Virulence Genes and Antimicrobial Susceptibilities of Hemolytic and Nonhemolytic *Escherichia coli* Isolated from Post-Weaning Piglets in Central Thailand

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ABSTRACT. The purpose of this study was to compare the existence of virulence genes in hemolytic *Escherichia coli* (HEC) and non-hemolytic *E. coli* (NHEC) isolated from weaner pigs in Thailand, and to determine their susceptibility to 10 antimicrobial agents. A total of 304 *E. coli* isolates were obtained from 90 piglets with diarrhea and 110 healthy piglets. Of these, 74 HEC isolates were obtained from 70 pigs with diarrhea, and 4 were obtained from 4 healthy pigs, while 190 and 40 NHEC were recovered from 110 healthy and 20 pigs with diarrhea, respectively. A ten digoxigenin (DIG)-labeled probe system was utilized for detecting genes encoding virulence-associated toxins and proteins in these isolates, and the minimal inhibitory concentration values against 10 antimicrobials were determined by means of the agar dilution technique. In total, 70.3% of the HEC isolates contained an exotoxin gene, *lth*, *estp* or *stx2e*, whereas 2.6% of the NHEC isolates hybridized with a gene probe for *estp* or *stx2e*. Over 90% of the isolates were resistant to most agents other than colistin and halquinol. The MIC₉₀ values of the HEC isolates for halquinol and colistin were 4 and 8 times greater than those of the NHEC isolates, respectively. The results represent the first characterization of resistant pathogenic *E. coli* distributed in the Thai pig industry. Amongst the HEC isolates, there appeared to be an association between the presence of some exotoxin genes, including *lth*, *estp* and *stx2e*, and reduced antimicrobial susceptibility.

KEY WORDS: antimicrobial resistance, *Escherichia coli*, hemolysis, swine, virulence gene.

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Escherichia coli is regarded as a commensal bacterium of the gastrointestinal tract, but individual strains may also cause a broad variety of intestinal and extraintestinal diseases in animals and humans [24, 32]. According to their possession of distinct virulence genes, pathogenic *E. coli* strains are classified into several major categories, i.e., enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic/shiga toxin producing *E. coli* (EHEC/STEC), enteroinvasive (EIEC), enteroaggregative (EAaggEC) and the diffusely-adherent group (DAEC) [15, 31]. In piglets, ETEC predominantly cause neonatal and postweaning diarrhea by producing heat-labile (LTh) and heat-stable (ST) enterotoxins [38, 41]. LTh toxin shares 82% amino acid homology with cholera toxin, which leads to activation of adenyl cyclase and chloride ion efflux [19]. ST is a methanol soluble heat-stable toxin which binds to membrane bound guanylyl cyclase C (GC-C) on the intestinal epithelium leading to increases in cGMP concentration, thereby causing loss of fluid in the intestinal lumen [34]. Shiga toxin2e (Stx2e) is produced by STEC strains responsible for edema disease in pigs [12]. Besides these toxins, the β -hemolytic property is thought to be an important virulence factor in pathogenic strains of *E. coli* [16]. In extraintestinal infections, hemolysin is a component necessary for com-

plete virulence in pneumonic lesions in a rat model and increases sepsis of subcutaneous healing in a mouse model [17].

Antimicrobials are regularly used to control diarrhea in Thai pig farming. In general, the disease is routinely controlled by supplementing antimicrobial agents in the feed of weaned piglets for three to six weeks, depending on the clinical signs in the pigs. In developing countries including Thailand, routine detection of suspected pathogenic *E. coli* strains is primarily accomplished by hemolysin production on blood agar media, and the strains are subsequently used for susceptibility testing. The aims of this study were to characterize the virulence gene profiles of HEC and NHEC isolates obtained from post-weaning piglets in commercial swine herds in Thailand and to determine their minimal inhibitory concentration (MIC) values for 10 antimicrobial agents.

MATERIALS AND METHODS

Sample collection: Fecal samples for bacterial isolation were collected from 12 swine commercial herds in Thailand during a three-year period from January 2006 to December 2008. The herds were located in the Chancherngsao, Nakhonpratom and Rajchaburi provinces in central Thailand, and all herds comprised over 1,000 sows. In these herds, endemic colibacillosis in post-weaning pigs had been diagnosed by isolation of β -hemolytic *E. coli*. The fecal samples were collected from a total of 200 post-weaning piglets aged

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4 to 8 weeks (90 piglets with diarrhea and 110 piglets without enteric symptoms) by direct rectal sampling of each individual piglet. Approximately 10 grams of feces was obtained, kept at 4°C and submitted to the laboratory within 12 hr. Data about each piglet including herd, age, clinical symptoms and antibiotics used in its feed were also recorded.

Bacterial isolation: In cases where HEC was present on the primary plate as the major population, one colony of HEC was collected for characterization. Additionally, one NHEC was also collected when it was observed together with HEC on the same plate. Where there was no HEC on the plate, at least one colony of NHEC was selected. Bacterial isolation and primary identification were performed according to routine microbiological diagnostic methods, including conducting the indole test, methyl red test, growth condition on eosin methylene blue agar, citrate utilization and a Voges-Proskauer (VP) test [20]. Beta-hemolytic *E. coli* was characterized by complete lysis of red blood cells on the agar.

Hybridization for DIG-labeled probes: Plasmid DNA from *E. coli* K12 containing virulence DNA fragments were extracted, purified and labeled by a random-primed assay using a digoxigenin labeling kit (Roche Applied Sciences, Munich, Germany). These Dig-labeled DNA probes were used for detecting genes for human heat-labile enterotoxin (*elth*); human heat-stable enterotoxin (*esth*); porcine heat-stable enterotoxin (*estp*); shiga toxin 1 (*stx1*); shiga-like toxin type2e (*stx2e*); invasion-associated locus of the invasion plasmid in EIEC (*ial*); bundle-forming pilin A (*bfpA*); enteroadherent factor plasmid (*eaf*), effacing and attachment factor (*eae*); and aggregative adherent factors (*aaf*), using the standard operating procedures of the Department of Enteric Diseases of the Armed Forces Research Institute of Medical Science, Thailand [2, 9]. Briefly, the bacterial colonies were spotted and cultured on an N+ nylon membrane (Amersham, Braunschweig, Germany) on dry Trypticase Soy agar plates. After cell lysis and fixation of the DNA samples, the samples were prehybridized with standard prehybridization buffer. Then, 10–20 ng/ml of the Dig-labeled DNA probes in hybridization buffer were reacted with the DNA samples on a nylon membrane at 42°C overnight. After hybridization, positive spots were visualized by colorimetric detection using a digoxigenin detection system (Roche Applied Sciences).

Seven reference strains harboring virulence plasmids or virulence DNA fragments, 933-J (*stx1*), P27 phage (*stx2e*), AS-04-1 (*eae*), pMSD 207 (*bfpA*), pMAR-22 (*eaf*), *E. coli* No. 2 (*ial*), and pCVD432 (*aaf*), were included as positive controls for STEC, EPEC, EIEC and EAaggEC, respectively. Three reference strains harboring enterotoxin genes, pEWD299 (*elth*), pDAS100 (*estp*) and pDAS101 (*esth*), were included as ETEC controls. *E. coli* XAC was used as a negative control.

Antimicrobials: The antimicrobials tested were from Sigma (St. Louis, MO, U.S.A.) except for halquinol, which was from Novartis (Basel, Switzerland). They included

colistin sulfate, streptomycin sulfate, amoxicillin trihydrate, enrofloxacin-d5 hydrochloride, nalidixic acid, doxycycline hyclate, chlortetracycline hydrochloride, tetracycline hydrochloride, and sulfamethoxazole-trimethoprim lactate salt and halquinol. These were dissolved in accordance with the Clinical Laboratory Standards Institute guidelines [7, 8]. All suspensions were kept at 4°C and used within 72 hr.

In vitro susceptibility test: An agar dilution test was carried out to determine the MIC values among the 304 isolates for the 10 antimicrobials. The antimicrobial agents were diluted from 1.25 to 1,280 µg/ml and mixed in Mueller-Hinton Agar (Difco, Detroit, MI, U.S.A.) with a final concentration of 0.12 to 128 µg/ml. The inoculum size was equivalent to a 0.5 McFarland concentration (1.5×10^8 CFU/ml) [7]. After inoculation, all plates were incubated at 37°C for 18–24 hr. The concentrations of the antimicrobials inhibiting visible growth of 50 and 90% of the *E. coli* isolates were interpreted as the MIC₅₀ and MIC₉₀, respectively. The resistance breakpoints for the isolates were those described by the National Committee for Clinical Laboratory Standards (NCCLS) [28], excepted for the breakpoints for streptomycin (64 µg/ml) and colistin (8 µg/ml), which were based on previously agreed values [4, 11]. *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference strains. For MIC determination, *E. coli* field isolates were cultured on Trypticase Soy agar at 37°C for 24 hr for preparation of the inoculum and then grown on Muller-Hinton agar [10].

Statistical analysis: The statistical analyses were carried out using Statistical Analysis System (SAS) version 9.0 (SAS Institute, Cary, NC, U.S.A.). The association between hemolytic activity (HEC and NHEC) and the expression of enteric virulence factors (LTh, STp, Stx2e and STp+Stx2e) was analyzed using Fisher's exact test. The rates of antimicrobial resistance were compared between HEC and NHEC using the Chi-squared test. $P < 0.05$ was regarded as indicating statistical significance.

RESULTS

Bacterial isolation: Seventy HEC isolates were obtained from piglets with symptoms of colibacillosis, while only four HEC isolates were obtained from four normal piglets. A total of 230 NHEC isolates were obtained from 40 and 190 isolates of piglets with and without diarrhea, respectively.

DNA probe hybridization: The distributions of the exotoxin genes in the HEC and NHEC isolates are presented in Table 1. DNA-encoding virulence genes were found in 70.3% (54/74) of the HEC isolates and in 2% (6/230) of the NHEC isolates ($P < 0.001$). The presence of *estp* in combination with *stx2e* was detected in 27% of the HEC isolates. In contrast, the genes encoding *esth*, *stx1*, *ial*, *bfpA*, *edf*, *eae* and *eag* were not detected.

Antimicrobial susceptibility: The MIC values and MIC distributions of the 304 isolates against the 10 antimicrobial agents are shown in Table 2 and Table 3, respectively. Over

Table 1. Comparison of the numbers and percentages of exotoxin genes detected amongst 74 hemolytic *E. coli* (HEC) and 230 non hemolytic *E. coli* (NHEC) isolates

Exotoxins	Bacterial isolations		Significance
	HEC	NHEC	
LTh	12 (16.2%)	0 (0.0)	$P<0.001$
Single STp	8 (10.8%)	4 (1.7%)	$P=0.002$
Single Stx2e	12 (16.2%)	2 (0.8%)	$P<0.001$
STp+Stx2e*	20 (27.0%)	0 (0.0)	$P<0.001$
Total	52 (70.2%)	2 (2.6%)	$P<0.001$

Abbreviations: LT, heat labile toxin; STp, porcine heat stable toxin; Stx2e, shiga-like toxin type variant 2e.

90% of the HEC and NHEC isolates had MIC values higher than the resistance breakpoints of amoxicillin, chlortetracycline, tetracycline, nalidixic acid and sulfamethoxazole/trimethoprim. The MIC₅₀ and MIC₉₀ values for NHEC were less than the breakpoints of colistin and enrofloxacin, but had an intermediate range for doxycycline. The values for HEC were higher than those of the NHEC. When considering HEC/NHEC isolated from the same pig, the MIC values of the HEC were higher than those of the NHEC. The MIC₅₀ and MIC₉₀ values were 2–32- and 2–4-fold higher in the HEC population, respectively. Compared with NHEC, HEC had a higher rate of antimicrobial resistance for streptomycin ($P<0.001$), colistin ($P<0.001$), doxycycline ($P=0.027$) and enrofloxacin ($P<0.001$). For other anti-

Table 2. Antimicrobial susceptibilities of the 74 HEC and 230 NHEC isolates against 10 antimicrobial agents

Antimicrobials	MIC ₅₀ (μg/ml)		MIC ₉₀ (μg/ml)		MIC ranges		Resistance Breakpoint μg/ml	Resistance rate		
	HEC	NHEC	HEC	NHEC	HEC	NHEC		HEC	NHEC	Total
Amoxicillin	>256	128	>256	128	>256	128–>256	16	100%	100%	100%
Chlortetracycline	>256	128	>256	128	>256	64–>256	16	100%	100%	100%
Nalidixic	>256	128	>256	128	>256	16–>256	32	100%	80%	90.1%
Streptomycin	128	8	256	32	16–256	0.25–>256	64	93.3%	24%	40.8%
Tetracycline	128	64	128	128	64–128	4–256	16	100%	85.7%	89.1%
Trimethoprim/Sulfamethoxazole	>8/152	>8/152	>8/152	>8/125	4/76–>8/152	0.06/1.2–>8/152	4/76	100%	91.8%	93.8%
Colistin ^{a)}	8	1	16	2	1–32	0.25–32	8	51.3%	5.2%	16.4%
Doxycycline ^{b)}	32	16	64	32	4–128	0.125–128	16	68.9%	54.3%	57.9%
Enrofloxacin ^{a)}	16	0.5	32	16	0.25–128	0.25–128	2	87.8%	35.6%	48.4%
Halquinol	32	8	32	8	8–32	8–32	–*	–	–	–

MIC₅₀: minimal inhibitory concentration value of the agents inhibiting 50% of the number of isolates.

MIC₉₀: minimal inhibitory concentration value of the agents inhibiting 90% of the number of isolates.

HEC: hemolytic *E. coli*.

NHEC: non-hemolytic *E. coli*.

Names in bold indicate the agents effective against *E. coli* isolates.

* Breakpoint is not available.

a) indicates a significant difference of resistance rate between HEC and NHEC at $P<0.001$.

b) indicates a significant difference of resistance rate between HEC and NHEC at $P=0.027$.

Table 3. Distribution of the MIC values of the HEC (n=74) and NHEC (n=230)

Antimicrobial agents	Concentrations of MIC distribution $\mu\text{g/ml}$)																															
	0.125		0.25		0.5		1		2		4		8		16		32		64		128		256		>256							
	HEC	NHEC	HEC	NHEC	HEC	NHEC	HEC	NHEC	HEC	NHEC	HEC	NHEC	HEC	NHEC	HEC	NHEC	HEC	NHEC	HEC	NHEC	HEC	NHEC	HEC	NHEC	HEC	NHEC						
Amoxicillin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	34	0	40	74	156						
Chlortetracycline	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	27	10	40	23	3	2	4	177						
Nalidixic	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	30	0	28	0	24	0	128	0	13	74	7						
Streptomycin	0	0	0	14	0	5	0	20	0	15	0	29	0	50	5	52	16	25	12	5	20	11	21	14	0	0						
Tetracycline	0	0	0	0	0	0	0	0	0	0	8	0	25	0	12	0	40	34	52	40	69	0	14	0	10							
Colistin	0	0	0	15	0	47	23	60	10	86	3	10	30	9	4	2	4	1	0	0	0	0	0	0	0	0						
Doxycycline	0	2	0	0	0	5	0	35	0	30	4	21	19	12	5	54	20	51	20	12	6	8	0	0	0	0						
Enrofloxacin	1	44	6	48	2	26	0	30	0	12	6	34	5	1	19	29	32	3	0	0	3	3	0	0	0	0						
Halquinol	0	0	0	0	0	0	0	0	0	0	0	0	70	10	1	15	3	205	0	0	0	0	0	0	0	0						
Concentrations ($\mu\text{g/ml}$)									0.06/1.2		0.125/2.4		0.25/4.8		0.5/9.5		1/19		2/38		4/76		8/152		>8/152							
Trimethoprim/Sulfamethoxazole									0		1		0		15		0		0		1		9		4		15		69		187	

The MIC susceptibility breakpoint value of each antimicrobial is indicated in bold.

crobials, i.e., amoxicillin, chlortetracycline, nalidixic acid, tetracycline and trimethoprim/sulfamethoxazole, the resistance rates were not significantly different between NHEC and HEC ($P > 0.05$; Tables 2 and 3).

DISCUSSION

β -hemolysin is a pore-forming cytotoxin causing cells to swell and eventually burst [21]. Even though the hemolysis traits of *E. coli* isolates have been used as presumptive evidence of virulence, the pathogenesis involves exotoxin-mediated secretion into the gut lumen [3, 26, 33]. Therefore, routine diagnosis of pathogenic field strains of *E. coli* on the basis of hemolysis might not provide sufficient evidence of their pathogenic potential. Many virulence factors have been identified and are mainly encoded on plasmids or on chromosomal regions that are detectable by DNA probe hybridization [5, 14, 30]. In the current study, over 70% of the HEC isolates tested harbored genes encoding for LTh, STp and Stx2e, indicating the existence of endemic ETEC and STEC in our study area. These isolates were strongly associated with watery diarrhea based on the clinical history, but no necropsies were performed on the affected pigs. A few of the NHEC isolates were shown to have genes encoding enterotoxins, indicating that they were ETEC and STEC, respectively. On the other hand, about 30% of the HEC isolates had no detectable toxin genes. Therefore, these findings confirmed that not all the HEC isolates were entirely representative of the pathotypes present [18, 40]. Interestingly, we also found hybridization with combinations of both *estp* and *stx2e* among the HEC isolates, and this might indicate a highly pathogenic endemic strain in our study area.

Using DIG-labeled probes, the number of strains that hybridized with the *elt* gene probe was lower than in previous studies, even in healthy pigs [5, 6]. On the other hand, only 32% of *E. coli* isolates derived from diarrheal pigs in Zimbabwe were found to possess enterotoxin and fimbrial genes by using multiplex PCR, respectively [22]. In Brazil, toxin genes (*elt*, *estp*, *stx1*, *stx2* and *eaeA*), either individually or combined, were present in most of the strains of *E. coli* from pigs with diarrhea and in 42.8% of the strains from pigs without diarrhea [23]. In Guwahati, *E. coli* derived from pigs with diarrhea possessed *stx2* and *eae*, causing edema lesion and becoming a public health concern [1]. On the other hand, typing of diarrheagenic *E. coli* (DEC) from human patients with diarrhea showed that EAST1EC (EAggEC with heat-stable enterotoxin 1) had the highest prevalence, followed by EPEC, EHEC, EAggEC and ETEC, respectively [13]. The prevalence was related to the finding in pigs that EAST1EC is distributed among porcine ETEC strains [39]. The virulence genes defining EAggEC, EPEC and EIEC could not be detected in the tested porcine strains, but ETEC was clearly confirmed as the common pathotype among the Thai porcine isolates in the current study. However, the finding that six NHEC isolates from non-diarrheal pigs contained *estp* or *stx2e* suggests that they

could be a source of distribution of these genes [27]. In Thailand, combinations of antimicrobials are used as feed additives as recommended by the manufacturers. The common antimicrobials used in sow feed both during gestation and the lactation period are chlortetracycline, sulfonamide/trimethoprim, amoxicillin, tiamulin and/or tylosin. Piglets are given feed that also routinely contains 1–3 kinds of antimicrobial drugs from 4–9 weeks of age. The antimicrobials are given to piglets to control diarrhea and respiratory problems during the postweaning period. In this study, most of the *E. coli* isolates were resistant not only to amoxicillin, chlortetracycline and sulfamethoxazole/trimethoprim, which are related to the routine antimicrobials used, but also to streptomycin and nalidixic acid. Previously, use of oxytetracycline and chlortetracycline in pig farms was closely associated with a high prevalence of resistance to chloramphenicol and sulfisoxazole [29]. Therefore, our results confirm the variant resistance patterns induced by routine use of antimicrobial administration. *In vitro*, colistin was the most effective antimicrobial for the tested isolates, followed by halquinol, enrofloxacin and doxycycline.

Recently, colistin, polymyxin E, was found to be satisfactory for controlling multi-resistant Gram-negative bacilli obtained from patients in Canadian hospitals [37], but it might not be useful in all porcine strains because 24% of the isolates were resistant to colistin in this study. Most of the HEC isolates containing virulence factors seemed to have a greater ability to tolerate this antimicrobial selective pressure than the NHEC isolates. Previously, a weak positive correlation was reported between hemolysin production and resistance to tetracycline and chloramphenicol in chicken strains of *E. coli* [42]; the genes for these phenotypes were located on the same plasmid, and this was also detected in an isolate from a patient with EHEC [36]. Moreover, the resistance phenotype was associated with biofilm formation, resulting in increased pathogenicity [35]. With regard to enteric pathogens, a possible explanation for the reduced sensitivity of *E. coli* to multiple antimicrobials is that the organisms are involved in a high rate of plasmid exchange. The gut is colonized with abundant bacterial species in close proximity, resulting in an increased probability of interspecies conjugation, such as with the plasmid RepFI of the family *Enterobacteriaceae* [25].

In conclusion, *E. coli* derived from Thai pigs with and without diarrhea was characterized. Genes encoding LTh, STp and Stx2e were detected in 74.3% of the HEC isolates, whereas only 2.6% of the NHEC isolates had *estp* and *stx2e*. Over 90% of the isolates were resistant to most of the antimicrobials examined in this study.

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