detection rate, including intact ETT2 or its deletion mutants, was 85.87% in the pathogenic *E. coli* isolates of porcine origin, which was significantly higher than that (47.37%) in the *E. coli* isolates of bovine origin (Table 4). Among different pathotypes of *E. coli* of porcine origin, prevalence of the ETT2 was 77.27, 89.13, and 83.33% in ETEC, STEC, and STEC/ETEC isolates, respectively.

Isoforms of ETT2 in Pathogenic *E. coli* Isolates

The gene-specific PCR amplification showed that the ETT2 in 168 *E. coli* isolates could be divided into 11 different isoforms, including the intact type containing the whole 35 genes (designated as type A) and 10 gene deletion mutants (designated as types B, C, D, E, F, G, H, I, J, and K). The schematic structures of 11 ETT2 genotypes are shown in Fig. 2. Type A was the complete form of ETT2 island with 35 genes; types B, C, D, E, and F were absence of 6, 7, 7, 8, and 9 genes in the ETT2 locus right (*Ecs3737*) regions, respectively; type G was absence of 8 genes in the right and 8 genes in the left (*Ecs3703*) region; type H was absence of 3 genes in the right, 8 genes in the middle, and 8 genes in the left region; form I was absence of 3 genes in the right and 13 genes in the middle region; and type J was absence of 3 genes in the right, 2 genes in the middle, and 8 genes in the left region; while type K was lost the great majority of genes of the locus.

For the pathogenic *E. coli* isolates from pig samples, five different ETT2 types could be detected, among which the types A (49.37%), B (12.66%), E (30.38%), F (5.06%), and G (2.53) were more prevalent form. For ETEC isolates, five different ETT2 types could be detected, among which the type A (15.79%), B (5.26%), E (47.37%), F (21.05%), and G (10.53%) were more prevalent. For STEC isolates, only two ETT2 types were detected, including the genotypes A (82.50%) and E (17.50%). For STEC/ETEC isolates, three different ETT2 types could be detected, including types A (15.00%), B (45.00%), and E (40.00%). For the pathogenic *E. coli* isolates from cow samples, eight ETT2 types (B, C, D, E, H, I, J, and K) were found and six of them (C, D, H, I, J, and K) were not found in the *E. coli* isolates of porcine origin. Among these the type D was the most prevalent

Table 2 The parameters for PCR detection of ETT2-associated genes

Primer set	Denaturation	Annealing	Extension	Cycles
ECs3703	94°C/40 s	58°C/40 s	72°C/40 s	30
ECs3704	94°C/40 s	57°C/40 s	72°C/45 s	30
ECs3705	94°C/30 s	56°C/30 s	72°C/30 s	30
ECs3706	94°C/30 s	52°C/30 s	72°C/30 s	30
ECs3707	94°C/30 s	54°C/30 s	72°C/30 s	30
ECs3708	94°C/30 s	54°C/30 s	72°C/30 s	30
ECs3709	94°C/60 s	56°C/60 s	72°C/60 s	30
ECs3710	94°C/30 s	52°C/30 s	72°C/30 s	30
ECs3711	94°C/40 s	55°C/40 s	72°C/35 s	30
ECs3712	94°C/40 s	57°C/40 s	72°C/40 s	30
ECs3713	94°C/40 s	51°C/40 s	72°C/35 s	30
ECs3744	94°C/30 s	52°C/30 s	72°C/30 s	30
ECs3715	94°C/50 s	53°C/50 s	72°C/45 s	30
ECs3716	94°C/50 s	54°C/50 s	72°C/45 s	30
ECs3717	94°C/30 s	55°C/30 s	72°C/30 s	30
ECs3718	94°C/30 s	52°C/30 s	72°C/30 s	30
ECs3719	94°C/40 s	56°C/40 s	72°C/40 s	30
ECs3720	94°C/40 s	55°C/40 s	72°C/33 s	30
ECs3721	94°C/60 s	54°C/60 s	72°C/70 s	30
ECs3722	94°C/30 s	52°C/30 s	72°C/30 s	30
ECs3723	94°C/30 s	55°C/30 s	72°C/30 s	30
ECs3724	94°C/30 s	56°C/30 s	72°C/30 s	30
ECs3725	94°C/60 s	57°C/60 s	72°C/60 s	30
ECs3726	94°C/60 s	55°C/60 s	72°C/60 s	30
ECs3727	94°C/30 s	55°C/30 s	72°C/30 s	30
ECs3728	94°C/30 s	53°C/30 s	72°C/30 s	30
ECs3729	94°C/40 s	53°C/60 s	72°C/35 s	30
ECs3730	94°C/60 s	58°C/60 s	72°C/60 s	30
ECs3731	94°C/60 s	57°C/60 s	72°C/60 s	30
ECs3732	94°C/60 s	57°C/60 s	72°C/60 s	30
ECs3733	94°C/60 s	55°C/60 s	72°C/60 s	30
ECs3734	94°C/50 s	56°C/50 s	72°C/45 s	30
ECs3735	94°C/30 s	52°C/30 s	72°C/30 s	30
ECs3736	94°C/60 s	55°C/60 s	72°C/60 s	30
ECs3737	94°C/30 s	54°C/30 s	72°C/35 s	30

Table 3 The identity of the 35 PCR regions shared with the reference sequence

PCR region	Identical to NC_002695 (%)
ECs3703	99.5
ECs3704	99.2
ECs3705	98.2
ECs3706	98.3
ECs3707	97.4
ECs3708	97.4
ECs3709	97.4
ECs3710	97.8
ECs3711	95.0
ECs3712	98.4
ECs3713	95.8
ECs3714	96.2
ECs3715	98.0
ECs3716	98.8
ECs3717	98.0
ECs3718	98.6
ECs3719	96.9
ECs3720	97.0
ECs3721	98.2
ECs3722	100
ECs3723	100
ECs3724	96.4
ECs3725	97.4
ECs3726	96.6
ECs3727	99.1
ECs3728	97.2
ECs3729	97.7
ECs3730	98.1
ECs3731	99.4
ECs3732	99.2
ECs3733	98.8
ECs3734	99.7
ECs3735	100
ECs3736	98.9
ECs3737	98.6

(63.89%) and only two isolates contained types B or E, while there is no single isolates carried types A, F, and G.

Association of ETT2 with Other Virulence Factors

The virulence properties of the 168 bacteria under investigation are summarized in Table 4. Among the 92 *E. coli* isolates of porcine origin, 92.31% ETT2 type A isolates were Stx2e-positive, 100 or 90.00% type B isolates were ST1- or Stx2e-positive, and 60.87, 43.48, or 21.74% type E isolates were Stx2e-, ST2- or ST1-positive. Among the 76 *E. coli* of bovine origin, however, no distinct association was found between the ETT2 and other virulence factors, including LT1, ST2, Cnf2, Tra, HPI, Hly, and F17a.

Discussions

E. coli strains often acquire new complex pathogenic phenotypes by the acquisition of pathogenicity islands, which contain virulence genes clustered on the chromosome and which are acquired en bloc by horizontal gene transfer [8]. The type III secretion system (T3SS) is an important virulence factor used by several gram-negative

Table 4 Prevalence and isoforms of the ETT2 islands among different pathogenic phenotypes *E. coli*

Animal	Virulence factor(s)	Total	A	B	C	D	E	F	G	H	I	J	K	Subtotal
Piglet	ST1 + ST2 + LT1	6					5							5
	ST1 + ST2	1		1										1
	ST1	3	3											3
	ST2	4					2		2					4
	ST2 + LT1	8					2	4						6
	Stx2e	46	33				7							40
	Stx2e + ST1	18	3	5			7							15
	Stx2e + ST1 + LT1	5		4										4
	Stx2e + ST2 + LT1	1					1							1
Cow	HPI	8		1		5								6
	HPI + Cnf2	6				3				1		2		6
	HPI + Tra	3				1								1
	Tra	15				5					3		1	9
	Cnf2	8				2								2
	ST2 + Cnf2	3				2								2
	ST2	6			2	1								3
	ST2 + F17a	1				1								1
	LT1 + ST2 + Hly +Cnf2	3				2								2
	LT1 + ST2 + Cnf2	2				1								1
	LT1 + ST2	9					1							1
	ST2 + Hly + Cnf2	2			1									1
	Hly	3											1	1
	LT1 + ST2 + F17a + Tra	1												0
	LT1 + ST2 + Hly	1												0
	ST2 + Tra	2												0
	ST2 + HPI	1												0
	LT1	2												0
Total		168	39	11	3	23	25	4	2	1	3	2	2	115

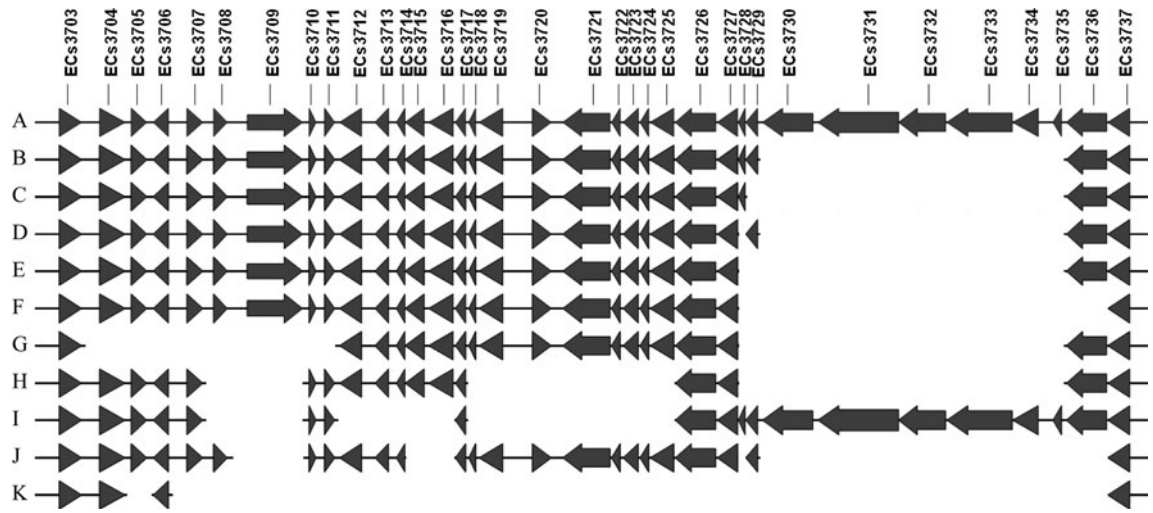

Fig. 2 The intact ETT2 and its deletion types derived from the data of this research

bacteria to deliver effector proteins which subvert host cellular processes [10, 13]. ETT2 is an additional T3SS and has been found in many *E. coli* strains [10, 12–14], but its in vivo role is not known.

To investigate the prevalence of the ETT2 in pathogenic *E. coli* isolates of different animal origins, the PCR targeting to 35 individual genes of the ETT2 locus were established and were submitted to detection of the presence of ETT2 in 168 *E. coli* isolates. The experimental data suggest that the ETT2, including the intact form or 10 different gene deletion mutants, was significantly more prevalent in 92 *E. coli* isolates from weaned piglets with edema and/or diarrhea than that in 76 *E. coli* isolates from cows with mastitis. In addition, the intact ETT2 was the dominant genotype in the pathogenic *E. coli* isolates of porcine origin with a closer association with shigatoxin type 2e, while the deletion mutants were more prevalent in the *E. coli* isolates of bovine origin without distinct association with other virulence factors. Although the detailed reason(s) remains to be defined, it is possible that such difference could be due to different tissue origins (intestine vs. mammary gland) for bacterial isolation. This claim remains to be verified by further investigation.

Among 10 ETT2 deletion mutants, most of them had 6–9 gene deletions in their right regions, which encode the proteins responsible for bacterial invasion and/or intracellular survival [18]. These data indicate that ETT2 is a pathogenicity island not only for *E. coli* isolates of human origin [18], but also for those of animal origins, which will be further investigated in our future studies using gene deletion technique.

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