Antibiotic resistant *Escherichia coli* from diarrheic piglets from pig farms in Thailand that harbor colistin-resistant *mcr* genes

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Antibiotic-resistant *Escherichia coli* is one of the most serious problems in pig production. This study aimed to determine the antibiotic susceptibility and genotypes profiles of diarrhoeagenic *E. coli* that causes diarrhea in piglets. Thirty-seven pathogenic *E. coli* strains were used in this study. These were isolated from rectal swabs of diarrheic piglets from farms in Thailand from 2018 to 2019. *Escherichia coli* isolates were highly resistant to amoxicillin (100%), followed by oxytetracycline (91.9%), enrofloxacin (89.2%), trimethoprim/sulfamethoxazole (86.5%), amoxicillin: clavulanic acid (81.1%), colistin and gentamicin (75.7%), ceftriaxone and ceftiofur (64.9%), ceftazidime (35.1%) and 97.3% showed multidrug-resistance (MDR). There were 8 (21.6%) *mcr-1* carriers, 10 (27.0%) *mcr-3* carriers and 10 (27.0%) co-occurrent *mcr-1* and *mcr-3* isolates. The phenotype-genotype correlation of colistin resistance was statistically significant (performed using Cohen’s kappa coefficient (κ = 0.853; *p* < 0.001)). In addition, PCR results determined that 28 of 37 (75.7%) isolates carried the *int1* gene, and 85.7% *int1*-positive isolates also carried the *mcr* gene. Genetic profiling of *E. coli* isolates performed by ERIC-PCR showed diverse genetics, differentiated into thirteen groups with 65% similarity. Knowledge of the molecular origins of multidrug-resistant *E. coli* should be helpful for when attempting to utilize antibiotics in the pig industry. In terms of public health awareness, the possibility of transmitting antibiotic-resistant *E. coli* from diarrheic piglets to other bacteria in pigs and humans should be of concern.

Abbreviations

| AMC | Amoxicillin: clavulanic acid |
| AMX | Amoxicillin |
| ATCC | American type culture collection |
| CAZ | Cefazidime |
| CEF | Ceftiofur |
| CLSI | Clinical and Laboratory Standards Institute |
| CN | Gentamicin |
| CRO | Ceftriaxone |
| CT | Colistin |
| ENR | Enrofloxacin |
| DAEC | Diffusely adherent *E. coli* |
| EAEC | Enteroaggregative *E. coli* |
| EIEC | Enteroinvasive *E. coli* |
| EPEC | Enteropathogenic *E. coli* |
| ERIC-PCR | Enterobacterial repetitive intergenic consensus polymerase chain reaction |
| ETEC | Enterotoxigenic *E. coli* |
| MCRPE | *mcr*-Positive *Escherichia coli* |
| MDR | Multidrug resistance |

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such as plasmids, transposons, and integrons that facilitate the integration and spread of resistance genes. The but it is more rapid than simple mutations. Resistance genes are often associated with mobile genetic elements. Enterobacteriaceae, including E. coli, are separated into non-pathogenic and pathogenic groups. The non-pathogenic E. coli is a commensal present in the intestinal lumen. Pathogenic E. coli are causative agents of intestinal and extraintestinal diseases in humans and animals. Escherichia coli strains are classified into different "pathotypes" based on the presence of virulence factors. There are six types of diarrhoeagenic E. coli, including enterotoxigenic E. coli (ETEC), enteropathogenic E. coli (EPEC), Shiga toxin-producing E. coli (STEC), enteroinvasive E. coli (EIEC), enteraggregative E. coli (EAEC), and diffusely adherent E. coli (DAEC) that causes diarrhea in young pigs. An outbreak of E. coli frequently requires quick action, and therefore antibiotics are used in the pig industry to control diarrhea caused by E. coli.

Unfortunately, using antibiotics for decades led to developed antimicrobial resistance in pathogens and caused risks of transmitting antimicrobial resistance genes transmission in the environment. Presently, antimicrobial resistance (AMR) is rising to dangerously high levels, while it is causing public health issues worldwide. For example, antibiotics are becoming less effective in the treatment of common infectious diseases in humans. The type of antibiotics frequently used in diarrhea caused by E. coli in pigs are β-lactam antibiotics (amoxicillin and a combination containing amoxicillin/clavulanic acid), cephalosporins (ceftiofur, ceftazidime), aminoglycosides (ampicillin, neomycin, gentamicin), aminocyclitols (spectinomycin), sulfonamide combined with trimethoprim (such as trimethoprim/sulfamethoxazole), fluoroquinolones (enrofloxacin, marbofloxacin and danofloxacin), quinolones (flumequine) and polymyxins (colistin sulfate).

Colistin (also known as polymyxin E) is an antibiotic commonly used against Gram-negative bacteria in pig production to prevent and treat diarrhea caused by E. coli. Excessive utilization of colistin in animal production has created selective pressure contributing to the increased resistance to colistin. In fact, colistin resistance brought about colistin-resistant genes in the mcr family. Since the first mcr-1 gene was reported in 2016, so far ten mcr variants have been described. Among them, E. coli was reported to harbor the mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5 genes. The mcr-1 gene is the most frequently detected in different animal food species and from Enterobacteriaceae infections in humans. Strains carrying antibiotic-resistance genes can transmit resistance genes to other pathogens. Horizontal gene transfer is a major mechanism for increased antimicrobial resistance, but it is more rapid than simple mutations. Resistance genes are often associated with mobile genetic elements such as plasmids, transposons, and integrons that facilitate the integration and spread of resistance genes. The class 1 integron-integrase gene (int1) plays a major role in the dissemination of antibiotic resistance. It is a genetic mechanism that allows bacteria to acquire, store and express new genes.

Resistance of E. coli isolates from pig farms to a wide range of antimicrobial agents has dramatically increased over several years worldwide. Studies from pig production farms by García et al. showed 91.6% resistance to at least three different antimicrobial classes, van Breda et al. showed that over 70% of isolates were resistant to antibiotics commonly used in veterinary practice, and Khine et al. showed 86.2% multidrug-resistance (MDR), and MHB (Mueller Hinton broth), MIC (Minimal inhibitory concentration), OTC (Oxytetracycline), PCR (Polymerase chain reaction), STEC (Shiga toxin-producing E. coli), SXT (Trimethoprim/sulfamethoxazole), TBE (Tris–borate-EDTA), UPGMA (Unweighted pair-group method using arithmetic averages).

Materials and methods

Escherichia coli collection and virulence genes detection. Thirty-seven pathogenic E. coli isolates were previously isolated and identified in routine microbiology service at the Laboratory of Bacteria, Veterinary Diagnostic Center, Faculty of Veterinary Science, Mahidol University. The isolates were obtained from rectal swabs of diarrheic piglets from farms in Thailand during Edema disease outbreak from 2018 to 2019. All isolates were stored at −80 °C at the Veterinary Diagnostic Center, Faculty of Veterinary Science, Mahidol University, Thailand. Nineteen specific virulence genes of pathogenic E. coli, including 7 toxins genes (lt, sth, stp, stx1A, stx2A, stx2e and astA), 7 adhesin genes (bfpA, eaeA, ipaH, aggR, pCDV432, paa and aidA) and 5 fimbriae genes (F4, F5, F6, F18 and F41) were detected using multiplex PCR. Two groups of primer sets were used to detect and discriminate virulence genes in E. coli isolates as listed in Supplementary Table S1. Multiplex PCR was carried out using a BiometraTOne96G thermocycler (AnalytikJena, Germany). For the group A primer (1A and 2A), amplification was performed with an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. In the case of amplification with the group B primer (1B, 2B and 3B), PCR was conducted with an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 61 °C, 57 °C, and 56 °C for fimbriae, toxins, and alternate adhesins, respectively, for 45 s, extension at 72 °C for 1 min, and a final extension step at 72 °C for 7 min. The PCR products were separated using 1.5% agarose gel electrophoresis, stained with 1X GelRed (Sigma Aldrich, USA), and visualized under a UV transilluminator UVP GelStudio (AnalytikJena, USA).
Antimicrobial susceptibility testing. All isolates were tested for antimicrobial susceptibility by broth microdilution according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (VET01S)\textsuperscript{17,18}. Broth microdilution in 96-well microdilution plates was used to determine minimal inhibitory concentrations (MICs). The antibiotic stock solution (256 µg/mL) was diluted by serial two-fold dilutions in Mueller Hinton broth (MHB) and a quality control was composed of media without antibiotic. The following antibiotics were tested: amoxicillin (AMX), amoxicillin: clavulanic acid (AMC), cefotiofure (CEF), ceftazidime (CAZ), ceftriaxone (CRO), colistin (CT), enrofloxacin (ENR), gentamicin (CN), oxytetracycline (OTC), trimethoprim/sulfamethoxazole (SXT). The inoculum was prepared by taking colonies from nutrient agar (NA) by a sterile swab and preparing a McFarland standard. The inoculum was dispensed into the microdilution plate with the serially diluted antibiotic and incubated at 37 °C for 16–20 h. Escherichia coli ATCC 25922 was used in each assay as a quality control. Results were recorded as the lowest concentration of an antimicrobial that inhibited visible growth of a microorganism.

The resistance breakpoints of the E. coli isolates were as follows: amoxicillin (AMX) (≥ 32 µg/mL), amoxicillin: clavulanic acid (AMC) (≥ 32 µg/mL), cefotiofure (CEF) (≥ 8 µg/mL), ceftazidime (CAZ) (≥ 16 µg/mL), ceftriaxone (CRO) (≥ 4 µg/mL), colistin (CT) (≥ 4 µg/mL), enrofloxacin (ENR) (≥ 2 µg/mL), gentamicin (CN) (≥ 16 µg/mL), oxytetracycline (OTC) (≥ 16 µg/mL) and trimethoprim/sulfamethoxazole (SXT) (≥ 8 µg/mL) according to CLSI guidelines (VET01S, M100)\textsuperscript{17,18}. Multidrug resistance (MDR) was defined “as non-susceptibility to at least one microdilution according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (VET01S)\textsuperscript{17}. Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) for E. coli isolates. ERIC-PCR was performed on a total of 37 E. coli isolates with primers ERIC-1 (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) and ERIC-2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′) that were described in previous studies\textsuperscript{25}. Genomic DNA was extracted from 1 mL of overnight culture using Qiaprep Spin Miniprep Kit (Qiagen, Germany) following the manufacturer’s instructions. PCR reactions were performed in a total volume of 20 µL containing 0.4 µM of each forward and reverse primer, 20 ng of DNA template and 1× Green PCR master mix kit (Biotechrabbit, Germany). The amplification steps were accomplished using a Biometra-TOne96G thermocycler (AnalytikJena, Germany) with the following thermal cycles: the initial denaturation at 94 °C for 5 min, followed by 25 cycles (denaturation at 94 °C for 30 s, annealing at 58 °C for 90 s, and extension at 72 °C for 60 s), and a final extension step at 72 °C for 5 min. The PCR products were separated using 1.5% agarose gel electrophoresis, stained with 1× GelRed (Sigma Aldrich, USA) and then visualized under a UV transilluminator UVP GelStudio (AnalytikJena, USA).

Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) for E. coli isolates. ERIC-PCR was performed on a total of 37 E. coli isolates with primers ERIC-1 (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) and ERIC-2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′) that were described in previous studies\textsuperscript{25}. Genomic DNA was extracted from 1 mL of overnight culture using a G-spin\textsuperscript{™} Genomic DNA Extraction Kit (iNtRON, Korea) and following the manufacturer’s instructions. ERIC-PCR was performed in a total volume of 20 µL containing 0.4 µM concentrations of each forward and reverse primer, 20 ng of DNA template and 1× Green PCR master mix kit (Biotechrabbit, Germany). The amplification steps were completed using a BiometraTOne96G thermocycler (AnalytikJena, Germany) with the following thermal cycles: the initial denaturation at 94 °C for 3 min, followed by 25 cycles (denaturation at 94 °C for 30 s, annealing at 58 °C for 90 s, and extension at 72 °C for 60 s), and a final extension step at 72 °C for 5 min. The PCR products were separated using 1.5% agarose gel electrophoresis, stained with 1× GelRed (Sigma Aldrich, USA) and then visualized under a UV transilluminator UVP GelStudio (AnalytikJena, USA).

Detection of virulence genes in E. coli. The distribution of virulence genes in each E. coli isolate are presented in Table 1. Among the 37 E. coli isolates examined, 30 (81.1%) were found to harbor virulence genes. Ten genes encoding astA, F4, F18 and F41 were detected. The prevalence of toxins genes, astA, F4, F18 and F41 were 53.1, 24.3, 18.9, 5.4 and 2.7%, respectively. The prevalence of the adhesin genes, eaeA, F18, F4, F41 were 16.2, 2.7 and 2.7%, respectively. In contrast, the genes encoding stx1A, stx2A, ipaH, bfpA, pCVD432, aggR, paa, F5 and F6 were not detected in any of the 37 isolates examined.

Detection of mcr and int1 genes using multiplex polymerase chain reaction (mPCR). Plasmid-mediated colistin-resistant genes mcr-1, mcr-2, mcr-3, mcr-4, mcr-5, mcr-6, mcr-7, mcr-8, mcr-9 and mcr-10 were detected by simplex PCR and the class 1 integron-integrase gene (int1) was detected by simplex PCR. mPCR was performed in 2 reactions including reaction 1 (for detecting mcr-1, mcr-4, mcr-5, mcr-7, mcr-8 and mcr-10) and reaction 2 (for detecting mcr-2, mcr-3, mcr-5, mcr-6 and mcr-9), and all reactions were performed with a positive control (contained genes of mcr-1 to 10). Plasmid DNA was extracted from 1 mL of overnight culture using QIAprep Spin Miniprep Kit (Qiagen, Germany) following the manufacturer’s instructions. PCR reactions were performed in a total volume of 20 µL containing 0.4 µM of each forward and reverse primer, 20 ng of DNA template and 1× Green PCR master mix kit (Biotechrabbit, Germany). The amplification steps were accomplished using a Biometra-TOne96G thermocycler (AnalytikJena, Germany) with the following thermal cycles: the initial denaturation at 94 °C for 5 min, followed by 35 cycles (denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min), and a final extension step at 72 °C for 5 min. The PCR products were separated using 1.5% agarose gel electrophoresis, stained with 1× GelRed (Sigma Aldrich, USA) and then visualized under a UV transilluminator UVP GelStudio (AnalytikJena, USA).

Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) for E. coli isolates. ERIC-PCR was performed on a total of 37 E. coli isolates with primers ERIC-1 (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) and ERIC-2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′) that were described in previous studies\textsuperscript{25}. Genomic DNA was extracted from 1 mL of overnight culture using a G-spin\textsuperscript{™} Genomic DNA Extraction Kit (iNtRON, Korea) and following the manufacturer’s instructions. ERIC-PCR was performed in a total volume of 20 µL containing 0.4 µM concentrations of each forward and reverse primer, 20 ng of DNA template and 1× Green PCR master mix kit (Biotechrabbit, Germany). The amplification steps were completed using a BiometraTOne96G thermocycler (AnalytikJena, Germany) with the following thermal cycles: the initial denaturation at 94 °C for 5 min, followed by 35 cycles (denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 5 min), and a final extension step at 72 °C for 10 min. PCR products were separated using 2.0% agarose gel electrophoresis, stained with 1× GelRed (Sigma Aldrich, USA), and visualized under a UV transilluminator UVP GelStudio (AnalytikJena, USA). ERIC-PCR results were analyzed by online data analysis services (insilico.ehu.es). ERIC profiles were compared using the Dice coefficient method, and a dendrogram was made via the unweighted pair-group method using arithmetic averages (UPGMA).

Statistical analysis. Genotype–phenotype correlations of harboring mcr genes and colistin resistance in E. coli isolates were performed using Cohen’s kappa coefficient in SPSS version 23 (IBM Corp. in Armonk, NY). p value of < 0.05 were considered statistically significant.

Ethics approval and consent to participate. The study was carried out in compliance with the ARRIVE guidelines. This research project was approved by the Faculty of Veterinary Science–Animal Care and Use Committee (FVS-ACUC-Protocol No. MUVS-2019-06-31 and MUVS-2021-10-40). All methods were performed in accordance with the relevant guidelines and regulations.

Results Detection of virulence genes in E. coli. The distribution of virulence genes in each E. coli isolate are presented in Table 1. Among the 37 E. coli isolates examined, 30 (81.1%) were found to harbor virulence genes. Ten genes encoding astA, F4, F18 and F41 were detected. The prevalence of toxins genes, astA, F4, F18 and F41 were 53.1, 24.3, 18.9, 5.4 and 2.7%, respectively. The prevalence of the adhesin genes, eaeA, F18, F4, F41 were 16.2, 2.7 and 2.7%, respectively. In contrast, the genes encoding stx1A, stx2A, ipaH, bfpA, pCVD432, aggR, paa, F5 and F6 were not detected in any of the 37 isolates examined.
Antimicrobial susceptibility testing. Thirty-seven pathogenic E. coli isolates were tested for antimicrobial susceptibility to 10 different antibiotics (Table 1). All of the samples were resistant towards amoxicillin (100%) followed by oxytetracycline (91.9%), enrofloxacin (89.2%), trimethoprim/sulfamethoxazole (86.5%), amoxicillin:clavulanic acid (81.1%), colistin and gentamicin (75.7%), ceftriaxone and ceftiofur (64.9%) and ceftazidime (35.1%) (Fig. 1). The results showed that 36 of 37 isolates (97.3%) were resistant to at least four different antimicrobial classes, which indicates multidrug-resistance (MDR). About 75.68% showed resistance to β-lactams, fluoroquinolone and aminoglycosides/polymyxin E, and 45.95% of isolates were resistant to all seven antimicrobial classes with different patterns (Table 2).

Table 1. Characteristics and antimicrobial susceptibility against 10 antimicrobial agents of E. coli isolated from the rectal swabs of diarrheic piglets of farms in Thailand during 2018 to 2019. Virulence factors included in this data table: shiga-toxin (stx2e), heat-labile enterotoxin (lt), heat-stable enterotoxin (stp, sth), enteroaggregative E. Coli heat-stable enterotoxin 1 (astA), intimin (eaeA), adhesin (aidA), fimbriae (F4, F18, F41). The values were below or above the dilution range marked with the sign “≤” or “>”. MICs highlighted in light grey represent resistance according to CLSI (VET01S, M100) guidelines. NA means not analyzed.

![Figure 1. The antimicrobial-resistance percentages of E. coli strains (N = 37). Antimicrobial susceptibility was performed by MIC assay and analyzed based on the resistance breakpoints according to CLSI (VET01S, M100) guidelines.](https://example.com/figure1.png)
The majority of E. coli isolates (29 of 37) were from Western Thailand, which has the highest pig farm density in Thailand. Minority isolates were from Central and Eastern Thailand (i.e., 2 and 6 isolates, respectively). The result of antimicrobial susceptibility showed resistance to amoxicillin, amoxicillin: clavulanic acid, colistin, enrofloxacin, oxytetracycline, trimethoprim/sulfamethoxazole, which is common across all three regions from 66.7 to 100% resistance (Fig. 2). Isolated E. coli (2/2) from Central Thailand were resistant to ceftiofur, ceftriaxone and susceptible to gentamicin, ceftazidime. All of the samples from Eastern Thailand were resistant to gentamicin with 83.3% and 100% of samples (6/6) susceptible towards third-generation cephalosporins including ceftiofur, ceftazidime, and ceftriaxone. Escherichia coli isolates from Western Thailand were resistant towards gentamicin (79.3%), ceftiofur (75.9%), ceftriaxone (75.9%) and ceftazidime (44.8%).

Colistin-resistance genes (mcr) and the class 1 integron-integrase gene (int1). A total of 37 pathogenic E. coli isolates were determined for colistin-resistance genes mcr-1 to 10 by multiplex PCR. A total of 28 (75.7%) isolates carried mcr-1 and mcr-3 genes (Table 3). None of the isolates carried mcr-2, mcr-4, mcr-5, mcr-6, mcr-7, mcr-8, mcr-9 and mcr-10 genes. mcr-1 gene was found in 18 of 37 (48.6%) isolates and the mcr-3 gene was found in 20 of 37 isolates (54.1%). Among 28 mcr-positive isolates, 10 (27.0%) isolates carried both mcr-1 and mcr-3 genes. The correlation between genotypes and phenotypes of colistin resistance are seen in Table 4. PCR results also determined that 28 of 37 (75.7%) isolates carried the int1 gene (Table 1). Almost all int1-positive isolates (24 isolates) carried the mcr gene and only four int1-positive isolates did not contain the mcr gene.

Genetic profiling of E. coli isolates. ERIC sequences of 37 isolates were amplified using PCR with ERIC-1 and ERIC-2 primers. Bands for each sample were recorded according to their molecular weights based on a molecular marker (100 bp DNA Ladder). All 37 pathogenic E. coli isolates had bands and were genotyped. The dendrogram from ERIC-PCR banding pattern was analyzed and the isolates were differentiated into thirteen groups with 65% similarity (Fig. 3). ERIC-PCR profile of some isolates showed a difference from others resulting in separate groups with only one isolate in each group (II, V, IX, X and XII). However, groups IV, VII and VIII had a greater number of isolates with 5, 8 and 7 isolates, respectively.

Discussion
Diarrheal disease caused by E. coli is one of the most common diseases in neonatal and weaned piglets. Although there are many approaches to prevent pathogenic E. coli infection in piglets, antibiotics are still commonly used to treat enteric colibacillosis in swine16. However, using antibiotics to control infection led to increased selection pressure and resulted in the selection of antibiotic-resistant bacteria. Governments sector worldwide, including Thailand, issued regulations on the use of antibiotics in livestock; however, the risk of antimicrobial resistance gene transmission is still a great global concern. Escherichia coli that carry the antimicrobial resistance gene

| Pattern | Profile | Number of resistance antimicrobials | Isolate(s) |
|---------|---------|-----------------------------------|------------|
| A       | AMX-AMC-ENR-OTC-SXT-CT-CN-CRO-CEF-CAZ | 10         | 5          |
| B       | AMX-AMC-ENR-OTC-SXT-CN-CRO-CEF-CAZ | 9          | 2          |
| C       | AMX-AMC-ENR-OTC-CT-CN-CRO-CEF-CAZ | 9          | 1          |
| D       | AMX-AMC-ENR-OTC-SXT-CT-CN-CRO-CEF | 9          | 3          |
| E       | AMX-ENR-OTC-SXT-CT-CN-CRO-CEF-CAZ | 9          | 4          |
| F       | AMX-AMC-ENR-OTC-SXT-CN-CRO-CEF | 8          | 1          |
| G       | AMX-AMC-ENR-OTC-CT-CN-CRO-CEF | 8          | 3          |
| H       | AMX-AMC-ENR-OTC-CT-CN-CRO-CEF | 8          | 1          |
| I       | AMX-ENR-SXT-CT-CN-CRO-CEF-CAZ | 8          | 1          |
| J       | AMX-ENR-SXT-CT-CN-CRO-CEF | 7          | 1          |
| K       | AMX-AMC-ENR-OTC-SXT-CN | 7          | 4          |
| L       | AMX-OTC-SXT-CT-CN-CRO-CEF | 7          | 1          |
| M       | AMX-AMC-ENR-OTC-CRO-CEF | 6          | 1          |
| N       | AMX-AMC-ENR-OTC-SXT-CN | 6          | 1          |
| O       | AMX-AMC-ENR-OTC-SXT-CN | 6          | 1          |
| P       | AMX-AMC-ENR-OTC-CRO-CEF | 6          | 1          |
| Q       | AMX-AMC-OTC-SXT-CN | 5          | 2          |
| R       | AMX-AMC-ENR-OTC-CRO-CEF | 5          | 1          |
| S       | AMX-AMC-ENR-OTC-CRO-CEF | 5          | 1          |
| T       | AMX-ENR-OTC-SXT-CN-CN | 5          | 1          |
| U       | AMX-AMC-OTC | 3          | 1          |

Table 2. Antibiograms based on the MIC results of 37 E. coli strains distributing into 21 pattern types (A-U). AMX amoxicillin, AMC amoxicillin: clavulanic acid, CEF ceftiofur, CAZ ceftazidime, CRO ceftriaxone, CT colistin, ENR enrofloxacin, CN gentamicin, OTC oxytetracycline, SXT trimethoprim/sulfamethoxazole.
may transfer these to other pathogens and human pathogens, particularly the \textit{mcr} gene which mediates colistin resistance in animals and humans\(^6\). Genotype and antimicrobial susceptibility-profiles of \textit{E. coli} would be helpful for the clinical use of antibiotics. In this study, we investigated whether or not \textit{E. coli} found in rectal swabs of diarrheic piglets from farms in Thailand were resistant to antimicrobes and if they harbored colistin-resistance \textit{mcr} genes. Our data showed that most of these strains contain one or more virulence factor genes (Table 1).

The result of antimicrobial susceptibility testing showed that 37 \textit{E. coli} isolates were resistant to at least three antibiotics with 21 different patterns. The resistance rate to ceftazidime were the lowest with 35.1% and the resistance rate to amoxicillin were the highest with 100%. 97.3% of the isolates were multidrug-resistant (MDR), which means resistant to at least one agent in three or more antimicrobial categories. Among them, the resistance to

![Figure 2. The MIC profiles of 37 \textit{E. coli} isolates from diarrheic piglets. The right vertical axis lists the geographical regions of Thailand and the horizontal axis is labeled with the antibiotics. The color bar represents the antimicrobial susceptibility results.](image)

| \textit{mcr} genes | No. of isolates | Different MIC of colistin (%) |
|-------------------|----------------|------------------------------|
|                   | 1 \textmu{g}/mL | 2 \textmu{g}/mL | 4 \textmu{g}/mL | 8 \textmu{g}/mL |
| \textit{mcr-1}    | 8              |            |            | 5 (17.9%) | 3 (10.7%) |
| \textit{mcr-3}    | 10             |            |            | 8 (28.6%) | 2 (7.1%)  |
| \textit{mcr-1} + \textit{mcr-3} | 10 | 1 (3.6%) |            | 2 (7.1%) | 7 (25.0%) |
| Total             | 28 (100%)      | 1 (3.6%)   | 0           | 15 (53.6%) | 12 (42.9%) |

Table 3. The distribution of \textit{E. coli} strains (\textit{N} = 28/37) harboring colistin-resistance genes at different MICs of colistin.
Table 4. Comparison between genotypes and phenotypes of colistin resistance using mPCR and MIC assays from 37 E. coli isolates, which are based on the resistance breakpoints according to CLSI (VET01S, M100) guidelines.

| Colistin Resistance phenotype | Resistance genotype | Non-resistance genotype | Total |
|------------------------------|---------------------|-------------------------|-------|
| Resistance phenotype         | 27                  | 1                       | 28    |
| Non-resistance phenotype     | 1                   | 8                       | 9     |
| Total                        | 28                  | 9                       | 37    |

Figure 3. Dendrogram of 37 E. coli isolates from ERIC-PCR banding pattern. Similarity analysis was performed by Dice coefficient and UPGMA method. The original ERIC-PCR gels are presented in Supplementary Fig. S1.
β-lactams and fluoroquinolone antimicrobial were the most frequent. Consequently, a diarrheal disease caused by multidrug-resistant E. coli (MDR-E. coli) can be difficult to treat in pig farms. These results contribute to the overall picture of antimicrobial resistance in E. coli in the pig industry. Studies from many countries showed that MDR-E. coli isolates from pigs were 86.2% in the US (between November 2013 and December 2014)\(^8\), 81.1% in Vietnam (from July to September 2019)\(^2\), 47% in Uganda\(^2\), 57.3% in Indonesia (between March 2017 and February 2018)\(^3\), 72.5% in Brazil\(^2\), and 81% in China\(^3\). In Spain, colistin-resistant E. coli isolates from pig farms were 91.6% resistant to at least three different antimicrobial classes\(^4\); in addition, 100% of mcr-positive E. coli isolated from fecal samples of healthy pigs are MDR\(^5\).

In this study, colistin-resistance genes were detected in 28 isolates (75.7%). In these 28 mcr-positive E. coli, the mcr-1 and mcr-3 genes were detected whereas mcr-2, mcr-4, mcr-5, mcr-6, mcr-7, mcr-8, mcr-9 and mcr-10 genes were not detected. Eight (21.6%) were mcr-1 carriers, 10 (27.0%) were mcr-3 carriers and 10 (27.0%) demonstrated co-occurrence of mcr-1 and mcr-3 isolates. The mcr-1 is the first gene reported and is globally distributed whereas others are less distributed\(^6\). In recent studies, the mcr-3 was also found along with mcr-1 0.43% and 3% respectively in E. coli isolates from pig farms in Thailand and Vietnam\(^6\). This is the first result that showed the mcr-3 gene found with a high rate (54%), which should be an alert for the rapid emergence of mcr-3-mediated colistin resistance in pig farm in Thailand.

On the other hand, one of the 28 colistin-resistant isolates did not carry the mcr gene (tested mcr genes) in this study. On the contrary, one mcr-positive (mcr-1 and mcr-3) isolate was susceptible to colistin, which is in agreement with an earlier report by Garcia et al.\(^4\) in which six of 143 colistin-resistant isolates did not contain mcr genes and three mcr-positive isolates were susceptible to colistin. This inactive form of mcr might be explained by the occurrence of an insertion of a 1.7 Kb IS1294b element into mcr-1\(^7\). It does not escape our attention that there was one mcr-negative colistin-resistant isolate. There might possibly be an alternative colistin-resistant mechanism that does not involve the mcr gene. Such mechanism would be of interest for further study. Nonetheless, the correlation between genotypes and phenotypes of colistin resistance was considered strongly statistically significant (κ = 0.853; p < 0.001). The high proportion of mcr-positive isolates (75.7%) is a risk for public health. In addition, 75.7% of isolates carried the \(int\)1 gene and 24/28 (85.7%) \(int\)-positive isolates carried the mcr gene. It has been reported that the presence of the \(int\)1 gene (a mobile genetic element) in pathogens, may increase the ability to transfer resistance genes to other bacteria in the environment\(^27\). ERIC-PCR profiles of isolated E. coli showed that isolates have diverse genetic structures. A total of 37 isolates were differentiated into thirteen groups with 65% similarity. Beside various gene sources, the rapid and easy genetic modification to adapt in E. coli may also cause genetic diversity. Frequent use of antibiotics in pig farms creates selective pressure leading to genetic modification in E. coli. In addition, each pig farm may have its own treatment approach for the selection of antibiotics in order to control piglet diarrhea. This can also result in the genetic diversity of isolates among pig farms\(^28\). In addition, Guenther et al. showed that antibiotic-resistant strains can transmit resistance genes to other pathogenic bacteria, especially to human pathogens\(^29\). It is worth noting that colistin is an antibiotic that is widely used in both humans and animals. Thus, the possibility of spreading the mcr gene from animals to humans is a serious public health concern. Bacteria carrying mcr gene have been found in humans and animals in many countries. Recently, it has been demonstrated that E. coli strains carrying mcr-1 and mcr-3 genes were found not only in pig feces, but also from contaminated pig carcasses and pork\(^30\). This indicates a high risk of spreading bacteria-harboring mcr genes to humans and other environments.

**Conclusion**

In the present study, multidrug-resistance (MDR) E. coli was found in a high proportion of fecal sample (97.3%) isolated from diarrheic piglets from pig farms in Thailand. From 10 mcr (1–10) genes tested, a large number of isolates harbored colistin-resistance genes mcr-1 and mcr-3. The correlation between the colistin resistance phenotype and genotype among isolates was significant (p < 0.001). Taken together, the results of this study provide informative scientific evidence regarding bacterial resistance to antibiotics in pig farms, and it should also raise public health awareness regarding transmitting resistance gene from animals to humans.

**Data availability**

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Received: 16 December 2021; Accepted: 23 May 2022

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Acknowledgements

We thank Dr. Anusak Kerdsin, Faculty of Public Health, Kasetsart University, Chalermphrakiat Sakon Nakon Province Campus, Sakon Nakhon, Thailand for mcr 1-10 reference strains.

Author contributions

L.N. performed the methods, data collection, analysis and prepared the first manuscript. Kr.K. performed laboratory works and prepared the bacterial material. K.K. conceptualized, supervised, edited the manuscript and secured funding. N.N. conceived of the idea, investigated, supervised, analyzed data, edited the manuscript, and managed the project. All authors read and approved the final manuscript.

Funding

This work was financially supported by Royal Golden Jubilee Ph.D. Programme (RGJ Ph.D.) under Grant number PHD/0074/2561 and Mahidol University, Thailand.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-13192-3.

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