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Original Article

Presence and characterization of *bla*$_{NDM-1}$-positive carbapenemase-producing *Klebsiella pneumoniae* from outpatients in Thailand

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Abstract *Background:* Presently, community-associated carbapenemase-producing Enterobacterales (CPE) remains largely unknown and require public attention. This study aimed to investigate the presence of CPE from outpatients in Thailand.
Methods: Non-duplicate stool (n = 886) and urine (n = 289) samples were collected from outpatients with diarrhea and urinary tract infection, respectively. Demographic data and characteristics of patients were collected. Isolation of CPE was performed by plating enrichment culture on agar supplemented with meropenem. Carbapenemase genes were screened by PCR and sequencing. CPE isolates were phenotypically and genotypically characterized.
Results: Fifteen samples (1.3%, 14 stool and 1 urine) yielded *bla*$_{NDM-1}$-positive carbapenemase-producing *Klebsiella pneumoniae* (CPKP). Additional resistance to colistin and tigecycline was observed in 53.3% and 46.7% of isolates, respectively. Age >60 years was identified as a risk factor for patients with CPKP (P < 0.001, adjusted odds ratio = 11.500, 95% confidence

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interval = 3.223–41.034). Pulsed field gel electrophoresis revealed genetic diversity of CPKP isolates; however, clonal spread has been observed. ST70 (n = 4) was common, followed by ST147 (n = 3). bla_{NDM-1} from all isolates were transferable and mainly resided on IncA/C plasmid (80%). All bla_{NDM-1} plasmids remained stable in bacterial host for at least 10 days in antibiotic-free environments, regardless of replicon types.

Conclusion: This study demonstrates that the prevalence of CPE among outpatients in Thailand remains low and the spread of bla_{NDM-1}-positive CPKP may be driven by IncA/C plasmid. Our results emphasize the need for a large-scale surveillance study to limit further spread of CPE in community.

Introduction

Carbapenem-resistant Enterobacterales (CRE) possesses a global problem in human medicine and public health. Infections with these organisms result in treatment difficulty and high mortality rates, primarily because there are only a few alternative drugs left to treat CRE infections.[1,2] CRE are listed, by the Center for Disease Control and Prevention, as one of the public health threats that require urgent and aggressive action.[3] Data from the Global Surveillance Program on CRE revealed that the overall percentage of meropenem-nonsusceptible Enterobacterales increased from 2.7% in 2012–2014 to 3.8% in 2015–2017.[4] Mechanisms of carbapenem resistance in Gram-negative bacteria are usually caused by (i). expression of extended-spectrum- or AmpC β-lactamase combined with porin deficiency and (ii). production of carbapenemases. The latter is considered the most common mechanism found in several species in Enterobacterales.[2] Several types of carbapenemases have been reported, including serine-type enzymes (KPC, OXA-48) and metallo β-lactamases (NDM, IMP, VIM). By far, NDM is the most prevalent carbapenemase in Southeast Asia.[2] NDM is able to hydrolyze almost all β-lactams but not aztreonam and its activity is not inhibited by commercially available β-lactamase inhibitors such as clavulanate, sulbactam and tazobactam.[5] Several NDM variants have been documented however, NDM-1 is the most prevalent and widely distributed enzyme. Most bla_{NDM} genes are frequently associated with conjugative plasmids which usually harbor additional resistant genes. These plasmids can be readily transferred among several bacterial species, resulting in multidrug-resistant strains.[5]

Carbapenemase-producing Enterobacterales (CPE) has been detected in several continents, particularly in Europe where CPE cases have been reported from 37 countries.[4,6] The outbreak or occurrence of CPE in many Asian countries, including Thailand, has been reported.[7] Recently, a study in clinical Enterobacterales isolates in 8 countries in the Asia–Pacific region demonstrated that Thailand ranked first for the prevalence of CPE, predominantly NDM-1-positive *Klebsiella pneumoniae*.[8] It is also worrying to observe that mortality rate of CPE infections in Thailand was as high as 47.6%.[9]

Most CPE isolates have been detected from a variety of human clinical specimens from hospital-acquired infections. CPE isolates have also been reported from long-term care facilities, healthy population, animals and environments, suggesting that CPE has already spread beyond hospital settings.[10–12] However, data on CPE in community is limited. To date, the prevalence of community-associated CPE remained low. Studies in Spain and the Netherlands showed no CPE in community settings, while the prevalence of community-associated CPE in Tunisia and Shangdong (China) were 0.33% and 2.3%, respectively.[12–15] In addition, direct transmission of NDM-producing *Escherichia coli* between household family members, their backyard animals, and farm environment has been documented.[12]

According to the National Antimicrobial Resistance Surveillance Center, Thailand, carbapenem resistance in *K. pneumoniae* and *E. coli* has increased from 1.6% in 2016 to 13.5% in 2020.[16] Infections caused by CPE in Thailand have also been rising and several studies have identified the two most common carbapenemase genes among Thai patients, bla_{NDM} and $bla_{OXA-48-like}$.[9,17] While, the incidence of hospital-associated CPE is on the rise, data on community-associated CPE in Thailand are limited. Therefore, to obtain a better understanding of CPE in a Thai community, this study was conducted to investigate the presence of CPE isolates from outpatients in Phitsanulok province, Lower Northern Thailand. Characterizations of CPE isolates were also carried out.

Methods

Ethical approval

The study was approved by the Naresuan University Institutional Review Board (COA No. 178/2018 and 011/2021). Written informed consent was obtained from patients prior to participating in this study.

Study settings and sample collections

This study was conducted as part of the antimicrobial resistance surveillance study in community settings in Phitsanulok province, Lower Northern Thailand, from November 2018 to August 2020. A total of 1387 non-duplicate stool and urine samples were investigated. These included stool (n = 1038) and urine (n = 349) samples of patients with diarrhea and urinary tract infection (UTI), respectively, who visited outpatient departments of the two local hospitals. Participants who were <15 years

old were excluded. In case of samples from the same patients revisited during the study period, only samples from the first visit were investigated.

Demographic characteristics of the patients were obtained using a structured questionnaire. The following data were collected: age, gender, education, occupation, number of family members, income, living place, pets, backyard poultry, type of drinking water, consumption of undercooked meat, underlying diseases, previous antibiotic usage and history of hospitalization.

Screening for carbapenem-resistant Enterobacterales

Stool and urine samples were enriched (1:10 ratio) in Enterobacteriaceae Enrichment broth (Oxoid Ltd., Basingstoke, UK) and incubated at 37 °C overnight. Enrichment broth was cultured on MacConkey agar supplemented with 0.5 μg/mL meropenem and incubated at 37 °C for 24 h. Growth of lactose-fermenting colonies was observed and colonies were selected (1 colony/sample). All isolates were subjected to susceptibility test by disk diffusion method, using imipenem and meropenem disks, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[18] Isolates that showed resistance to either imipenem or meropenem were considered CRE. All CRE isolates were confirmed to the species level by API 20E (BioMérieux SA, Marcy-l'Etoile, France) and 16S rDNA sequencing.[19]

Susceptibility test and carbapenemase production

Susceptibility to third generation cephalosporins (cefotaxime and ceftazidime), imipenem, meropenem, ciprofloxacin and colistin were performed by broth microdilution method as recommended by CLSI.[18] Tigecycline susceptibility was determined using MIC strip test (0.016—256 μg/mL) (Liofilchem, Roseto degli Abruzzi, Italy). MICs for all antibiotics, except tigecycline, were interpreted according to CLSI guideline. MIC interpretation of tigecycline followed the European Committee on Antimicrobial Susceptibility testing.[20] Production of carbapenemase was investigated by modified carbapenem inactivation method (mCIM) according to the CLSI.[18] *E. coli* DMST4212, obtaining from the Department of Medical Sciences, Ministry of Public Health, Bangkok, was used as a control strain.

Screening for carbapenemase genes

All carbapenemase-producing isolates were screened for the presence of five carbapenemase genes (bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM} and bla_{OXA-48}), by multiplex PCR as previously described.[21] *E. coli* isolates carrying those genes (kindly provided by Prof. Timothy Walsh, Oxford University, UK) were used as positive controls. Identification of bla_{NDM} alleles was performed using primers 5′- ATGATGACTCAGAGCATT CG-3′ and 5′-TTATTGCATCAGAAACCGTG-3′ (812 bp). The PCR conditions were 5 min of initial denaturation at 94 °C, followed by 30 cycles at 94 °C for 45 s, 52 °C for 30 s, and 72 °C for 30 s and a final extension at 72 at 94 °C for 5 min. PCR products were purified using a GF-1 PCR purification kit (Vivantis Technologies Sdn. Bhd., Selangor, Malaysia) and sequenced by First BASE Laboratories Sdn. Bhd. (Selangor, Malaysia). The obtained sequences were compared with those available in the GenBank database using the BLAST algorithm available on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov).

Clonal relatedness

To study the genetic relationship of bla_{NDM-1}-positive isolates, pulsed field gel electrophoresis (PFGE) was performed. Chromosomal DNA of *K. pneumoniae* in agarose plugs was prepared and digested with *Xba*I (Thermo Fisher Scientific, MA, USA). Plugs were then subjected to PFGE analysis in 1% agarose gel (Pulsed Field Certified™ agarose; Bio-Rad Laboratories, CA, USA) and 0.5X Tris-borate-EDTA buffer using a CHEF Mapper® XA System (Bio-Rad Laboratories). The gels were run at 6.0 V/cm with an angle of 120 at 14 °C for 20 h. *Saccharomyces cerevisiae* chromosomal DNA (Bio-Rad Laboratories) was used as a molecular size standard. PFGE profiles were visually analyzed and interpreted as described previously.[22]

Multilocus sequence typing analysis (MLST)

MLST was performed by amplification and sequencing of seven house-keeping genes (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB* and *tonB*) according to the protocols from *K. pneumoniae* MLST website (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

Conjugation experiments and PCR-based replicon typing

To investigate the transferability of bla_{NDM-1}, conjugation experiments were carried out by broth mating method using sodium azide-resistant *E. coli* J53 as a recipient. Cultures of donor and recipient cells were mixed (1:1) and incubated at 37 °C overnight without shaking. Transconjugants were spread on Tryptic Soy agar (TSA) supplemented with sodium azide (150 μg/mL) and meropenem (0.5 μg/mL), and incubated at 37 °C overnight. Conjugation frequency was expressed as the number of transconjugants divided by the number of donor cells. The presence of bla_{NDM-1} in transconjugants was confirmed by PCR. MICs of transconjugants were determined by broth microdilution method. PCR-based replicon typing (PBRT) was used to identify the plasmid incompatibility groups.[23]

Plasmid stability testing

Stability of bla_{NDM-1} plasmids was assessed as previously described with slight modification.[24] *E. coli* J53 transconjugants carrying bla_{NDM-1} were cultured in Tryptic Soy Broth (TSB) and incubated at 37 °C overnight. Then, 100 μL cultures were serially diluted and spread on TSA (day 0). Cultures were then serially passaged for 10 consecutive days (1:10^4 dilution) in antimicrobial-free TSB. On days 2, 4, 6, 8 and 10, 100 μL cultures were collected, serially diluted and spread on antibiotic-free TSA. Approximately 50 colonies were randomly chosen and spotted on TSA supplemented with 0.5 μg/mL meropenem. Twenty colonies

grown on meropenem-supplemented agar were randomly selected to confirm the presence of bla_{NDM-1} by PCR. The percentage of plasmid stability was calculated by comparing the number of bla_{NDM-1}-positive colonies and the number of selected colonies on meropenem-supplemented agar. Experiments were performed in triplicate.

Statistical analysis

Data were collected and analyzed using SPSS version 17.0 (SPSS, Chicago, IL, USA). Categorical data were expressed as counts and percentages and continuous variables were presented as mean ± SD. The incidence rate of CPE was calculated by dividing the number of CPE-positive patients by the total number of patients. Univariate analysis was performed using chi-square or Fisher's exact test as appropriate for categorical variables, and Mann–Whitney U test for continuous variables. All variables with $P < 0.05$ in univariate analysis were included in a backward stepwise multivariate logistic regression analysis to determine risk factors associated with CPE. The results were presented as adjusted odds ratio (aOR) with 95% confidence intervals (CI). A P value of <0.05 was considered significant.

Results

Characteristics of outpatients and screening for CPE

A total of 1387 outpatients with diarrhea and UTI agreed to participate in this study. Of these, 212 participants were excluded and 1175 participants (886 diarrheagenic patients and 289 UTI patients) were included in further analysis (Fig. 1). Demographic data and characteristics of outpatients are provided in Table 1. Most patients were in the working-age population with a mean age of 46.1 years (range = 15–99 years) and 58.5% were female. Patients were more likely to live in rural area (68.1%) and in a household of 1–5 persons (72.0%). Three quarters of the patients (75.7%) had a basic level of education (primary and secondary schools). Slightly more than half of the participants (56.6%) were unemployed or worked as labors. Family income of most participants (87.9%) was below the average monthly income in the studied area (10,000 Thai baht). Hypertension was the most common underlying condition (19.1%), followed by diabetes (10.8%). Raising dog or cat at home was observed in 28.5% of patients. Filtered water was

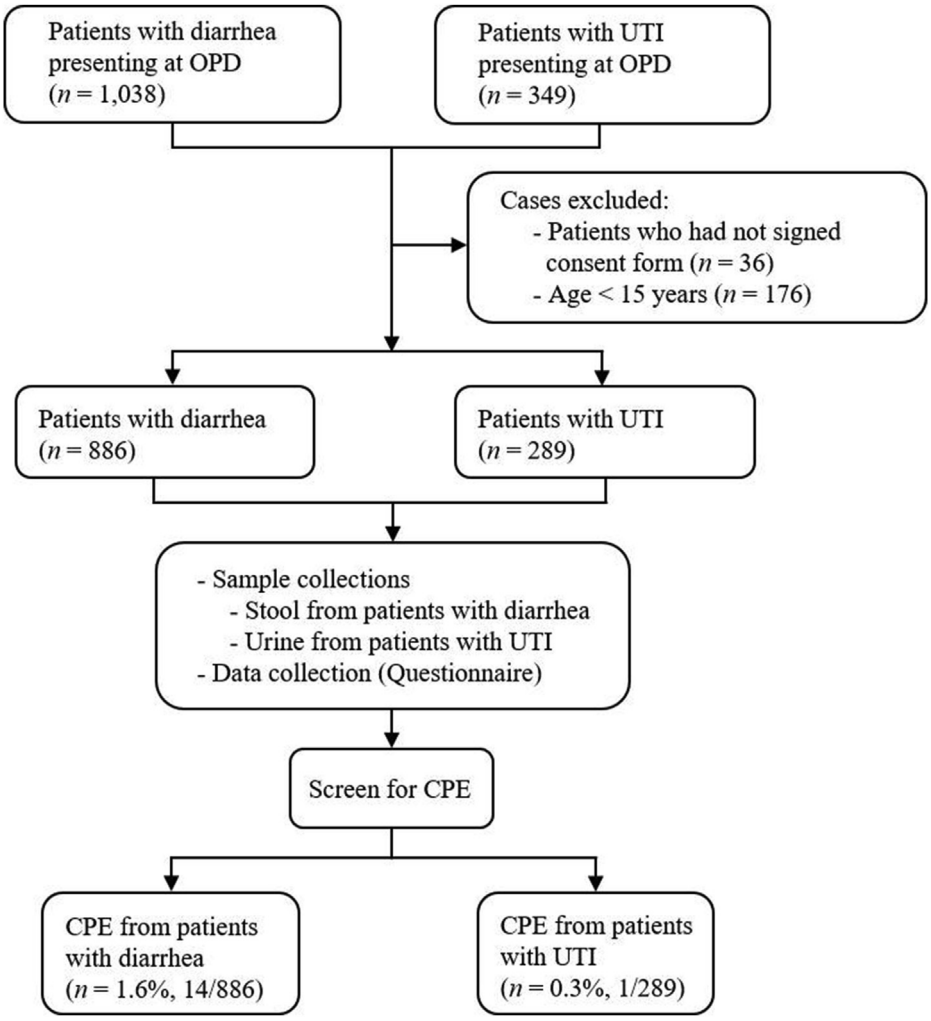

Figure 1. Flowchart of this study. OPD, outpatient department; UTI, urinary tract infection; CPE, carbapenemase-producing Enterobacterales.

Table 1 Characteristics of participants carrying carbapenemase-producing *K. pneumoniae*.

Variables	Total patients (n = 1175), No. (%)	No. of patients with CPKP (n = 15), No. (%)	No. of patients with non CPKP (n = 1160), No. (%)	Univariate analysis P value[a]	Multivariate logistic regression analysis		
					P value[b]	aOR	95% CI
Female patients	687 (58.5)	8 (53.3)	679 (58.6)	0.682			
Age (mean)	46.1 ± 18.6	69.4 ± 12.8	45.8 ± 18.4	<0.001			
Age ≤20 years	78 (6.8)	0 (0)	78 (6.8)	0.617			
Age 21–30 years	223 (19.1)	0 (0)	223 (19.4)	0.090			
Age 31–40 years	221 (19.0)	0 (0)	221 (19.2)	0.089			
Age 41–50 years	168 (14.4)	1 (6.7)	167 (14.5)	0.710			
Age 51–60 years	167 (14.3)	2 (13.3)	165 (14.3)	1.000			
Age >60 years[c]	309 (26.5)	12 (80.0)	297 (25.8)	<0.001	<0.001	11.500	3.223–41.034
Living in rural area	795 (68.1)	11 (73.3)	784 (68.0)	0.786			
No. of family member							
1–2 persons	240 (20.6)	2 (13.3)	238 (20.7)	0.749			
3–5 persons	598 (51.4)	9 (60.0)	589 (51.3)	0.503			
6–10 persons	132 (11.3)	4 (26.7)	128 (11.1)	0.080			
>10 persons	188 (16.2)	0 (0)	188 (16.4)	0.149			
Education[d]							
Uneducated	44 (3.8)	2 (13.3)	42 (3.6)	0.107			
Primary school	329 (28.2)	7 (46.7)	322 (28.0)	0.145			
Secondary school	554 (47.5)	5 (33.3)	549 (47.7)	0.270			
College and university or higher	240 (20.6)	1 (6.7)	239 (20.7)	0.331			
Occupation							
Unemployed	425 (36.3)	8 (53.3)	417 (36.1)	0.168			
Labor	238 (20.3)	3 (20.0)	235 (20.3)	1.000			
Government officer & company employee	223 (19.1)	1 (6.7)	222 (19.2)	0.328			
Personal business	157 (13.4)	1 (6.7)	156 (13.5)	0.708			
Farmer	124 (10.6)	2 (13.3)	122 (10.6)	0.668			
Family incomes[e]							
No income	382 (32.7)	7 (46.7)	375 (32.5)	0.272			
1–10,000 baht	645 (55.2)	8 (53.3)	637 (55.2)	0.885			
10,001–20,000 baht	58 (5.0)	0 (0)	58 (5.0)	1.000			
>20,000 baht	88 (7.5)	0 (0)	88 (7.6)	0.620			
Animals at home							
Dog or cat	333 (28.5)	6 (40.0)	327 (28.3)	0.387			
Chicken or duck	68 (5.8)	2 (13.3)	66 (5.7)	0.215			
Swine or cow	5 (0.4)	0 (0)	5 (0.4)	1.000			
Drinking water							
Filtered water	700 (60.3)	9 (60.0)	691 (60.3)	0.981			
Bottled water	307 (26.4)	5 (33.3)	302 (26.4)	0.559			
Tap water	168 (14.5)	1 (6.7)	167 (14.6)	0.710			
Consumption of undercooked fish	98 (8.4)	0 (0)	98 (8.5)	0.239			
Consumption of undercooked meat							
Chicken meat	95 (8.1)	0 (0)	95 (8.2)	0.247			
Pork or beef	147 (12.6)	0 (0)	147 (12.7)	0.140			
Underlying diseases							
Hypertension	223 (19.1)	5 (33.3)	218 (18.9)	0.182			
Diabetes	126 (10.8)	5 (33.3)	121 (10.5)	0.017	0.247	1.952	0.628–6.065
Dyslipidemia	86 (7.4)	1 (6.7)	85 (7.4)	1.000			
Respiratory disease	33 (2.8)	2 (13.3)	31 (2.7)	0.065			
Cardiovascular disease	11 (0.9)	1 (6.7)	10 (0.9)	0.133			

Table 1 (*continued*)

Variables	Total patients (n = 1175), No. (%)	No. of patients with CPKP (n = 15), No. (%)	No. of patients with non CPKP (n = 1160), No. (%)	Univariate analysis P value[a]	Multivariate logistic regression analysis		
					P value[b]	aOR	95% CI
Liver disease	12 (1.0)	0 (0)	12 (1.0)	1.000			
Other (tumor, gout, thyroid, thalassemia, allergy, AIDS, etc.)	45 (3.9)	0 (0)	45 (3.9)	1.000			
Antibiotic usage within previous 3 months	50 (4.3)	0.0 (0)	50 (4.3)	1.000			
Hospitalization within previous 6 months	160 (13.7)	4 (26.7)	156 (13.5)	0.137			

[a] A P value of <0.05 was included in multivariate logistic regression analysis.
[b] A P value < 0.05 was considered statistically significant.
[c] Thailand's official retirement age is 60 at government agencies and many companies.
[d] Basic education in Thailand consists of 6 years of primary school education and 6 years of secondary school education.
[e] In the studied area, the average monthly income is 10,000 baht.
Abbreviations: CPKP, carbapenemase-producing *K. pneumoniae*; aOR, adjusted odds ratio; CI, confidence interval.

the main drinking water (60.3%) and consumption of undercooked fish or meat was noted in 8.1–12.6% of patients. Previous antibiotic usage and history of hospitalization were noted in 4.3% and 13.7% of patients, respectively.

Overall, CRE were found in 15 non-duplicate samples. All 15 CRE isolates produced carbapenemases, as judged by mCIM test, resulting in 1.3% (15/1175) prevalence of CPE among outpatients. Fourteen CPE isolates (1.6%) were obtained from diarrheagenic patients while a single CPE isolate (0.3%) was recovered from a UTI patient (Fig. 1 and Table 2). All CPE isolates were identified as *K. pneumoniae* and were positive for bla_{NDM-1}. No other carbapenemase genes were detected.

During the study period, 6.7% (n = 1) and 13.3% (n = 2) of patients with bla_{NDM-1}-positive carbapenemase-producing *K. pneumoniae* (CPKP) were detected in 2018 and 2020, respectively, while 80% (12/15) of CPKP-positive cases were detected in 2019 (Table 2). Specifically, a high incidence of CPKP-positive patients (66.7%, 5/15) was noted in July 2019. The average age of patients with CPKP was significantly higher than those without CPKP (69.4 vs 45.8, P < 0.001) (Table 1). Based on univariate analysis, only age >60 years and diabetes were significantly associated with CPKP-positive patients. These two variables were further included in multivariate logistic regression analysis. The result showed that age >60 years was the only independent risk factor for patients with CPKP (P < 0.001, aOR = 11.500, 95% CI = 3.223–41.034) (Table 1).

Susceptibility test

All bla_{NDM-1}-positive CPKP isolates exhibited high-level resistance to cefotaxime and ceftazidime (MICs >128 μg/mL) and carbapenems (MICs of 32 − > 32 μg/mL) (Table 2). Resistance to ciprofloxacin, colistin and tigecycline was observed in 66.7% (10/15, MICs = 4 − > 32 μg/mL), 53.3% (8/15, MICs = 4 − >16 μg/mL) and 46.7% (7/15, MICs = 0.75–1 μg/mL) of isolates, respectively.

PFGE and MLST

Genetic relationship among bla_{NDM-1}-positive CPKP isolates (n = 15) was investigated by PFGE (Table 2 and Fig. 2). Eight PFGE profiles, designated I–VIII, were observed. Identical PFGE profiles were noted. Profiles I, VII and VIII comprised 2, 3 and 3 isolates, respectively, from diarrheagenic patients. Profile IV included 2 and 1 isolates from patients with diarrhea and UTI, respectively (Fig. 2, Lanes 5–7). CPKP isolates from diarrheagenic patients (strains GM3, GM7, GM9, GM10) showed unique PFGE profiles.

All 15 bla_{NDM-1}-positive CPKP isolates were analyzed for their sequence types (ST). ST70 was common (n = 4), followed by ST147 (n = 3). Other STs, including ST16, ST247, ST11, ST231, ST485 and ST540, were detected at lower frequencies (1–2 isolate each) (Table 2).

Transferability of bla_{NDM-1} and plasmid replicon types

Conjugation experiments were performed with all bla_{NDM-1}-positive CPKP isolates as donors (n = 15). Within 24 h of mating, all isolates were able to transfer bla_{NDM-1} to the recipient, *E. coli* J53, with the conjugation frequencies ranging from 3.4×10^{-7} to 1.4×10^{-2} transconjugants per donor cell (Table 3). Imipenem and meropenem MICs were 4–32 μg/mL for all *E. coli* transconjugants carrying bla_{NDM-1}, except the transconjugant carrying bla_{NDM-1} from strain GM1.

PBRT analysis of 15 bla_{NDM-1}-carrying transconjugants revealed that most bla_{NDM-1} genes were located on IncA/C plasmids (80%, n = 12) (Table 3). IncFIA and IncN plasmids were found in CPKP isolates from patients with diarrhea (1 isolate each) while plasmid with IncI1 replicon type was found in CPKP isolate from a UTI patient.

Stability of bla_{NDM-1} plasmids

Five *E. coli* J53 transconjugants carrying bla_{NDM-1} plasmids with different replicon types were selected for stability

Table 2 Phenotypic and genotypic characteristics of carbapenem-resistant *K. pneumoniae*.

Sources	Bacterial strains	Isolation date	MICs (μg/mL)[a]							mCIM[b]	Sequence Type (ST)	PFGE profiles[c]
			CTX	CAZ	IPM	MEM	CIP	COL	TIG			
Stool samples from diarrheagenic patient	*K. pneumoniae* GM1	16/2/19	>128	>128	>32	>32	>32	8	1	+	ST231	VIII
	K. pneumoniae GM2	22/3/19	>128	>128	>32	>32	4	>16	0.75	+	ST16	I
	K. pneumoniae GM3	1/7/19	>128	>128	>32	>32	4	8	0.75	+	ST247	II
	K. pneumoniae GM6	11/7/19	>128	>128	>32	>32	4	8	0.38	+	ST16	I
	K. pneumoniae GM7	11/7/19	>128	>128	>32	32	4	4	0.75	+	ST247	III
	K. pneumoniae GM8	17/7/19	>128	>128	>32	>32	>32	4	0.38	+	ST147	IV
	K. pneumoniae GM9	18/7/19	>128	>128	>32	32	2	2	0.5	+	ST540	V
	K. pneumoniae GM10	25/8/19	>128	>128	>32	>32	<0.125	>16	0.25	+	ST70	VI
	K. pneumoniae GM11	25/8/19	>128	>128	>32	>32	2	4	0.75	+	ST70	VII
	K. pneumoniae GM12	25/8/19	>128	>128	>32	>32	2	2	0.5	+	ST70	VII
	K. pneumoniae GM16	17/10/19	>128	>128	>32	>32	2	2	0.75	+	ST70	VII
	K. pneumoniae GM18	30/10/19	>128	>128	>32	>32	>32	1	1	+	ST147	IV
	K. pneumoniae GM21	26/2/20	>128	>128	32	32	>32	2	0.25	+	ST485	VIII
	K. pneumoniae GM22	4/8/20	>128	>128	>32	>32	>32	1	0.38	+	ST11	VIII
Urine sample from urinary tract infection patient	*K. pneumoniae* UM	30/11/18	>128	>128	>32	>32	>32	2	0.19	+	ST147	IV
–	*E. coli* DMST4212	–	0.5	0.5	0.25	0.25	0.5	0.25	0.19	–	–	–

[a] Broth microdilution method was used to determine MICs for CTX, CAZ, IPM, MEM, and COL. MICs for tigecycline were examined using MIC test strip (LiofilChem, Italy). The CLSI breakpoints for resistance to CTX, CAZ, IPM, MEM, and COL are ≥4, ≥16, ≥4, ≥4, ≥4 and ≥ 4 μg/mL respectively.[18] EUCAST interpretative MIC breakpoint for tigecycline resistance was >0.5 μg/mL[20]

[b] mCIM (modified carbapenem inactivation method) is a phenotypic method for the detection of carbapenemase production as recommended by CLSI.[18]

[c] PFGE profiles were designated by a roman number. Isolates with indistinguishable banding patterns were assigned to the same PFGE profile.

Abbreviations: CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin, COL, colistin and TIG, tigecycline.

study. Transconjugants carrying pNDM-1-GM7-IncA/C and pNDM-1-GM22-IncA/C were randomly selected as representatives of bla_{NDM-1} IncA/C plasmids. Transconjugants carrying pNDM-1-GM1-IncFIA, pNDM-1-UM-IncI1 and pNDM-1-GM8-IncN were included. The results showed that all bla_{NDM-1} plasmids were stably maintained in *E. coli* J53 for at least 10 days in an antibiotic-free environment (Fig. S1).

Discussion

Among CRE isolates, much attention has been paid on CPE because CPE have been associated with higher meropenem MICs and mortality rates than non carbapenemase-producing CRE.[25] CPE isolates are frequently detected in healthcare settings; however, dissemination of CPE from hospitals to communities has been documented.[10] Variation on the prevalence of community-associated CPE in Asian countries was noted, for instance 0.5% in Hong Kong and 1.1% in 19 provinces across China.[26,27] In contrast, community-associated CPE in Myanmar and Pakistan were up to 8.7% and 14.4%, respectively.[28,29] Our study showed that the prevalence of CPE among outpatients was 1.3%, which is similar to that in our neighboring country, Cambodia (1%).[30] These differences may, to some extent, be attributed to the variations in study designs, study settings and the number of participants. Overall, we obtained 15 non-duplicate bla_{NDM-1}-positive CPKP isolates which possessed ≥ 8-fold higher in imipenem and meropenem MICs compared to the CLSI breakpoints (≥32 μg/mL vs 4 μg/mL). Resistance to colistin and tigecycline was observed in 53.3% and 46.7% of isolates, respectively, which is of concern since they were considered as the last few drugs for the treatment of CRE infections.[1,2]

$bla_{NDM-1}$was the only carbapenemase gene found in our study, consistent with the fact that bla_{NDM} is the frequent carbapenemase gene reported in Southeast Asia.[2] In addition to bla_{NDM-1}, bla_{OXA-48} and $bla_{OXA-48-like}$ ($bla_{OXA-181}$ and $bla_{OXA-232}$) are widespread in Thailand.[9,17] However, bla_{OXA-48} usually exhibits weak carbapenemase activities and high-level expression of bla_{OXA-48} could be achieved in combination with permeability defect which has also been noted in carbapenem-resistant *K. pneumoniae* isolates in Thai patients[31,32] Low-level expression of bla_{OXA-48} may therefore, be problematic for *in vitro* detection and may result in the low prevalence of CPE found in our study.

Our study revealed that age >60 years is an independent risk factor associated with CPKP among outpatients ($P < 0.001$, Table 1), in line with the previous study by Tang et al.[33] who showed that CRE from community settings in Taiwan were more likely to be isolated from elderly participants. Diabetes was significantly associated with CPKP-positive patients ($P = 0.017$), although it was not identified as a risk factor in a final multivariate logistic regression

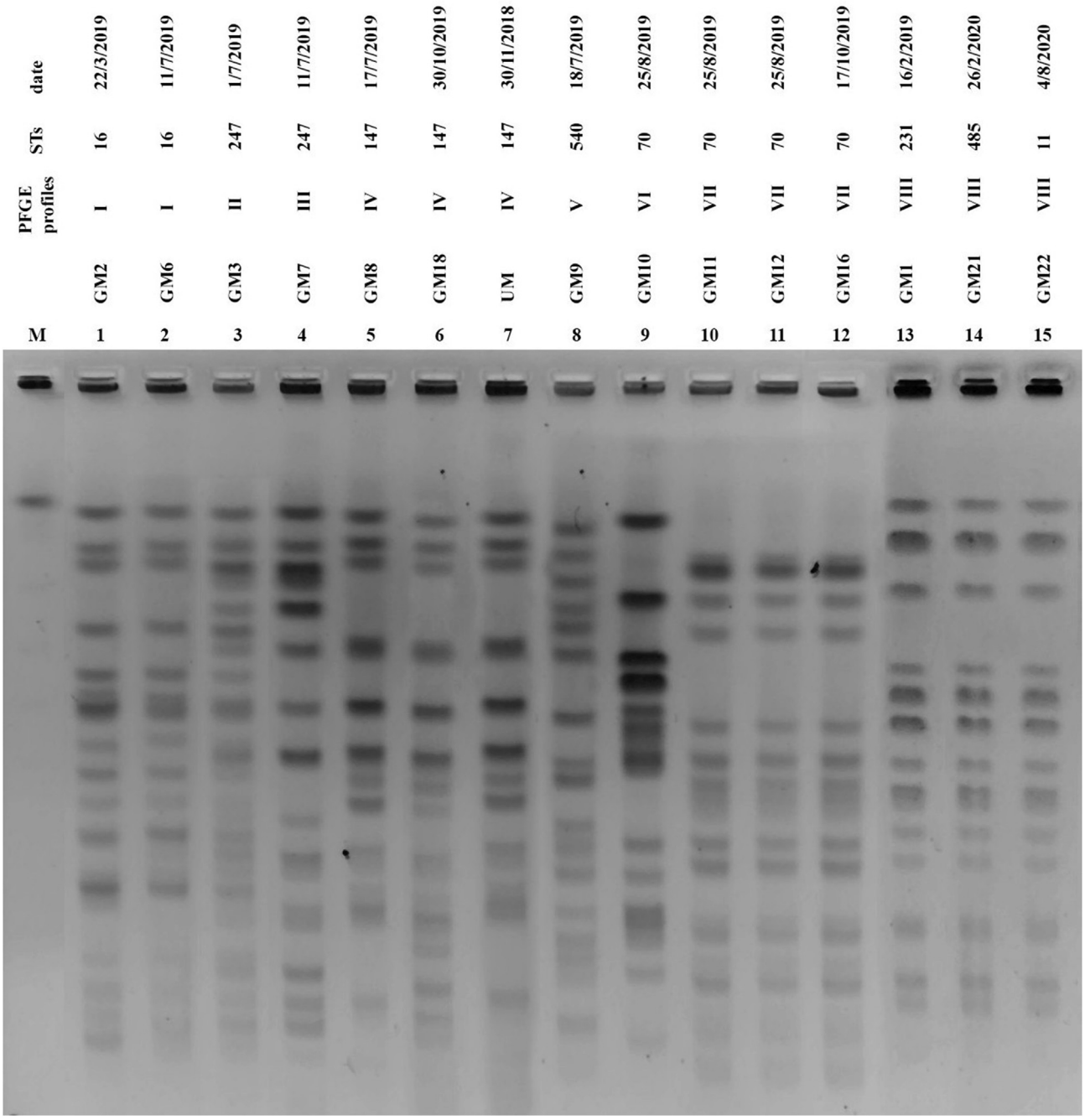

Figure 2. DNA macrorestriction profiles of bla_{NDM-1}-positive *K. pneumoniae* isolates. Chromosomal DNA was prepared and digested with *Xba*I and was subjected to pulsed-field gel electrophoresis (PFGE). PFGE profiles were designated by a roman number. Isolates with indistinguishable banding patterns were assigned to the same PFGE profile. M, *Saccharomyces cerevisiae* chromosomal DNA (Bio-Rad Laboratories Inc., Hercules, CA), Lanes 1—15, bla_{NDM-1}-positive *K. pneumoniae* isolates.

analysis. This result corresponded to the previous literature suggesting that diabetes is one of the most common comorbidities among CRE in community settings.[10]

A small number of CPKP-positive patients (6.7%) were detected in 2018 and increased sharply in 2019 (80%). The reason for this is not known. However, these data coincided with the carbapenem-resistant rates in *K. pneumoniae* obtained from outpatients in Thailand which increased from 5.7 to 5.9% in 2018 to 8.5—8.9% in 2019.[16] The incidence of CPKP-positive patients was clustered over an area with a 27 km radius (Fig. 3). It is a rural area where people mostly work in the agricultural sector, both plantation and food animal farming. CPE may be transmitted from environment to human via food chain and, consequently, become part of gut microbiota in healthy individual and probably spread within the community.[1] Genotypic analysis by PFGE revealed the genetic diversity among bla_{NDM-1}-positive CPKP isolates. Nevertheless, we observed 4 cases of clonal relatedness of isolates from patients who were unrelated and lived far away from each other. For

Table 3 Transferability of bla_{NDM-1}, plasmids replicon types and carbapenem MICs.

bla_{NDM-1}-positive *K. pneumoniae*	Conjugation			
	Conjugation frequency[a]	Plasmid replicon types	MIC (μg/mL) in transconjugants[b]	
			IPM	MEM
K. pneumoniae GM1	1.7×10^{-6}	IncFIA	0.5	0.5
K. pneumoniae GM2	4.4×10^{-5}	IncA/C	16	16
K. pneumoniae GM3	3.0×10^{-3}	IncA/C	16	8
K. pneumoniae GM6	3.3×10^{-5}	IncA/C	4	4
K. pneumoniae GM7	3.2×10^{-5}	IncA/C	16	16
K. pneumoniae GM8	3.4×10^{-7}	IncN	32	16
K. pneumoniae GM9	2.8×10^{-4}	IncA/C	16	4
K. pneumoniae GM10	8.3×10^{-6}	IncA/C	4	8
K. pneumoniae GM11	1.8×10^{-5}	IncA/C	32	32
K. pneumoniae GM12	6.1×10^{-5}	IncA/C	32	32
K. pneumoniae GM16	2.0×10^{-4}	IncA/C	32	32
K. pneumoniae GM18	5.8×10^{-4}	IncA/C	8	8
K. pneumoniae GM21	4.9×10^{-4}	IncA/C	32	32
K. pneumoniae GM22	1.4×10^{-2}	IncA/C	32	32
K. pneumoniae UM	1.4×10^{-2}	IncI1	16	32
E. coli J53[c]	—	—	0.25	0.25

[a] Conjugation frequency was expressed as the number of transconjugants divided by the number of donor cells.
[b] MIC breakpoints for resistance to imipenem and meropenem were ≥4 μg/mL[18]
[c] Sodium azide-resistant *E. coli* J53 was used a recipient strain.
Abbreviations: IPM, imipenem and MEM, meropenem.

examples, isolates belonging to PFGE profiles I, VII and VIII were recovered from stool samples of participants who lived from 2 to as far as 92 km apart (Fig. 3). Moreover, CPKP isolates belonging to PFGE profiles I and VII shared the same sequence types, ST16 and ST70, respectively (Table 1). In another case, 3 isolates (GM8, GM18 and UM), belonging to ST147, revealed identical PFGE profile (profile IV). However, they were isolated from different sample types (2 stool and 1 urine samples) from participants who live ~55–100 km away from each other. In addition, CPKP isolates, belonging to PFGE profiles I, IV, VII and VIII, were recovered approximately 4, 3–11, 2 and 6–18 months apart, respectively (Table 2). These data suggest that bla_{NDM-1}-positive CPKP isolates have been circulating within a Thai community for some time.

Previous literature suggested that NDM-producing *K. pneumoniae* distributed across a large number of STs.[5] Our study showed that the most common ST among bla_{NDM-1}-positive CPKP was ST70; however, *K. pneumoniae* ST70 carrying bla_{NDM-1} has rarely been reported. To our knowledge, it has been reported from hospitalized patients in Greece in 2015 and 2016.[34] In addition, two isolates of bla_{NDM-1}-positive CPKP ST70 has been reported from patients in northeastern Thailand in 2016 and 2017.[35] Notably, our study found 3 bla_{NDM-1}-positive CPKP isolates belonging to ST147, one of the most prevalent type carriers of bla_{NDM}.[5] This becomes a matter of concern because ST147 is considered as one of the highly resistant lineages that contributes to disease burden worldwide.[36] In addition, ST11, ST16, ST147 and ST231 have been identified as hosts of bla_{NDM-1} in clinical isolates of CPKP in Thailand. Particularly, ST16 has been associated with invasive diseases and poor outcomes among Thai patients.[9]

Previous reports from Thailand revealed that bla_{NDM-1} could be found on either chromosome or plasmid.[35,37] In this study, bla_{NDM-1} in all CPKP isolates was successfully transferred to *E. coli* J53, suggesting that bla_{NDM-1} resided on conjugative plasmids. Almost all *E. coli* transconjugants carrying bla_{NDM-1} plasmids demonstrated at least 16-fold increase in imipenem and meropenem MICs compared with those in the recipient strain (Table 3). However, transconjugant carrying bla_{NDM-1} from strain GM1 was defined as susceptible (carbapenem MICs = 0.5 μg/mL), implying that bla_{NDM-1} may not be fully expressed. The variable levels of carbapenem resistance (susceptible, resistance) among transconjugants carrying bla_{NDM-1} were in accordance with those previously reported for other bla_{NDM-1} plasmids.[38] IncA/C was the most common plasmid replicon type (80%) associated with bla_{NDM-1} while IncFIA, IncN and IncI1 were detected at low frequencies (Table 3). Following conjugation, bla_{NDM-1}-carrying plasmids remained stable in bacterial host for at least 10 days in antibiotic-free environments, regardless of plasmid replicon types. The predominate IncA/C plasmid among bla_{NDM-1}-positive CPKP differed from the previous study in Thai patients which showed that bla_{NDM-1} resided on IncN2 plasmids.[35] Moreover, bla_{NDM-1}-carrying IncA/C plasmid was found among CPKP with different genotypes, suggesting that IncA/C plasmid may facilitate the spread of bla_{NDM-1} among multiclonal CPKP isolates within community. This is particularly of concern because IncA/C is a broad host range plasmid that can be transferred among several bacterial species. Furthermore, bla_{NDM-1}-carrying IncA/C plasmid has apparently been shown to cause outbreak of NDM-1-producing *K. pneumoniae* and *E. coli* in several countries such as India, UK and Canada.[39,40]

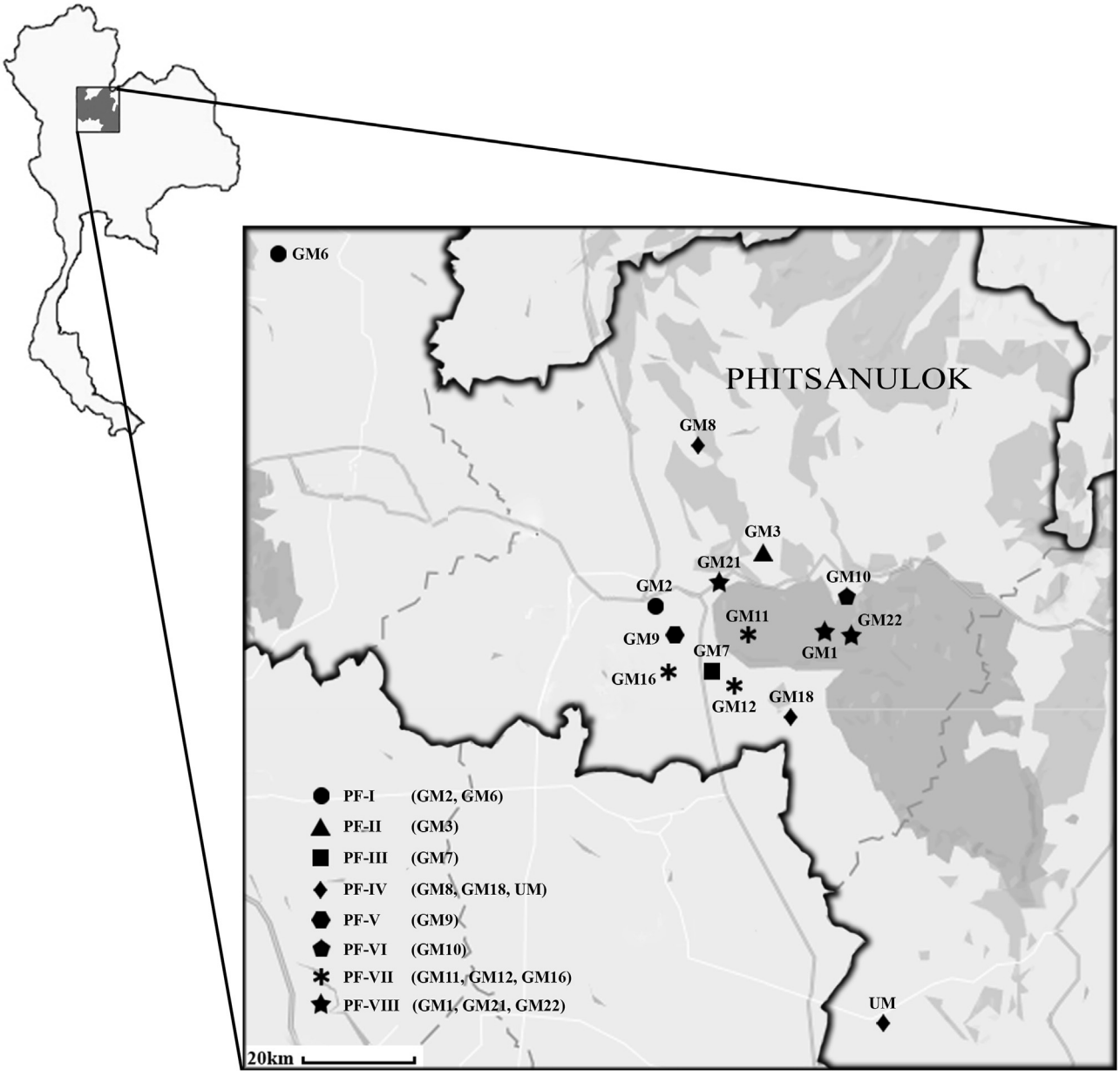

Figure 3. Location of participants who were positive for *bla*$_{NDM-1}$-carrying *K. pneumoniae*. Each symbol (●, ▲, ■, ♦, ⬟, ⬣, ✱ and ★) represents a different PFGE profile (PF). The distances between origins of isolates in each PF are as follows: -PF-I (●): 92 km (GM2 & GM6), -PF-IV (♦): 54.5 km (GM8 & GM18), 55.5 km (GM18 & UM) and 100 km (GM8 & UM)., -PF-VII (*): 8.6 km (GM11& GM12), 8.4 km (GM12 & GM16) and 13 km (GM11 & GM16)., -PF-VIII (✱): 18 km (GM1 & GM21), 20 km (GM21 & GM22) and 2 km (GM1 & GM22).

In conclusion, this is the first study demonstrating the presence of CPE among outpatients in Thailand. Although, a low prevalence was observed, our study confirms that community-associated CPE does exist and warrants further vigilance. Furthermore, the emergence of *K. pneumoniae* ST70, the uncommon NDM-positive *K. pneumoniae* lineage, emphasizes the need for a large-scale surveillance study on CPE to limit the spread of CPE in a Thai community.

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Author contributions

PRN and TL contributed to conception and design of the study. KA, NS and PT performed sample collection and data acquisition. KA, AN, TL and PRN performed data analysis and interpretation. PRN wrote the original draft. PRN and TL reviewed and edited the manuscript and funding acquisition. All authors involved in discussion and approved the manuscript.

Declaration of competing interest

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2023.01.018.