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Original Article

Clonal dissemination of drug-resistant *Acinetobacter baumannii* in Thailand: insights from nationwide molecular typing

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Abstract

Introduction: Drug-resistant *Acinetobacter baumannii* poses a global health crisis, especially in Asia. It has a propensity to become clonally endemic in healthcare settings. However, its clonal distribution in a broad geographic area is unclear.

Methodology: The clonality of *A. baumannii* was characterized nationwide by collecting 572 drug-resistant *A. baumannii* from 18 hospitals across Thailand regions between 2017–2018 and genotyping them by random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) in association with carbapenemase genes data.

Results: The results depicted 12 types of RAPD banding. Strikingly, two types were predominant in all hospitals (79%). Of those, 96% harbored the *blaOXA-23* gene. The banding pattern matched the preexisting strain in the institution, suggesting an ongoing nationwide circulation of the resistant clone. Interestingly, a unique banding type was identified in high proportion in two nearby hospitals in the northern region (21%, 53/252). Two isolates with the same banding pattern were also identified in a hospital in Bangkok, suggesting the possibility of transfer between regions. Most of the subset of isolates analyzed belonged to sequence type (ST) 2, the most prominent ST in the Asia-Pacific region.

Conclusions: This study demonstrated continuous dissemination of predominating *A. baumannii* clones across the country, and the emergence of endemic hospital-specific clones, all with high burdens of *blaOXA-23*; suggesting a strong selection for these resistance determinants. In addition, genotyping with RAPD can be a simple and cost-effective epidemiological tool with efficient discriminatory power for *A. baumannii* in developing countries.

Key words: *Acinetobacter baumannii*; carbapenem resistance; RAPD; Thailand.

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Introduction

Acinetobacter baumannii is currently one of the most concerning pathogens and is frequently isolated in the intensive care unit (ICU) where it is responsible for severe infections such as ventilator-associated pneumonia, sepsis, and urinary tract infection. *A. baumannii* can be disseminated in healthcare settings facilitated by the capacity to acquire resistant genes and persist in the environment; thus, raising concerns globally. The rapid expansion of multi-drug- (MDR) and extremely drug-resistant (XDR) *A. baumannii* and the prevalence of the international clone II among international hospitals demonstrates the importance of surveillance to control transmission [1,2]. The resistance gene dissemination can be through colonized patients traveling between regions or through contaminated hospital equipment and poor hygiene [3].

Persistent use of broad-spectrum antibiotics has increased resistance selection, rendering even the last resort drugs, such as colistin, ineffective [4]. Thus, carbapenem resistant *A. baumannii* (CRAB) is considered the highest priority by the World Health Organization (WHO) and is identified as a critical pathogen [5]. Carbapenem resistance in *A. baumannii* is derived from OXA oxacillinases and metallo-beta-lactamases [6]. These enzymes are encoded by acquired resistance genes, e.g., *blaOXA-23*, *blaOXA-24*, and *blaOXA-58*. Resistance genes are mobilized by plasmids or transposons between different or the same strains [7]. The emergence of XDR *A. baumannii* is a major concern for Asian countries compared to other European and North American countries [8]. The impact of the XDR *A. baumannii* in hospital settings prolongs hospital stay, increases the mortality rate, and affects the economy of developing countries [9]. According to the National Antimicrobial Resistant

Surveillance Center's Thailand report, there was an increase in resistance to all antibiotics along with the emergence of XDR *A. baumannii* in all regions of Thailand in 2022 [10], with > 60% mortality during nosocomial outbreaks [11].

Based on molecular characterization, clonal dissemination mainly facilitates the spread of the carbapenem-resistant gene. Globally, there are two circulating clones: Global Clone (GC) 1 and 2. In Asian countries, GC2 is the most prevalent type. The dissemination of certain clones of *A. baumannii* in tertiary care hospitals in Thailand and the possible clonal variation in other general hospitals in comparison to large university hospitals were previously speculated [12]. Another study conducted at Siriraj Hospital in Bangkok during 2003–2008 demonstrated a persistent dominant *A. baumannii* clone circulating in the hospital over the years [13]. A previous study conducted in Hua Hin hospital, Thailand, concluded that clonal relatedness was evident between large and satellite hospitals, reflecting the clonality of *A. baumannii* in a wider area [12]. A study in a tertiary care hospital in southern Thailand also demonstrated clonal dissemination within and between the wards [14,15]. However, the overall picture of the country is unclear due to the lack of sampling across regions. Therefore, the research question in this study was, whether this pattern could be observed in other hospitals where a hospital-specific *A. baumannii* clone is selected and maintained, or if there are only a few clones disseminating among hospitals region-wide or even country-wide.

In this context, the clonal relatedness of XDR/MDR *A. baumannii* across hospitals of different sizes and in difference regions of Thailand were determined to elucidate clonal similarity countrywide. Sequence-based typing, e.g., multilocus sequence typing (MLST), is a standard; although it is costly and time-consuming. Alternatively, random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) is useful in a low-resource setting for investigating a large number of samples with good reproducibility and is comparatively faster than other typing methods [16]. In this study, 572 XDR/MDR *A. baumannii* isolates from 18 hospitals across Thailand were investigated to determine the clonality by RAPD-PCR and the associated dissemination of carbapenem resistance genes.

Methodology

Bacterial isolates

Five hundred and seventy-two MDR and XDR isolates of *A. baumannii* (CRAB) were obtained from the Thailand Research University Network (RUN) collection [17]. The isolates were originally collected during 2017–2018 from hospitals covering all regions of Thailand (Central, Northern, Northeastern, and Southern regions). The hospitals included several types of establishments, such as, university, regional, general, military, and private hospitals; and healthcare levels from secondary to supra-tertiary care (Table 1). All isolates were genotypically confirmed for the presence of the *blaOXA-51* gene. The sources of the clinical isolates included blood, urine, respiratory system (sputum and tracheal aspirate), and sterile area (cerebrospinal fluid, ascites fluid, pleural effusion, and peritoneal fluid). All isolates were previously determined for antimicrobial susceptibility; and presence of carbapenemase genes i.e., *blaOXA-23*, *blaOXA-24*, *blaOXA-58*, *blaIMP*, and *blaVIM* [17]. The data on the isolates can be provided at request.

Table 1. List of hospitals included in the study and the number of isolates collected from each.

Regions	Hospital number	Hospital type	Healthcare level	Number of beds	Number of isolates
Northern	1	University	Supra-tertiary	1,282	156
	2	General	Secondary	440	20
	3	Regional	Tertiary	700	21
	4	Regional	Tertiary	743	29
	5	Regional	Tertiary	858	5
	6	General	Secondary	420	5
Northeastern	7	Regional	Tertiary	1,387	58
Central	8	University	Supra-tertiary	2,221	102
	9	Military	Tertiary	774	18
	10	Private	N/A	100	8
	11	Regional	Tertiary	722	24
	12	Regional	Tertiary	611	17
	13	Private	N/A	486	6
	14	Regional	Tertiary	700	15
	15	Bangkok Metropolitan	Tertiary	393	27
	16	Private	N/A	207	29
	17	Regional	Tertiary	400	7
Southern	18	Regional	Tertiary	553	25
		Total number of isolates			572

Genotypic determination

Genomic DNA was extracted using the boiling method [18]. Purified DNA was quantified by the NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) at 260/280nm, and further diluted with TE buffer to 50 ng/µL. In order to compare with previous results, RAPD-PCR was performed with a 10 bp single primer R003 (CCTTGACGCA) and the PCR conditions were optimized according to the previous study conducted at Siriraj Hospital to improve the clarity of bands [14]. Briefly, 0.2 µM of primer, 100 ng of template, and 1 U of *Taq* enzyme were used. The cycle comprised of 94 °C for 10 min; followed by 40 cycles of 94 °C for 10s, 36 °C for 30 seconds, and 72 °C for 1 min; and then 72 °C for 2 min. AB 5073 (USA) was used as a control strain.

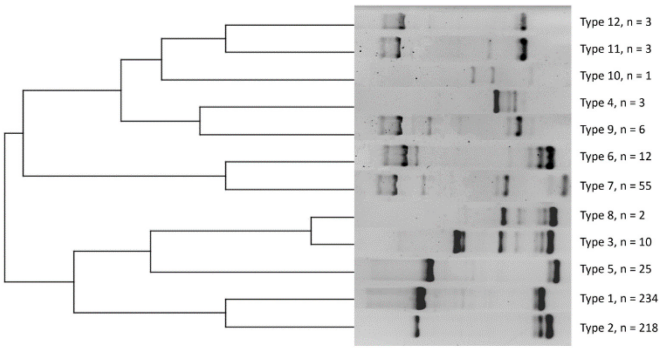
Cluster analysis

Gel electrophoresis was conducted in 2% agarose at 95 V for 75 min, with the Gene Ruler 1 kb DNA Ladder (Thermo Fisher Scientific, Vilnius, Lithuania) for comparison. The image was analyzed using PyElph [19]. The lane detection was performed with the option (define lane width) set to a 70% threshold value to eliminate background noise. The dendrogram based on the clustering method applied to the distance matrix was computed with the neighbor-joining unweighted pair group with arithmetic mean (UPGMA) method and plotted to compare the phylogenetic relatedness. The heatmap was generated using Heatmapper [20].

Multilocus sequence typing (MLST)

In silico MLST was performed with 42 isolates to compare the RAPD fingerprints. The isolates were chosen based on RAPD fingerprints, hospital distributions, and antimicrobial resistance genes combinations. DNA of the selected isolates were extracted with ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, USA) and purified with Agencourt AMPure beads (Beckman Coulter Life Sciences, Indianapolis, USA) according to the manufacturers' instructions. The purified DNA were 150 bp-paired-end sequenced with an Illumina HiSeq 2500 (Genewiz, Suzhou, China). The MLST analysis was carried out in silico using the Pasteur scheme with 7 housekeeping genes (*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD*) [21]. The representative isolates will be further analyzed in future studies using the whole genome sequence analysis approach.

Figure 1. Isolates computed with the neighbor-joining unweighted pair group with arithmetic mean (UPGMA) method showing the relatedness of each of the RAPD types. The number of isolates is shown along with each RAPD type.



Ethical approval

This study was approved by the Institution Review Board of the Faculty of Medicine Siriraj Hospital, Mahidol University (COA no. Si 366/2017).

Results

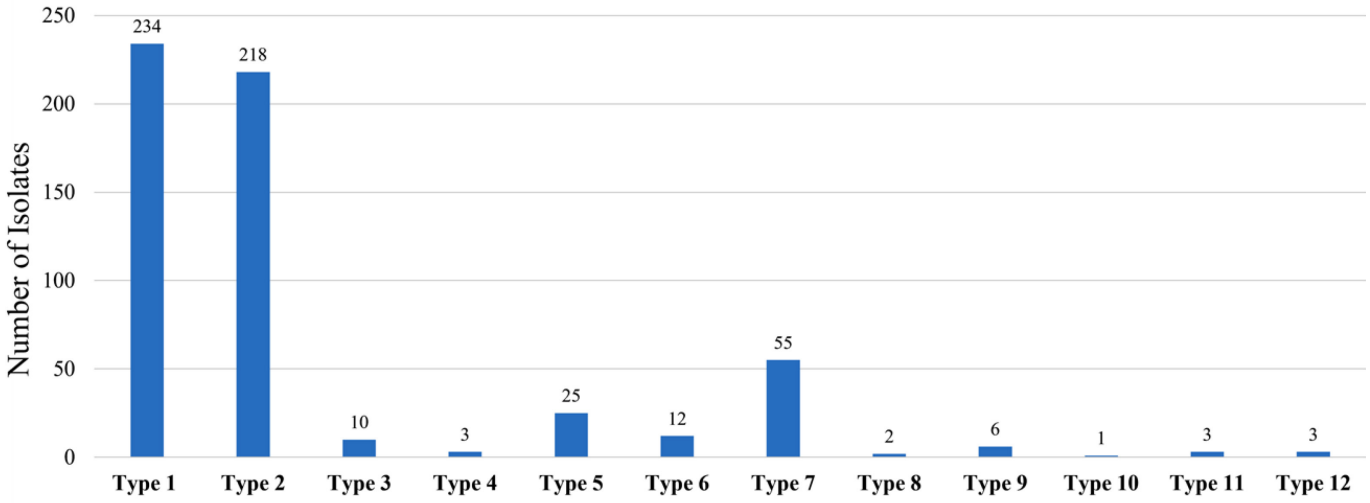
Epidemiology of A. baumannii isolates

In this study, 572 *A. baumannii* isolates were collected from 18 hospitals; all of which tested positive for the carbapenemase gene. The isolates originated from various clinical sources; i.e. 44% (n = 250) from respiratory specimens, 26% (n = 147) from urine, 21% (n = 119) from blood, and 10% (n = 56) from sterile sites. Geographically, the isolates were distributed across regions as follows: Northern (44%, n = 252), Northeastern (10%, n = 58), Central (43%, n = 244), and Southern (3%, n = 18). Only 8% (n = 43) of the isolates were from private hospitals. Regarding the level of healthcare, 43% (n = 248) of the isolates were from supra-tertiary, 45% (n = 257) were from tertiary, and 4% (n = 24) were from secondary care hospitals (Table 1).

RAPD profiles

The RAPD-PCR analysis of the isolates yielded 12 banding patterns clustered in three clades (Figure 1). Most patterns exhibited shared bands, including a high degree of clonal relatedness among the isolates. The types of banding patterns were arbitrarily assigned by numbers 1 to 12. Types 1 and 2 were the most prevalent occurring in 41% (n = 234) and 38% (n = 218) of isolates, respectively; followed by type 7 (10%, n = 55) and type 5 (4%, n = 25) (Figure 2). The RAPD types 1 and 2 differed only in a few bands and shared a common dendrogram node (Figure 1). On the other hand, a few unique banding patterns were observed, i.e., types 3, 4, and 11, that circulated in certain regions.

Figure 2. Total number of isolates of each RAPD type. The number of isolates is shown on the vertical axis.



Regional distribution of RAPD types

Regionally type 1 was the most common in the Southern region, comprising 61% of the isolates, followed by Northeastern and Northern (Figures 3A, 3B). In contrast, type 2 was most prevalent in the Central region. Notably, the data for the Southern and Northeastern regions were derived from one hospital in each region. Type 7 had a high prevalence in the Northern region (21%), while it was rare in the Central region (1%), and absent in the others (Figure 3A). Among the unique banding types, RAPD type 5 was detected in all regions, with the highest prevalence in the Northeastern region (7%), followed by the Southern (6%) and Central regions (5%).

Distribution of RAPD types among different hospital levels

The types 1 and 2 were the most common in tertiary hospitals, accounting for 46% (117/257; Figure 4B). The unique banding types in the tertiary hospitals included type 3 and type 12, both presented at 1% (3 out of 257). A similar pattern was observed in the supra-tertiary hospitals, where types 1 and 2 were predominant at 40% (99/248) and 26% (65/248),

Figure 3 A. Map of Thailand showing the location of hospitals and the prevalence of RAPD types in each region. The dark blue, green, and red circles represent the supra-tertiary, tertiary, and secondary hospitals, respectively. **B.** Heatmap showing the presence of carbapenemase genes in relation to RAPD type, source of samples, region, and hospital type. The red box indicates the most common RAPD type 1 and 2 isolates, which were present in all regions and hospital types. In contrast, the blue box indicates RAPD type 7, which was almost exclusively identified in a university hospital in the Northern region.

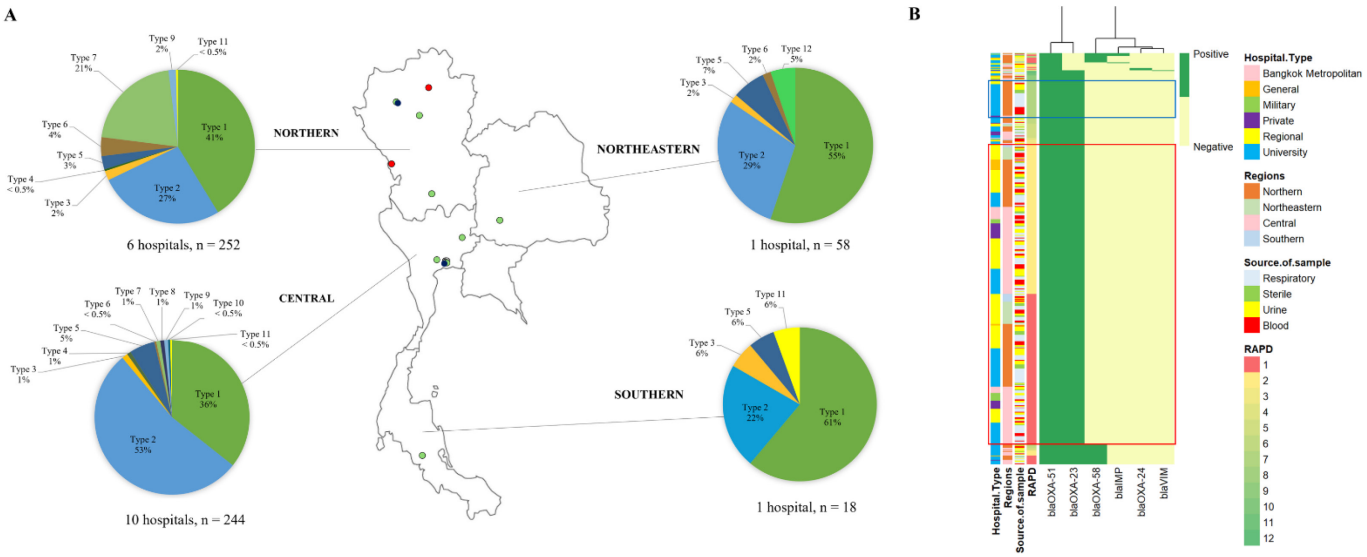
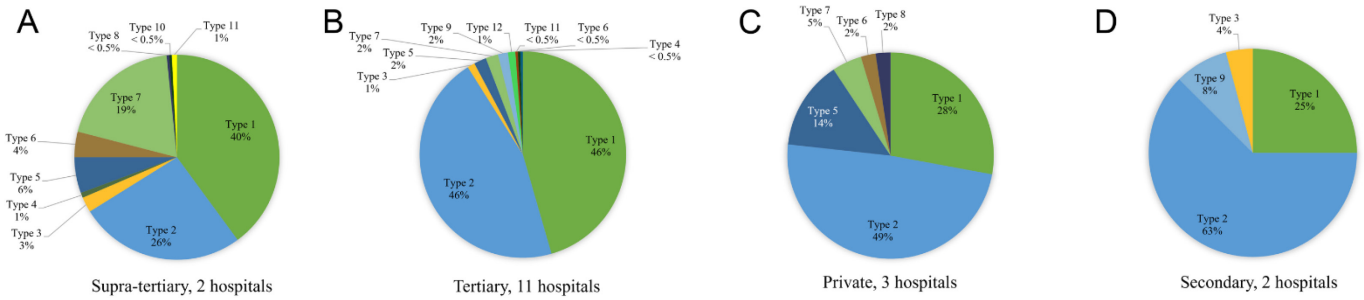


Figure 4. Prevalence of RAPD types at different hospital levels. **A.** supra-tertiary; **B.** tertiary; **C.** private; **D.** secondary hospitals.



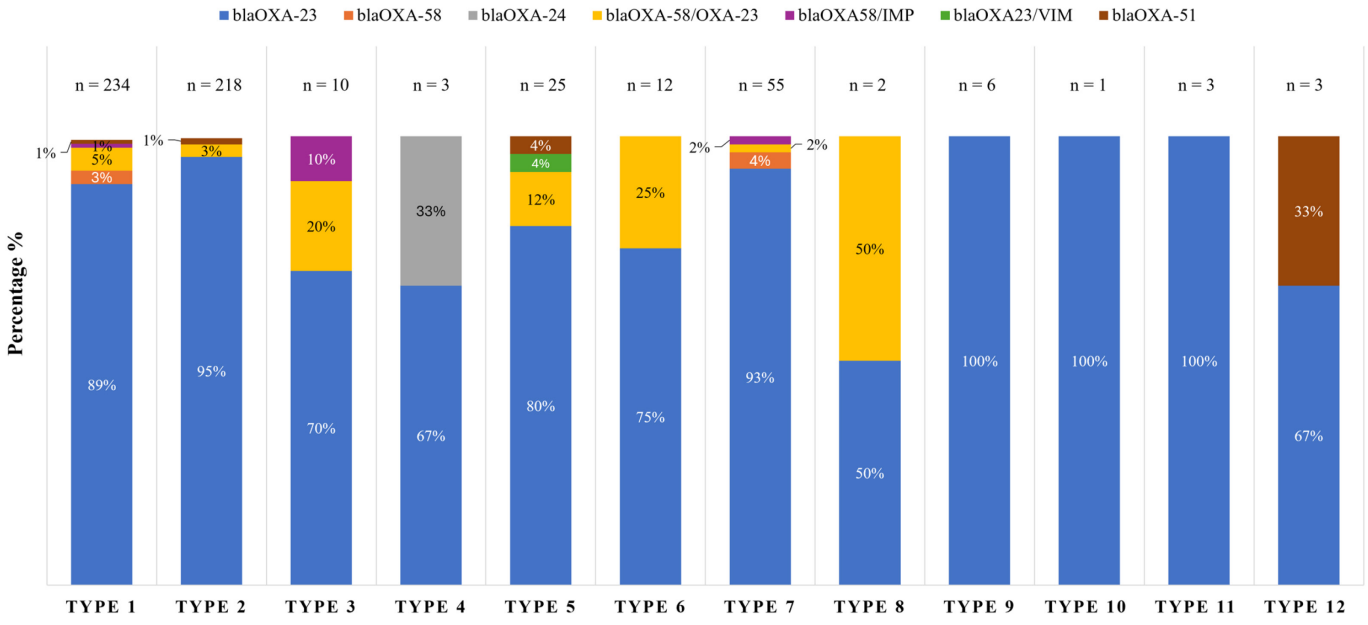
respectively (Figure 4A). However, type 7 was found to be more common in the supra-tertiary hospitals at 19% (48/248) of isolates (Figure 4A). Additionally, type 7 was found predominantly in the Northern region, and, to a lesser extent in a nearby tertiary hospital in proximity (6.9 km) and in a private hospital in Bangkok (581 km away) (Figures 4A, 4B, 4C). Similarly, type 5 was observed mainly in both the supra-tertiary hospitals, 6% (14/248), and 14% (6/43); and in the private hospital in Bangkok (Figures 4C, 4D). Type 9 was found only in the secondary and tertiary hospitals at 8% (2/24) and 2% (4/257), respectively. In contrast, the secondary and private hospitals had a higher prevalence of type 2 at 63% (15/24) and 49% (21/43), respectively.

RAPD types and their associated carbapenem-resistant genes

Besides the *blaOXA-51* carbapenemase gene which was present in all isolates, *blaOXA-23* was the most predominant across all RAPD types (Figure 2). Most type 1 and type 2 isolates harbored a single-presence of the *blaOXA-23* gene with a prevalence of 89% (209/234) and 95% (208/218), respectively (Figure 5). The single-presence of *blaOXA-58* was evident among type 1 at 3% (7/234) and type 7 at 4% (2/55) (Figure 5). However, the co-presence of genes was also detected in a minority of isolates. The *blaOXA-23*/*blaOXA-58* was observed in 5% (28/572) of the isolates, mostly in type 1. The *blaIMP*/*blaOXA-58* and *blaVIM*/*blaOXA-23* gene combinations were also identified in a very low proportion. Notably, 1% (7/572) isolates harbored only *blaOXA-51* but were phenotypically multi-drug resistant. This phenomenon was found in type 2 (3/218), type 1 (2/234), type 5 (1/25), and type 12 (1/3) isolates. A trace of *blaOXA-24* was observed in type 4, followed by type 2 and type 1. Phenotypically, type 7 isolates were XDR (90%).

The single-presence of *blaOXA-23* carbapenemase gene was highly prevalent in 41% (232/572) respiratory

Figure 5. Distribution of carbapenemase genes in association with the RAPD types. The number of isolates of each RAPD type are shown on top of the bars. Isolates labeled with blaOXA-51 were positive for only blaOXA-51.



samples, followed by urine and blood at 22% (124/572) and 20% (113/572) respectively. The isolates harboring only *blaOXA-51* were mostly obtained from urine.

Comparison of the efficiency of RAPD with MLST

The most common ST observed was ST2 (43%, 18/42), which aligned with the predominant RAPD types. While almost all type 2 and type 5 were ST2, type 1 isolates showed diversity. Notably, all type 7 isolates were classified as ST2 (Figure 6).

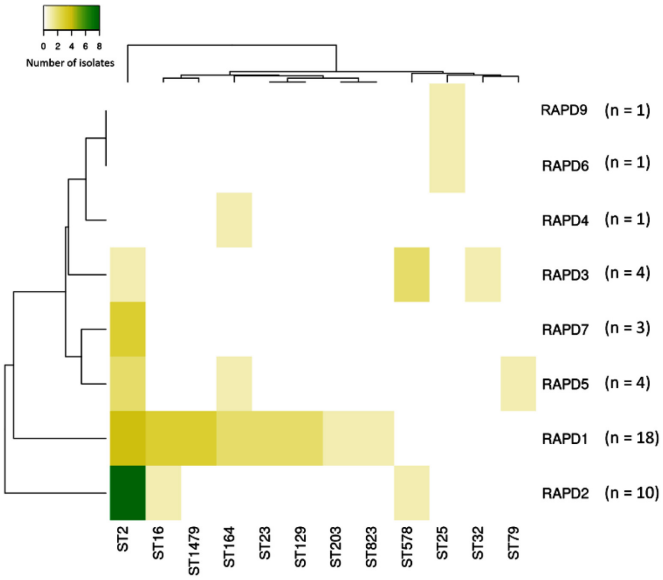
Discussion

The primary aim of this study was to determine the clonality and relationship of *A. baumannii* isolates among hospitals across regions of Thailand. The results demonstrated the countrywide clonal dissemination of MDR/XDR *A. baumannii*, and the distribution of RAPD banding types over regions depicted a possible countrywide dissemination of certain *A. baumannii* clones. There were 12 RAPD banding patterns, and the supra-tertiary hospitals hosted the most diverse types as they are the highest-level referral centers which accept referred patients from the others. Interestingly, *A. baumannii* isolates with similar banding patterns were previously observed indicating that types 1 and 2 have been circulating at Siriraj Hospital since 2009 [13]. Such evidence of endemic clonal persistence of *A. baumannii* has also been observed globally, especially in association with the prevalence of *blaOXA-23* [22]. A possible outbreak was observed in Ohio, USA, where the same ST2 had been present for 11 years along with the emergence of new STs in association with increased carbapenem resistance [23].

Type 7 isolates were mainly identified in nearby tertiary hospitals of the Northern region, suggesting a possible local transmission. Additionally, it was not found in the other regions, except in a private hospital in Bangkok. The banding pattern of type 7 was clustered separately and was drastically different from type 1 and type 2. Therefore, type 7 may not be a subclone of type 1 or type 2, but an endemic clone in the Northern regions with possible long-distance transmission to Bangkok [3]. All of the isolates in type 7 were XDR. All except two isolates, regardless of the hospitals, harbored only *blaOXA-23*. These findings imply that when the resistance is extensive, antibiogram or resistance gene profiling cannot not distinguish the isolates and thus are not very useful for epidemiological investigations. This fact warrants the need for genotyping.

Among other circulating types, type 5 clustered with type 1 in the dendrogram, and type 3 and type 8

Figure 6. Average linkage hierarchical clustering showing the number of isolates in concordance between RAPD types and sequence types (ST).



had a unique banding pattern, suggesting that these were also unique circulating clones separated from type 1 and type 2 [12]. Type 3 was present even in the Northeastern and Southern regions, where there was only one representative hospital, indicating the wide distribution of type 3, which was previously identified at Siriraj Hospital in the central region [24]. The other types were found sporadically in a small number. Type 9 was only found in a tertiary and secondary hospital in the Central and Northern region, while type 11 was present in supra-tertiary hospitals in the Northern and Central regions, and in a tertiary hospital in the Southern region. Analysis of more isolates is needed to determine whether these are regional endemic types.

A study conducted during 2013–2015 in Thailand showed that *blaOXA-23* was the most prevalent carbapenemase gene in *A. baumannii*, and the finding remains consistent in this study [12]. The *blaIMP* was previously reported in 2006 in a hospital in Phitsanulok, located 350 km north of central Bangkok [25]. In this study, all isolates except for one carried *blaIMP*. However, in another study conducted in 2013–2015 in Thailand, none of the isolates carried *blaIMP* [7]. A study of 183 CRAB isolates conducted in Thailand that included 11 tertiary care hospitals from 2016–2017 reported 1.09% of *blaOXA-58* from the Central and Northeastern regions, and only one isolate carried *blaOXA-23*/*blaOXA-58* [26]; which is comparable with the results of this study. However, the occurrence of *blaOXA-24* was rare in this study. Although, type 7 belongs to a different lineage than types 1 and 2; it

possesses the same resistance gene profile, which reflects the extensiveness of gene transfer possibly in response to similar antibiotic selection pressure.

A. baumannii ST2 is predominant in Thailand and the Asia-Pacific regions [27], which was also reflected in the results of this investigation. However, type 1 showed a certain degree of heterogeneity with ST2 indicating that MLST could not distinguish the region-specific type 7. This finding highlighted the limitation of a single typing method, although the result should be considered with caution due to the limited number of isolates tested. In this study, the RAPD method was able to classify the clonal variations among *A. baumannii* successfully. In addition, region-specific type 7 could either have emerged locally or transferred from elsewhere, and this requires further genomic investigation. However, previous studies observed persistence *A. baumannii* in the environment which may facilitate possible countrywide transmission [24,28]. Therefore, periodic surveillance with genomic data could depict the transmission dynamics and endemicity over time to inform stakeholders to take control measures.

Conclusions

This study demonstrated that the circulating RAPD types represent the clones that have successfully adapted under antibiotic selection, and are capable of spreading over time and geographic region. The rapid spread of XDR *A. baumannii* clonal variants is a concern for global healthcare systems. New variants could emerge in the presence of established XDR clones, and successively dominate within healthcare facilities, giving rise to the observation in this study. These findings emphasize the importance of robust surveillance for antibiotic-resistant isolates.

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Data availability

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

Authors' contributions

IT, TA, conceptualization and methodology; TA, PN, formal analysis and investigation; TA, manuscript-original draft; WK, TW, CC, PK, IT, manuscript-review and editing; PK, funding acquisition and supervision; TY, resources. All authors read and approved the final manuscript.

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Conflict of interest

No conflict of interest is declared.

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