OCCURRENCE OF NDM-5 AND ANTIBIOTIC RESISTANCE GENES AMONG *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* IN COMPANION ANIMALS IN THAILAND

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Abstract. *Escherichia coli* and *Klebsiella pneumoniae* are infectious agents of concern in causing a variety of diseases, including diarrhea, respiratory diseases and septicemia, leading to morbidity and mortality in both humans and companion animals. Molecular characteristics of extended-spectrum β-lactamase (ESBL)- and carbapenemase-producing *E. coli* and *K. pneumoniae* in companion animals were investigated in 30 *E. coli* and 14 *K. pneumoniae* isolates from companion animals at the Veterinary Teaching Hospital, Faculty of Veterinary Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand during August 2016 to January 2018. ESBL-producing *E. coli* harbored bla_{TEM} ($n = 2$) and $bla_{CTX\text{-}M}$ ($n = 4$), while ESBL-producing *K. pneumoniae* harbored $bla_{TEM} + bla_{SHV} + bla_{CTX\text{-}M}$ ($n = 12$). Carbapenemase-producing *E. coli* ($n = 3$) and *K. pneumoniae* ($n = 8$) carried $bla_{NDM\text{-}5}$ gene and AmpC-producing *E. coli* ($n = 6$) carried bla_{AmpC}. Plasmid-mediated quinolone resistance gene, *qnrS*, was predominantly detected in 7 *E. coli* strains, while that carrying *aac(6')-lb-cr* in 10 *K. pneumoniae* isolates. Phylogeny analysis demonstrated *E. coli* strains distributed into pathogenic groups B2 (27%) and D (20%), and commensal groups A (30%) and B1 (23%), while all *K. pneumoniae* strains belonged to KpI group. ERIC-PCR indicated high diversity of *E. coli* strains, while 11 *K. pneumoniae* originated from the same clone. Multilocus sequence typing of NDM-5-producing strains revealed in *E. coli* were ST224 ($n = 1$) and ST410 ($n = 2$) and in all *K. pneumoniae* ST147. In conclusion, as *E. coli* ST410 and *K. pneumoniae* ST147 are considered high-risk strains, companion animals might play a significant role in the dissemination of antibiotic resistance bacteria.

Keywords: *Escherichia coli*, *Klebsiella pneumoniae*, antibiotic resistance, carbapenemase, companion animal, ESBL, NDM-5, ST147, ST410.

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INTRODUCTION

Emergence of extended-spectrum β-lactamase (ESBL) and carbapenemase-producing bacteria are global public health problems in both human and

veterinary medicine due to limitations of treatment and subsequently leading to therapeutic failure (Wilson and Török, 2018). Another essential and longstanding concern is that the occurrence of these multidrug-resistant (MDR) bacteria in companion animals may pose potential reservoirs for human infection.

In veterinary medicine, β-lactam antibiotics are commonly used to treat various infections due to their effectiveness and fewer side effects (Rubin and Pitout, 2014). However, carbapenems are not widely used in treating companion animals as human breakpoints are applied to animal use and thereby leading to development of antibiotic resistance (Melo *et al*, 2017). Mechanism of resistance to β-lactams is mainly mediated by production of β-lactamases, which can be divided into four classes based on amino acid sequences, namely, Classes A, C, and D that have a serine in the active site, and Class B that requires an active site zinc ion (Ambler, 1980). Among β-lactamases, ESBLs and carbapenemases have continued to be a significant challenge in clinical settings worldwide (Sawa *et al*, 2020). ESBLs, members of Ambler Class A, hydrolyze penicillins, third-generation cephalosporins, except for cephamycins and carbapenems (Bonnet, 2004). ESBLs are classified into three main classes, namely, CTX-M, SHV and TEM, with CTX-M-14 and CTX-M-15 being most prevalent (Bevan *et al*, 2017). In addition, β-lactamase-producing bacteria can be co-resistant to other antibiotics, including fluoroquinolones (Wiener *et al*, 2016). Mechanism of resistance to fluoroquinolones is mainly mediated by mutations in target genes *gyrA* and *parC*. Although acquisition of plasmid-mediated quinolone resistance (PMQR) genes is a minor proportion of resistance mechanisms, it is important in dissemination of fluoroquinolone resistance (Strahilevitz *et al*, 2009). Carbapenemases hydrolyze almost all β-lactams, including carbapenems, and are classified into Class A KPC type, Class B type (IMP, NDM and VIM) and Class D OXA-48 type (Queenan and Bush, 2007).

During dissemination of mobile genetic elements, such as plasmids, transposons and integrons, various resistance genes are transferred at the same time resulting in MDR bacteria and leading to treatment failure (Jacoby and Sutton, 1991). Several studies have demonstrated resistance determinants and MDR clones are shared among companion animals and their owners (Gronthal *et al*, 2018). Spread of ESBL- and carbapenemase-producing Enterobacteriaceae, in particular *Escherichia coli* and *Klebsiella pneumoniae*, in companion animals, livestock and wildlife has been reported (Guerra *et al*, 2014), and these isolates showed closely clonally relatedness to human isolates (Gronthal *et al*, 2018).

The study determined antimicrobial susceptibility, characterized ESBLs and carbapenemases and analyzed clonal relationship among carbapenemase-producing *E. coli* and *K. pneumoniae* obtained from companion animals in the Veterinary Teaching Hospital, Prince of Songkla University, Thailand. This study is the first report of carbapenemase-producing Enterobacteriaceae in companion animals in Thailand, which should contribute to epidemiologic management and improvement in infection control.

MATERIALS AND METHODS

Sample sources and bacteria identification

Clinical specimens including urine

and swabs from wound, respiratory tract, genital, pus, rectum, feces, abdominal cavity and urinary bladder were obtained from diseased companion animals (n = 168) comprising of 96 dogs and 72 cats examined at the Veterinary Teaching Hospital, Prince of Songkla University (August 2016 - January 2018). Specimens were streaked onto MacConkey agar (Beckton Dickinson, MD) and up to three morphologically different lactose-fermenting colonies were collected. Isolates were screened using biochemical tests and confirmed as *E. coli* and *K. pneumoniae* using PCR (Heininger *et al*, 1999). In brief, bacterial DNA was extracted by a boiling method and PCR amplification was performed using *uidA*-primers and16S rRNA primers as internal control for detection of *E. coli*, and *khe*-primers and cognate 16S rRNA primers for detection of *K. pneumoniae*.

Phylogenetic grouping

Bacterial DNA extracted as described above was subjected to PCR amplification of *E. coli*-specific *chuA*, *yjaA* and TspE4.C2 and phylogenetic groups of *E. coli* were interpreted depending on the presence of these genes as previously described (Clermont *et al*, 2000).

Phylogenetic groups of *K. pneumoniae* were determined by *gyrA* PCR-RFLP (restriction fragment length polymorphism). Briefly, *gryA* was amplified and subsequently digested by *Taq*I and *Hae*III (Thermo Fisher Scientific, Waltham, MA). Three phylogenic groups, KpI, KpII and KpIII, were assigned based on the RFLP profiles (Brisse *et al*, 2004).

Antibiogram profiling

Susceptibility to 11 antibiotics of all isolates was determined using a disk diffusion method in accordance with the Clinical Laboratory Standards Institute (CLSI) criteria (CLSI, 2016). Individual disc (Oxoid, Basingstoke, UK) contained ampicillin (20 μg), amikacin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), cotrimoxazole (trimethoprim/sulphamethoxazole 1:19, total 25 μg), cefoxitin (30 μg), gentamicin (10 μg), meropenem (10 μg), imipenem (10 μg), and piperacillin/tazobactam (100 μg/10 μg). *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference strains. MDR was defined as resistance to ≥3 antimicrobial categories (Magiorakos *et al*, 2012).

Phenotypic test for ESBL, AmpC and carbapenemase production

Production of ESBLs, AmpC and cabapenemases was detected by combined disc methods (antibiotics versus antibiotics plus their inhibitors) and was considered when inhibition zone diameters around the antibiotic disc+ its inhibitor increased to $\geq$ 5 mm as compared to the inhibition zone diameters around the antibiotic disc alone.

Two combined disc methods were performed to identify ESBL-producing strains: cefotaxime (30 μg), cefotaxime/clavulanate (30 μg/10 μg), ceftazidime (30 μg) and ceftazidime/clavulanate (30 μg/10 μg) according to CLSl guidelines (CLSI, 2016). To determine AmpC production, discs containing combined cefoxitin (30 μg) and cefoxitin/cloxacillin (30 μg/750 μg) were used (Jones-Dias *et al*., 2014). Discs containing meropenem (MEM) (10 μg) and MEM supplemented with different inhibitors were used for detection of carbapenemase classes: MEM/phenyl boronic acid (PBA) (10 μg/400 μg) for Class A carbapenemase, MEM/dipicolinic acid (DPA) (10 μg/1,000 μg) for Class B carbapenemase and MEM/PBA/DPA (10 μg/400 μg/1,000

μg) for co-production of Classes A and B carbapenemases (van Dijk *et al*, 2014).

Detection of β-lactam resistance and integrase genes, and integron sequences

PCR amplification was employed to determine the presence of bla_{AmpC}, bla_{CTX-M}, bla_{IMP}, bla_{NDM}, bla_{OXA-48}, bla_{SHV}, bla_{TEM}, bla_{VIM} (Mammeri *et al*, 2004; Monstein *et al*, 2007; Poirel *et al*, 2011), and PMQR genes (*aac(6')-Ib-cr*, *qepA*, *qnrA*, *qnrB*, and *qnrS*) (Cattoir *et al*, 2007; Kim *et al*, 2009). Isolates harboring any resistance genes were further analyzed for the presence of Class 1, 2 and 3 integrase genes and integron variable regions using PCR as previously described (Levesque *et al*, 1995; Goldstein *et al*, 2001). Integron variable regions with approximately 1700 base pairs were subjected to sequencing.

In order to subtype New Delhi metallo-beta-lactamase (NDM), amplicons of carbapenemase genes were purified by Presto™ Mini gDNA Bacteria Kit (Geneaid, Taipei, Taiwan ROC), sequenced (Macrogen Inc, Seoul, Republic of Korea) and nucleotide sequences analyzed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Determination of bla_{NDM-5} location

For determination bla_{NDM-5} location, plasmid DNA was extracted using PureLink™ Quick Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA) and subjected to PCR amplification of bla_{NDM} (Poirel *et al*, 2011). Amplicon was analyzed by 1% agarose gel-electrophoresis, purified by Presto™ Mini gDNA Bacteria Kit (Geneaid, Taipei, Taiwan ROC) and sequenced (Macrogen Inc, Seoul, Republic of Korea).

Clonal relatedness

For enterobacterial repetitive intergenic consensus (ERIC) PCR analysis, bacterial genomic DNA was isolated using Presto™ Mini gDNA Bacteria Kit (Geneaid, Taipei, Taiwan ROC), subjected to PCR using ERIC primer and separated by 1% agarose gel-elctrophoresis (Rivera *et al*, 1995). Cluster analysis was conducted using BioNumerics Software (version 7.0) (Applied Maths, Sint-Martens-Latem, Belgium) with Unweighted Pair Group Method Arithmetic Averages (UPGMA) at 75% similarity cut-off using a Dice correlation coefficient.

Pulsed-field gel-electrophoresis (PFGE) of *Xba*I (Thermo Fisher Scientific, Waltham, MA) -digested DNA fragments was performed according to Han *et al* (Han *et al*, 2013) and DNA profiles were analyzed by BioNumerics Software (version 7.0) (Applied Maths, Sint-Martens-Latem, Belgium) at 75% similarity cut-off.

Multilocus sequence typing (MLST) of *E. coli* and *K. pneumoniae* isolates was performed by PCR amplification of seven *E. coli* housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*) and seven *K. pneumoniae* housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *ropB*, and *tonB*) followed by sequencing (Macrogen Inc, Seoul, Republic of Korea) (Diancourt *et al*, 2005)2005. ST types were identified using MLST database (https://pubmlst.org/mlst/).

RESULTS

Characteristics and phylogenetics of bacterial strains

During an eighteen-month period (August 2016 - January 2018), 240 species of Enterobacteriaceae were identified using biochemical tests. Thirty *E. coli* and 14 *K. pneumoniae* isolates obtained from 31 dogs and 13 cats were confirmed by PCR. Samples were from urine (n = 8) and swabs of wound (n = 20), respiratory tract (n = 4), genital (n = 5), pus (n = 2),

rectum (n = 2), abdominal cavity (n = 2) and urinary bladder (n = 1). Phylogenetic grouping revealed *E. coli* isolates belonged to phylogroup A (n = 9, 30%), group B1 (n = 7, 23%), group B2 (n = 8, 27%) and group D (n = 6, 20%) (Fig 1), and all *K. pneumoniae* isolates belonged to KpI group.

Antibiogram profiles and detection of resistance genes

Employing a disk diffusion assay, among *E. coli* strains, resistance to ampicillin (83%) was the highest, while that to carbapenems, imipenem (3%) and meropenem (10%), the lowest (Table 1). Similarly, among *K. pneumoniae* strains, resistance to ampicillin (100%) was the highest and that to carbapenems, imipenem (57%) and meropenem (57%), the lowest. In general, percent resistance to all 11 antibiotics was higher among *K. pneumoniae* compared to *E. coli* strains. Percent MDR *E. coli* strains were higher in commensal phylogenetic groups A and B1 (69%) than those of pathogenic phylogenetic groups B2 and D (29%). Percent of MDR *K. pneumoniae* was 86. Combined disc diffusion test revealed *E. coli* (n = 6, 20%) and *K. pneumoniae* (n = 12, 86%) strains produced ESBLs, and AmpC production in *E. coli* (n = 6, 12%) isolates but not in any *K. pneumoniae* isolate. Combination disc method for detecting carbapenemases showed *E. coli* (n = 3, 10%) and *K. pneumoniae* (n = 8, 57%) isolates class B (metallo-β-lactamase) producers.

Employing PCR-based assay, among ESBL-producing *E. coli* isolates, four carried $bla_{\text{CTX-M}}$ and two bla_{TEM}, while all ESBL-producing *K. pneumoniae* isolates carried $bla_{\text{CTX-M}}$ + bla_{SHV} + bla_{TEM} (Table 2). As expected, presence of bla_{AmpC} was only observed in *E. coli* isolates producing AmpC, and *E. coli* and *K. pneumoniae*

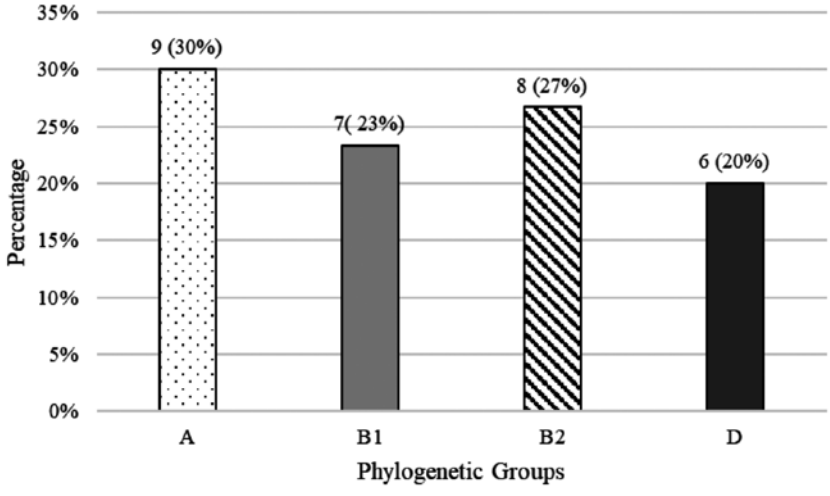

Fig 1-Distribution of phylogenetic groups of *Escherichia coli*

Phylogenetic groups of *E. coli* isolates from companion animals were determined by PCR amplification of the *chuA* and *yjaA* and DNA fragment TspE4.C2 primers and categorized into A, B1, B2 and D according to Clermont *et al* (2000). Data are given as numbers of isolates in each group (percentage in parentheses). The percentage is calculated as the number of isolates in each group over the total number of isolates belonging to each phylogenetic group.

Table 1
Antibiogram profiles of *Escherichia coli* and *Klebsiella pneumoniae* isolates in cats and dogs examined at the Veterinary Teaching Hospital, Prince of Songkla University, Thailand (August 2016 - January 2018).

Organism	AMP *n* (%)	TZP *n* (%)	FOX *n* (%)	CTX *n* (%)	CAZ *n* (%)	IPM *n* (%)	MEM *n* (%)	CN *n* (%)	AK *n* (%)	CIP *n* (%)	SXT *n* (%)
E. coli (n = 30)	25 (83)	3 (10)	6 (20)	12 (40)	10 (33)	1 (3)	3 (10)	20 (67)	10 (33)	18 (60)	12 (40)
K. pneumoniae (n = 14)	14 (100)	11 (79)	9 (64)	12 (86)	12 (86)	8 (57)	8 (57)	11 (79)	12 (86)	12 (86)	12 (86)

AK: amikacin; AMP: ampicillin; CAZ: ceftazidime; CIP: ciprofloxacin; CN: gentamicin; CTX: cefotaxime; FOX: cefoxitin; IPM: imipenem; MEM: meropenem; SXT: sulphamethoxazole/trimethoprim; TZP: tazobactam/piperacillin.

class B (metallo-β-lactamase) producers carried $bla_{\text{NDM-5}}$, located on a plasmid in one *E. coli* strain (2.18) and the remaining chromosome-located (data not shown). It is worth noting no ESBL-producing *E. coli* harbored carbapenemase gene, while all ESBL-producing *K. pneumoniae* carried carbapenemase gene. Among five PMQR genes examined, *E. coli* isolates carried *qnrS* (n = 4, 13%), *qnrB* + *qnrS* (n = 2, 7%), *qnrS* + *aac(6')-Ib-cr* (n = 1, 3%) and *qnrS* + *qepA* (n = 1, 3%), while *K. pneumoniae* isolates carried only *aac(6')-Ib-cr* (n = 10, 83%). No significant correlation between carbapenemase and any PMQR gene was detected.

Among the bacteria strains harboring ESBL, AmpC, carbarpenemase, and PMQR genes, *E. coli* carried only *int1* (n = 4, 21%) and *K. pneumoniae* both *int1* (n = 9, 75%) and *int1* + *int3* (n = 3, 25%) (Table 2). Amplification of integron variable regions generated a variety of amplicons, ranging 150-1,700 bp and subsequent sequencing of 1,700-bp amplicon of *K. pneumoniae* 3.13 showed the presence of two antimicrobial resistance genes, *aadA2* (encoding aminoglycoside resistance) and *dfrA12* (encoding trimethoprim resistance) (Genbank accession no. MT437391).

Clonal relatedness

ERIC-PCR of *E. coli* isolates with a 75% similarity cut-off formed nine distinct clusters, while *K. pneumoniae* isolates formed two clusters (Fig 2). In *K. pneumoniae* cluster I, 8/14 isolates harbored $bla_{\text{NDM-5}}$ and were collected at different times. *K. pneumoniae* isolates that showed 100% similarity by ERIC-PCR were subsequently subjected to PFGE, demonstrating only *K. pneumoniae* 3.5 had 90% similarity (data not shown). MLST analysis showed NDM-5-producing *E. coli* strains belonged to ST224 (n = 1) and

Table 2
Characteristics of ESBL-, AmpC- and carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from cats and dogs examined at the Veterinary Teaching Hospital, Prince of Songkla University, Thailand (August 2016 - January 2018).

Date of collection	Animal	Isolate ID (n = 26)	Organism	Phylogenetic group	Beta-lactamase gene			PMQR gene	Integrase gene
					ESBL*	AmpC*	Carbapenemase*		
August 2016	Dog	1.1	*E. coli*	A	-	bla_{AmpC}	-	*qnrB, qnrS*	*int1*
August 2016	Dog	1.2	*E. coli*	A	-	bla_{AmpC}	-	*qnrB, qnrS*	*int1*
December 2016	Dog	1.21	*E. coli*	A	$bla_{\text{CTX-M}}$	-	-	-	*int1*
February 2017	Dog	2.9	*E. coli*	D	-	bla_{AmpC}	-	-	*int1*
March 2017	Dog	2.11	*E. coli*	B2	$bla_{\text{CTX-M}}$	-	-	-	int1
March 2017	Dog	2.12	*E. coli*	B2	$bla_{\text{CTX-M}}$	-	-	-	-
March 2017	Dog	2.18	*E. coli*	A	-	-	$bla_{\text{NDM-5}}$	-	-
March 2017	Dog	2.20	*E. coli*	B1	bla_{TEM}	-	$bla_{\text{NDM-5}}$	-	int1
March 2017	Dog	2.21	*E. coli*	A	bla_{TEM}	-	-	-	-
June 2017	Dog	2.23	*E. coli*	D	-	bla_{AmpC}	-	-	-
September 2017	Cat	3.12	*E. coli*	B2	-	-	$bla_{\text{NDM-5}}$	-	-
September 2017	Dog	3.14	*E. coli*	B2	-	bla_{AmpC}	-	-	-
September 2017	Dog	3.16	*E. coli*	B2	-	bla_{AmpC}	-	-	-
October-2017	Dog	3.18	*E. coli*	D	$bla_{\text{CTX-M}}$	-	-	-	-
December 2016	Cat	1.17	*K. pneumoniae*	KpI	bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$	-	-	-	int1
February 2017	Dog	2.6	*K. pneumoniae*	KpI	bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$	-	-	*aac(6')-Ib-cr*	int1
March 2017	Cat	2.13	*K. pneumoniae*	KpI	bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$	-	-	-	int1

Table 2 (Continued)

Date of collection	Animal	Isolate ID (n = 26)	Organism	Phylogenetic group	Beta-lactamase gene			PMQR gene	Integrase gene
					ESBL*	AmpC*	Carbapenemase*		
March 2017	Dog	2.19	*K. pneumoniae*	KpI	bla_{TEM}, bla_{SHV}, bla_{CTX-M}	-	-	*aac(6′)-Ib-cr*	int1
March 2017	Dog	2.22	*K. pneumoniae*	KpI	bla_{TEM}, bla_{SHV}, bla_{CTX-M}	-	bla_{NDM-5}	aac(6′)-Ib-cr	int1
March 2017	Cat	2.24	*K. pneumoniae*	KpI	bla_{TEM}, bla_{SHV}, bla_{CTX-M}	-	bla_{NDM-5}	*aac(6′)-Ib-cr*	int1
September 2017	Cat	3.13	*K. pneumoniae*	KpI	bla_{TEM}, bla_{SHV}, bla_{CTX-M}	-	bla_{NDM-5}	*aac(6′)-Ib-cr*	int1
September 2017	Dog	3.15	*K. pneumoniae*	KpI	bla_{TEM}, bla_{SHV}, bla_{CTX-M}	-	bla_{NDM-5}	*aac(6′)-Ib-cr*	int1
November 2017	Dog	3.19	*K. pneumoniae*	KpI	bla_{TEM}, bla_{SHV}, bla_{CTX-M}	-	bla_{NDM-5}	*aac(6′)-Ib-cr*	
November 2017	Cat	3.21	*K. pneumoniae*	KpI	bla_{TEM}, bla_{SHV}, bla_{CTX-M}	-	bla_{NDM-5}	*aac(6′)-Ib-cr*	*int1*, *int3*
December 2017	Dog	3.22	*K. pneumoniae*	KpI	bla_{TEM}, bla_{SHV}, bla_{CTX-M}	-	bla_{NDM-5}	*aac(6′)-Ib-cr*	*int1*, *int3*
January 2018	Dog	3.24	*K. pneumoniae*	KpI	bla_{TEM}, bla_{SHV}, bla_{CTX-M}	-	bla_{NDM-5}	*aac(6′)-Ib-cr*	*int1*, *int3*

*Bacteria producer; ESBL: extended-spectrum β-lactamase; PMQR: plasmid-mediated quinolone resistance.

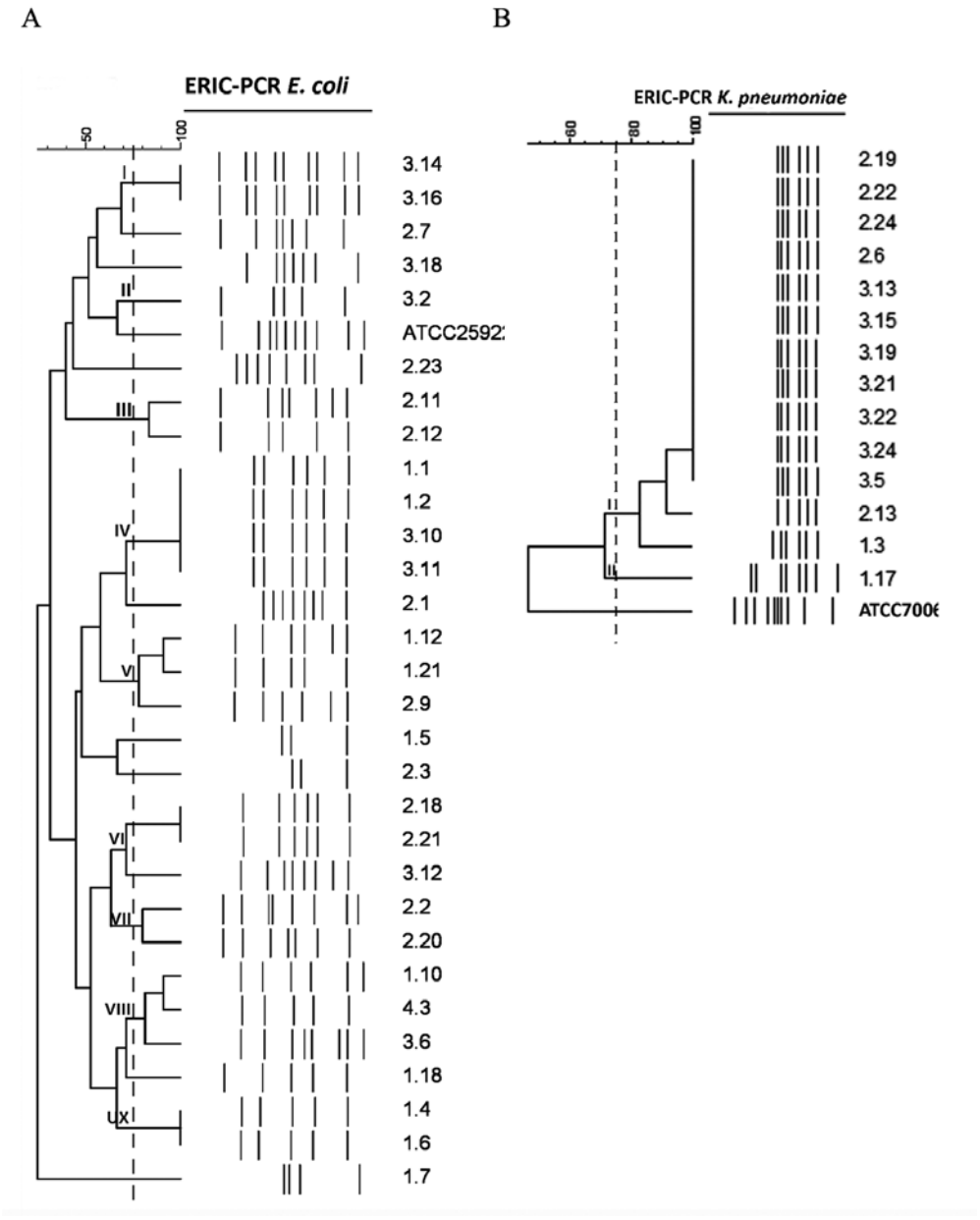

Fig 2-Clonal relationship by enterobacterial repetitive intergenic consensus (ERIC) PCR (shown diagrammatically) of *Escherichia coli* (A) and *Klebsiella pneumoniae* (B) strains from cats and dogs examined at the Veterinary Teaching Hospital, Prince of Songkla University, Thailand (August 2016 - January 2018)

Genomic DNA was amplified using ERIC primer and separated by 1.3% agarose gel-elctrophoresis (Rivera *et al*, 1995). Cluster analysis was conducted using BioNumerics software (version 7.0) with Unweighted Pair Group Method Arithmetic Averages at 75% similarity cut-off (vertical dash line). Cluster group is indicated by Roman numeral. Scale represents percent similarity.

ST410 (n = 2) and *K. pneumoniae* strains to ST147 (n = 8).

DISCUSSION

Antibiotic resistance in companion animals has drawn attention as a public health threat since antibiotic resistance genes have been shown to circulate between humans and companion animals. Gronthal *et al* (2018) reported several bacteria isolated from pets and their owners have similar antibiograms with presence of the same antibiotic gene variants and the clonal relatedness demonstrated by PFGE and MLST, indicating these isolates are genetically related. Other studies reported ESBL- and carbapenemase-producing isolates recovered from humans; however, few showed the antimicrobial resistance in companion animals (Rubin and Pitout, 2014).

The present study is the first in Thailand to conduct molecular characterization of ESBL- and carbapenemase-producing *E. coli* and *K. pneumoniae* strains in companion animals (cats and dogs). Phylogenetic groups of *E. coli* were diverse with MDR phenotype present in most commensal and non-pathogenic phylogenetic groups A and B1. These findings suggest commensal or non-pathogenic *E. coli* were able to be selected under antimicrobial selective pressure and evolve MDR phenotype. On the other hand, *K. pneumoniae* isolates belonged to KpI phylogenetic group, similar to human isolates (Pons *et al*, 2015).

The high prevalence of resistance to ampicillin in *E. coli* and *K. pneumoniae* might be attributable to the presence of class A β-lactamase in bacteria chromosomes (Haeggman *et al*, 2004) and high MDR prevalence to selective pressure from use of several classes of drugs. A correlation between presence of PMQR and ESBL genes has been observed in several studies (Donati *et al*, 2014; Harada *et al*, 2016) in agreement with the present study. The possibility that *aac(6')-Ib-cr* and ESBL genes exist on the same plasmid can be tested by performing conjugation and hybridization experiments. The occurrence of ESBL-producers among both *E. coli* and *K. pneumoniae* strains was higher than that from companion animals reported in Japan (34.8%) for *K. pneumoniae* (Harada *et al*, 2016), Taiwan (3.3% for *E. coli* and 22.7% for *K. pneumoniae* (Kuan *et al*, 2016) and USA (3.8% for *E. coli*) (Liu *et al*, 2016).

Until now, there were only 11 reports on carbapenemase-producing Enterobacteriaceae in companion animals (Shaheen *et al*, 2013; Stolle *et al*, 2013; Schmiedel *et al*, 2014; Abraham *et al*, 2016; González-Torralba *et al*, 2016; Liu *et al*, 2016; Yousfi *et al*, 2016; Ewers *et al*, 2017; Melo *et al*, 2017; Cui *et al*, 2018; Pruthvishree *et al*, 2018; Yousfi *et al*, 2018), and, to the best of our knowledge, the present study is the first such report in companion animals in Thailand. Frequency of carbapenem resistance in *K. pneumoniae* was higher than that in *E. coli*, and both higher than in previous reports from Africa, America, Asia, Australia, and Europe (Kock *et al*, 2018). Carbapenems are not approved to be used on animals but under some jurisdiction, infection with multidrug resistant bacteria, they can be prescribed legally (Torren-Edo *et al*, 2015). The findings in pets as outpatients indicated improper use of antibiotics in veterinary practice and / or transmission of resistance genes or bacteria from infected owners. In Thailand, surveillance on carbapenem-resistant Enterobacteriaceae has been limited only to human isolates, with a

prevalence of 5.9 and 11.1% for *E. coli* and *K. pneumoniae* reported from a hospital in Bangkok (Netikul and Kiratisin, 2015) and 0.14 and 0.32% from a provincial hospital in a northeastern region of the country (Rimrang *et al*, 2012).

Among the 11 $bla_{\text{NDM-5}}$-producing bacteria strains, $bla_{\text{NDM-5}}$ was plasmid-borne in only a single *E. coli* strain, indicating a possibility of horizontal transfer to other bacteria. In addition, this strain was clustered in the same group containing strains lacking $bla_{\text{NDM-5}}$, suggesting either absence of horizontal transfer or curing of this particular plasmid. It is of interest *K. pneumoniae* 3.13 and *E. coli* 3.12 collected in September 2017 from pus of the same cat harbored chromosome-located $bla_{\text{NDM-5}}$. *K. pneumoniae* 3.13 carried *int1* but *E. coli* 3.12 did not. Analysis of *K. pneumoniae* 3.13 class I integron cassette failed to detect $bla_{\text{NDM-5}}$, suggesting another integron might be involved.

MLST analysis of these NDM-5-producing strains revealed *E. coli* strains were assigned to ST224 and ST410 and all *K. pneumoniae* strains to ST147. *E. coli* ST224 has been reported as an MDR strain found in both humans and animals in Asia, Europe and South America, and is associated presence of ESBL genes (Silva *et al*, 2018). *E. coli* ST410 and *K. pneumoniae* ST147 are recognized as high-risk clones, *ie* having (i) ability to be distributed globally, (ii) association with various antimicrobial resistance genes, (iii) ability to persist for prolonged periods within the hosts, (iv) ability to be transmitted effectively among hosts, (v) enhancement in fitness and virulence, and (vi) ability to cause severe or recurrent infections (Baquero and Coque, 2011). *E. coli* ST410 has been detected worldwide, causing interspecies transmission in humans, animals and the environment (Roer *et al*, 2018), while *K. pneumoniae* ST147 has been associated with different bla_{NDM} variants including $bla_{\text{NDM-5}}$ and $bla_{\text{OXA-181}}$ in human isolates in many countries (Peirano *et al*, 2011; Lascols *et al*, 2013; Shin *et al*, 2016). A tigecycline-resistant *K. pneumoniae* ST147 strain was detected for the first time in companion animals in Spain (Ovejero *et al*, 2017), suggesting these NDM-producing bacteria might be interspecies transmissible, leading to morbidity and mortality in both humans and animals.

PFGE demonstrated an outbreak of *K. pneumoniae* ST147 in the Veterinary Teaching Hospital, Prince of Songkla University and the strain remained for a lengthy period (from 2017 to 2018). As all bacterial isolates were obtained from companion animals treated at the hospital as outpatients, the risk of becoming infected from direct transmission from animal to animal was considered low. Thus, infection by the same strain might occur due to the hospital environment or be carried by companion animal care workers. However, further research is needed to elucidate the cause of the outbreak. Strict hospital environmental disinfection and appropriate hygiene policy should be implemented to prevent future occurrences.

In summary, the study demonstrates the occurrence of NDM-5-producing multidrug-resistant *E. coli* and *K. pneumoniae* in companion animals treated at the Veterinary Teaching Hospital, Prince of Songkla University. The prevalence of carbapenem resistance was higher than in previous studies conducted in companion animals but also in humans. *E. coli* ($n = 2$) and *K. pneumoniae* ($n = 8$) were assigned to high-risk ST410 and ST147 strains respectively. The presence of these strains in companion animals

suggests possible transmission between humans and companion animals, and it would be advisable to implement public health surveillance in veterinary hospitals to minimize infection of multidrug-resistant bacteria in companion animals. Appropriate antibiotic management programs in companion animals need to be carried out to reduce development of antibiotic resistance, in particular multidrug-resistance, because health of animals is intimately linked to the health of humans and the environment according to the concept of One Health.

ACKNOWLEDGEMENTS

The work was supported by the Faculty of Science Research Fund (grant no. SCI6304010S), Prince of Songkla University, by Thailand Education Hub for Southern Region of ASEAN Countries Project Office of the Higher Education Commission (grant no. 0954/2015) and by a research grant for thesis financial support for the fiscal year 2018 of Prince of Songkla University. The authors thank the Veterinary Teaching Hospital, Prince of Songkla University for providing bacteria isolates.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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