Research article

OXA-48-positive carbapenem-resistant *Enterobacteriaceae* in a farrow-to-finish pig farm: First report in Thailand

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Abstract

Carbapenem-resistant *Enterobacteriaceae* (CRE) have emerged as an urgent threat to public health. This study aimed to determine the occurrence of CRE and the carbapenemase genes in a farrow-to-finish pig farm, and to investigate carriage proportion and maintenance of CRE during the pig production cycle. We conducted a cross-sectional study by collecting 200 rectal swabs from healthy pigs of 5 groups: gilts, sows, piglets, weaners, and fatteners. In the longitudinal study, 20 healthy pigs were followed from 2 to 26 weeks old, and rectal swabs were collected from each pig for 5 times. Samples were screened for CRE using MacConkey agar supplemented with meropenem at 0.5 µg/mL. Identification and antimicrobial susceptibility pattern of the recovered isolates were determined using an automated system. PCR was used to detect carbapenemase genes. The occurrence of *Enterobacteriaceae* isolates with the carbapenem resistant phenotype and/or harboring the *bla*<sub>OXA-48</sub> gene was 3% (6/200) in the cross-sectional study. Groups of sows and piglets had the same occurrence rate at 5% (2/40), while weaner and fatter groups had 2.5% (1/40). In the longitudinal study, CRE were not detected in pigs at an early age; however, two isolates were detected at the age of finishing. This study is the first report of *Enterobacteriaceae* with a carbapenem resistant phenotype and/or carrying *bla*OXA-48 gene in pigs in Thailand. Finding CRE in pigs at all age categories including finisher in the study farm underscores the need for active monitoring and surveillance studies to determine the occurrence of CRE in pig farms in Thailand.

Keywords: Carbapenem resistance Enterobacteriaceae, Carbapenemase genes, Pigs, Thailand
INTRODUCTION

Antimicrobial resistance (AMR) is an important global health threat. Carbapenems are one of the few last-line antibiotics for treatment of multi-drug resistance (MDR) Gram-negative bacterial infection (Bassetti et al., 2019; Nicolau, 2008). Thus, emergence of carbapenem-resistant bacteria is considered as a serious threat to public health globally. With their high level of resistance to almost all easily available antibiotics and their association with high mortality, carbapenem-resistant Enterobacteriaceae (CRE) are among pathogens listed in a group of critical priority for research and development of new antibiotics by the World Health Organization (WHO, 2017). The increasing occurrence of CRE infections in humans and the widespread dissemination of CRE in humans and human environments pose a serious problem to public health (Logan and Weinstein, 2017). In Thailand, CRE also poses a great threat to human health. Based on surveillance data from 69 hospitals in Thailand, numbers of patients with CRE infections have been increasing since 2015, from 118 cases in 2015 to 1,960 cases in 2017 (NARST, 2019). In addition to humans, the occurrence and dissemination of CRE in animals, including pets, wildlife, and livestock, have been increasingly reported (Köck et al., 2018). The expansion of CRE threatens the effectiveness of antibiotic treatment for infections due to their resistance to all or nearly all available antibiotics, including carbapenems (Zhou et al., 2020; Li et al., 2018).

Three major mechanisms of decreased susceptibility or resistance to carbapenems are the production of carbapenemase enzymes, porin alteration, and increase of efflux pump activity (Papp-Wallace et al., 2011). Of these, carbapenemase enzyme production is the main resistance mechanism (Papp-Wallace et al., 2011). Carbapenemases can hydrolyze carbapenems using active catalytic substrates including serine and zinc (Papp-Wallace et al., 2011). These enzymes are encoded by several genes that can be located on either the chromosome or mobile genetic elements such as plasmids, transposons and integrons (Diene and Rolain, 2014). Among carbapenemase genes, \( \text{bla}_{\text{KPC}} \), \( \text{bla}_{\text{VIM}} \), \( \text{bla}_{\text{IMP}} \), \( \text{bla}_{\text{NDM}} \), and \( \text{bla}_{\text{OXA-48}} \) are frequently detected, and are mostly located on mobile genetic elements, enabling them to spread efficiently in a bacterial community (Diene and Rolain, 2014). In addition, co-harboring of carbapenemase and mobile colistin resistance (\( \text{mcr} \)) genes in CRE isolates has been reported previously (Huang et al., 2020). As colistin is an important “last-line” treatment for infections caused by CRE, spreading of CRE strains carrying \( \text{mcr} \) genes could pose a serious problem to human health due to limited treatment options and increased mortality in patients infected with colistin resistant CRE.

Although carbapenems are restricted for use in food-producing animals including pigs, there have been several reports of CRE in swine farms in many countries including China, Germany, USA, Italy and India (Irrgang et al., 2020a; Mollenkopf et al., 2018; Nirupama et al., 2018; Pruthvishree et al., 2017; Pulss et al., 2017; Zhang et al., 2019). After the first report of CRE harboring \( \text{bla}_{\text{VIM-1}} \) in Germany in 2012 (Fisher et al., 2012), CRE carrying other carbapenemase genes including \( \text{bla}_{\text{NDM}} \), \( \text{bla}_{\text{IMP}} \), \( \text{bla}_{\text{KPC}} \), \( \text{bla}_{\text{OXA}} \), and \( \text{bla}_{\text{GES}} \) have been detected (Irrgang et al., 2020a; Irrgang et al., 2020b; Mollenkopf et al., 2018; Nirupama et al., 2018; Pruthvishree et al., 2017; Pulss et al., 2017; Zhang et al., 2019). Selective pressure from using other beta-lactams such as
ceftiofur is hypothesized to play an important role in development of CRE (Mollenkopf et al., 2018). Beta-lactams including amoxicillin and ceftiofur have been used on medicated feed, or as oral and injectable antibiotics for prevention and treatment of diseases in pig farms in Thailand (Lekagul et al., 2020).

With a significant trend of increasing CRE infections in humans and few studies of CRE in pigs in Thailand, there is a need for active surveillance studies of CRE in pig production systems. In this study, we aimed to determine the occurrence of CRE and major carbapenemase genes in a farrow-to-finish pig farm in Thailand, and to investigate carriage proportion and maintenance of CRE during the production cycle of pigs. Findings in this study will provide an important information of the on-farm epidemiology of CRE that may lead to development of effective strategies for control of CRE that may disseminate in pig farms.

**MATERIALS and METHODS**

**Study design and sample collection**

An accessible, intensive farrow-to-finish pig farm with the use of beta-lactams was selected for this study. Data on antimicrobial use in the study farm was collected by interviewing the farm owner. To reveal the occurrence of CRE, and to investigate carriage proportion and maintenance of CRE during the production cycle of pigs, cross-sectional and longitudinal studies were designed. In the cross-sectional study, rectal swab samples were collected from 5 different age groups of healthy pigs including gilts, sows, piglets, weaners, and fatteners in February 2019. The sample size was calculated using the formula, 

\[ N = \left( \frac{Z_{1-\alpha/2}^2 P (1 - P)}{e^2} \right) \]

(Fosgate, 2009), with an expected prevalence of 5% based on our pilot study, and a confidence level of 95%. The calculated sample size was at least 73 samples. However, the sample size was adjusted to 200 samples in which 40 rectal swab samples per each group of pigs were collected to provide greater accuracy and more reliable results.

In the longitudinal study, a confidence level of 90% was used to calculate the sample size due to limitations of farm management. Twenty healthy pigs were followed from 2 weeks old to 26 weeks old. These 20 pigs were derived from 4 sows (5 piglets per sow) and were raised in 2 pens (10 pigs per pen) from weaning to finishing stages. Rectal swabs were collected from each pig at 5 time points in 2019: 1) 2-week-old piglets; 2) 4-week-old piglets; 3) 8-week-old weaners; 4) 16-week-old fatteners; and 5) 26-week-old finishers. Total number of rectal swabs from these pigs was 100 samples. All samples were collected using sterile cotton swabs and were kept in Cary Blair transport medium (Oxoid™, UK). These were stored at 4°C in an ice box during transportation to the laboratory for further processing. This study was approved by the Animal Ethics Committee of Khon Kaen University (IACUC KKU) (no. 660201.2.11/50).
Isolation Procedure

Each swab sample was enriched in 10 mL buffered peptone water (Hardy Diagnostics™, USA) and incubated at 37°C overnight. Ten microliters of the enriched samples were plated onto MacConkey agar containing 0.5 µg/mL meropenem (Sigma™, USA) and incubated at 37°C for 18-24 hours for screening of carbapenem resistance. Colonies with a presumptive Enterobacteriaceae morphology were sub-cultured onto MacConkey agar (Hardy Diagnostics™, USA) and Trypticase soy agar (Difco™, USA).

Identification and Antimicrobial Susceptibility Testing of bacterial isolates

Biochemical identification and antimicrobial susceptibility tests (AST) were performed via the automated BD Phoenix M50 system, using the NMIC/ID-95 panel and following the manufacturer’s instruction (Becton Dickinson Phoenix™ ID & AST System, USA). Isolates were examined for their susceptibility to 22 antimicrobial drugs, including amoxicillin/Clavulanate (AMC), ampicillin (AMP), Aztreonam (ATM), pipercillin/Tazobactam (TZP), cefepime (FEP), cefoxitin (FOX), ceftazidime (CAZ), ceftriaxone (CRO), cefuroxime (CXM), cephalothin (CEF), ertapenem (ETP), imipenem (IPM), meropenem (MEM), chloramphenicol (CHL), colistin (CST), ciprofloxacin (CIP), norfloxacin (NOR), tobramycin (TOB), gentamicin (GEN), nitrofurantoin (NIT), trimethoprim (TMP) and trimethoprim/Sulfamethoxazole (SXT). Escherichia coli (ATCC® 25922™) was used as the quality control strain. Susceptibility or resistance to antimicrobial agents was identified in compliance with the recommendation of the Clinical and Laboratory Standards Institute (CLSI, 2017). Isolates that exhibited resistance to three or more classes of antimicrobial agents were defined as multidrug-resistant strains (MDR). A broth microdilution method to determined meropenem minimum inhibitory concentration (MIC) was performed according to CLSI (2018).

Detection of carbapenemase encoding genes using PCR

Bacterial DNA was extracted from each isolate using a rapid boiling method (Dashti, 2009). Six major carbapenemase genes, including bla_KPC, bla_IMP, bla_OXA-48, bla_NDM, bla_VIM and bla_GES, commonly found in Enterobacteriaceae isolated from humans and pigs, were studied. Simplex PCRs targeting the 16S rRNA gene (Greisen et al., 1994) and six major carbapenemase genes were performed as previously described (Tijet et al., 2011; Dallenne et al., 2010). The primers for PCR amplifications are shown in Table 1. The final volume of each reaction mixture was at 25 µL containing 12.5 µL of 2× GoTaq® Green Master Mix (Promega™, USA), 0.2 µM of each primer of a specific primer pair for each gene, and 5 µL of extracted DNA. No template DNA was included as a negative control and bacterial DNA containing the carbapenemase gene was included as a positive control. The PCR conditions consisted of an initial denaturation step at 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds, and a final elongation step at 72°C for 10 minutes. PCR products were separated by electrophoresis with 1.5% agarose gel in 1×TAE buffer and were visualized under UV light using a gel documentation system (Bio-Rad™, USA).
Confirmation of \textit{bla}_{OXA-48} containing isolates

The presence of \textit{bla}_{OXA-48} was verified by sequencing of amplified PCR products using next generation sequencing technology by a commercial company (U2Bio™, Korea). The obtained sequence data were examined for homology against the GenBank database using the \textit{BlaST} algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

| Target         | Primer name | DNA sequence (5' to 3') | References                  |
|----------------|-------------|-------------------------|-----------------------------|
| 16S rRNA       | DG74        | AGGAGGTTGATCCAACCGCA    | Greisen et al., 1994        |
|                | RW01        | AACTGGAAGGAAGTGGGGAT    |                             |
| NDM-1          | NDM-F       | AATGGAATTGCCAAATATTAGC  | Tijet et al., 2011          |
|                | NDM-R       | CGAAAGTACGGCTGTGTTGC    |                             |
| OXA-48-like    | MultiOXA-48 for | GCTTGATGCCCCCCCGATT  | Dallenne et al., 2010       |
|                | and GES-11  | GATTTGGCTCCGTGGCGGAAA   |                             |
| VIM variants   | MultiVIM for | GATGGGTGTTGTCGCTATA    | Dallenne et al., 2010       |
|                | MultiVIM rev | CGAATGCGCAGCACCAAG     |                             |
| KPC-1 to KPC-5 | MultiKPC for | CATCAGGGCTTCCTTGCTGC   | Dallenne et al., 2010       |
|                | MultiKPC rev | ACGACGGCATAGTCATTTCGC  |                             |
| IMP            | MultiIMP for | GGAATAGAGTGCTTTAYTCTCT | Dallenne et al., 2010       |
|                | MultiIMP rev | GGTATTAAAAAACAAACACC   |                             |
| GES-1 to GES-9 | MultiGES for | AGTCGGCTAGACCGGAAAAG   | Dallenne et al., 2010       |
|                | and GES-11  | GCTTGATGCGCCCTCGATT    |                             |
| \textit{mcr}-1 | CLR F       | CGGTCAGCCGTGTTGTTCC    | Liu et al., 2016            |
|                | CLR R       | CTTGCTCGGCTTGTAGGG     |                             |
| \textit{mcr}-2 | MCR2-1F     | TGGTGTCTTGGCGGATAGGGA  | Xavier et al., 2016         |
|                | MCR2-1R     | AGATGGTATTTGGTGGGGTG    |                             |
| \textit{mcr}-3 | MCR3-qf     | ACCCTCCAGCGTAGGATGTTCCACMR | Li et al., 2017           |
|                | MCR3-qr     | GCCGTTCCCCAACCACGACAGCA   |                             |
| \textit{mcr}-4 | MCR-4F      | TCACTTCATCACCTGGCGGTC    | Rebelo et al., 2018         |
|                | MCR-4R      | TGTGCTCCATGCACTCAATG    | Rebelo et al., 2018         |
| \textit{mcr}-5 | MCR-5F      | ATGCGGTGTTGCTATTATC     |                             |
|                | MCR-5R      | CTTGTGGTGCTGTCCCTATT    |                             |
| \textit{mcr}-6 | mcr-6_mp_fw | AGCTATGGCTCAATTCCGAGAT  | Borowiak et al., 2020      |
|                | mcr-6_mp_rev | ATGGCTAGGTTTGCAATC      |                             |
| \textit{mcr}-7 | mcr-7_mp_fw | GCCCTCTCTCTCTGTTGTT     | Borowiak et al., 2020      |
|                | mcr-7_mp_rev | GCTTTGTCCTCTTTCTG       |                             |
| \textit{mcr}-8 | mcr-8_mp_fw | TCAACAATTCTCAAAAGCGTG   | Borowiak et al., 2020      |
|                | mcr-8_mp_rev | AATGCTGCGCAAGTGAAG      |                             |
| \textit{mcr}-9 | mcr-9_mp_fw | TTCCCTTGTGCTGCTG        | Borowiak et al., 2020      |
|                |             | GCAGGTAATAAGGTCGTC      |                             |
Detection of colistin resistance genes \textit{mcr}-1 to \textit{mcr}-9 by PCR

Colistin resistance genes \textit{mcr}-1 to \textit{mcr}-9 were detected by simplex PCR as previously described (Liu et al., 2016; Xavier et al., 2016; Li et al., 2017; Rebelo et al., 2018, Borowiak et al., 2020). The primers are shown in Table 1. Bacterial DNA containing the \textit{mcr} gene was included as a positive control, and no template DNA was included as a negative control.

RESULTS

\textit{Enterobacteriaceae} isolates with carbapenem resistant phenotype

The operation of the farrow to finish farm in our study includes approximately 1,000 sows. Amoxicillin and tiamulin are used in medicated feed for controlling subclinical infections until pigs reach a body weight of 60 kg. Amoxicillin is also given to pigs after castration. Furthermore, amoxicillin, enrofloxacin, gentamicin and ceftiofur are administered orally or by injection for treatment of diseased pigs. To recover CRE, rectal swabs samples were screened for \textit{Enterobacteriaceae} with decreased susceptibility to meropenem by using MacConkey agar supplemented with 0.5 µg/mL. Of 200 rectal swab samples collected in the cross-sectional study, 28 isolates were recovered from all groups of pigs, except gilts (Table 2). In the longitudinal study, 4 isolates with reduced susceptibility to meropenem were obtained from 1 piglet and 3 finishers. These 32 isolates were identified as \textit{Enterobacter cloacae} (n=3), \textit{Escherichia coli} (n=2), \textit{Klebsiella aerogenes} (n=5), \textit{Klebsiella ozaenae} (n=1), \textit{Klebsiella pneumoniae} (n=1), \textit{Proteus mirabilis} (n=9), \textit{Proteus penneri} (n=1), \textit{Proteus vulgaris} (n=2), \textit{Morganella morganii} (n=4), and \textit{Providencia alcalifaciens} (n=4). Antimicrobial susceptibility testing results revealed that all isolates were susceptible to meropenem and piperacillin-tazobactam (data not shown). The meropenem MIC values of all isolates were at 0.5 µg/mL as determined by broth microdilution. Four carbapenem-resistant isolates were obtained, including 3 isolates of \textit{K. aerogenes} with the imipenem MIC value of 4 µg/mL, and 1 \textit{E. cloacae} isolate with the ertapenem MIC value of >1 µg/mL (Table 3). Two \textit{K. aerogenes} isolates and an \textit{E. cloacae} isolate were recovered from a sow, a piglet, and a weaner of the cross-sectional study, respectively. Another \textit{K. aerogenes} isolate with CRE phenotype was obtained from fecal sample of a finisher of the longitudinal study (Table-3). Of 32 isolates, 27 (84.4%) were MDR \textit{Enterobacteriaceae}, exhibiting resistance to 3 or more classes of antimicrobials (data not shown).
Table 2 Identified *Enterobacteriaceae* isolates with reduced susceptibility to meropenem (MIC value of 0.5 µg/mL)

| Species                  | Cross-sectional study (number of isolates) | Longitudinal study (number of isolates) | Total |
|--------------------------|--------------------------------------------|-----------------------------------------|-------|
|                          | Sow | Piglet | Weaner | Fattener | Piglet | Finisher |       |
| *Enterobacter cloaca*    | 2   | -      | 1      | -        | -      | -        | 3     |
| *Escherichia coli*       | -   | 2      | -      | -        | -      | -        | 2     |
| *Klebsiella aerogenes*   | 1   | 1      | -      | -        | -      | -        | 5     |
| *Klebsiella ozaenae*     | 1   | -      | -      | -        | -      | -        | 1     |
| *Klebsiella pneumonia*   | -   | 1      | -      | -        | -      | -        | 1     |
| *Morganella morganii*    | 1   | -      | -      | 2        | 1      | -        | 4     |
| *Providencia alcalifaciens* | -   | -      | -      | 4        | -      | -        | 4     |
| *Proteus mirabilis*      | 4   | 4      | -      | 1        | -      | -        | 9     |
| *Proteus penneri*        | 1   | -      | -      | -        | -      | -        | 1     |
| *Proteus vulgaris*       | 1   | 1      | -      | -        | -      | -        | 2     |
| **Total**                | 11  | 9      | 1      | 7        | 1      | 3        | 32    |
Table 3  Carbapenem-resistant *Enterobacteriaceae* isolated from a farrow-to-finish pig farm, Thailand.

| SampleID/Isolate ID | Group  | Type of study | Sample type | Species               | MIC values (µg/mL) | Carbapenemase gene | Resistance pattern                                                                                                                                 |
|---------------------|--------|---------------|-------------|-----------------------|--------------------|---------------------|--------------------------------------------------------------------------------------------------------------------------------------------------|
| SW02/19             | Sow    | Cross-sectional | Rectal swab | *Klebsiella aerogenes* | 0.5 4 0.5          | *bla*<sub>OXA-48</sub> | IPM-AMC-AMP-FOX-CEF                                                                                                                                  |
| SW10/21             | Sow    | Cross-sectional | Rectal swab | *Proteus mirabilis*   | 0.5 -a 1           | *bla*<sub>OXA-48</sub> | AMP-FEP-CRO-CXM-CEF-CIP-NOR-NIT-CST-TOB-GEN-CHL-SXT-TMP                                                                                           |
| PG11/45             | Piglet | Cross-sectional | Rectal swab | *Klebsiella aerogenes* | 0.5 4 ≤0.25        | -                   | IPM-AMC-AMP-FOX-CEF                                                                                                                                  |
| PG07/42             | Piglet | Cross-sectional | Rectal swab | *Proteus mirabilis*   | 0.5 -a 0.5         | *bla*<sub>OXA-48</sub> | AMP-CEF-NIT-CST-GEN-CHL-SXT-TMP                                                                                                                  |
| WN04/51             | Weaner | Cross-sectional | Rectal swab | *Enterobacter cloaca* | 0.5 ≤1 >1          | *bla*<sub>OXA-48</sub> | ETP-AMC-AMP-FOX-CXM-CEF-CTB-GEN-CHL-SXT-TMP                                                                                                       |
| FN13/66             | Fattener | Cross-sectional | Rectal swab | *Proteus mirabilis*   | 0.5 -a ≤0.25       | *bla*<sub>OXA-48</sub> | AME-CEF-NIT-CST                                                                                                                                    |
| 5F12/06             | Finisher | Longitudinal  | Rectal swab | *Klebsiella aerogenes* | 0.5 2 ≤0.25        | *bla*<sub>OXA-48</sub> | AME-CEF-FOX-CXM-CEF-CHL-SXT-TMP                                                                                                                  |
| 5F12/07             | Finisher | Longitudinal  | Rectal swab | *Klebsiella aerogenes* | 0.5 4 ≤0.25        | -                   | IPM-AMC-AMP-FOX-CEF-CHL-SXT-TMP                                                                                                                  |

*a* Data not shown due to intrinsic resistance.

*b* Negative for *bla*<sub>VIM</sub>, *bla*<sub>KPC</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>GES</sub>, and *bla*<sub>NDM</sub>

MEM: meropenem; IPM: imipenem; ETP: ertapenem; AMC: amoxicillin-clavulanate; AMP: ampicillin; FOX: cefoxitin; FEP: cefepime; CRO: ceftriaxone; CXM: cefuroxime; CEF: cephalothin; CST: colistin; CHL: chloramphenicol; CIP: ciprofloxacin; NOR: norfloxacin; TOB: tobramycin; GEN: gentamicin; NIT: nitrofurantoin; TMP: trimethoprim; SXT: trimethoprim-sulfamethoxazole
**Enterobacteriaceae isolates harboring carbapenemase gene**

To determine the occurrence of *Enterobacteriaceae* harboring carbapenemase genes which can be found in bacterial isolates with reduced susceptibility to carbapenems, 6 major carbapenemase genes, including \( \text{bla}_{\text{VIM}} \), \( \text{bla}_{\text{KPC}} \), \( \text{bla}_{\text{IMP}} \), \( \text{bla}_{\text{OXA-48}} \), \( \text{bla}_{\text{GES}} \) and \( \text{bla}_{\text{NDM}} \) were investigated by PCR. The presence of bacterial DNA extracted from colonies of each strain was verified by detection of 16S rRNA gene, and all samples were found to be positive. PCR screening of 6 major carbapenemase genes in 32 isolates with decreased susceptibility to meropenem revealed that none of \( \text{bla}_{\text{VIM}} \), \( \text{bla}_{\text{KPC}} \), \( \text{bla}_{\text{IMP}} \) \( \text{bla}_{\text{G}	ext{-ES}} \) and \( \text{bla}_{\text{NDM}} \) was detected. Molecular characterization by PCR and DNA sequencing showed that 6 isolates harbored \( \text{bla}_{\text{OXA-48}} \) gene (Table 3). Two of the \( \text{bla}_{\text{OXA-48}} \) positive isolates were *K. aerogenes* and *E. cloacae*, with phenotypic resistance to imipenem or ertapenem, respectively. Another \( \text{bla}_{\text{OXA-48}} \) carrying isolate was *K. aerogenes* exhibiting intermediate susceptibility to imipenem, with an MIC value of 2 µg/mL. The other 3 \( \text{bla}_{\text{OXA-48}} \) positive isolates were *P. mirabilis* showing susceptibility to all carbapenems tested. Of 6 \( \text{bla}_{\text{OXA-48}} \) carrying isolates, 1 isolate was obtained from a 26-week-old finisher of the longitudinal study. The other 5 isolates were from rectal swab samples of the cross-sectional study including 3 *P. mirabilis* isolates from a sow, a piglet and a fattener; 1 *K. aerogenes* isolate from a sow; and 1 *E. cloacae* isolate from a weaner (Table-3). Results of antimicrobial susceptibility testing and PCR detection of carbapenemase genes in the cross-sectional study demonstrated that the occurrence of *Enterobacteriaceae* isolates with carbapenem resistant phenotype and/or harboring \( \text{bla}_{\text{OXA-48}} \) gene was 5% (2/40) in the groups of sows and piglets, and 2.5% (1/40) each for the weaner and fattener groups.

**Antimicrobial susceptibility patterns of carbapenem-resistant Enterobacteriaceae**

The antimicrobial susceptibility profiles of the isolates with phenotypic resistance to one of the carbapenems, and \( \text{bla}_{\text{OXA-48}} \) positive isolates with a decreased susceptibility phenotype, were investigated. Isolates showed significant variation in antimicrobial resistance profiles; 7 distinct resistance profiles were revealed (Table 3). All 8 isolates were found to be non-susceptible to more than 3 classes of antimicrobials and categorized as multidrug resistant (MDR). All isolates were resistant to cephalothin and almost all isolates (87.5%, 7/8) were resistant to ampicillin (Table 4). The majority of isolates were resistant to amoxicillin-clavulanate (75%, 6/8), cefoxitin (62.5%, 5/8), chloramphenicol (62.5%), trimethoprim (62.5%), trimethoprim-sulfamethoxazole (62.5%); colistin resistance was found in 4 isolates (50%). All isolates remained susceptible to meropenem, aztreonam, ceftazidime, and piperacillin-azobactam (Table 4).
Table 4 Carbapenem-resistant *Enterobacteriaceae* isolated from a farrow-to-finish pig farm, Thailand.

| Isolate ID | Group | Species       | AMC  | AMP  | ATM  | FEP  | FOX  | CAZ  | CRO  | CXM  | CEF  | CHL  | CIP  |
|------------|-------|---------------|------|------|------|------|------|------|------|------|------|------|------|
|            |       |               | <16/8| >16 R| ≤2 S | >16 R| ≤2 S | ≤1 S | >16 R| >16 R| >16 R| >16 R| 2 I  |
| 51         | Weaner| *E. cloacae*  |      | >16 R|      | >16 R|      |      | >16 R| >16 R| >16 R|      |      |
| 06         | Fattener| *K. aerogenes*| >16 R| >16 R| ≤2 S | >16 R| ≤2 S | ≤1 S | >16 R| >16 R| >16 R| 1 S  |      |
| 07         | Fattener| *K. aerogenes*| >16 R| >16 R| ≤2 S | >16 R| ≤2 S | ≤1 S | >16 R| >16 R| >16 R| >0.5 S |      |
| 19         | Sow    | *K. aerogenes*| >16 R| >16 R| ≤2 S | >16 R| ≤2 S | ≤1 S | >16 R| >16 R| >16 R| 8 S  | ≤0.5 S|
| 45         | Piglet | *K. aerogenes*| >16 R| >16 R| ≤2 S | >16 R| ≤2 S | ≤1 S | >16 R| >16 R| >16 R| >0.5 S|      |
| 21         | Sow    | *P. mirabilis*| >16 R| >16 R| ≤2 S | >16 R| ≤2 S | ≤1 S | >16 R| >16 R| >16 R| >0.5 S|      |
| 42         | Piglet | *P. mirabilis*| ≤4/2 S| >16 R| ≤2 S | >16 R| ≤2 S | ≤1 S | >16 R| >16 R| >16 R| >0.5 S|      |
| 66         | Fattener| *P. mirabilis*| >16 R| <4 S | <2 S | >16 R| ≤2 S | ≤1 S | >16 R| >16 R| >16 R| >0.5 S|      |

| Isolate ID | Group | Species       | CST | ETP | IPM | MEM | NIT | GEN | NOR | TZP | TOB | TMP | SXT |
|------------|-------|---------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|            |       |               | <4 R| >1 R| <1 S| <1 S| ≤16 S| >8 R| 4 S | 64/4 I | >8 R | >8 R | >2/38 R |
| 51         | Weaner| *E. cloacae*  |      | >1 R| <1 S| <1 S| <16 S| >8 R| 4 S | 64/4 I | >8 R | >8 R | >2/38 R |
| 06         | Fattener| *K. aerogenes*| ≤1 S| ≤0.25 S | 2 I | <1 S| <16 S| <2 S| ≤2 S| ≤4/4 S| ≤2 S| >8 R | >2/38 R |
| 07         | Fattener| *K. aerogenes*| ≤1 S| ≤0.25 S | 4 R | <1 S| <16 S| <2 S| ≤2 S| ≤4/4 S| ≤2 S| >8 R | >2/38 R |
| 19         | Sow    | *K. aerogenes*| ≤1 S| 0.5 S | 4 R | <1 S| <16 S| <2 S| ≤2 S| ≤4/4 S| ≤2 S| <1 S | ≤0.5/9.5 S|
| 45         | Piglet | *K. aerogenes*| ≤1 S| ≤0.25 S | 4 R | <1 S| <16 S| <2 S| ≤2 S| ≤4/4 S| ≤2 S| <1 S | ≤0.5/9.5 S|
| 21         | Sow    | *P. mirabilis*| >4 R| 1 I | N/A | ≤1 S| >64 R| >8 R | >8 R | <4/4 S| >8 R | >8 R | >2/38 R |
| 42         | Piglet | *P. mirabilis*| >4 R| 0.5 S | N/A | ≤1 S| >64 R| >8 R | >8 R | <4/4 S| 8 I  | >8 R | >2/38 R |
| 66         | Fattener| *P. mirabilis*| >4 R| ≤0.25 S | N/A | ≤1 S| >64 R| >8 R | >8 R | <4/4 S| 8 I  | ≤1 S | ≤0.5/9.5 S|

S: susceptible; I: intermediate; R: resistant; N/A: not available

AMC: amoxicillin-clavulanate; AMP: ampicillin; ATM: aztreonam; FEP: cefepime; FOX: cefoxitin; CAZ: ceftazidime; CRO: ceftriaxone; CXM: cefuroxime; CEF: cephalothin; CHL: chloramphenicol; CIP: ciprofloxacin; CST: colistin; ETP: ertapenem; IPM: imipenem; MEM: meropenem; NIT: nitrofurantoin; GEN: gentamicin; NOR: norfloxacin; TZP: piperacillin-tazobactam; TOB: tobramycin; TMP: trimethoprim; SXT: trimethoprim-sulfamethoxazole
Colistin resistance genes, mcr, in carbapenem-resistant 
Enterobacteriaceae

Based on antimicrobial susceptibility testing results, 4 isolates with phenotypic resistance to carbapenems, and/or harboring \( \text{bla}_{\text{OXA-48}} \) gene were discovered to be resistant to colistin, with an MIC value of more than 4 µg/mL. To investigate whether colistin resistance phenotypes of these isolates were mediated by mobile colistin resistance genes (mcr), PCR detection of mcr-1 to mcr-9 genes was performed. None of the isolates were found to harbor mcr genes by PCR.

DISCUSSION

In this study, Enterobacteriaceae carrying the \( \text{bla}_{\text{OXA-48}} \) gene were detected in pigs of all age groups. There are a few reports of the presence of \( \text{bla}_{\text{OXA-48}} \) in CRE isolates from humans in Thailand (Köck et al, 2018; Lunha et al., 2016; Srijan et al., 2018). To the best of our knowledge, this is the first report of CRE harboring \( \text{bla}_{\text{OXA-48}} \) in pigs in Thailand.

The OXA-48 enzyme hydrolyses penicillins at a high level, while it has a low to moderate hydrolytic activity on carbapenems and poorly hydrolyzes expanded-spectrum β-lactamase (Zurfluh et al., 2015). In our study, all isolates carrying the \( \text{bla}_{\text{OXA-48}} \) gene exhibited a resistance phenotype to antibiotics belonging to the penicillin class and the first-generation cephalosporin, and all but one of the isolates were susceptible to ceftazidime. Only an E. cloaca and a K. aerogenes isolate were found to be resistant to carbapenems, indicating weak carbapenemases activities of the other 5 isolates harboring \( \text{bla}_{\text{OXA-48}} \) gene. Interestingly, two K. aerogenes isolates with phenotypic resistance to imipenem did not harbor the \( \text{bla}_{\text{OXA-48}} \) gene nor any carbapenemase genes tested. These isolates may possess other carbapenemase genes or may have other resistance mechanisms to carbapenems. The \( \text{bla}_{\text{OXA-48}} \) gene currently spreading is mostly linked to the frequently reported plasmid in Enterobacteriaceae, IncL/M plasmid type, which carries antimicrobial resistance genes (Carattoli, 2009). However, chromosomal acquisition of the \( \text{bla}_{\text{OXA-48}} \) gene through insertion of transposons was reported (Beyrouthy et al., 2013). The gene location and possible horizontal gene transfer mechanisms of the \( \text{bla}_{\text{OXA-48}} \) gene in the isolates recovered in this study will need to be further investigated.

There were two previous reports of OXA-48 carbapenemases in pigs. Nirupama (2018) reported \( \text{bla}_{\text{OXA-48}} \)-carrying E. coli from diarrheic piglets in India. The isolates were MDR, exhibiting resistance to many antibiotic drugs including penicillins, penicillins and beta-lactamase inhibitors, cephalosporins, monobactams, fluoroquinolones, tetracycline, trimethoprim-sulfamethoxazole, aminoglycosides, and carbapenems. More recently, Irrgang (2020a) reported an OXA-48-producing E. coli isolate in a German pig farm that was found to harbor the \( \text{bla}_{\text{OXA-48}} \) gene on the IncL/M plasmid. The isolate was also resistant to several antibiotics including ampicillin, cefepime, ertapenem, cefotaxime, imipenem, meropenem and temocillin. Colistin resistance of \( \text{bla}_{\text{OXA-48}} \)-carrying E. coli isolates was not reported in these studies. Similar to the previous reports, Enterobacteriaceae carrying \( \text{bla}_{\text{OXA-48}} \) isolates in our study were resistant to more than 3 classes of antibiotics and determined as MDR. Notably, we found that a majority of the isolates were non-susceptible to colistin,
an important “last-line” treatment for infections caused by CRE. Co-harboring of carbapenemase and mcr-1 genes in CRE isolates has been reported previously (Huang et al., 2020). However, we did not find the existence of colistin-resistance mcr-1 to mcr-9 genes in our isolates. Further work will need to be done to determine the genetic basis and mechanisms of colistin resistance.

Antimicrobial use in food-producing animals has been a common practice to control subclinical infection and to prevent disease, particularly when animals are under stressful situations such as moving between pens, and post vaccination or castration (Landers et al., 2012, Nuangmek et al., 2020). Antimicrobials can be applied in pigs by mixing into feed or drinking water (Callens et al., 2012). In Thailand, the national pig herd was 9.5 million and number of pigs per person was 0.14 in 2016 (Coyne et al., 2019). The amounts of antimicrobials of medicated feed for pigs in Thailand was 842 tons in 2017 (Lekagul et al., 2020). In the study farm in this report, amoxicillin and tiamulin are used in medicated feed for disease prevention. In addition, amoxicillin, enrofloxacin, gentamicin and ceftiofur are used for controlling subclinical infection and treatment of disease. Use of carbapenem is prohibited in food-producing animals in Thailand and carbapenems has not been used in the study farm. However, CRE were found to disseminate in this pig farm. Use of β-lactams such as cephalosporins has been hypothesized to be a selective pressure that contributes to maintenance and amplification of CRE in pigs (Mollenkopf et al., 2018). The use of amoxicillin and veterinary extended-spectrum cephalosporins, ceftiofur, may contribute to the occurrence and persistence of CRE isolates in the study farm. Direct or indirect transmission from human, other domestic animals, and flies to pigs may also be possible. A spillover of CRE strains from human to pigs has been demonstrated previously (Irrgang et al., 2020a).

In the cross-sectional study, CRE isolates were detected from all groups of healthy pigs during the pig production, except gilts. Based on antimicrobial resistance profiles and the presence of the blaOXA-48 gene, the occurrence of Enterobacteriaceae isolates with a carbapenem resistant phenotype and or harboring blaOXA-48 gene was 5% (2/40) in the groups of sows and piglets, and 2.5% (1/40) in the weaner and fattener groups. In this study farm, gilts were housed in separate pens, and were separated from the other groups. They may have a lower risk of exposure to CRE than weaners and fatteners in which 10-20 pigs were raised in a pen, providing opportunities for the exchange of bacteria and resistance genes among pigs. The findings that more isolates were recovered from sows and piglets than weaners and fatteners, although not significantly different, suggested that sows may be the reservoirs of CRE and the blaOXA-48 gene in this farm. Previous study also indicated sows as potential reservoirs for CRE in pig farms, and piglets as transient carriers of CRE (Mollenkopf et al., 2018). We cannot be certain whether carriage of CRE in pigs is transient or persistent throughout the pig production cycle since pigs at an early age in our longitudinal study did not carry CRE. Recovery of CRE in a finisher in the longitudinal study suggested that pigs may acquire CRE from humans or other animal sources in the farm, or de novo under selective antibiotic pressure. Lack of detailed administration of antibiotics in the study group is the limitation of our study. The presence of CRE in finishing pigs is an alarming signal of risk to public health since inappropriate slaughtering may spread CRE through food chains.
CONCLUSION

This study is the first report of Enterobacteriaceae with a carbapenem resistant phenotype and/or bla_{OXA-48} genotype in pigs in Thailand. Use of beta-lactam antibiotics may be responsible for the presence of CRE in this farm. Reports of CRE in food-producing animals are still rare and the means for the spread of CRE in animals is also unclear. Further investigation and characterization of CRE in pigs is required for a better understanding of their acquisition and spread in pig farms, which would help to develop appropriate management strategies for controlling CRE.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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