Detection and characterization of ESBL-producing *Escherichia coli* and additional co-existence with *mcr* genes from river water in northern Thailand

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Background. Extended-spectrum β-lactamase producing *Escherichia coli* (ESBL-producing *E. coli*) have emerged, causing human and animal infections worldwide. This study was conducted to investigate the prevalence and molecular genetic features of ESBL-producing and multidrug-resistant (MDR) *E. coli* in river water. **Methods.** A total of 172 *E. coli* samples were collected from the Kok River and Kham River in Chiang Rai, Thailand, during a 10-month period (2020-2021). **Results.** We detected 45.3% of *E. coli* to be MDR. The prevalence of ESBL-producers was 22%. Among those ESBL-producing strains, CTX-M-15 (44.7%) was predominantly found, followed by CTX-M-55 (26.3%), CTX-M-14 (18.4%), and CTX-M-27 (10.5%). The $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-116}}$ genes were found to be co-harbored with the $bla_{\text{CTX-M}}$ genes. Mobile elements, i.e., IS*Ecp*1 and Tn*3*, were observed. Twelve plasmid replicons were found, predominantly being IncF (76.3%) and IncFIB (52.6%). Whole genome sequencing of ten selected isolates revealed the co-existence of ESBL with *mcr* genes in two ESBL-producing *E. coli*. A wide diversity of MLST classifications was observed. An *mcr-1.1-pap2* gene cassette was found to disrupt the PUF2806 domain-containing gene, while an *mcr-3.4* contig on another isolate contained the *nimC*/*nimA-mcr-3.4-dgkA* core segment. **Discussion.** In conclusion, our data provides compelling evidence of MDR and ESBL-producing *E. coli*, co-existing with *mcr* genes in river water in northern Thailand, which may be disseminated into other environments and so cause increased risks to public health .

Detection and characterization of ESBL-producing *Escherichia coli* and additional co-existence with *mcr* genes from river water in northern Thailand

Short title: ESBL-*E.coli* in River Water

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Abstract

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Methods. A total of 172 *E. coli* samples were collected from the Kok River and Kham River in Chiang Rai, Thailand, during a 10-month period (2020-2021).

Results. We detected 45.3% of *E. coli* to be MDR. The prevalence of ESBL-producers was 22%. Among those ESBL-producing strains, CTX-M-15 (44.7%) was predominantly found, followed by CTX-M-55 (26.3%), CTX-M-14 (18.4%), and CTX-M-27 (10.5%). The $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-116}}$ genes were found to be co-harbored with the $bla_{\text{CTX-M}}$ genes. Mobile elements, i.e., IS*Ecp*1 and Tn*3*, were observed. Twelve plasmid replicons were found, predominantly being IncF (76.3%) and IncFIB (52.6%). Whole genome sequencing of ten selected isolates revealed the co-existence of ESBL with *mcr* genes in two ESBL-producing *E. coli*. A wide diversity of MLST classifications was observed. An *mcr-1.1-pap2* gene cassette was found to disrupt the PUF2806 domain-containing gene, while an *mcr-3.4* contig on another isolate contained the *nimC*/*nimA*-*mcr-3.4-dgkA* core segment.

Discussion. In conclusion, our data provides compelling evidence of MDR and ESBL-producing *E. coli*, co-existing with *mcr* genes in river water in northern Thailand, which may be disseminated into other environments and so cause increased risks to public health.

Key words: Extended-spectrum β-lactamase producing *Escherichia coli,* River water, *mcr*, multidrug-resistant

Introduction

Escherichia coli (*E. coli*) are commensal bacteria in humans and animals. However, *E. coli* is a commonly implicated bacteria, that can cause a variety of diseases, including diarrhea, septicemia, and urinary tract infection. Because *E. coli* can acquire antimicrobial resistant genes via horizontal gene transfer, therefore, multidrug-resistant *E. coli* have been extensively found *(Razavi et al., 2020)*. These infections are frequently associated with high morbidity and mortality in affected patients. The presence of *E. coli* expressing extended-spectrum β-lactamases (ESBLs) activity in patients, healthy carriers, and the environment has been reported in Thailand, *(Runcharoen et al., 2017; Saekhow & Sriphannam 2021; Thamlikitkul et al., 2019)*.

The ESBLs phenotype, which can be produced by gram-negative bacteria, mediates the resistance to third generation cephalosporins and monobactams. CTX-M has emerged as the most common ESBL type, displacing TEM-1 and -2 and SHV-1 *(Ruppé et al., 2015)*. CTX-M enzymes are composed of five groups, groups 1, 9, and 2 are commonly found in hospital settings and communities *(Bonnet 2004)*.

Regarding epidemiological studies of ESBL-producing *E. coli*, tracking the route and spread in different environments, several studies focused on its presence, particularly in pigs and chickens *(Lay et al., 2021; Nahar et al., 2018; Seenama et al., 2019)*. Because of the widespread misuse of antibiotics in farming, pork meat was also studied *(Tansawai et al., 2018)*. It has been found that contamination in farm wastewater could also occur *(Saekhow & Sriphannam 2021)*. Contamination of ESBL-producing *E. coli* in cultivated soils demonstrated their ability to survive for extended periods of time *(Hartmann et al., 2012)*. Outbreaks due to surface water contamination in association with extreme precipitation were implicated as a public health concern *(Curriero et al., 2001)*. Thus, water could be the source of dissemination of ESBL-producing *E. coli* over extensive areas, including water sources for human drinking water *(Mahmud et al., 2020)*. Several reports showing the presence of antibiotic-resistant *E. coli*, including ESBL isolates from water environments, have been published in other countries *(Banu et al., 2021; Hassen et al., 2020; Murugadas et al., 2021)*. Nonetheless, the epidemiological data available for the contamination of ESBL-producing *E. coli* in water rivers is still limited in Thailand.

The Kok River, which has its source in Myanmar and flows through Chang Rai and Chiang Mai provinces in northern Thailand, is a 285 km tributary river (leading to the larger Mekong River). Most of its length in Thailand is in Chiang Rai province, where it receives inputs from urban catchments in Mueang Chiang Ria district. The Kham River originates in Chiang Rai province and flows through to the Mekong River (85 km). Both rivers are used in agriculture, especially rice and various crops; this is the major land use in Chiang Rai *(Chantima et al., 2020)*. Hence, the Kok and Kham Rivers provide the site for an epidemiological study of the

multidrug-resistant (MDR) *E. coli* as this relates to the main use of water resources for people in many activities in Chiang Rai and may be the source of water-borne diseases. Therefore, this study aimed to determine the prevalence of MDR and ESBL-producing *E. coli* in river water. Furthermore, plasmid profiling and resistant genes were also characterized to clarify the possibility and extent of dissemination.

Materials & Methods

Study Area and Sample Collection

Water samples were collected from the two main rivers in Chiang Rai, Thailand (Kok River and Kham River). Sampling was performed on 3 sites at each river (Fig. 1). Site A.1 was located close to agricultural areas upstream of the Mueang Chiang Rai district. Site A.2 was located on the route of river flow close to the center of Chiang Rai city. Site A.3 was located downstream of the Kok River, after its passage through the main city to the urban areas with agricultural activity taking place alongside the river. For sampling at the Kham River, site B.1 was located near the transition to the agricultural areas above Mae Chan district. Site B.2 was located close to the community areas of the residents of the Mae Kham sub-district, where both urban and agricultural activities were taking place. Site B.3 was located downstream, being more agricultural in nature. The study design and field experiments were approved by the Research Council of Mae Fah Luang University (project number:641C08004).

Between December 2021 and September 2022, water samples were obtained from sites 1-6 monthly. Water samples were collected at a depth of 30 cm below the surface of water with sterile bottles (500 ml/bottle) in triplicate at each sampling site. During the transportation, all samples were kept on ice and processed within 6 h of collection.

Bacterial Enumeration, *E. coli* Identification and DNA Isolation

Water samples were processed as described previously *(Purohit et al., 2020)*. Briefly, ten-fold serial dilutions were prepared in sterile 0.9% normal saline and processed by standard membrane filtration technique using 47 mm in diameter and a pore size of 0.45µm membrane filters (Merck Millipore, Germany). After that, the membranes were placed on Coliform agar (Merck Millipore, Germany) for 24 hours at 37°C for cultivation and manual counting of colonies. Three independent assays were performed for each sampling site, and technical triplicates were used. For the selection of ESBL-producing *E. coli*, water samples were inoculated on the selective medium CHROMagar ESBL (dark pink-red colony; CHROMagar, Paris, France) for 24 hours at 37°C. The total coliform count was enumerated in colony-forming units (CFUs)/100 ml. The identification of 6-10 *E. coli* isolates per water-river sampling site was followed by biochemical tests (Indole, motile, citrate, methyl red, and Voges-Proskauer test) and PCR amplification of *yaiO* and *uidA* genes *(Molina et al., 2015)*. Genomic DNA was extracted using the boiling method, while plasmid DNA was extracted using the Nucleospin plasmid extraction kit (Macherey-Nagel, Duren, Germany). The DNA was stored at -20°C and subjected to a PCR-based assay.

Antimicrobial Susceptibility Testing

The confirmed *E. coli* colonies were subjected to antibiotic susceptibility testing with eight commonly used classes of antibiotics by the Kirby Bauer disc diffusion test on Muller Hinton Agar (Himedia, Mumbai, India). The antimicrobials selected were ciprofloxacin, nalidixic acid, chloramphenicol, streptomycin, gentamicin, meropenem, ertapenem, tetracycline, amoxicillin-clavulanic acid, ampicillin, trimethoprim/sulfamethoxazole, cefoxitin, cefepime, ceftazidime, and cefotaxime (Oxoid, Hampshire, England). The procedure and interpretation were performed according to the Clinical and Laboratory Standard Institute guidelines *(CLSI 2020)*. Intermediate results were categorized as resistant. Multidrug resistance (MDR) was confirmed by resistance to three or more antimicrobial classes. For quality control, the *E. coli* reference strain ATCC 25922 was used. ESBL-producing strains were confirmed by the combination disc diffusion test, where an increase in the inhibition zone diameter of 5 mm for a combination disc versus either ceftazidime or ceftriaxone confirmed ESBL production. A CLSI broth microdilution was used to determine the MIC of colistin in isolates expressing *mcr* genes.

Plasmid Replicon Typing

Plasmid typing was characterized by five multiplex (M)-PCR, including multiplex 1 for HI1, HI2 and I1-Iγ, multiplex 2 for X, L/M and N, multiplex 3 for FIA, FIB and W, multiplex 4 for Y, P and FIC, and multiplex 5 for A/C, T and FIIAs. Three simplex PCRs were detected for F, K, and B/O *(Carattoli et al., 2005)*.

Phylogenetic Typing, β-lactamase Gene, Integrons and Mobile Genetic Elements

Phylogenetic groups of *E. coli* (A, B1, B2, C, D, E, F, and Escherichia cryptic clade I) were classified by PCR as described previously *(Clermont et al., 2013)*. The $bla_{\text{CTX-M}}$ (group 1, 2, and 9) genes were detected via M-PCRs *(Dallenne et al., 2010)*, and the bla_{TEM} and bla_{SHV} genes were detected using a single PCR *(Pitout et al., 1998)*. The presence of integrons, *intI1* and *int2* *(Kurekci et al., 2017)*; transposon Tn*3 (Gregova et al., 2021)*; and insertion sequence IS*Ecp1* *(Eckert et al., 2006)*. DNA sequencing of PCR products (CTX-M1, M9, and TEM) was used.

Whole Genome Sequencing and Analysis

Whole-genome sequencing was performed by Macrogen (Seoul, Korea). AxyPrep Bacterial Genomic DNA (Axygen Biosciences, Hangzhou, China) was used to perform DNA extraction from an overnight culture of all selected *E. coli* isolates. The sequencing DNA library was prepared using the TruSeq Nano DNA Kit (Illumina, San Diego, CA, USA). Whole genome sequencing was performed on the Illumina MiSeq with 101 bp paired-end reads. The average number of assembled contigs per sample was 127 (range 67 to 220), the average N50 was 185 kb (range 93 kb to 242 kb), and the total assembly length was 4.7 to 5.4 megabases (Mb). Raw read quality was checked using FASTQC software *(Wingett & Andrews 2018)* and the adaptors and poor-quality reads were removed by using Fastp *(Chen et al., 2018)*. Complete genome assemblies were performed using Unicycler *(Wick et al., 2017)* and annotated with Prokka *(Seemann 2014)* at default settings. Genome assemblies were evaluated for quality by Quast *(Gurevich et al., 2013)*. Antimicrobial resistance genes were identified by ABRicate, which included the databases of Resfinder *(Zankari et al., 2012b)*, PointFinder *(Zankari et al., 2012a)*, CARD *(Alcock et al., 2020)*, PlasmidFinder *(Carattoli et al., 2014)*, and SerotypeFinder *(Joensen*

et al., 2015). All gene predictions were called by applying a select threshold for identification and a minimum length of 95 and 80%, respectively. For sequence type analysis, raw data generated from the Illumina platform were submitted to Enterobase (https://enterobase.warwick.ac.uk/), and the multilocus sequence typing (MLST) was determined with MLST 2.0 *(Larsen et al., 2012)*. The phylogenetic relationship of the extracted canonical wgMLST (cano-wgMLST) gene of selected 10 ESBL-producing *E. coli* isolates was conducted using cano-wgMLST_BacCompare *(Liu et al., 2019)*.

The complete sequence of all ten selected whole genome sequenced in this work has been deposited under the BioProject accession number PRJNA846957 with BioSample accessions: SAMN28906491-SAMN28906500 (https://www.ncbi.nlm.nih.gov/sra/PRJNA846957).

Statistical Analysis

An Unpaired *t*-test was applied to compare the means of the CFU/ml between each site of the river water collected in each month ($p < 0.05$). Independent *t*-test was performed where the sample mean values were normally distributed. Three replicates (independent experiments) were performed for all assays. Descriptive statistical parameters, such as the mean and standard deviation were applied to the data.

Results

Distribution of Coliform Bacteria

The level of coliform bacteria CFU/ml for the three sites of the Kok River was between 14.78 x 10^3 and 109.00 x 10^3 (mean 54.23 ± 23.31 x 10^3) and the Kham River was between 6.56 x 10^3 and 137.33 x 10^3 (mean 59.08 ± 35.54 x 10^3). Overall, the number of coliform bacteria peaked in June (mean total 96.52 ± 8.85 x 10^3 CFU/ml) and August (mean total 123.37 ± 10.85 x 10^3 CFU/ml) for the Kok River and Kham River, respectively, this being the rainy season. Generally, the number of coliform bacteria was not different for each sampling site at Kok River (Fig. 2A). In January, the colony count at site A.1 (30.89 x 10^3 CFU/ml) was lower than at site A.3 (57.33 x 10^3 CFU/ml, $p<0.01$), while in February, site A.1 (37.33 x 10^3 CFU/ml) was lower than site A.2 (86 x 10^3 CFU/ml, $p<0.01$). On the other hand, in March, the colony count at site A.1 (53.44 x 10^3 CFU/ml) was higher than at site A.3 (37.89 x 10^3 CFU/ml, $p=0.03$). Moreover, in September, CFU/ml of coliform bacteria were higher at site A.1 (91.67 x 10^3 CFU/ml) than at sites A.2 (79.33 x 10^3 CFU/ml, $p<0.01$) and A.3 (58.56 x 10^3 CFU/ml, $p<0.01$).

In December, January, March, and April, the colony count at sites B.1 of the Kham River (6.89 x 10^3, 6.56 x 10^3, 7.22 x 10^3, and 9.22 x 10^3 CFU/ml, respectively) (Fig. 2B) had a lower number than sites B.2 (21.11 x 10^3 ($p<0.001$), 31.11 x 10^3 ($p<0.01$), 29.33 x 10^3 ($p<0.01$), and 35.78 x 10^3 ($p<0.001$) CFU/ml, respectively and B.3 (23.78 x 10^3 ($p<0.001$), 88.56 x 10^3 ($p<0.001$), 28.33 x 10^3 ($p<0.01$), and 58.33 x 10^3 ($p<0.001$) CFU/ml, respectively) (Fig. 2B). In addition, in July and August, the colony count at sites B.1 (74.44 x 10^3 and 110.89 x 10^3 CFU/ml, respectively) had a lower number than sites B.3 (111.33 x 10^3 ($p<0.01$) and 137.33 x 10^3 ($p=0.02$) CFU/ml, respectively). In June, however, the CFU/ml observed at site B.2 (79.56 x 10^3 CFU/ml) was lower than at site B.1 (82.44 x 10^3 CFU/ml, $p=0.01$).

E. coli and Antibiotic Susceptibility Test

A total of 172 *E. coli* isolates were collected, including 74 isolates from the Kok River and 98 isolates from the Kham River. Of the *E. coli* isolates obtained from both rivers, 45.3% (78/172) were positive for MDR. Most isolates from the 2 rivers were resistant to ampicillin (71.5%, 123/172), followed by tetracycline (46.5%, 80/172), streptomycin (32.6%, 56/172), amoxicillin-clavulanic acid (29.7%, 51/172), ciprofloxacin (26.7%, 46/172), cefotaxime (25.6%, 44/172) and cefepime (24.4%, 42/172). A few isolates were resistant to nalidixic acid (20.9%, 36/172), trimethoprim/sulfamethoxazole (19.2%, 33/172), ceftazidime (18%, 31/172), chloramphenicol (16.3%, 28/172), gentamicin (9.9%, 17/172), meropenem (2.3%, 4/172), cefoxitin (1.7%, 3/172), and ertapenem (0.6%, 1/172). No pan-drug resistance was observed. The percentage of antibiotic resistant *E. coli* from each river is shown in Fig. 3. Overall, a total of 79 antibiogram profiles were obtained (Table S1). Furthermore, a total of 22.1% (38/172) of ESBL-producing *E. coli* isolates were collected. ESBL-producing *E. coli* isolates from the Kok River and Kham River were 3.1% (23/74) and 15.3% (15/98), respectively. Of note, all ESBL isolates were sensitive to ertapenem but resistant to ampicillin. Resistance to ciprofloxacin, tetracycline, and streptomycin was the most common trait (Table 1).

Phylogenetic Grouping

Phylogenetic typing revealed that phylogroup B1, A, and C were the predominant types and were detected in 46.5% (80/172), 17.4% (30/172), and 16.3% (28/172), respectively. Other phylogroups were found at a lower frequency, including phylogroups E (8.7%, 15/172), B2 (4.7%, 8/172), D (4.1%, 7/172), and F (2.3%, 4/172) (Fig. 4).

Characterization of β-lactamase Gene and Genetic Elements

All 38 ESBL isolates contained $bla_{\text{CTX-M}}$, consisting of $bla_{\text{CTX-M-15}}$ (44.7%, 17/38), $bla_{\text{CTX-M-55}}$ (26.3%, 10/38), $bla_{\text{CTX-M-14}}$ (18.4%, 7/38), and $bla_{\text{CTX-M-27}}$ (10.5%, 4/38). $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-116}}$ genes were co-harbored with the $bla_{\text{CTX-M}}$ gene in 23.7% (9/38) and 2.6% (1/38), respectively, whereas bla_{SHV} was not detected. The presence of integrase genes was found to be 55.3% of *Int*1 genes (21/38) and 5.3% of *Int*2 genes (2/38), and one isolate contained both *Int*1 and *Int*2 genes. IS*Ecp1* and Tn*3* genes were found at 55.3% (21/38) and 21.1% (8/38), respectively (Table 1).

Plasmid Replicon Typing

In total, twelve plasmid replicons were detected in the present work. The predominant types were F, FIB, I1-Iγ, Y, and K, which were detected in 76.3% (29/38), 52.6% (20/38), 34.2% (13/38), 34.2% (13/38), and 26.3% (10/38), respectively (Fig. 5). Plasmid replicon types L/M, N, P, T, and W were not detected in this study. Other replicons were found with low prevalence, including FIA (18.4%, (7/38), B/O (15.8%, 6/38), HI2 (13.2%, 5/38), FIIAs (7.9%, 3/38), HI1 (7.9%, 3/38), X (2.0%, 2/38), A/C (2.6%, 1/38), and FIC (2.6%, 1/38).

Whole Genome Sequencing

Ten ESBL-producing *E. coli* were selected for whole genome sequencing (WGS) analysis to identify the genes and plasmid types that are responsible for resistance. All ten ESBL-producing *E. coli* contained more than five different types of acquired resistance genes as well as at least one resistant plasmid (Table 2). The other antimicrobial resistance genes in the ESBL-producing

E. coli, including aminoglycosides, fluoroquinolones, macrolides, chloramphenicol, polymyxin, sulfonamide, tetracycline, and trimethoprim, are shown in Table 2. Moreover, quinolone resistance was observed due to mutations in chromosomal genes *gyrA* (S83L, D87N), *parC*(S80I, E84K, E84V), and *parE*(S458A, I529L). Substitution at S83L and D87N in *gyrA* was predominant. The isolates EK2501, EK2504, and EK9101 did not contain quinolone resistance due to mutation.

Additionally, the co-occurrence of *mcr-1.1*, $bla_{\text{TEM-1}}$, and $bla_{\text{CTX-M55}}$ was found in the EH2301 isolate, while *mcr-3.4*, $bla_{\text{TEM-1}}$, and $bla_{\text{CTX-M55}}$ were both found in the EK9101 isolate (Table 2). These two isolates exhibited phenotypic resistance to colistin, by broth microdilution, showing that the minimal inhibitory concentration (MIC) values of these *mcr*-harboring isolates were 4 μg/ml (the MIC value of ≥ 4 μg/ml confirmed resistance according to the 2020 CLSI M100-30 guidelines). The *mcr-1.1* gene in the EH2301 isolate was in a contig that is presumed to be part of an IncX4 plasmid. However, the *mcr-3.4* gene could not be predicted on the contig because the plasmid marker was not observed by PlasmidFinder 2.0.1. The genetic organization of the *mcr* genes in these isolates is outlined in Fig. 6. The genomic context of the *mcr-1.1-pap2* cassette in EH2301 disrupted a pre-existing DUF2806-demain containing gene and contained the flanking upstream and downstream regions with a DUF2726 domain-containing gene and pseudo-methyltransferase, respectively. The genomic cassette demonstrated 100% nucleotide identity (BLAST aligned with Accession number CP063335). The upstream and downstream genetic organization of the *mcr-3.4* gene was different from that of the *mcr-1.1* gene, which the organization of the *mcr-3.4* gene in the EK9101 isolate was located between *nimC*/*nimA* and diacylglyceral kinase (*dgkA*) genes (Fig. 6).

As shown in Table 2, of the 10 isolates, two ESBL-producing *E. coli* carried $bla_{\text{OXA-1}}$. The EH2102 isolate carried $bla_{\text{CTX-M15}}$ and $bla_{\text{OXA-1}}$, whereas the EH9101 isolate contained $bla_{\text{CTX-M15}}$, $bla_{\text{TEM-1}}$, and $bla_{\text{OXA-1}}$. Nine different serotypes and eight sequence types (STs) were found. The genetic relationship based on the integration of the extraction of the whole genomes and the identification of the most discriminatory loci is demonstrated in Fig. 7. The top 25 discriminatory refinement loci among the 10 *E. coli* genomes used for constructing the canonical wgMLST tree are shown in Table S2. The isolates collected from different rivers at different time points were in the same cluster (ST224 in EK1201 and EH1201, and ST5218 in EK9101). The two isolates (EH1201 and EK1201) classified as ST224, however, carried different *bla* genes ($bla_{\text{CTX-M14}}$ and $bla_{\text{CTX-M55}}$, respectively).

Discussion

The levels of coliform bacteria were high in June and August in both the Kok River and Kham River, respectively, which was during the wet season in Thailand. Water flow during the monsoon may deliver soil and microorganisms to the river. The predominant agriculture during the wet season along both the Kok and Kham rivers was in-season rice, maize, and cassava. Similarly, studies from the Chao Phraya River (central Thailand) demonstrated a strong trend of fecal-coliform concentrations during the wet season *(Huang et al., 2019; Singkran et al., 2018)*.

Gao *et al*. reported the transmission of antibiotic resistant bacteria from swine manure to the environment *(Gao et al., 2015)* and rainfall and runoff were found to be associated with the spread of those bacteria to water *(Curriero et al., 2001)*. Overall, total coliform bacteria collected at each site of the Kok River did not show any difference, except in January and February (dry season), in which sites A.3 and A.2 were found to have a higher number than that of site A.1, respectively. Differences were also found in the Kham River in December, January, March, and April, both in sites B.2 and B.3, when compared to site B.1. When compared to site B.1, the cumulative number of coliform bacteria at both sites during the dry season could be attributed to waste from urban and rural communities or agricultural processes along the river. During November and January, off-season rice farming begins along the Kok River and Kham River in Chiang Rai, this being harvested by April, at the latest.

The occurrence of MDR *E. coli* was moderate in both rivers (45.3%). This circumstance may increase the incidence of transfer of resistant genes from non-clinical settings to a wide range of bacteria species in aquatic environments via horizontal gene transfer *(Taylor et al., 2011)*. In this study, most *E. coli* stains were resistant to ampicillin and tetracycline. This is in agreement with previous studies related to *E. coli* isolated from patients in a tertiary care hospital in Phayao and wastewater from dairy farms in Chiang Mai, both of which are close to Chiang Rai province *(Saekhow & Sriphannam 2021; Srimora et al., 2021)*. A study in ESBL-producing *E. coli* from vegetables in Chiang Rai demonstrated that most isolates were resistant to aztreonam, gentamicin and trimethoprim/sulfonamide *(Chotinantakul et al., 2022)*, while ESBL isolates in this work were occasionally resistant to gentamicin and trimethoprim/sulfonamide. Most *E. coli* observed in this study belong to the phylogroup B1 (46.5%), A (17.4%), and C (16.3%), in accordance with a previous study *(Chotinantakul et al., 2022)*. On the other hand, phylogroup A was the predominant type isolated from dairy farm wastewater in Chiang Mai *(Saekhow & Sriphannam 2021)*. Phylogroups A and B1 are ubiquitous in humans and animals, respectively *(Berthe et al., 2013)* and an infrequent phylogenetic group C is closely related to phylogroup B1 *(Moissenet et al., 2010)*. Strains belonging to phylogroups B2, D, and F are related to extraintestinal *E. coli* infection *(Clermont et al., 2013)*. The data here suggests that a high proportion of phylogroup B1 would be from the contamination of organic manure that is commonly used in farming, and phylogroups A and C would possibly be from human contamination. Although some phylogroups are considered commensal, they could be converted to pathogens when receiving some antibiotic resistant determinants or virulence factor genes from the pathogenic ones.

The prevalence of ESBL-producing *E. coli* in this study was present at a lower rate (22.1%) when compared to previous studies in Thailand *(Boonyasiri et al., 2014; Saekhow & Sriphannam 2021)*, Tunisia *(Hassen et al., 2020)*, and Ghana *(Banu et al., 2021)*, but present at a higher rate than in France *(Girlich et al., 2020)* and Tanzania *(Kimera et al., 2021)*. The discrepancies could be due to many factors (e.g., geographical variations, atmospheric conditions, human activities, and manipulation of the farm with insecticide and manure, including antimicrobial usage). All ESBL isolates in this work carried the bla_{CTX} gene, with sporadic coexistence with the $bla_{\text{TEM-1}}$

gene, in accordance with a previous study *(Hassen et al., 2020)*. However, the characterization of *E. coli* from dairy farm wastewater and pigs in northern Thailand demonstrated a higher rate of $bla_{\text{CTX-M-positive}}$ *E. coli* in combination with the $bla_{\text{TEM-1}}$ gene *(Lay et al., 2021; Saekhow & Sriphannam 2021)*. One ESBL-positive strain in this work contained both $bla_{\text{CTX-M-55}}$ and $bla_{\text{TEM-116}}$ genes. TEM-116 is thought to have evolved from TEM-1 *(Usha et al., 2008)*. A study in Thailand reported the co-presence of $bla_{\text{TEM-1}}$ with $bla_{\text{TEM-116}}$ genes and $bla_{\text{CTX-M-15}}$ with $bla_{\text{TEM-116}}$ genes in clinical isolates of *E. coli* and *K. pneumoniae (Pornsinchai et al., 2015)*, while another study reported the occurrence of the $bla_{\text{TEM-116}}$ gene from *E. coli* in poultry meat *(Tansawai et al., 2018)*. There is no report of both $bla_{\text{CTX-M-55}}$ and $bla_{\text{TEM-116}}$ genes co-harboring in *E. coli* in Thailand, but it has been shown in piglets in Taiwan and environments in India *(Lee & Yeh 2017; Murugadas et al., 2021)*. TEM-116 may be transferred between intraspecies or interspecies via conjugation in the environment *(Lahlaoui et al., 2011)*. Among ESBL-positive isolates in the present work, the $bla_{\text{CTX-M-15}}$ gene was predominant, followed by $bla_{\text{CTX-M-55}}$, $bla_{\text{CTX-M-14}}$, and $bla_{\text{CTX-M-27}}$ genes. On the other hand, a previous report demonstrated a high prevalence of $bla_{\text{CTX-M-55}}$ followed by $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ genes in ESBL-producing *E. coli* isolated from farm waste and canals in eastern Thailand *(Runcharoen et al., 2017)*. In northern Thailand, CTX-M-55 and CTX-M-14 were prevalent in ESBL isolates from healthy humans and pigs *(Lay et al., 2021; Seenama et al., 2019)*. Furthermore, CTX-M-55 was found in ESBL-producing *E. coli* cultured from fresh vegetables *(Chotinantakul et al., 2022)*. The occurrence of ESBL-positive strains emphasizes the importance of antimicrobial resistant bacteria that can be distributed in the main rivers of Chiang Rai. Those rivers are used for consumption and farming activities, but by chance may increase the risk of MDR dissemination in humans, causing harmful diseases. Besides the finding in the river water, other environments such as soil, wastewater from hospitals and factories, and manure should be further monitored to explore the source of contamination.

Integrons play a key role in the dissemination and spread of antibiotic resistance by their ability to excision and integrate gene cassettes carrying antibiotic resistant genes *(Deng et al., 2015)*. Integrons are widely spread in association with mobile DNA elements, i.e., transposons or plasmids *(Deng et al., 2015)*. A high proportion of ESBL-producing *E. coli* in the present work harbored the *int1* gene, in which some isolates were associated with either transposon Tn*3* or insertion sequence IS*Ecp1* genes on the plasmids. Two ESBL isolates harbored both class 1 and class 2 integrons. The presence of transposons and insertion sequences suggested the ability to mobilize many genes, particularly antibiotic resistance genes *(Razavi et al., 2020)*. Previous work demonstrated the abundance of Tn*3* and IS*Ecp1* in most environments, i.e., rivers, industrial pollutants, wastewater, marine, soil, and sediment *(Razavi et al., 2020)*. Various plasmid replicons were identified in this study, supporting the assumption that plasmids found in ESBL isolates could play a role in the dissemination of antibiotic resistance, including other virulence genes.

Our study revealed eight ESBL-producing STs from ten selected isolates (ST69, ST131, ST224, ST603, ST648, ST1421, ST5218, and ST13160). ST131, ST69, and ST648 are the

predominant extraintestinal pathogenic *E. coli* isolates worldwide *(Manges et al., 2019)*. ST131, which carried ESBL genes, was found in the Thai patients and environmental isolates *(Runcharoen et al., 2017)*. On rare occasions, ESBL-producing *E. coli* belonging to ST224, ST603, ST1421, and ST5281 were found in humans and animals *(Apostolakos et al., 2017; Prapasawat et al., 2017; Qiu et al., 2019; Silva et al., 2016)*. A newly identified ST in the present work was ST13160, which carried $bla_{\text{CTX-M-14}}$. The phylogenetic analysis did not show the unique genes found in each river or at any time point of collection. The ST224 and ST5218 isolates were found in the same cluster, but the two ST224 isolates carried different *bla* genes.

The emergence of plasmid-mediated colistin resistance genes is of global concern. The distribution of *mcr-1* is more frequent than other types (*mcr-1* through *mcr-10*), particularly in food animals than in humans and food products, suggesting the role of foodborne transmission *(Elbediwi et al., 2019)*. In this study, $bla_{\text{CTX-M55}}$ and $bla_{\text{TEM-1B}}$ were found with *mcr-1* in *E. coli*. The *mcr-1* gene was carried on IncX4, which is the most common type of plasmid replicon. IncX4, IncI2, and IncHI2 have been shown to be the predominant plasmid types carrying *mcr-1* spreading worldwide, including in Thailand *(Paveenkittiporn et al., 2020; Wu et al., 2018)*. These plasmid replicons carrying the *mcr-1* gene could improve host fitness and co-selection, allowing *E. coli* to disseminate globally *(Wu et al., 2018)*. The genomic context of the *mcr-1.1-pap2* cassette was present in this work. A variety of *mcr-1.1-pap2* cassette compositions have been shown, suggesting the ability of *mcr-1* to mobilize across the genes *(Girardello et al., 2021; Snesrud et al., 2016)*. IS*Apl1* flanking the *mcr-1.1-pap2* cassette conferred the transposition and was found to be lost after mobilization *(Snesrud et al., 2016)*. However, this IS element was not observed in the present work. The disruption of the DUF2806-domain containing gene by the *mcr-1.1-pap2* identified in this work was previously described in chicken meat and slaughterhouse (Accession numbers: MK875286.1; CP053735.1). The presence of a flanking DUF2726-domain containing gene upstream of the *mcr-1.1-pap2* cassette was discovered in clinically *mcr*-harboring carbapenem-resistant *E. coli* and *Klebsiella pneumoniae* isolates in Thailand *(Paveenkittiporn et al., 2020)*. The MICs of colistin-resistant isolates were found near to be the resistance breakpoint (≥ 4 μg/ml), as previously described *(Lee et al., 2019)*. Additionally, co-expression of the *mcr-3.4* gene and the $bla_{\text{CTX-M55}}$ gene was found in the present work. The *mcr-3.4* gene is a variant of *mcr-3.1* and was first reported in *E. coli* in China *(Xu et al., 2018)*. The phenomenon of *mcr-3* gene distribution has been shown in several sources, including water, animals, food, and humans *(Elbediwi et al., 2019)*. A previous study demonstrated the co-existence of ESBL ($bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-55}}$) and *mcr* genes (*mcr-1.1* and *mcr-3.1*) in pigs in Thailand *(Trongjit & Chuanchuen 2021)*. To our knowledge, the *mcr-3.4* variant has never been reported in Thailand, and our data revealed for the first time that the *mcr-3.4* gene co-occurred with the $bla_{\text{CTX-M}}$ gene in the river water.

Conclusions

In conclusion, MDR *E. coli* was found in two main rivers, the Kok River and the Kham River, in Chiang Rai, Thailand. ESBL-producing *E. coli* was sporadically found, which mostly

contained CTX-M-15. Co-occurrence of *mcr* genes (*mcr-1.1* and *mcr-3.4*) and ESBL genes were discovered, which were found in the river water. Integrons, transposons, and insertion sequences were also found in combination with the $bla_{\text{CTX-M}}$ genes, suggesting their role in disseminating the antibiotic resistant genes in the environment and possibly causing increasing risks to public health. Further findings of ESBL-producing *E. coli* should be extended to samples collected from soil and farmers near the rivers, including manures that are used in the agriculture. Wastewater from hospitals and small factories in the city should also be observed to find out the possibility of spreading those drug-resistant bacteria into the environment.

Acknowledgements

We would like to thank Dr. Roger Callaghan for English-editing assistance.

Additional Information and Declarations

Funding

This work was supported by Mae Fah Luang University, Thailand (641C08004).

Grant Disclosures

The Mae Fah Luang University grant information: 641C08004 was disclosed by the authors.

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

KC contributed with study design, conduction of experiments, data analysis, writing and revision of the manuscript, and approved the final draft.
PC performed the laboratory experiments, analyzed the data, and approved the final draft.
SO contributed with study design, writing the manuscript, supervision, and approved the final draft.

Data Availability

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

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Figure 1

Figure 1. Map of location of the sampling sites at the Kok River and Kham River in Chiang Rai province.

A small box indicates the location of Chiang Rai province in northern Thailand (highlight area). Samples were collected from three sampling sites on each river.

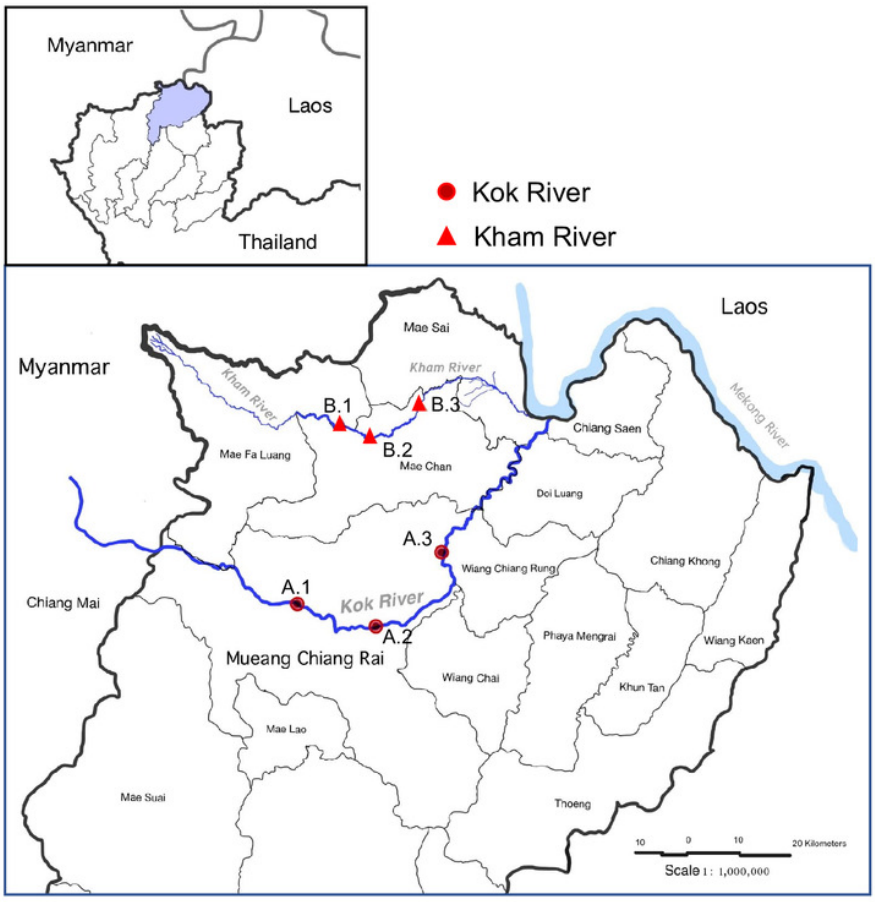

Figure 1. Map of location of the sampling sites at the Kok River and Kham River in Chiang Rai province. A small box indicates the location of Chiang Rai province in northern Thailand (highlight area). Samples were collected from three sampling sites on each river.

Figure 2

Figure 2. Total coliform bacteria in the (A) Kok River and (B) Kham River in northern Thailand.

CFUs were counted from each collecting site. The columns represent the mean plus or minus standard deviation of three independent experiments, with triplicates. Statistical differences were analyzed with an unpaired t-test. Values that are significantly different are indicated by asterisks as follows: $*p < 0.05$, $**p < 0.01$ when compared to site 1; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ when compared to site 2.

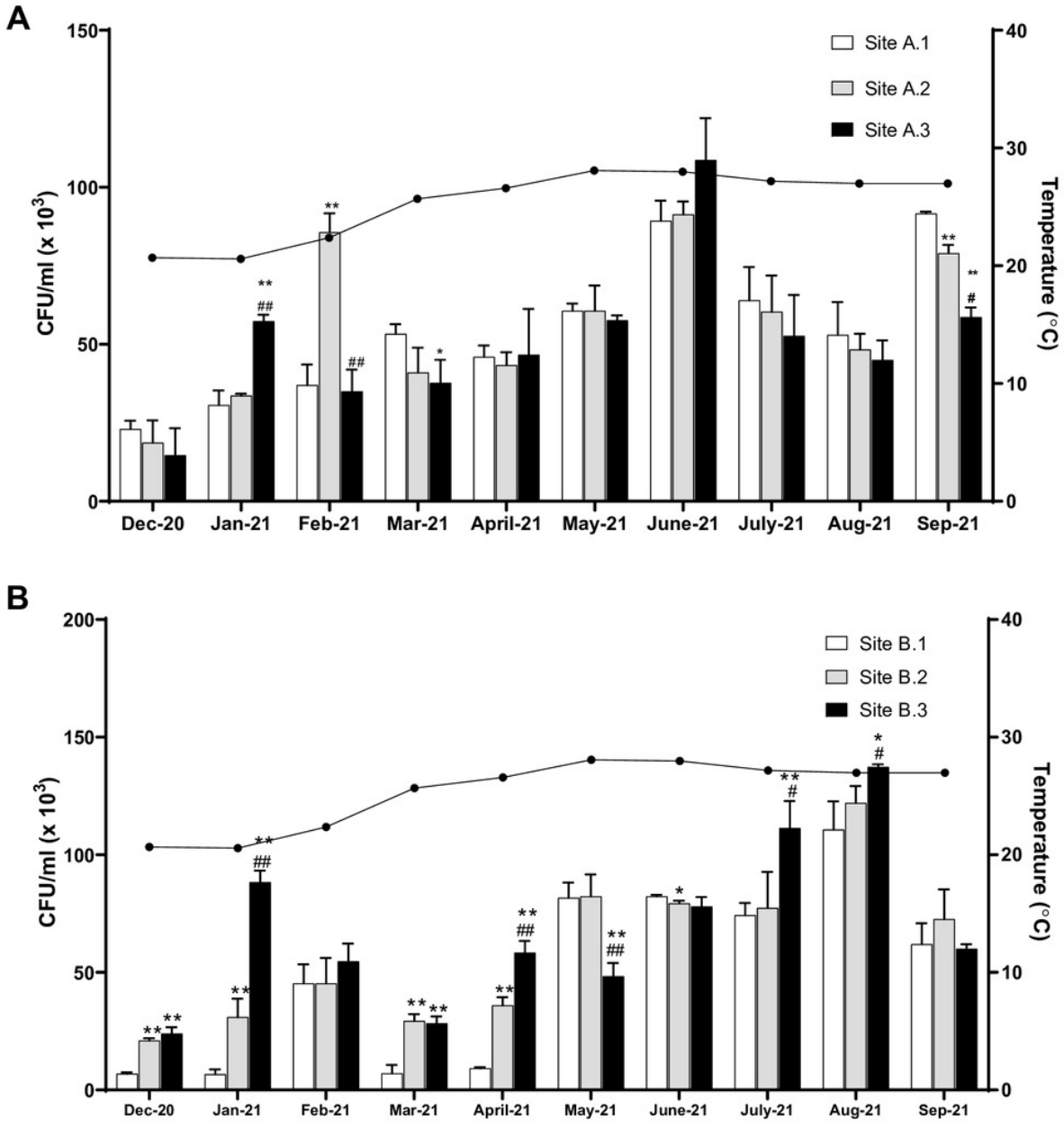

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Figure 3

Figure 3. Antibiotic resistance of E. coli from the Kok River and Kham River in northern Thailand.

Cip, ciprofloxacin; NA, nalidixic acid; C, chloramphenicol; S, streptomycin; CN, gentamicin; MEM, meropenem; ETP, ertapenem; TE, tetracycline; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; STX, trimethoprim/sulfamethoxazole; FOX, cefoxitin; FEP, cefepime; CAZ, ceftazidime; CTX, cefotaxime.

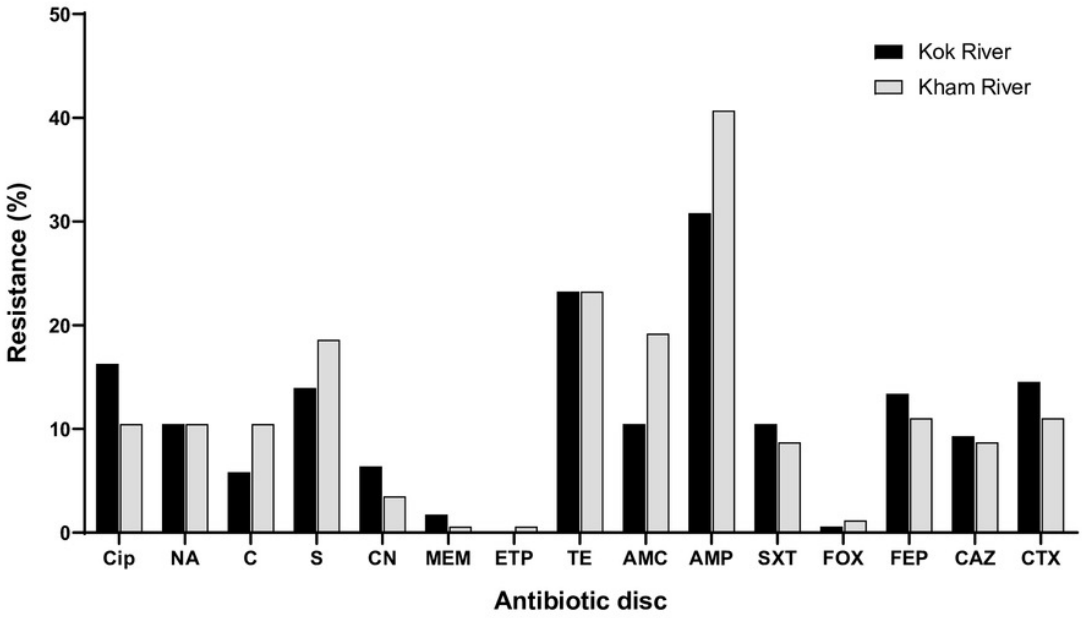

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Figure 4

Figure 4. Phylogroup typing of E. coli isolates from rivers in northern Thailand.

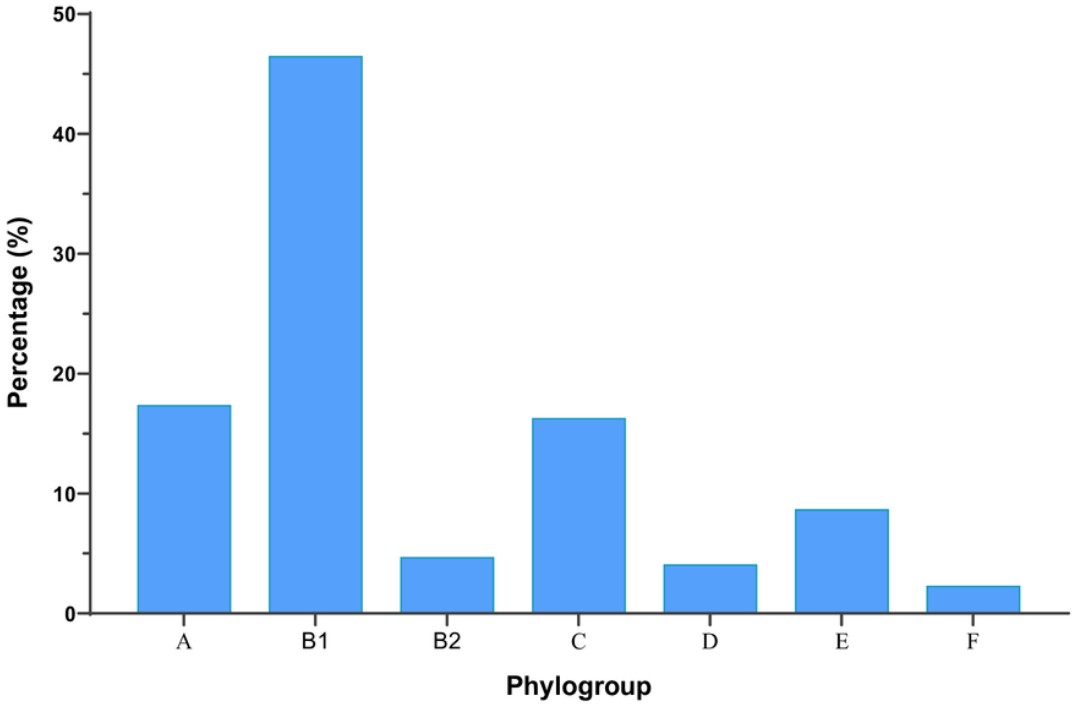

Figure 4. Phylogroup typing of *E. coli* isolates from rivers in northern Thailand.

Figure 5

Figure 5. Plasmid replicon types among ESBL-producing E. coli from rivers in northern Thailand.

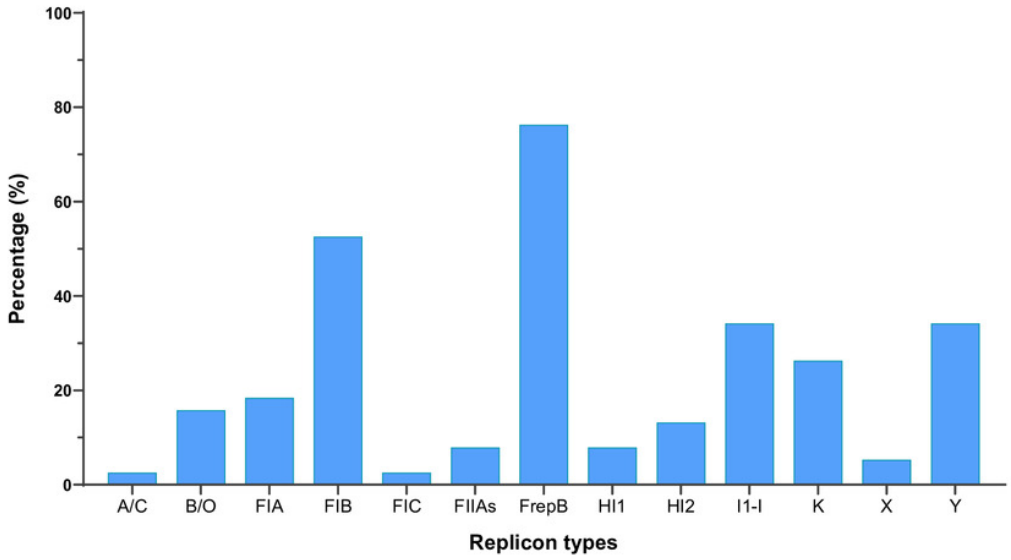

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Figure 6

Figure 6. Genomic content of EH2301 and EK9101 isolates carrying mcr-1.1 and mcr-3.4, respectively.

The schematic shows the genes flanking the mcr genes in each isolate.

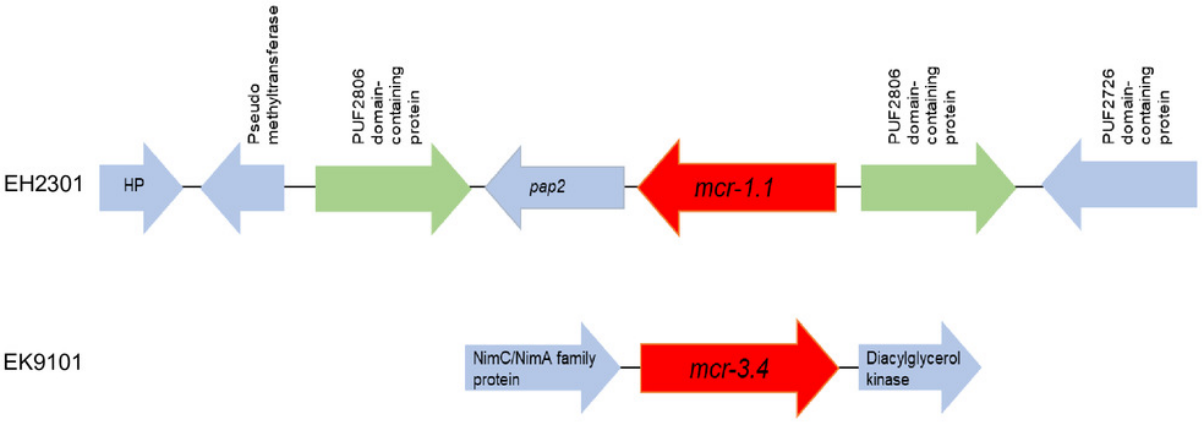

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Figure 7

Figure 7. Phylogenetic tree of selected 10 isolates based on (A) the whole genome MLST (wgMLST) and (B) the most discriminatory refinement loci (canonical wgMLST) using the web server, cano-wgMLST_BacCompare.

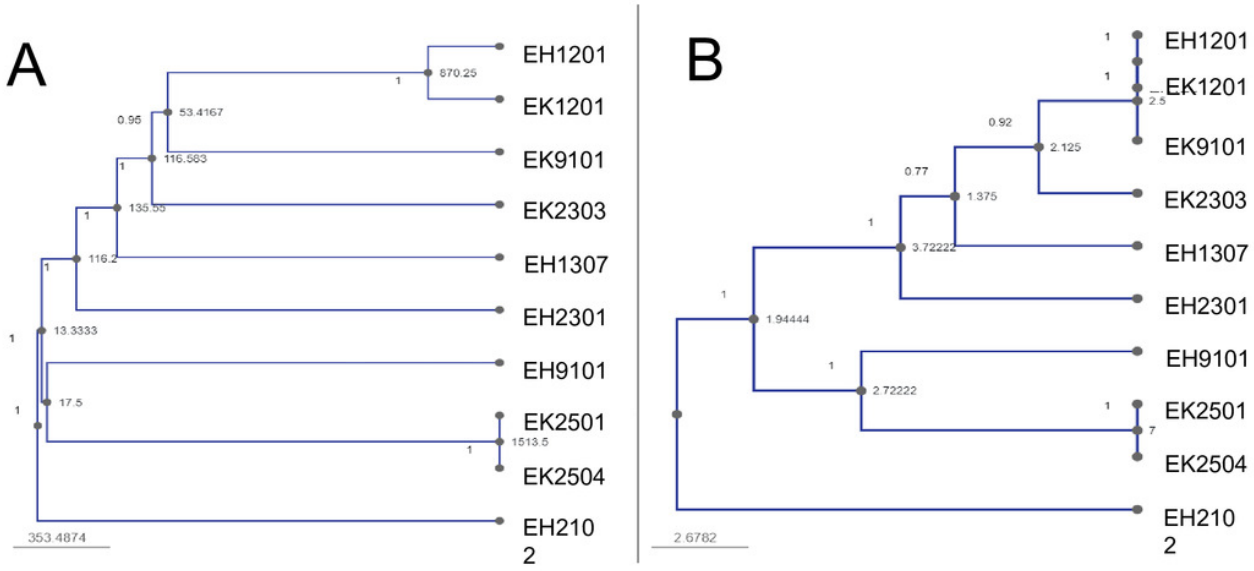

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Table 1(on next page)

Table 1. Characteristics of ESBL-producing *E. coli.*

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Strains	Month	Site	Phylogroup[a]	Beta-lactamases[b]	Integrons/ transposons[c]	Plasmid Replicon[d]
EH2101	Dec-20	Kok River	A	CTX-M-15	IS*Ecp1*	F, FIB, I1-Iγ, K, B/O
EH2102	Dec-20	Kok River	B2	CTX-M-15	*Int1*	F, FIA, I1-Iγ
EH3101	Dec-20	Kok River	D	CTX-M-15	IS*Ecp1*	F
EH1201	Jan-21	Kok River	B1	CTX-M-14	IS*Ecp1*	F, FIB, I1-Iγ, FIIAs, X
EH1203	Jan-21	Kok River	B1	CTX-M-15	-	F, FIB, I1-Iγ, Y, K, B/O
EH2201	Jan-21	Kok River	B2	CTX-M-27	*Int1*	F, FIA, I1-Iγ
EH2204	Jan-21	Kok River	A	CTX-M-14	-	F, HI1, K
EH3201	Jan-21	Kok River	B1	CTX-M-15	-	F, I1-Iγ, K, B/O
EH1303	Feb-21	Kok River	B1	CTX-M-15	IS*Ecp1*	F, FIA, Y, K
EH1306	Feb-21	Kok River	B1	CTX-M-14	*Int1*, IS*Ecp1*	F, FIB, HI2, K
EH1307	Feb-21	Kok River	C	CTX-M-14	*Tn3*, IS*Ecp1*	F, FIB, I1-Iγ, A/C, B/O, FIIAs
EH2301	Feb-21	Kok River	A	CTX-M-55, TEM-1	*Int1*, Tn*3*, IS*Ecp1*	F, HI2, X
EH3301	Feb-21	Kok River	A	CTX-M-15	-	F, Y, K, B/O
EH3302	Feb-21	Kok River	A	CTX-M-14	Tn*3*	F, FIB, Y
EH1401	Mar-21	Kok River	B1	CTX-M-15, TEM-1	*Int1*, Tn*3*, IS*Ecp1*	-
EH1402	Mar-21	Kok River	A	CTX-M-55	*Int1*	F, FIB, HI1
H2404	Mar-21	Kok River	B1	CTX-M-15	IS*Ecp1*	F, FIB, HI1, I1-Iγ
EH2401	Mar-21	Kok River	B2	CTX-M-15	*Int1*	F, FIA, I1-Iγ
EH2402	Mar-21	Kok River	B1	CTX-M-55, TEM-1	*Int1*, Tn*3*, IS*Ecp1*	F, FIB, I1-Iγ
EH6303	May-21	Kok River	D	CTX-M-15	-	F, I1-Iγ, B/O
EH8203	Jul-21	Kok River	D	CTX-M-15, TEM-1	*Int1*, *Int2*, IS*Ecp1*	F, HI2, K
EH9101	Aug-21	Kok River	D	CTX-M-15, TEM-1	*Int1*, *Int2*, IS*Ecp1*	F, FIB
EH9102	Aug-21	Kok River	C	CTX-M-27	*Int1*	FIA, FIB
EK3101	Dec-20	Kham River	B1	CTX-M-55, TEM-116	*Int1*, IS*Ecp1*	Y
EK1201	Jan-21	Kham River	B1	CTX-M-55	*Int1*, IS*Ecp1*	Y
EK1301	Feb-21	Kham River	B1	CTX-M-15	IS*Ecp1*	FIA
EK1302	Feb-21	Kham River	F	CTX-M-55, TEM-1	*Int1*, IS*Ecp1,* Tn*3*	F, FIB, I1-Iγ
EK2302	Feb-21	Kham River	B1	CTX-M-27	-	F, FIB, I1-Iγ
EK2303	Feb-21	Kham River	B1	CTX-M-14, TEM-1	*Int1*, IS*Ecp1*, Tn*3*	F, FIA, FIB, Y, K
EK3304	Feb-21	Kham River	E	CTX-M-55	-	F, FIB
EK3305	Feb-21	Kham River	B1	CTX-M-55	IS*Ecp1*	F, FIB
EK1401	Mar-21	Kham River	B1	CTX-M-55	*Int1*	F, FIB, HI2
K1506	Apr-21	Kham River	C	CTX-M-14	*Int1*	Y
EK2501	Apr-21	Kham River	D	CTX-M-15, TEM-1	*Int1*, IS*Ecp1*, Tn*3*	Y
EK2503	Apr-21	Kham River	E	CTX-M-27	*Int1*	F, FIA, FIB
EK2504	Apr-21	Kham River	D	CTX-M-15, TEM-1	*Int1*, IS*Ecp1*, Tn*3*	Y
EK8301	Jul-21	Kham River	B1	CTX-M-15	*ISEcp1*	FIIAs
EK9101	Aug-21	Kham River	B1	CTX-M-55	*Int1*	F, FIB, HI2, Y

[a]Phylogroup characterized by clement typing.[b]beta-lactamase, [c]integrons/transposon, [d]plasmid replicon were determined using PCR.

Table 2(on next page)

Table 2. Molecular characteristics of ten ESBL-producing *E. coli.*

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Isolate	MLST[a]	Serotypes[b]	Resistance genes[c]								Plasmids[d]
			Aminoglycoside	Beta-lactam	Quinolone resistance gene/ point mutation	Macrolide, Lincosamind, Streptogramin B	Phenicol	Polymyxin	Sulfonamide	Tetracycline	Trimethoprim	
EH1201	224	O8:H23	*aac(3)-IId, ant(3'')-Ia*	*bla*$_{CTX-M-14}$	*gyrA*(S83L, D87N), *parC*(S80I), *parE*(S458A)	*mdf(A), erm(42), erm(B), mph(A)*	*floR*		*sul2*	*tet(X)*		IncFIC(FII), IncX1, IncFIB(AP001918), Col(MG828)
EH1307	13160	-:H4	*aac(3)-IId, aph(3'')-Ib, aph(6)-Id*	*bla*$_{CTX-M-14}$	*qnrS1*, *gyrA*(S83L)	*mdf(A)*	*floR*		*sul2*	*tet(A)*		p0111, IncFIC(FII), IncA/C2, IncB/O/K/Z, IncFIB(AP001918), ColpVC, Col(MG828)
EH2102	131	O25:H4	*aadA5*	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$	*aac(6')-Ib-cr*, *gyrA*(S83L, D87N), *parC*(S80I, E84V), *parE*(I529L)	*mdf(A), mph(A)*			*sul1*	*tet(A)*	*dfrA17*	IncFII, IncFIA, Col156, Col(BS512), Col(MG828)
EH2301	1421	O9:H4	*aac(3)-IId, aadA2, ant(3'')-Ia, aph(3'')-Ib*	*bla*$_{CTX-M-55}$, *bla*$_{TEM-1B}$	*qnrS1*, *gyrA*(S83L), *parC*(S80I)	*mdf(A), lnu(F)*	*cmlA1, floR*	*mcr-1.1*	*sul2, sul3*	*tet(A), tet(X)*	*dfrA12*	IncR IncX1, IncX4,
EH9101	648	O102:H6	*aac(3)-IIa, aph(6)-Id*	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$	*aac(6')-Ib-cr*, *gyrA*(S83L, D87N), *parC*(S80I), *parE*(S458A)	*mdf(A)*			*sul2*	*tet(A)*	*dfrA14*	IncFII(pRSB107), IncFIB(AP001918), Col(BS512), Col(MG828)
EK1201	224	O8:H23	*aac(3)-IIa, aadA2, ant(3'')-Ia, aph(3'')-Ib, aph(6)-Id*	*bla*$_{CTX-M-55}$	*gyrA*(S83L, D87N), *parC*(S80I), *parE*(S458A)	*mdf(A)*	*cmlA1, floR*			*tet(A), tet(M)*	*dfrA12*	IncFIB(pHCM2), IncFIA(HI1), IncFIB(K), IncX1, Col440I
EK2303	603	O175:H16	*aac(3)-IId, aadA2, aph(3')-Ia, aph(3'')-Ib, aph(6)-Id*	*bla*$_{CTX-M-14}$, *bla*$_{TEM-1B}$	*gyrA*(S83L, D87N), *parC*(E84K)	*mdf(A), lnu(F)*			*sul2*	*tet(B)*		IncFIA, IncFIB(AP001918), IncY, Col156, Col(MG828)
EK2501	69	O17 or O44 or O77:H18	*aph(3'')-Ib, aph(6)-Id*	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$	*qnrS1*	*mdf(A)*			*sul2*	*tet(A)*	*dfrA14*	IncY
EK2504	69	O17 or O44 or O77:H18	*aph(3'')-Ib, aph(6)-Id*	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$	*qnrS1*	*mdf(A)*			*sul2*	*tet(A)*	*dfrA14*	IncY
EK9101	5218	O3:H7	*aac(3)-IId, aadA2, ant(3'')-Ia, aph(3'')-Ib, aph(6)-Id*	*bla*$_{CTX-M-55}$	*qnrS1*	*mdf(A)*	*catA2, cmlA1*	*mcr-3.4*	*sul2, sul3*			P0111, IncFIB(AP001918), IncI1, IncHI2A, IncHI2

[a]MLST determined by https://enterobase.warwick.ac.uk/.

[b]Serotype determined by https://cge.food.dtu.dk/services/SerotypeFinder/.

[c]The antibiotic resistance genes were search with ResFinder 4.1.

[d]The plasmid markers were identified by PlasmidFinder 2.1.