ANTIBIOGRAMS AND VIRULENCE GENES OF *ESCHERICHIA COLI* AND *SALMONELLA* SP FROM FARM PIGS IN CENTRAL THAILAND

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Abstract. The increasing global trend of antimicrobial resistance (AMR), especially in pig production, can pose a threat to human health. The study investigated the prevalence of AMR in *Escherichia coli* and *Salmonella* serovars from farm pigs in central Thailand. *E. coli* and *Salmonella* serovars from pig stool specimens were identified by standard methods, susceptibility to antimicrobials by the disk agar diffusion method and pathogenic *E. coli* virulence genes by multiplex PCR. *E. coli* isolates harboring *astA* were the most predominant (27%), followed by *eaeA* (9%). *E. coli* isolates with virulence genes were present significantly more in pigs with loose stool (70%) than those with normal stool (40%) (p-value <0.001). The overall prevalence of *Salmonella* sp isolates in pigs was 44.3%, with *S.* Agona serovar predominant (27%). *E. coli* and *Salmonella* sp isolates demonstrated predominant resistance to penicillin (71 and 79% respectively), followed by tetracycline (67.5 and 73.1%), then streptomycin (52 and 51%), and sulfonamide (45 and 36%), with multidrug resistance (MDR) (≥3 antimicrobial classes) in 66 and 74% *E. coli* and *Salmonella* sp isolates, respectively. *E. coli* harboring a single *astA* was significantly associated with MDR phenotype compared to those without any virulence gene (odds ratio = 2.16, 95% confidence interval: 1.09-4.29). In conclusion, the study indicates that farm pigs in central Thailand could be potential sources of foodborne MDR *E. coli* and *Salmonella* sp.

Keywords: *Escherichia coli*, *Salmonella* serovar, antimicrobial resistance, farm pig, stool, virulence gene, Thailand

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INTRODUCTION

Pathogenic *Escherichia coli* and *Salmonella* spp are zoonotic pathogens, which cause foodborne infections of major public health concern (Tauxe *et al*, 2010; Tadee *et al*, 2015). Bacteria with and without antimicrobial resistance (AMR) are frequently found in farm pigs and porcine production system, resulting in infection of both animals and humans (Chlebicz and Slizewska, 2018; Zhang *et al*, 2019).

Several pathogenic *E. coli* strains cause diarrhea in farm pigs resulting in morbidity and mortality, which can lead to significant economic loss (Fairbrother *et al*, 2005; Nagy and Fekete, 2005). Pathogenic *E. coli* strains are classified into seven major pathotypes based on their virulence factors, clinical manifestations of disease, epidemiology, and phylogenetic profile, namely, 1) enteropathogenic *E. coli* (EPEC), 2) enterohemorrhagic *E. coli* (EHEC) or Shiga toxin-producing *E. coli* (STEC) or verocytotoxin-producing *E. coli*, 3) enteroinvasive *E. coli* (EIEC), 4) enteroaggregative *E. coli* (EAEC), 5) enterotoxigenic *E. coli* (ETEC), 6) diffusely adherent *E. coli* (DAEC), and 7) adherent invasive *E. coli*. Human infections from EIEC, atypical EPEC, ETEC, and STEC present in animal reservoirs stem from fecal/oral transmission, direct contact and/or consumption of contaminated recreation/drinking water (Croxen *et al*, 2013).

Farm pigs constitute reservoirs of pathogenic *E. coli* and *Salmonella* spp that can cause severe illness in humans (Mathole *et al*, 2017; Chlebicz and Slizewska *et al*, 2018). In addition to treating infected-farm animals, antibiotics are widely used to prevent microbial infection and enhance animal growth, resulting in rapid development of AMR bacteria that adversely affect food safety. In addition, antimicrobial resistance genes can be disseminated to other intestinal microorganisms (Lay *et al*, 2012; WHO, 2014). Several virulence genes, *eg, eae, F4, lt, stx*, and *stx2*, are present in diarrheal pigs (Bessone *et al*, 2017).

Currently, large-scale pig farms for breeding, fattening and provision of pork and pork products are widely distributed in central

Thailand, particularly in suburban areas surrounding cities, including Bangkok Metropolitan region, owing to convenience in transporting pork products to the main markets and ready access to pig feed (Thanapongtharm *et al*, 2016). In Pathum Thani Province, adjacent to Bangkok, pig commercial farming consists of 3 major types of pig farming system. Those are finishing systems (66.2%), followed by small-holders system (26.5%) and breeding systems (7.3%). About half (50.8%) of pig farms in Thailand are small scale holdings raising 50-500 pigs per farm (Department of Livestock Development, 2020). Moreover, a study reported antibiotic use mostly in finishing farms (68.5%), and commercial farms (62.1%) (Lekagul *et al*, 2020). If AMR bacteria are prevalent in a pig farm, there is a high chance of these bacteria being transmitted to other pig farms, those working on the farms and ultimately to consumers, resulting in outbreaks.

Data on virulence genes and AMR in *E. coli* and *Salmonella* serovars in central Thailand are scarce even though there have been many reports in other parts of the country (Dawangpa *et al*, 2022). Thus, this study investigated prevalence of *E. coli* strains and *Salmonella* serovars, presence of virulence genes and their antibiograms in farm pigs specifically from finishing system in Pathum Thani Province, central Thailand following the evidence of prominent antibiotic use in pig farm as mentioned earlier. The findings can specify antibiograms in this area where pig farming is overwhelmed to indicate the level of risk to human. A regular monitoring based on the findings could be performed in order to control and prevent the risk in transferring affected bacteria from swine to human as an ultimate goal.

MATERIALS AND METHODS

Study sites

The study sites were located in Muang Pathum Thani and Klongluang Districts, Pathum Thani Province, central Thailand, where there is a high density of small-scale pig farms (50-500 pigs per farm) (Thanapongtharm *et al*, 2016). Pig stool specimens were collected in July 2015.

Sample size calculation

Based on an estimated prevalence of 50% (highest value), the required sample size to obtain 10 % absolute precision and 95 % significance is calculated to be 96 samples (Thrusfield, 2005). A two-stage cluster sampling was performed and the required sample size for multi-stage sampling is twice the calculated sample size using the usual design effect of cluster sampling (2.0) (WHO, 2005), resulting in a minimum sample size calculation of 192 samples within 20 clusters, regarded as the minimum number of clusters. Pig fecal samples (n = 10-12)

were randomly collected from each farm (n = 20) (WFP, 2004).

Samples collection

Pig farms were randomly chosen for the study. Fattening pigs were selected study animals as they constitute the major source of bacterial transmission to humans. At each farm, pigs (n = 2) from pens (n = 5) were randomly selected, and each pig was handled according to the 3Rs (Fenwick *et al*, 2009) to reduce stress. Pig defecation were stimulated manually (gloved hands) and approximately 10-20 g of stool were collected per animal (which was then marked to avoid duplicate stool collection). Each stool sample was placed in a covered container, immediately placed on ice and transferred to the laboratory within 6 hours for bacterial isolation. Loose stool is defined as liquid stool with no solid form at the time of specimen collection.

Study protocols were approved by the Institutional Animal Ethics Committee, Thammasat University (project no. 008/2558). All pig farm owners were provided with information regarding the study, and prior written informed consent was obtained from each farm owner.

Isolation and identification of *E. coli* and *Salmonella* sp

Pig fecal samples were directly steaked onto MacConkey (MC) agar (Nissui, Tokyo, Japan) for isolation of *E. coli*. Suspected typical colonies positive for lactose fermentation on MC were picked and further inoculated into Triple Sugar Iron and Indole-Methyl red-Voges Proskauer-Citrate (IMViC) solutions for biochemical confirmation of *E. coli* (Adams and Moss (2000). For isolation of *Salmonella* sp, the same samples were placed in Buffer-Peptone-Water (BPW) solution (Oxoid Ltd, Basingstoke, United Kingdom) and incubated at 37°C for 18-24 hours. Then an aliquot of each culture was transferred onto modified semi-solid Rappaport Vassiliadis (MSRV) agar (Oxoid Ltd, Basingstoke, United Kingdom) (Suthienkul *et al*, 1995). For biochemical confirmation, the swarming growth on MSRV isolates were selected from each individual sample for further biochemical identification using the Triple Sugar Iron (Oxoid Ltd, Basingstoke, United Kingdom), Lysine-Indole Motile (Oxoid Ltd, Basingstoke, United Kingdom), and urease tests (Oxoid Ltd, Basingstoke, United Kingdom) (Adams and Moss, 2000). Cultures identified as *Salmonella* were subsequently serotyped as described below. Each *E. coli* and *Salmonella* isolate was kept as stocks in 20 % glycerol at -80°C until used.

***Salmonella* serotyping**

Salmonella serogroups were determined using polyvalent antisera against O and H antigens (Serotest Reagent Inc, Bangkok, Thailand). *Salmonella* serovars were confirmed

by the WHO National *Salmonella* and *Shigella* Center, National Institute of Health, Department of Medical Sciences at Ministry of Public Health, Nonthaburi Province, Thailand.

Pathogenic *E. coli* virulence genes determination

DNA was isolated from *E. coli* isolate using a boiling method (Peng *et al*, 2013). In brief, test *E. coli* isolate was grown in Luria-Bertani (LB) broth (Difco, Detroit, MI) at 37°C for 18-24 hours. Then, one ml aliquot of culture was sedimented and pellet washed with TE buffer (Takara Bio Inc, Shiga, Japan) and then boiled at 100°C for 10 minutes. The tube was immediately placed on ice for 5 minutes and DNA pelleted by centrifugation at 10,000 × g for 2 minutes. DNA was dissolved in 0.2 ml of TE buffer and stored at -20°C until used.

Determination of *E. coli* virulence genes was performed using a multiplex PCR method employing 10 sets of primers to amplify the following virulence genes: DAEC *aidA*, EAEC *astA*, EHEC *eaeA* and *stx2A*, EPEC *eaeA* and *paa*, and ETEC *F4*, *F5*, *F41*, *lt*, and *stp* (Table 1). For detection of *eaeA*, *lt*, *stp*, and *stx2A*, PCR mixture contained 1X PCR buffer (Promega, Madison, WI), 2 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.5 µl of each primer (0.5 mM), 1.25 U *Taq* polymerase (Promega, Madison, WI), 10 µl of DNA template, and deionized water to make up final volume (50 µl). Thermocycling was carried out in a Px2 Hybrid Thermal Cycler (Thermo Fisher Scientific, Waltham, MA) as follows: 95°C for 5 minutes; 35 cycles of 95°C for 60 seconds, 52°C for 60 seconds and 72°C for 60 seconds; with a final step of 72°C for 5 minutes (Pachanon *et al*, 2013). For detection of *aidA*, *astA*, *F4*, *F5*, *F41*, and *paa*, the reaction mixture contained 10X PCR buffer (Promega, Madison, WI), 1 mM dNTPs, 0.4 pmol of each primer except 0.8 pmol each of EAST1-F and EAST1-R, 2.5 U of *Taq* polymerase (Promega, Madison, WI), 3 µl of DNA template, and deionized water to make up final volume (50 µl). Thermocycling was carried out as described above but with the following conditions: 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 57°C for *aidA*, *astA* and *paa* or 61°C for *F4*, F5, and *F41* for 30 seconds and 72°C for 60 seconds; with a final step of 72°C for 7 minutes (Lee *et al*, 2008; Prapasawat *et al*, 2017).

Amplicons (Table 1) were separated by 2.0% agarose gel-electrophoresis and visualized by staining with GelRed™ dye (Thermo Fisher Scientific, Waltham, MA) and recorded with a PrepOne™ system (Embi Tec, San Diego, CA).

Antibiogram profiling

Antimicrobial susceptibility was determined using a disk diffusion method according to the Clinical and Laboratory Standards Institute's guidelines (CLSI, 2013),

Table 1

Primers used for amplification of pathogenic *Escherichia coli* virulence genes

Subgroup	Pathotype	Virulence gene	Primer name	Sequence (5'→3')	Amplicon size (bp)	Reference
1A	Enteropathogenic *E. coli* (EPEC)	*eaeA*	eaeA/1	GCGATTACGCGAAAGATACC	677	Pachanon *et al* (2013)
	Enterohemorrhagic *E. coli* (EHEC)		eaeA/2	GATAACGGAACTGCATTGAGT		
2A	Enterohemorrhagic *E. coli* (EHEC)	*stx2*A	stx2A-F2	TTGACCATCTTCGTCTGATTATTG	542	Prapasawat *et al* (2017)
			stx2A-R2	CTGATGATGGCAATTCAGTATAAC		
1A	Enterotoxigenic *E. coli* (ETEC)	*lt*	LT-F	ATGACGGATATGTTTCCACTTCTC	393	Prapasawat *et al* (2017)
			LT-R	AACCTTGTGGTGCATGATGAATCC		
2A	Enterotoxigenic *E. coli* (ETEC)	*stp*	STp-F	TTAATAACATCCAGCACAGGCAGG	176	Prapasawat *et al* (2017)
			STp-R	TCCCCTCTTTTAGTCAGTCAACTG		
1B	Enterotoxigenic *E. coli* (ETEC)	*F4*	F4-F	GCCTGGATGACTGGTGATTT	706	Lee *et al* (2008)
			F4-R	TCTGACCGTTTGCAATACCC		
1B	Enterotoxigenic *E. coli* (ETEC)	*F5*	F5-F	TTGGGCAGGCTGCTATTAGT	222	Lee *et al* (2008)
			F5-R	TAGCACCACCAGACCCATTT		

Table 1 (cont)

Subgroup	Pathotype	Virulence gene	Primer name	Sequence (5'→ 3')	Amplicon size (bp)	Reference
1B	Enterotoxigenic *E. coli* (ETEC)	*F41*	F41-F	GGAGCGGGTCATATTGGTAA	941	Lee *et al* (2008)
			F41-R	CTGCAGAAACACCAGATCCA		
2B	Enteroaggregative *E. coli* (EAEC)	*astA*	EAST1-F	CCATCAACACAGTATATCGA	111	Chapman *et al* (2006)
			EAST1-R	GGTCGCGAGTGACGGCTTTGT		
3B	Enteropathogenic *E. coli* (EPEC)	*paa*	pAA-F	CCATAAAGACAGCTTCAGTGAAAA	162	Zhang *et al* (2007)
			pAA-R	GTATTACTGGTACCACCACCATCA		
3B	Diffusely adherent *E. coli* (DAEC)	*aidA*	AIDA-I-F	TGGTGGGAAAA CACTGCTA	771	Lee *et al* (2008)
			AIDA-I-R	TAGCCGCCATCACTAACCAG		

with disks containing the following antimicrobials (Becton Dickinson, Franklin Lakes, NJ): ampicillin (AM, 10 μg), chloramphenicol (C, 30 μg), cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), doxycycline (D, 30 μg), gentamicin (GM, 10 μg), kanamycin (KAN, 30 μg), nalidixic acid (NA, 30 μg), streptomycin (S, 10 μg), sulfamethoxazole-trimethoprim (SXT, 1.25/23.75 μg), and tetracycline (TE, 30 μg). *E. coli* ATCC 25922 was used as reference strain. Multidrug resistance (MDR) is defined as an isolate resistant to three or more separate classes of antimicrobials (Magiorakos *et al*, 2012).

Statistical analysis

Prevalence of virulence genes in *E. coli* and *Salmonella* sp were compared using Chi-square or Fisher's exact tests at 5% significance. Odds ratio of virulence gene profile in MDR isolate is calculated using R ver. 3.3.0 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Prevalence of *E. coli* and *Salmonella* sp in farm pig stool samples

From suspected isolates (n = 1,157) from 235 pig stools (189 normal and 46 loose) randomly collected from 20 farms in central Thailand, 930 (80.4%) isolates were biochemically confirmed to be *E. coli*, a prevalence in stool samples of 97% (229/235). Positivity rate of *E. coli* detection in normal and loose stool was 97 (183/189) and 100% (46/46) respectively

Of 394 suspected isolates, 260 (66.0%) were confirmed to be *Salmonella* sp, a prevalence in stool samples of 44% (104/235). Positivity rate of *Salmonella* sp detection in normal and loose stool was 42% (80/189) and 52% (24/46) respectively. Predominant *Salmonella* serovar was *S.* Agona, followed by *S.* 4,5,12:i:-, then *S.* Rissen, and *S.* Anatum (Table 2). Prevalence of *S.* Agona in loose stool is significantly higher compared to that in normal stool (p-value <0.01), but the converse relationship was observed for the other three predominant *Salmonella* serovars (p-value >0.1).

AMR among *E. coli* and *Salmonella* sp isolates

E. coli and *Salmonella* sp isolates were tested for their resistance to eleven antimicrobials from seven classes, the majority of *E. coli* and *Salmonella* sp isolates being resistant to penicillin (61-80%), followed by tetracycline group (39-75%), then quinolone (nalidixic acid) (29-42%), chloramphenicol (25-62%) and sulfonamide (21-47%), with lowest resistance to cephalosporin (ceftazidime) (4-29%) (Table 3). Loose stools had, on the whole, higher rates of AMR compared to normal stools. There is no statistical difference in antimicrobial resistance among *E. coli* isolates with and without virulence genes in both normal and loose stools.

Table 2

Prevalence of *Salmonella* serovars from fecal samples of farm pigs, Pathum Thani Province, central Thailand (July 2015)

Salmonella serovar	Number of Salmonella serovars (%)		
	Total (N = 104)	Normal stool (N = 80)	Loose stool (N = 24)
S. Agona	28 (27)	16 (20)	12 (50)
S. 4,5,12:i:-	16 (15)	14 (17.5)	2 (9)
S. Rissen	15 (14)	14 (17.5)	1 (4)
S. Anatum	13 (12)	11 (14)	2 (9)
S. Kentucky	5 (5)	4 (5)	1 (4)
S. Kedougou	4 (4)	4 (5)	0 (0)
S. Give	4 (4)	4 (5)	0 (0)
S. Schwarzenground	4 (4)	3 (4.5)	1 (4)
S. Stanley	3 (3)	3 (4.5)	0 (0)
S. Braenderup	2 (2)	1 (1)	1 (4)
S. Derby	2 (2)	2 (2)	0 (0)
S. 1,3,19 :-:1w	1 (1)	1 (1)	0 (0)
S. 4,3,19:i:-	1 (1)	0 (0)	1 (4)
S. 6,7:-: enz15	1 (1)	0 (0)	1 (4)
S. Indiana	1 (1)	1 (1)	0 (0)
S. Livingstone	1 (1)	1 (1)	0 (0)
S. Mbandaka	1 (1)	0 (0)	1 (4)
S. Newport	1 (1)	1 (1)	0 (0)
S. Weltevreden	1 (1)	0 (0)	1 (4)

E. coli and *Salmonella* sp isolates demonstrated a similar pattern of drug resistance: no resistance, 17 and 14%; resistance to one class of antimicrobials, 8 and 6% (6/104), resistance to two classes of antimicrobials, 9 and 6%; and MDR, 66 and 74%, respectively (Fig 1).

MDR was present in 74% (77/104) of *Salmonella* serovars. However, MDR

Table 3

Prevalence of antibiotic resistance in *Escherichia coli* and *Salmonella* sp isolates from fecal samples of farm pigs, Pathum Thani Province, central Thailand (July 2015)

Class (number) / antimicrobial	Normal stool			Loose stool		
	Resistant *E. coli* isolates w/o virulence gene (N = 134)	Resistant *E. coli* isolates with virulence gene (N = 76)	Resistant Salmonella isolates (N = 80)	Resistant *E. coli* isolates w/o virulence gene (N = 23)	Resistant *E. coli* isolates with virulence gene (N = 32)	Resistant Salmonella isolates (N = 24)
	n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Penicillin (269)						
Ampicillin	94 (70)	55 (72)	64 (80)	14 (60)	24 (75)	18 (75)
Quinolone (124)						
Nalidixic acid	45 (34)	*27 (35)*	23 (29)	7 (30)	12 (37)	10 (42)
Tetracycline (462)						
Doxycycline	69 (51)	*43 (57)*	54 (67)	9 (39)	15 (47)	17 (71)
Tetracycline	87 (65)	*55 (72)*	58 (72)	13 (56)	24 (75)	18 (75)
Cephalosporin (131)						
Cefotaxime	34 (25)	25 (33)	13 (16)	3 (13)	9 (28)	10 (42)
Ceftazidime	10 (7)	10 (13)	6 (7)	1 (4)	3 (9)	7 (29)
Sulfonamide (157)						
SXT	61 (45)	34 (45)	33 (41)	9 (39)	15 (47)	5 (21)

Table 3 (cont)

Class (number) / antimicrobial	Normal stool			Loose stool		
	Resistant *E. coli* isolates w/o virulence gene (N = 134)	Resistant *E. coli* isolates with virulence gene (N = 76)	Resistant Salmonella isolates (N = 80)	Resistant *E. coli* isolates w/o virulence gene (N = 23)	Resistant *E. coli* isolates with virulence gene (N = 32)	Resistant Salmonella isolates (N = 24)
	n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Phenicol (154)						
Chloramphenicol	61 (45)	31 (41)	20 (25)	11 (48)	20 (62)	11 (46)
Aminoglycoside (356)						
Gentamicin	39 (29)	27 (35)	10 (12)	6 (26)	9 (28)	8 (33)
Kanamycin	19 (14)	13 (17)	12 (15)	5 (22)	8 (25)	8 (33)
Streptomycin	67 (50)	42 (55)	39 (49)	10 (43)	20 (62)	14 (58)

SXT: sulfamethoxazole-trimethoprim; w/o: without

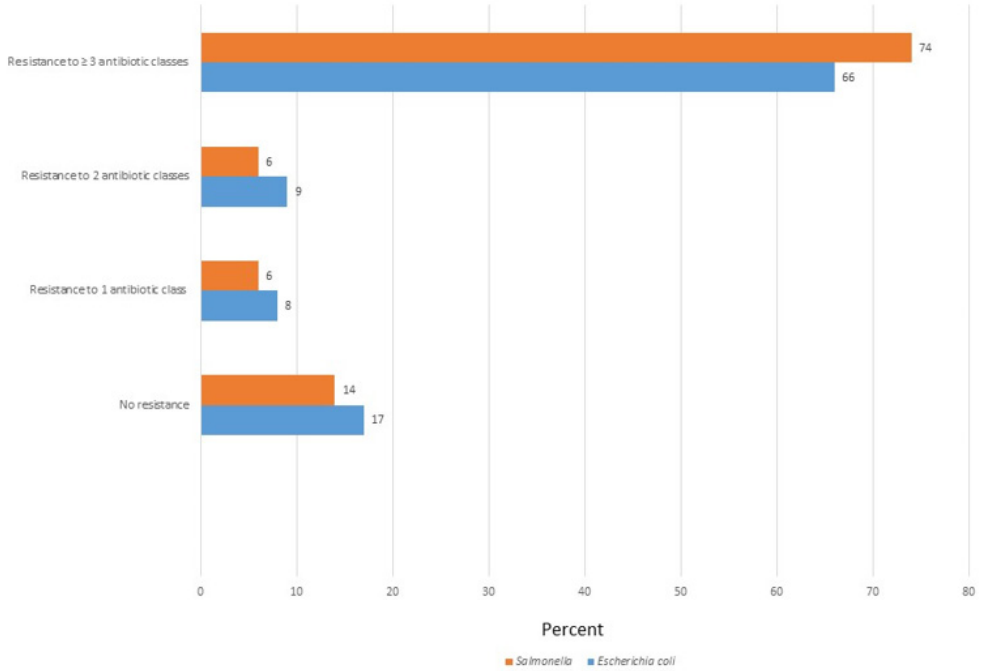

Fig 1 - Percent antimicrobial-resistant *Escherichia coli* (n = 265) and *Salmonella* sp (n = 104) isolates from fecal samples of farm pigs,

Pathum Thani Province, central Thailand (July 2015)

Note: Antimicrobial susceptibility was determined using a disk diffusion method against 11 antibiotics from 7 antimicrobial classes (CLSI, 2013).

prevalence of *S.* Agona, 82% (23/28), is not significantly different to the other serovars, 71% (54/76) (Table 4).

E. coli carriage of virulence genes

E. coli isolates (n = 265) were analyzed for presence of 10 virulence genes using multiplex PCR, results indicating 108 (41%) isolates harbored these genes, with 82 (76%), 23 (21%) and 3 (3%) isolates carrying a single gene, two different and three different genes, respectively (Table 5). Among the virulence genes, *astA* was the most prevalent among all isolates, 27% (71/265), followed by *eaeA*, 9% (24/265). *E. coli* isolates that possessed a single *astA* had a significantly higher MDR prevalence (79%, 48/61) than those without any virulence gene (63%, 99/157, p-value = 0.027) (Table 5).

Table 4

Presence of virulence genes and resistance to antibiotics of *Salmonella* serovars from fecal samples of farm pigs, Pathum Thani Province, central Thailand (July 2015)

Salmonella serovar	Number of isolates	Antibiotic class*								MDR (number of isolates)	Non-MDR (number of isolates)
		Neg	1	2	3	4	5	6	7		
S. Agona	28	4	1	0	1	5	6	11	0	23	5
S. 4,5,12:i:-	16	0	1	1	4	5	3	2	0	14	2
S. Rissen	15	1	1	1	10	2	0	0	0	12	3
S. Anatum	13	1	0	0	3	7	2	0	0	12	1
S. Kentucky	5	0	0	0	3	2	0	0	0	5	0
S. Kedougou	4	4	0	0	0	0	0	0	0	0	4
S. Give	4	0	1	0	1	0	1	0	1	3	1
S. Schwarzenground	4	0	0	0	1	3	0	0	0	4	0
S. Stanley	3	0	0	2	1	0	0	0	0	1	2
S. Braenderup	2	1	1	0	0	0	0	0	0	0	2
S. Derby	2	0	0	1	0	0	1	0	0	1	1
S. 1,3,19 :-:1w	1	0	0	0	0	0	0	1	0	1	0
S. 4,3,19:i:-	1	0	0	0	0	0	1	0	0	1	0
S. 6,7:-: enz15	1	1	0	0	0	0	0	0	0	0	1

Table 4 (cont)

Salmonella serovar	Number of isolates	Antibiotic class*								MDR (number of isolates)	Non-MDR (number of isolates)
		Neg	1	2	3	4	5	6	7		
S. Indiana	1	0	1	0	0	0	0	0	0	0	1
S. Livingstone	1	1	0	0	0	0	0	0	0	0	1
S. Mbandaka	1	1	0	0	0	0	0	0	0	0	1
S. Newport	1	0	0	1	0	0	0	0	0	0	1
S. Weltevreden	1	1	0	0	0	0	0	0	0	0	1
Total	104	15	6	6	24	24	14	14	1	77	27

*From Table 3

MDR: multidrug resistance (resistance to ≥3 antimicrobial classes); Neg: sensitive to all 7 antimicrobial classes; Non-MDR: resistance to <3 antimicrobial classes

Table 5

Presence of virulence genes and resistance to antimicrobials of Escherichia coli isolates from fecal samples of farm pigs, Pathum Thani Province, central Thailand (July 2015)

Number of virulence genes carried	Virulence gene		Number of isolates	Antimicrobial class†								MDR (Number of isolates)	Non-MDR (Number of isolates)	OR (95% CI)	*p*-value*
				Neg	1	2	3	4	5	6	7				
1	*aidA*		4	2	0	0	0	1	1	0	0	2	2	0.63 (0.04-8.31)	0.629
	astA		61	3	3	7	15	6	7	10	10	48	13	2.16 (1.09-4.29)	0.027
	eaeA		10	1	0	1	1	2	3	2	0	8	2	2.33 (0.44-23.3)	0.334
	paa		5	1	0	1	0	1	1	0	1	3	2	0.88 (0.1-10.8)	1.000
	F4		1	1	0	0	0	0	0	0	0	0	1	NA	NA
	stp		1	0	0	0	0	0	0	1	0	1	0	NA	NA
2	*eaeA*	*aidA*	3	2	0	0	0	1	0	0	0	1	2	0.29 (0.00-5.79)	0.557
	aidA	*astA*	3	0	0	0	0	2	1	0	0	3	0	NA	NA
	eaeA	*astA*	2	0	2	0	0	0	0	0	0	0	2	NA	NA
	astA	*paa*	1	0	0	0	0	1	0	0	0	1	0	NA	NA
	astA	*lt*	1	1	0	0	0	0	0	0	0	0	1	NA	NA
	stp	*stx2A*	1	0	0	0	0	1	0	0	0	1	0	NA	NA
	eaeA	*paa*	8	0	2	0	0	0	4	1	1	6	2	1.75 (0.30-18.3)	0.712
	paa	*stp*	1	0	1	0	0	0	0	0	0	0	1	NA	NA
	F5	*stp*	2	0	0	2	0	0	0	0	0	0	2	NA	NA
	F4	*F41*	1	0	0	0	1	0	0	0	0	1	0	NA	NA

Table 5 (cont)

Number of virulence genes carried	Virulence gene	Number of isolates	Antimicrobial class†								MDR (Number of isolates)	Non-MDR (Number of isolates)	OR (95% CI)	*p*-value*
			Neg	1	2	3	4	5	6	7				
3	*aidA astA lt*	1	0	0	0	0	0	1	0	0	1	0	NA	NA
	astA paa stp	1	0	0	0	0	1	0	0	0	1	0	NA	NA
	eaeA astA paa	1	0	0	1	0	0	0	0	0	0	1	NA	NA
+		108	11	8	12	17	16	18	14	12	77	31	1.45 (0.86-2.46)	0.163
-		157	34	12	12	15	18	29	23	14	99	58	-	ref

*Significant when $p<0.05$ compared to reference isolates

†From Table 3

CI: confidence interval; MDR: multidrug resistance (resistance to ≥3 antimicrobial classes); Neg: sensitive to all 7 antimicrobial classes; Non-MDR: resistance to <3 antimicrobial classes; NA: Not Applicable; OR: odds ratio; ref: reference

+: carriage of virulence gene(s); -: no carriage of virulent gene

DISCUSSION

The prevalence of *E. coli* (97%) and *Salmonella* sp (44%) in farm pig stools from central Thailand demonstrated in this study were higher than those found elsewhere in Thailand with the prevalence of *E. coli* accounting for 48-89% (Hanson *et al*, 2002; Sudatip *et al*, 2022), and 2-30% in *Salmonella* (Hanson *et al*, 2002; Tadee *et al*, 2014), and even in organic farm pigs where the prevalence of *Salmonella* accounted for 33% (Tadee *et al*, 2021). Variation of the prevalence could be accounted by differences in type of farming, geographical area, environment, and detection method (Wales *et al*, 2011; Gotter *et al*, 2012). As regards to *Salmonella* serovars, there were differences in the predominant serovars detected in the present study compared to previous studies, namely, *S.* 4,5,12:i:-, *S.* Rissen, and *S.* Anatum being the main serovars in pig and pork products in earlier work (Chuanchuen *et al*, 2008; Tadee *et al*, 2014), while *S.* Agona was the predominant serovar in the present study. Prevalence of *S.* Agona isolates in pigs with loose stool is significantly higher compared to that in normal stool (p-value <0.01). It is worth noting that the predominant *Salmonella* serovars present in farm pigs are generally mirrored by those detected in humans in the same region and similar period of investigation (Bangtrakulnonth *et al*, 2004; Ke *et al*, 2014).

The predominant virulence *astA* gene in *E. coli* from farm pig stools in the current study is associated with EAEC, responsible for persistent diarrhea among children as EAEC can evade the host immune system causing persistent infection, and, in addition, *astA* can be present in other diarrheagenic *E. coli* strains (Yatsuyanagi *et al*, 2003) and be found in both clinical and non-clinical settings (Zhang *et al*, 2016). In the present study *astA* was also present together with one or two other virulence genes, posing a potential risk of virulent infections in humans and animals (Trongjit *et al*, 2016) and dispersion of these highly pathogenic microorganisms into the environment, as reported in Thailand (Dawangpa *et al*, 2022), Southeast Asia (Lay *et al*, 2021) and Canada (Magaña-Lizárraga *et al*, 2022). The present finding of *astA* presence in normal and loose stools has been previously observed (Prapasawat *et al*, 2017). The present study shows that AMR *E. coli* harboring *astA* was 2-fold more frequent that those without any virulence genes. Further investigations on the relationships among carriage of virulence genes, pathogenicity and AMR phenotypes of *E. coli* from pig stools are warranted.

As regards *E. coli* AMR phenotypes, our findings were in agreement with a previous report (Strom *et al*, 2017) from pig farms in Northeastern Thailand, which shows a similar range in resistance rates to C (39.1-58%), CAZ

(3.7-4.3%) and NA (20.2-30.8%), but significantly higher resistance rates to AM (69.6-85.2%), S (37.6-76.5%), SXT (42-84%), and TE (75.3-86.3%), and lower resistance rates to CTX (1.2-1.4%) and GM (7.4-11.4%). Such differences may be attributed to variations in sample size (Love *et al*, 2015), differences in farm types (Strom *et al*, 2017) and extents of antimicrobials administration (Burow *et al*, 2014). A recent study in central region of Thailand reported 100% MDR *E. coli* in fattening pigs and piglets, particularly 100% resistance to AM and TE (Dawangpa *et al*, 2022).

Comparison between *Salmonella* sp isolated from pig farms in this study with those collected around the country by the National Institute of Animal Health in Bangkok from 2003 to 2005 shows similar resistance rates to C (25-45.8%), GM (12.5-33.3%) and TE (72.5-75%), but overall MDR rate is higher (74%) (Chuanchuen *et al*, 2008). Compared to the present study, *Salmonella* sp isolates from farm pigs in northern Thailand from 2011 to 2013 demonstrate no differences in resistance rates to AM (75-80%), C (25.0-45.8%), CTX (16.3-41.7%), SXT (20.8-41.3%), and TE (72.5-75.0%), but lower resistance rate to S (48.8-70.5%) and higher resistance rate to NA (28.8-41.7%) (Tadee *et al*, 2014). The reasons for these differences are probably similar to those described above for *E. coli*. However, our findings were not able to indicate prevalence of MDR *Salmonella* sp isolates in pork samples during the period 2014-2017, which has been shown to range 29.3-44.0% (Wongsrichai *et al*, 2021).

The study suffered from two major limitations. Firstly, there was a lack of information on antimicrobial use in the study pig farms and the addition of antimicrobials to feed is prohibited in the country (Thamlikitkul *et al*, 2015). Guidelines for antimicrobial use in pig farming are well established and described in the Notification of the Ministry of Agriculture and Cooperatives, which followed the most updated law for controlled drugs in animals as indicated in the Ministry of Public Health's announcement (Ministry of Public Health, 2019). Secondly, identification of *E. coli* virulence genes from farm pig stool was limited only to 10 main genes and their relationship with pathogenicity in the host was not investigated, only their association with antibiogram profiles.

In summary, the study reveals the high prevalence of *E. coli* virulence *astA* and *S.* Agona serovar in stool of farm pigs in central Thailand, and finds no association between the presence or absence of virulence genes and antibiogram profiles in *E. coli*. Monitoring of antimicrobial resistance in farm pig is crucial for early detection of resistant strains to enable appropriate and timely control of foodborne zoonotic pathogens. Future research should focus on

the development of whole genome sequencing to identify all virulence genes in commensal and pathogenic bacteria and determination their importance to human and farm animal health. Routine survey of prevalence of antimicrobial resistance and of virulence genes in foodborne pathogens will contribute to an understanding of the dynamics of antimicrobial resistance and virulence genes in pathogenic bacterial populations to enable development of model(s) for prediction of possible outbreaks of foodborne infections in various regions of the country.

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REFERENCES

Adams MR, Moss MO. Food microbiology. 2nd ed. Cambridge: The Royal Society of Chemistry; 2000.

Bangtrakulnonth A, Pornreongwong S, Pulsrikarn C, *et al*. *Salmonella* serovars from humans and other sources in Thailand, 1993-2002. *Emerg Infect Dis* 2004; 10: 131-6.

Bessone FA, Bessone G, Marini S, Conde MB, Alustiza FE, Zielinski G. Presence and characterization of *Escherichia coli* virulence genes isolated from diseased pigs in the central region of Argentina. *Vet World* 2017; 10: 939-45.

Burow E, Simoneit C, Tenhagen BA, Kasbohrer A. Oral antimicrobials increase antimicrobial resistance in porcine *E. coli* - a systematic review. *Prev Vet Med* 2014; 113: 364-75.

Chapman TA, Wu XY, Barchia I, *et al*. Comparison of virulence gene profiles of *Escherichia coli* strains isolated from healthy and diarrheic swine. *Appl Environ Microbiol* 2006; 72: 4782-95.

Chlebicz A, Slizewska K. Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: a review. *Int J Environ Res Public Health* 2018; 15: 863.

Chuanchuen R, Pathanasophon P, Khemtong S, Wannaprasert W, Padungtod P. Susceptibilities to antimicrobials and disinfectants in *Salmonella* isolates obtained from poultry and swine in Thailand. *J Vet Med Sci* 2008; 70: 595-601.

Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animal. Second Informational Supplement VET01-S2.

Wayne, PA: Clinical and Laboratory Standards Institute; 2013.

Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB. Recent advances in understanding enteric pathogenic *Escherichia coli. Clin Microbiol Rev* 2013; 26: 822-80.

Dawangpa A, Lertwatcharasarakul P, Boonsoongnern A, *et al*. Multidrug resistance problems targeting piglets and environmental health by *Escherichia coli* in intensive swine farms. *Emerg Contam* 2022; 8: 123-33.

Department of Livestock Development. Information on the number of livestocks in Thailand, 2020 [cited 2022 Jan 20]. Available from: URL: http://docimage.dld.go.th/FILEROOM/CABDLD_BOOKSHELF2/DRAWER26/GENERAL/DATA0000/00000082.PDF [in Thai]

Fairbrother JM, Nadeau E, Gyles CL. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Anim Health Res Rev* 2005; 6: 17-39.

Fenwick N, Griffin G, Gauthier C. The welfare of animals used in science: how the "Three Rs" ethic guides improvements. *Can Vet J* 2009; 50: 523-30.

Gotter V, Blaha T, Klein G. A case-control study on the occurrence of *Salmonella* spp. in the environment of pigs. *Epidemiol Infect* 2012; 140: 150-6.

Hanson R, Kaneene JB, Padungtod P, Hirokawa K, Zeno C. Prevalence of *Salmonella* and E. coli, and their resistance to antimicrobial agents, in farming communities in northern Thailand. *Southeast Asian J Trop Med Public Health* 2002; 33 (Suppl 3): 120-6.

Ke B, Sun J, He D, Li X, Liang Z, Ke CW. Serovar distribution, antimicrobial resistance profiles, and PFGE typing of *Salmonella* enterica strains isolated from 2007-2012 in Guangdong, China. *BMC Infect Dis* 2014; 14: 338.

Lay KK, Jeamsripong S, Sunn KP, *et al*. Colistin resistance and ESBL production in *Salmonella* and *Escherichia coli* from pigs and pork in the Thailand, Cambodia, Lao PDR, and Myanmar border area. *Antibiotics* 2021; 10: 657.

Lay KK, Koowattananukul C, Chansong N, Chuanchuen R. Antimicrobial resistance, virulence, and phylogenetic characteristics of *escherichia coli* isolates from clinically healthy swine. *Foodborne Pathog Dis* 2012; 9: 992-1001.

Lee SI, Kang SG, Kang ML, Yoo HS. Development of multiplex polymerase chain reaction assays for detecting enterotoxigenic *Escherichia coli* and their application to field isolates from piglets with diarrhea. *J Vet Diagn Invest* 2008; 20: 492-6.

Lekagul A, Tangcharoensathien V, Mills A, Rushton J, Yeung S. How antibiotics are used in pig farming: a mixed-methods study of pig farmers, feed mills and veterinarians in Thailand. *BMJ Glob Health* 2020; 5: e001918.

Love DC, Tharavichitkul P, Arjkumpa O, *et*

al. Antimicrobial use and multidrug-resistant *Salmonella* spp., *Escherichia coli*, and *Enterococcus faecalis* in swine from Northern Thailand. *Thai J Vet Med* 2015; 45: 43-53.

Magaña-Lizárraga JA, Gómez-Gil B, Rendón-Maldonado JG, Delgado-Vargas F, Vega-López IF, Báez-Flores ME. Genomic profiling of antibiotic-resistant *Escherichia coli* isolates from surface water of agricultural drainage in north-western Mexico: detection of the international high-risk lineages ST410 and ST617. *Microorganisms* 2022; 10: 662.

Magiorakos AP, Srinivasan A, Carey RB, *et al*. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012; 18: 268-81.

Mathole MA, Muchadeyi FC, Mdladla K, Malatji DP, Dzomba EF, Madoroba E. Presence, distribution, serotypes and antimicrobial resistance profiles of *Salmonella* among pigs, chickens and goats in South Africa. *Food Control* 2017; 72: 219-24.

Ministry of Public Health. Controlled drugs, 2019 [cited 2022 Jan 19]. Available from: URL: https://www.fda.moph.go.th/sites/drug/Shared%20Documents/Law03-TheMinistryOfHealth/SPC(50).pdf [in Thai]

Nagy B, Fekete PZ. Enterotoxigenic *Escherichia coli* in veterinary medicine. *Int J Med Microbiol* 2005; 295: 443-54.

Pachanon R, Ngamwongsatit P, Siripanichgon K, Suthienkul O. Detection of enterotoxin *Escherichia coli* in diarrheal piglets by polymerase chain reaction, 2013 [cited 2021 Dec 17]. Available from: URL: https://li01.tci-thaijo.org/index.php/gst/article/view/12433/11190

Peng X, Yu KQ, Deng GH, *et al*. Comparison of direct boiling method with commercial kits for extracting fecal microbiome DNA by Illumina sequencing of 16S rRNA tags. *J Microbiol Methods* 2013; 95: 455-62.

Prapasawat W, Intarapuk A, Chompook P, Nakajima C, Suzuki Y, Suthienkul O. Antimicrobial resistance, integron, virulence gene, and multilocus sequence typing of *Escherichia coli* isolates from postweaning piglets with and without diarrhea. *Southeast Asian J Trop Med Public Health* 2017; 48: 1042-55.

Strom G, Halje M, Karlsson D, *et al*. Antimicrobial use and antimicrobial susceptibility in *Escherichia coli* on small- and medium-scale pig farms in north-eastern Thailand. *Antimicrob Resist Infect Control* 2017; 6: 75.

Sudatip D, Tiengrim S, Chasiri K, *et al*. *One Health* surveillance of antimicrobial resistance phenotypes in selected communities in Thailand. *Antibiotics* 2022; 11: 556.

Suthienkul O, Siripanichgon K, Pariyanonda A, *et al*. Rapid salmonellae detection in frozen food by modified technique using modified semi-solid Rappaport-Vassiliadis medium. *Southeast Asian J*

Trop Med Public Health 1995; 26 (Suppl 2): 238-41.

Tadee P, Boonkhot P, Pornruangwong S, Patchanee P. Comparative phenotypic and genotypic characterization of *Salmonella* spp. in pig farms and slaughterhouses in two provinces in Northern Thailand. *PLoS One* 2015; 10: e0116581.

Tadee P, Kumpapong K, Sinthuya D, *et al*. Distribution, quantitative load and characterization of *Salmonella* associated with swine farms in upper-northern Thailand. *J Vet Sci* 2014; 15: 327-34.

Tadee P, Patchanee P, Pascoe B, *et al*. Occurrence and sequence type of antimicrobial resistant *Salmonella* spp. circulating in antibiotic-free organic pig farms of northern-Thailand. *Thai J Vet Med* 2021; 51: 311-9.

Tauxe RV, Doyle MP, Kuchenmuller T, Schlundt J, Stein CE. Evolving public health approaches to the global challenge of foodborne infections. *Int J Food Microbiol* 2010; 139 (Suppl 1): S16-28.

Thamlikitkul V, Rattanaumpawan P, Boonyasiri A, *et al*. Thailand Antimicrobial Resistance Containment and Prevention Program. *J Glob Antimicrob Resist* 2015; 3: 290-4.

Thanapongtharm W, Linard C, Chinson P, *et al*. Spatial analysis and characteristics of pig farming in Thailand. *BMC Vet Res* 2016; 12: 218.

Thrusfield M. Veterinary epidemiology. 3rd ed. Oxford, UK: Wiley-Blackwell; 2005.

Trongjit S, Angkittitrakul S, Chuanchuen R. Occurrence and molecular characteristics of antimicrobial resistance of *Escherichia coli* from broilers, pigs and meat products in Thailand and Cambodia provinces. *Microbiol Immunol* 2016; 60: 575-85.

Wales AD, Cook AJ, Davies RH. Producing *Salmonella*-free pigs: a review focusing on interventions at weaning. *Vet Rec* 2011; 168: 267-76.

Wongsrichai S, Phuektes P, Jittimanee S. Multidrug-resistance and mobile colistin resistance (*mcr*) genes of *Salmonella* isolates from pork in Thailand during 2014-2017: comparison between two different types of slaughterhouses and retails, 2021 [cited 2021 Dec 19]. Available from: URL: https://he02.tci-thaijo.org/index.php/vis/article/view/251163/170651

World Food Programme (WFP). Thematic Guidelines: Sampling guidelines for vulnerability analysis, 2004 [cited 2021 Dec 19]. Available from: URL: https://www.fsnnetwork.org/sites/default/files/thematic_guidelines.pdf

World Health Organization (WHO). Antimicrobial resistance: global report on surveillance, 2014 [cited 2021 Dec 19]. Available from: URL: https://apps.who.int/iris/bitstream/handle/10665/112642/9789241564748_eng.pdf?sequence=1&isAllowed=y

World Health Organization (WHO). Immunization coverage cluster

survey: reference manual, 2005 [cited 2021 Dec 19]. Available from: URL: https://apps.who.int/iris/bitstream/handle/10665/69087/WHO_IVB_04.23.pdf?sequence=1&isAllowed=y

Yatsuyanagi J, Saito S, Miyajima Y, Amano KI, Enomoto K. Characterization of atypical enteropathogenic *Escherichia coli* strains harboring the *astA* gene that were associated with a waterborne outbreak of diarrhea in Japan. *J Clin Microbiol* 2003; 41: 2033-9.

Zhang B, Ku X, Yu X, *et al*. Prevalence and antimicrobial susceptibilities of bacterial pathogens in Chinese pig farms from 2013 to 2017. *Sci Rep* 2019; 9: 9908.

Zhang R, Gu DX, Huang YL, Chan EW, Chen GX, Chen S. Comparative genetic characterization of enteroaggregative *Escherichia coli* strains recovered from clinical and non-clinical settings. *Sci Rep* 2016; 6: 24321.

Zhang WP, Zhao M, Ruesch L, Omot A, Francis D. Prevalence of virulence genes in *Escherichia coli* strains recently isolated from young pigs with diarrhea in the US. *Vet Microbiol* 2007; 123: 145-52.