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Research article

Genotypic and phenotypic mechanisms underlying antimicrobial resistance and synergistic efficacy of rifampicin-based combinations against carbapenem-resistant *Acinetobacter baumannii*

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ABSTRACT

Purpose: Carbapenem-resistant *Acinetobacter baumannii* (CRAB) is an urgent concern to public health. This study focuses on exploring the resistance mechanisms and the in vitro results of using rifampicin in combination with conventional antibiotics for the management of CRAB.
Methods: The synergistic and bactericidal effects of rifampicin with conventional antibiotics were evaluated using chequerboard assay and time-kill assay, while the phenotypic and genotypic characteristics of resistant determinants were performed by efflux pump detection and whole genome sequencing on 29 isolates from ICU patients with underlying health diseases.
Results: The isolates showed multidrug resistance, with over 60% showing addictive responses to rifampicin-based combinations at FICI ranging from 0.6 to 0.8. The time-kill assay revealed 99 % killing for rifampicin and minocycline combination in one isolate at 1/4 MIC rifampicin plus 1/4 MIC minocycline, while a bacteriostatic effect was observed at 1/2 MIC rifampici plus 1/2 MIC for a second isolate. Combination with tigecycline resulted in a 99% killing in two out of three isolates with a 2.5–3 log reduction in CFU at 1/4 MIC rifampicin plus 1/4 MIC tigecycline. Rifampicin plus colistin exhibited bactericidal activity against three out of four isolates. The combinations of rifampicin with ciprofloxacin, chloramphenicol, and trimethoprim-sulfamethoxazole were ineffective against the isolates. In addition, a 4-fold reduction in rifampicin MIC was observed in 2 out of 14 isolates in the presence of an efflux pump inhibitor. The pan-genome study demonstrated a progressive evolution with an accessory genome estimated to cover 58% of the matrix. Seven of the ten sequenced isolates belong to sequence type 2 (ST2), while one isolate each was assigned to ST164, ST16, and ST25. Furthermore, 11 plasmids, 34 antimicrobial resistance (AMR) genes, and 65 virulence-associated genes were predicted from the whole genome data. The $bla_{\text{OXA-23}}$ $bla_{\text{ADC-25}}$, $bla_{\text{OXA-66}}$, $bla_{\text{PER-7}}$, *aph(6)-Id*, *armA*, and *arr-3* were

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prevalent among the isolates. Sequence alignment of the bacteria genome to the reference strain revealed a deleterious mutation in the *rpoB* gene of 4 isolates.
Conclusion: The study suggests that rifampicin in combination with either minocycline, tigecycline, or colistin might be a treatment option for CRAB clinical isolates. In addition, genotypic analysis of the bacteria isolates may inform the clinician of the suitable drug regimen for the management of specific bacteria variants.

1. Introduction

Antibiotic resistance among clinically relevant isolates of *Acinetobacter baumannii* (*A. baumannii*) has become a global problem and a major challenge to public health. *Acinetobacter. baumannii* is a Gram-negative bacteria and naturally possesses a structural barrier that limits the influx of antibiotics promoting its adaptation to different microenvironments with the acquisition of virulence factors. *A. baumannii* is a major pathogen, associated with resistance to diverse antibiotics, with an estimated cost per infection of about $33,510 to $129,917 [1]. In 2019, carbapenem-resistant *A. baumannii* (CRAB) was linked with increased mortality and morbidity rate and an estimated $281 million in healthcare spending, 8500 hospitalized patients, and 700 deaths [2]. Recently, the USA Center for Disease Control and Prevention 2020 report listed carbapenem-resistant *Acinetobacter* species as the first Gram-negative bacteria among the 18 most alarming threats of antimicrobial resistance. Data collated from the National Antimicrobial Resistance Surveillance Center Thailand (NARST) in 2020 also reported 70.1% and 69% resistance to imipenem and meropenem, respectively [3].

A. baumannii is associated with infections such as bloodstream infection, respiratory tract infection, and urinary tract infection [4,5]. A study has suggested that *A. baumannii* infections are common among immunocompromised patients and critically ill patients on a mechanical ventilator, and other forms of medical intubations [6]. Also, patients suffering from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are often co-infected by other secondary infections such as *A. baumannii* [7].

Previous studies have highlighted different mechanisms of resistance of *A. baumannii* to antibiotics, including carbapenems, fluoroquinolone, aminoglycosides, and cephalosporin [8,9]. Whole-genome sequencing (WGS) of CRAB identified the *arr-3* gene which might confer resistance to rifampicin [10]. The mutation of the *rpoB* gene is another cause of rifampicin resistance. Furthermore, the synthesis of hydrolytic enzymes (carbapenemases) [11], the exclusion of antibiotics through efflux pumps or upregulation of efflux pump regulatory gene, the mutation of outer membrane protein [12], the presence of multidrug resistance plasmids, and the ability to form biofilms on hospital wares and devices [13], are common antimicrobial-resistant mechanisms associated with CRAB. The OXA-type carbapenemases genes including $bla_{\text{OXA-23-like}}$, and over-expression of $bla_{\text{OXA-51}}$ also mediate resistance to carbapenems [14].

The resistance of *A. baumannii* to carbapenems has led to the use of alternative treatment regimens including colistin, tigecycline, and minocycline, which are cytotoxic [15] as well as the repurposing of antibacterial agents that are not indicated for the treatment of GNB, such as fosfomycin. Combination therapy is one of the numerous strategies currently employed for the treatment of CRAB [16]. In previous studies, rifampicin combination therapy with colistin enhanced the antibacterial activity of the cationic peptide against *A. baumannii* [17]. The combination of rifampicin with polymyxin B exhibited bactericidal killing of extensively drug resistance (XDR) *A. baumannii* isolates [18]. Adjunctive rifampicin therapy with colistin and tigecycline also demonstrated synergism against *A. baumannii* isolates [19].

Although various antibiotic combinations have demonstrated synergistic activities, the effects of rifampicin-based combinations have only been investigated with colistin and a few other antibiotics. Thus, there is insufficient data on the activities of rifampicin with antibiotics classes such as aminoglycosides, carbapenems, glycylcyclines, fluoroquinolones, sulfonamide/dihydrofolate reductase inhibitors, and phosphonic antibiotic (fosfomycin), peptidyl-transferase inhibitor against *A. baumannii* isolates obtained from southern Thailand. This work aims to investigate the *in-vitro* outcome of combination therapies of rifampicin tigecycline, minocycline, fosfomycin, chloramphenicol, ciprofloxacin, amikacin, gentamycin, tobramycin, trimethoprim-sulfamethoxazole, polymycin B, and colistin for the management and treatment of carbapenem-resistant *A. baumannii*. It also investigates the mechanism mediating resistance to diverse antibiotics.

2. Material and methods

2.1. Bacterial isolates, antimicrobial agents, and media

The CRAB clinical isolates were obtained from our previous study [20]. All required antibiotics including, aminoglycosides (amikacin, gentamycin, tobramycin) Sulfonamide/Dihydrofolate reductase inhibitors (trimethoprim/sulfamethoxazole or cotrimoxazole), and Peptidyl-Transferase inhibitor (chloramphenicol) and carbonyl cyanide *m*-chlorophenyl hydrazone were purchased from Sigma-Aldrich, (Saint Louis, MO, USA). Fluoroquinolones (ciprofloxacin, and levofloxacin) were obtained from Siam Bheasach Co., Ltd. (Bangkok, Thailand). Glycylcyclines (tigecycline and minocycline) were delivered from Pfizer Inc. (Philadelphia, PA, USA). Rifampicin was supplied by HiMedia Laboratories Pvt. Ltd, Mumbai, India. Phosphonic antibiotic (fosfomycin) was procured from Meiji Seikakaisna, Ltd. (Tokyo, Japan). Culture media (Mueller Hinton agar and broth) were supplied by Becton Dickinson & Co. Difco (Franklin Lakes, NJ, USA) TIANamp bacteria DNA extraction kit was procured from Tiangen, Beijing China.

2.2. Collection of carbapenem-resistant A. baumannii clinical isolates

Two hundred and sixteen (216) clinical isolates of CRAB collected from hospitals in Southern Thailand were included in the study. Biochemical characterization of isolates revealed the following attributes: Gram-negative, oxidase-negative, nonmotile, non-fermenting coccobacill i [21], and by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) [22]. In this study, isolates were collected from the −80 °C storage facility and sub-cultured in tryptic soy agar (TSA) using the streak plate method. *A. baumannii* ATCC 19606 was used as a control.

2.3. Disc diffusion assay

The rifampicin sensitivity test was conducted using disc diffusion assay and rifampicin-resistant CRAB isolates were identified as previously described with slight modification [23]. Since rifampicin is not indicated for the treatment of *A. baumannii*, the Clinical Laboratory Standard Institute (CLSI), 2020 guideline for disc diffusion breakpoint for rifampicin against *Staphylococcus* spp. (susceptible: ≥20 mm, intermediate: 17–19 mm, and resistant: ≤16 mm) was employed. Briefly, a colony of each isolate was inoculated into a sterile 1.5-ml microcentrifuge tube containing Mueller Hinton broth and cultured to log phase for 3 h. The culture was converted to 0.5 MacFarland standard. For each isolate, the cotton swab was used to get the adjusted culture and streaked on the plate of Mueller Hinton agar. Rifampicin discs of 5 μg were properly stationed on each of the plates. All plates were then incubated at 37 °C for 18 h [20].

2.4. Broth microdilution assay

The representative isolates of rifampicin-resistant CRAB were treated with amikacin, gentamycin, tobramycin, minocycline, tigecycline, trimethoprim/sulfamethoxazole, ciprofloxacin, levofloxacin, fosfomycin, chloramphenicol, and rifampicin. The minimum inhibitory concentration (MIC) of each antibiotic was determined using broth microdilution assay as previously described [24]. In brief, serial two-fold dilutions of each antibiotic were performed in a 96-well microtiter plate containing Mueller–Hinton broth. Then 100 μL aliquots of bacteria culture 1×10^6 CFU/mL were placed in each well and incubated at 37 °C for 18 h. MIC was then expressed as the lowest concentration of the antibiotic without microbial growth and wells without antibiotics were used as control. Interpretation of the results was based on the recommendations of the CLSI, 2020. Tigecycline susceptibility was based on criteria of the United States Food and Drug Administration for Enterobacteriaceae (susceptible: ≤2 μg/mL, intermediate: 4 μg/mL, resistant: ≥8 μg/mL) [25]. Rifampicin breakpoints were ≥4 μg/mL for resistant, 2 μg/mL for intermediate, and ≤1 μg/mL for susceptible.

2.5. Chequerboard assay

A synergism study was conducted to investigate the combined effect of rifampicin with other antibiotics including amikacin, gentamycin, tobramycin, minocycline, tigecycline, ciprofloxacin, levofloxacin, fosfomycin and trimethoprim/sulfamethoxazole on 29 rifampicin-resistant and carbapenem-resistant isolates of *A. baumannii* using the chequerboard technique with slight modification [26]. Briefly, serial 2-fold dilution of 50 μL rifampicin was conducted in 50 μL of Mueller Hinton broth horizontally while 50 μL of different concentrations of the second antibiotics were distributed to each well vertically in 96 well microtiter plates at subinhibitory concentrations. Isolates were grown to log phase and 100 μl aliquot of 1×10^6 CFU/mL adjusted bacteria stock were placed in each well. The 96-well microplates were then incubated at 37 °C for 18 h. MICs of antibiotics in combination were defined as concentrations without growth based on the resazurin test. The antibacterial effects of single antibiotics were also tested as a control. The experiment was performed for three independent repeats. Antimicrobial efficacy of combination was defined as fractional inhibitory concentration index (FICI) as shown in the following equation: FIC A + FIC B which is given as.

$$\mathrm{FICI} = \frac{MIC\ of\ drug\ A\ in\ combination}{MIC\ of\ drug\ A\ alone} + \frac{MIC\ of\ drug\ B\ in\ combination}{MIC\ of\ drug\ B\ alone}$$

The FICI results for each combination were interpreted as follows: FICI ≤0.5, synergism; 0.5 < FICI <1, additive; 1 ≤ FICI <2, indifference; FICI ≥2, antagonism [27].

2.6. Time-kill assay

Time-kill assay was performed against 2 representative isolates of rifampicin-resistant CRAB as previously described with slight modification [28]. Overnight cultures of the isolates were adjusted to a concentration of 10^6 CFU/mL and then subjected to treatment with a combination of rifampicin plus ciprofloxacin, trimethoprim/sulfamethoxazole, minocycline, or tigecycline. The treatments were administered at concentrations of MIC + ½ MIC, ½ MIC + ½ MIC, ½ MIC + ¼ MIC, and ¼ MIC + ¼ MIC, respectively. The culture was monitored for 24 h at intervals (2, 4, 8, 12, and 24). Bacteria colonies were enumerated and the effect of antibiotics on the bacteria population was assessed. Reductions in viable cell count over 24 h were reported as log reductions in CFU/mL. Synergism was defined as a 2-log reduction in CFU/mL when compared with the most active single antibiotic treatment, whereas bactericidal and bacteriostatic activity was defined as a ≥ 3-log and 2-log reduction in CFU/mL when compared with the number of viable cells at time zero (0 h), respectively. All experiments were performed in duplicate.

2.7. Efflux pump detection

The phenotypic detection of the rifampicin-resistant efflux pump was performed in the presence of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) as previously described with modifications [29]. CRAB isolates that were resistant to rifampicin at MIC ≥16 μg/mL were cultured to log phase for 4 h. Then a serial 2-fold dilution was conducted with a 50 μL of rifampicin and 50 μL of CCCP (20 μg/ml) was placed in each well. Then, a 100 μL of adjusted bacteria culture at 10^6 CFU/mL was included in the wells. The plates were incubated for 18 h and the MIC of rifampicin against rifampicin-resistant CRAB isolates in the presence of CCCP were taken. The positive phenotype of overexpression of the efflux pump was defined as at least a 4-fold reduction of rifampicin MIC observed in the presence of CCCP.

2.8. Genome DNA extraction and whole genome sequencing

The short-read WGS of 20 rifampicin-resistant CRAB isolates were obtained from the previous study[10], and 10 isolates without genomic data were sequenced in this study. In brief, *A. baumannii* clinical isolates were grown on a TSA overnight then a single colony of each bacteria isolate was inoculated in a sterile tube containing LB broth and incubated at 37 °C for 24 h. Genomic DNA extraction of the isolates including SK024, SK052, SK065, ST002, SK068, TR125, TR131, TR009, and ST004 was performed using the TIANamp Bacteria DNA kit (Tiangen, Beijing, China), based on the manufacturer's instruction. All extracted DNA samples were preserved and further analyzed at the Beijing Genomics Institute (BGI) in China. The integrity check of all extracts was conducted and scored based on purity and concentration using the Agarose Gel electrophoresis and Qubit Fluorometer (Invitrogen), respectively. The qualified DNA samples were sequenced with the MGISEQ-2000 platform with 150-bp pair-end reads.

2.9. Genome assembly and annotation

A *de novo* assembly was further conducted on 10 isolates including the SK024, TR009, ST004, SK 065, SK052, ST002, TR125, TR131, ST011, and SK068 using SPAdes v3.12 [30]. Then the Quast v5.0.2 and Busco v5.1.2 were utilized to assess the quality and completeness of the bacteria genomes, respectively [31,32]. Genomic annotations of the assembled genomes of all isolates were performed using Prokka v1.12 [33].

2.10. Sequence analysis

The WGS of all rifampicin-resistant CRAB isolates was used for the identification of mechanisms of antibiotic resistance in the isolates. The multilocus sequence typing (MLST), acquired AMR genes, and plasmid types were identified using staramr v0.7.2 with databases of ResFinder [34] and PlasmidFinder [35] in the Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/) and the MLST in PubMLST (https://github.com/tseemann/mlst) [36]. Virulence-associated genes were also searched using blastn with the virulence factor database (VFDB) (http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Acinetobacter) [37]. Chromosomal mutations in the *rpoB* gene of the bacteria genomes were investigated by aligning to the reference sequence of the *A. baumannii* ATCC 19606 genome (NZ_CP015121.1) using Geneious prime® software. The effects of each variant were further analyzed using the Mutpred 2 software® (http://mutpred.mutdb.org). Furthermore, a study was conducted to assess the diversity of resistance and the spread of AMR genes. A comparative analysis of the genomic data of rifampicin-resistant CRAB clinical isolates from this study and previous WGS results [38] was conducted using Roary software® v3.13.0 [39]. The phylogenic tree with pan-genome matrix was visualized by the phandango software® (https://jameshadfield.github.io/phandango/#/) [40]. Data were summarized and presented as gene present and absent in a table and hitmaps.

3. Results

3.1. Demographic and medical details

A. baumannii clinical isolates were collected from 5 different hospitals in southern Thailand including Pattani, Phatthalung, Songkhla, Satun, and Trang hospitals. Patients were on admission in both the medical and surgical intensive care unit and were suffering from one or more of these disease conditions, diabetes mellitus, essential blood hypertension, chronic kidney disease, cerebrovascular disease, coronary heart disease, and pulmonary disease. All isolates were sourced from either the patient's sputum, urine, blood, abdomen, nasogastric tube, nasopharyngeal swab, skin, and pus. Only patients with prolonged hospital stay between 6 and 32 days and an APACHE II score of 8–27 were included in the study.

3.2. Antimicrobial susceptibility profiles of Acinetobacter baumannii clinical isolates

The antibiogram and disc diffusion assay results of rifampicin against 29 representative CRAB clinical isolates were assessed in our previous study [20]. In this study, most of the rifampicin-resistant isolates were also resistant to diverse classes of antibiotics. Among 29 isolates, 23 (77%) were susceptible to tigecycline, 5 (17%) were intermediate and 1 (3%) was resistant to tigecycline. With minocycline, 26 (90%) isolates were susceptible, 2 (7%) were intermediate, and 1 (3%) was resistant. The isolates were also multidrug-resistant to aminoglycoside, fosfomycin, chloramphenicol, ciprofloxacin, and trimethoprim/sulfamethoxazole. Most of the

isolates demonstrated increased MICs of ≥1024 μg/mL for gentamycin, tobramycin, and amikacin. The tested isolates were all resistant to tobramycin, gentamycin, and chloramphenicol. The isolates SK035, PA025, and ST002 displayed MIC of ≤2 μg/mL, while ST016 showed MIC of 512 μg/mL to amikacin, respectively. Precisely, 41% (12/29) of the isolates were resistant to levofloxacin, while 93% (27/29) were resistant to ciprofloxacin. Furthermore, the isolates demonstrated 24% (7/29) susceptibility and 76% (22/29) resistance to trimethoprim/sulfamethoxazole (Table 1).

3.3. Synergistic activity

All 29 rifampicin-resistant CRAB clinical isolates were further subjected to treatment with rifampicin combination therapy with other antibiotics. Rifampicin enhanced the antimicrobial efficacy of the used antibiotics resulting in 1-3-fold reduction in the MIC of mono-therapeutic antibiotics treatments. Only 10% (3/29) of the isolates demonstrated synergism with combination therapies. For rifampicin plus tigecycline, 24 (80%) were additive and 2 (7%) were indifferent. A similar result was obtained for rifampicin plus minocycline combination with 4 (14%), 23 (73%), and 4 (10%) showing synergism, additive, and indifferent, respectively. Overall, about 1–13% synergism, 66–89% addictive, and 3–27% indifferent responses were demonstrated by all isolates to adjunctive therapy of rifampicin (Table 2, Fig. 1).

Table 2 The fractional inhibitory concentration index of several antibiotics in combination with rifampicin against CRAB clinical isolates.

3.4. Time-kill kinetics

The time dependent killing of isolates TR045, SK009, TR131, TR023, PA037, TR123, and SK059 were investigated to confirm the most effective antibiotic combinations suitable for the management of rifampicin-resistant CRAB. The bactericidal and bacteriostatic activities of resistant antibiotics were demonstrated among isolates in combination with rifampicin. The isolates TR082, TR069, PT046, SK059 the exhibited increased resistance to almost all the antibiotics were treated with the most effective antibiotics colistin. Rifampicin plus minocycline combinations exhibited a bactericidal activity against TR045 at MIC rifampicin plus 1/2 MIC of

Table 1
The minimal inhibitory concentration of commonly used antibiotics against CRAB clinical isolates.

Isolates ID	MIC (μg/mL)													
	ZOI[a]	RIF[a]	TIG	MIN	CIP	LEV	FOS	AMK	GEN	TOB	TMZ/SMZ	CPH	COL	POL
ST002	18	2	2	0.13	16	2	128	≤2	ND	ND	0.24/1.19	64	ND	ND
ST004	NZ	64	0.13	256	2	512	128	2048	2048	2048	60.4/304	32	1	1
ST010	6	2	8	8	64	8	512	>2048	ND	ND	60.4/304	256	ND	ND
ST011	7	1	2	2	32	2	256	1024	ND	ND	0.95/4.75	128	ND	ND
PA025	18	1	1	0.13	8	2	128	≤2	ND	ND	0.116/0.59	32	ND	ND
PA037	17	2	4	2	32	2	256	ND	ND	ND	30.4/152	64	ND	ND
TR009	NZ	64	2	0.13	64	32	128	>2048	>2048	>2048	30.4/152	32	2	1
TR023	12	2	4	0.25	32	2	512	ND	ND	ND	30.4/152	64	ND	ND
TR045	8	2	2	4	32	4	256	ND	ND	ND	30.4/152	128	ND	ND
TR069	NZ	32	0.5	0.25	16	2	512	ND	>2048	>2048	122/608	128	4	2
TR082	NZ	32	0.5	0.25	32	4	256	ND	2048	>2048	122/608	128	4	2
TR123	7	8	2	1	16	2	256	ND	ND	ND	30.4/152	32	4	2
TR125	NZ	64	0.25	0.25	16	4	128	ND	>2048	>2048	122/608	64	2	2
TR131	10	4	4	8	128	8	512	ND	ND	ND	7.6/38	128	4	2
SK009	11	256	4	4	128	16	64	>2048	ND	ND	60.4/304	128	2	1
SK015	NZ	64	1	1	64	16	64	2048	>2048	>2048	30.4/152	64	2	1
SK024	NZ	16	0.5	1	16	2	128	>2048	>2048	>2048	15.2/76	64	2	2
SK025	NZ	16	2	2	32	2	256	2048	>2048	>2048	30.4/152	64	2	2
SK035	7	1	4	0.25	8	2	256	≤2	ND	ND	0.24/1.19	64	ND	ND
SK040	NZ	32	0.5	0.25	512	16	128	2048	>2048	>2048	30.4/152	16	2	2
SK052	NZ	64	0.5	1	512	32	64	2048	>2048	>2048	15.2/76	32	2	1
SK056	NZ	64	1	2	512	64	128	2048	>2048	>2048	15.2/76	64	2	2
SK059	NZ	64	1	0.13	128	8	128	>2048	>2048	>2048	60.4/304	32	4	2
SK065	NZ	32	2	1	128	32	128	>2048	2048	>2048	30.4/152	64	2	2
SK067	15	64	2	2	32	8	256	ND	ND	ND	30.4/152	64	2	2
SK068	15	1	4	2	16	2	128	>2048	ND	ND	0.95/4.75	32	ND	ND
PT004	NZ	32	0.125	0.13	16	2	128	>2048	>2048	>2048	60.4/304	32	2	1
PT046	NZ	32	0.125	0.13	32	4	128	>2048	>2048	>2048	7.6/38	16	4	2
PA033	NZ	32	0.06	0.03	2	0.5	128	>2048	>2048	>2048	60.4/304	32	4	1
RBT		≥4	≥8	≥16	≥4	≥8	≥256	≥64	≥16	≥16	≥4/76	≥8	≥4	≥4
%R		63	0	3	93	40	40	90	100	100	77	100		

[a] The results were obtained from a previous study [20]. Abbreviations: RBT, Resistant Breakpoint; %R, Percentage Resistant; ZOI, zone of inhibition; RIF, rifampicin[20]; TIG, tigecycline; MIN, minocycline; CIP, ciprofloxacin; LEV, levofloxacin; FOS, fosfomycin; AMK, amikacin; GEN, gentamycin; TOB, tobramycin; TMZ/SMZ, trimethoprim/sulfamethoxazole; CPH, chloramphenicol; COL, colistin; POL, polymyxin; ND, not determined; NZ, no zone inhibition.

Table 2
The fractional inhibitory concentration index of rifampicin combination with tigecycline, minocycline, Trimethoprim-sulfamethoxazole, levofloxacin, Fosfomycin, chloramphenicol, or colistin against CRAB clinical isolates.

Isolate ID	FICI							
	RIF + TIG	RIF + MIN	RIF + TMP/SMZ	RIF + LEV	RIF + CIP	RIF + FOS	RIF + CPH	RIF + COL
ST002	1 (Ind)	1 (Ind)	0.75 (Ad)	0.63 (Ad)	0.75 (Ad)	0.75 (Ad)	0.5 (Syn)	ND
ST004	0.63 (Ad)	0.75 (Ad)	0.75 (Ad)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.75 (Ad)	0.3 (Syn)
ST010	1 (Ind)	0.75 (Ad)	0.63 (Ad)	1 (Ind)	0.75 (Ad)	1 (Ind)	0.5 (Syn)	ND
ST011	0.63(Ad)	0.75 (Ad)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	ND
PA025	0.63 (Ad)	1 (Ind)	0.75 (Ad)	1(Ind)	0.63 (Ad)	1 (Ind)	0.63(Ad)	ND
PA037	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	1 (Ind)	0.63 (Ad)	0.63 (Ad)	0.75 (Ad)	ND
TR009	0.63 (Ad)	0.63 (Ad)	0.75 (Ad)	0.63 (Ad)	1 (Ind)	1 (Ind)	0.63 (Ad)	ND
TR023	0.75 (Ad)	0.63 (Ad)	0.64 (Ad)	0.75 (Ad)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	ND
TR045	0.75 (Ad)	0.75 (Ad)	0.75 (Ad)	0.75 (Ad)	0.63 (Ad)	0.75 (Ad)	0.75 (Ad)	ND
TR069	0.63 (Ad)	0.75 (Ad)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.75 (Ad)	0.75 (Ad)	0.3 (Syn)
TR082	0.63 (Ad)	0.75 (Ad)	1 (Ind)	1 (Ind)	0.75 (Ad)	0.63 (Ad)	0.75 (Ad)	0.3 (Syn)
TR123	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	1 (Ind)	0.75 (Ad)	0.3 (Syn)
TR125	0.63 (Ad)	0.63 (Ad)	0.75 (Ad)	0.63 (Ad)	0.75 (Ad)	0.63 (Ad)	0.63 (Ad)	ND
TR131	0.75 (Ad)	0.38 (Syn)	0.63 (Ad)	1 (Ind)	0.5 (Syn)	1 (Ind)	0.63 (Ad)	0.5 (Syn)
SK009	0.75 (Ad)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.75 (Ad)	0.75 (Ad)	0.6
SK015	0.5 (Syn)	0.5 (Syn)	0.75 (Ad)	0.75 (Ad)	0.63 (Ad)	0.75 (Ad)	0.63 (Ad)	ND
SK024	0.63 (Ad)	1 (Ind)	0.63 (Ad)	0.75 (Ad)	0.63 (Ad)	0.75 (Ad)	1 (Ind)	ND
SK025	0.38 (Syn)	0.5 (Syn)	0.63 (Ad)	1 (Ind)	0.75 (Ad)	0.75 (Ad)	0.5 (Syn)	ND
SK035	0.75 (Ad)	0.75 (Ad)	0.75 (Ad)	1 (Ind)	0.75 (Ad)	0.5 (Syn)	0.63 (Ad)	ND
SK040	0.63 (Ad)	0.5 (Syn)	0.63 (Ad)	0.5 (Syn)	0.63 (Ad)	0.75 (Ad)	0.63 (Ad)	ND
SK052	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.5 (Syn)	0.63 (Ad)	0.5 (Syn)	0.63 (Ad)	ND
SK056	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.75 (Ad)	0.63 (Ad)	0.75 (Ad)	0.63 (Ad)	ND
SK059	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.5 (Syn)	0.63 (Ad)	0.3 (Syn)
Sk065	0.75 (Ad)	0.63 (Ad)	0.75 (Ad)	0.75 (Ad)	1 (Ind)	0.63 (Ad)	0.75 (Ad)	0.5 (Syn)
Sk067	0.63 (Ad)	0.63 (Ad)	0.75 (Ad)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	ND
SK068	0.5 (Syn)	0.63 (Ad)	0.63 (Ad)	0.75 (Ad)	0.75 (Ad)	0.63 (Ad)	0.63 (Ad)	ND
PT004	0.75 (Ad)	0.63 (Ad)	0.75 (Ad)	1 (Ind)	0.63 (Ad)	0.75 (Ad)	0.75 (Ad)	0.3 (Syn)
PA033	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.63 (Ad)	0.75 (Ad)	0.63 (Ad)	1 (Ind)	ND
PT046	0.75 (Ad)	0.75 (Ad)	0.63 (Ad)	0.75 (Ad)	0.75 (Ad)	0.75 (Ad)	1 (Ind)	0.3 (Syn)

RIF, rifampicin; TIG, tigecycline; MIN, minocycline; TMP/SMZ, trimethoprim – sulfamethoxazole; LEV, levofloxacin; CIP, ciprofloxacin, FOS, Fosfomycin; CPH, Chloramphenicol; COL, colistin; FICI, Fractional inhibitory concentration index; Syn, synergy; Ad, additive; Ind, Indifferent.

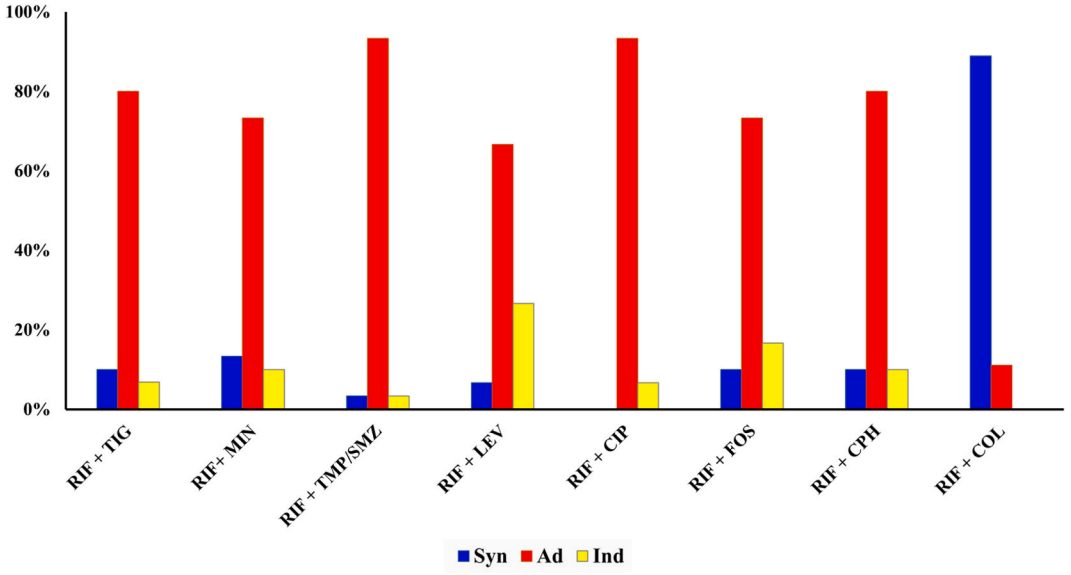

Fig. 1. Antibiotic response of 29 CRAB clinical isolates to adjunctive therapies with rifampicin. Syn, Synergy; Ad, additive; Ind, indifferent.

minocycline and at 1/2 MIC of rifampicin plus 1/2 MIC of minocycline. Combinations of the antibiotics at sub-inhibitory concentrations inhibited bacteria growth with a less than 2 log reduction in CFU (Fig. 2A). In addition, the treatment resulted in a bactericidal killing at all the combined concentrations of rifampicin and minocycline against TR131 (Fig. 2C). However, the combinations were ineffective against isolates SK009 (Fig. 2B). The combination of rifampicin with tigecycline, resulted in a 2–2.5 log reduction in CFU at

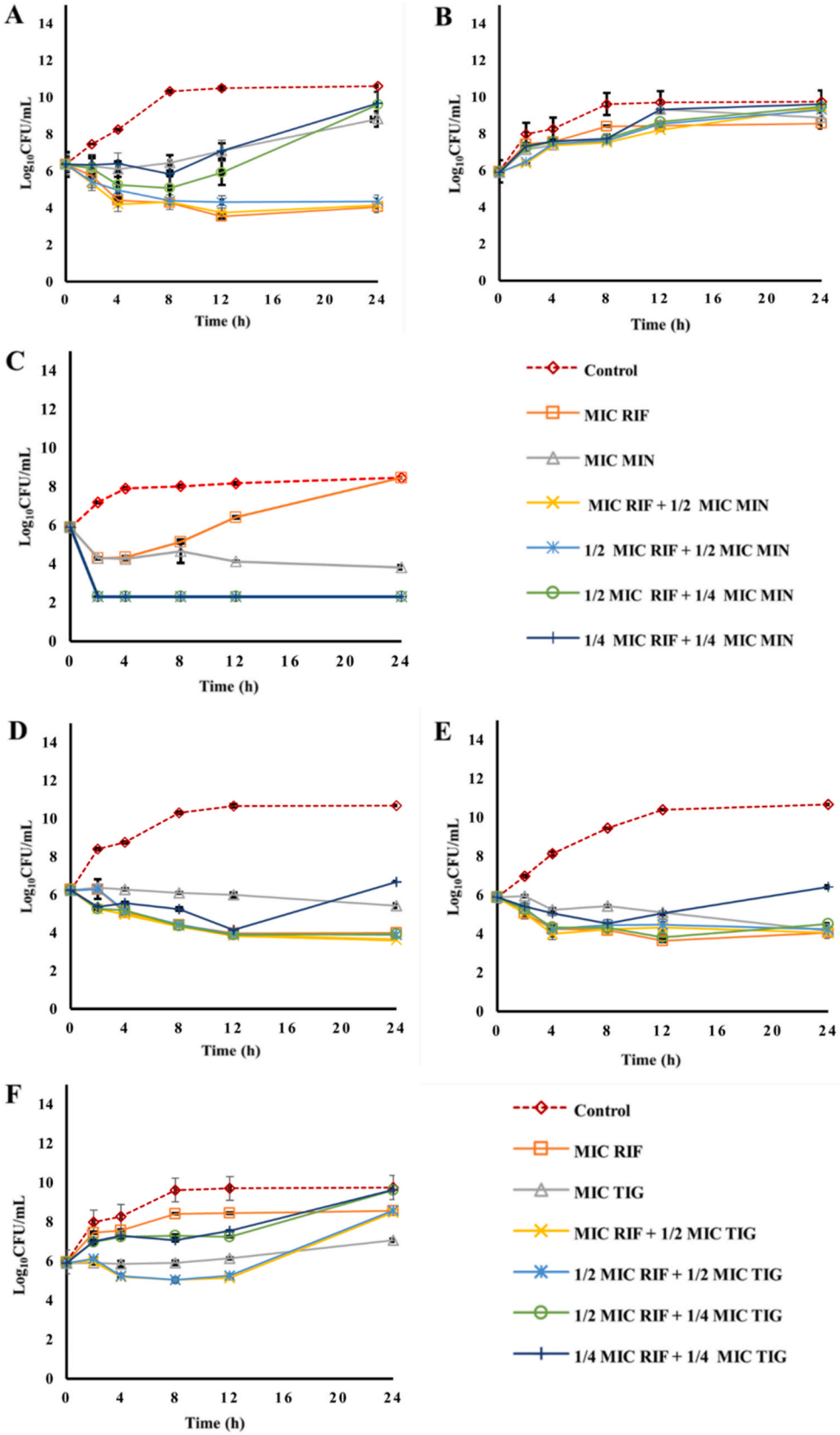

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Fig. 2. Time killing curve of rifampicin plus tigecycline, or minocycline against CRAB clinical isolates. (A) rifampicin combination with minocycline against TR045 (B) rifampicin combination with minocycline against SK009 (C) rifampicin combination with minocycline against TR131 (D) rifampicin combination with tigecycline against TR023 (E) rifampicin combination with tigecycline against PA037 (F) rifampicin combination with tigecycline against SK009. RIF, rifampicin; TIG, tigecycline; MIN, minocycline; MIC, minimum inhibitory concentration.

all the combined concentrations (Fig. 2D and E). For Fig. 2F, Combinations did not affect the growth of the isolate.

Combination of rifampicin and ciprofloxacin were bactericidal at MIC rifampicin plus 1/2 MIC of ciprofloxacin, 1/2 MIC of rifampicin plus 1/2 MIC of ciprofloxacin and at 1/2 MIC of rifampicin plus 1/4 MIC of ciprofloxacin on TR023 but bacteriostatic at 1/4 MIC of rifampicin plus 1/4 MIC of ciprofloxacin (Fig. 3A). Combination therapy also showed bactericidal activity at MIC rifampicin plus 1/2 MIC of ciprofloxacin (Fig. 3B and C). Similarly, at MIC rifampicin plus 1/2 MIC of chloramphenicol, a bactericidal effect was demonstrated against TR023 (Fig. 3D). Furthermore, a bactericidal activity was demonstrated at MIC rifampicin plus 1/2 MIC of chloramphenicol with a regrowth after 12 h whereas other combinations were inactive against the isolate (Fig. 3E).

The combination of rifampicin with trimethoprim-suphamethoxazole was less effective against the isolates resulting in a $\leq$2 log reduction in CFU in a dose dependent manner (Fig. 4A–C). The effect of the combination of rifampicin with fosfomycin was bactericidal at MIC rifampicin plus 1/2 MIC of fosfomycin but bacteriostatic at 1/2 rifampicin plus 1/2 MIC of fosfomuycin, 1/2 MIC rifampicin plus 1/4 MIC of fosfomycin and 1/4 MIC rifampicin plus 1/4 MIC of fosfomycin (Fig. 4 D & E).

The result of colistin and rifampicin combination showed synergistic activity at a sub-inhibitory concentration. Three isolates TR082, TR069, and PT046 were killed at 1/4 MIC of rifampicin and 1/4 MIC of colistin displaying a synergistic and bactericidal effect with $\geq$3 log reduction in CFU/mL (Fig. 5A–C). Furthermore, the fractional inhibitory concentration index (FICI) of the three isolates confirms the result of the time-kill assay. However, for isolate SK059 the results did not correlate to treatment at 1/4 MIC of rifampicin and 1/4 MIC of colistin (Fig. 5D) showing an inconsistent result compared with the FICI obtained from the checkerboard assay. Similarly, a previous study has reported a discrepancy between synergism and bactericidal activity of certain antibiotics in combination therapy (22).

3.5. Phenotypic detection of rifampicin-resistant efflux pump

Rifampicin resistance due to the presence of efflux pump phenotype was assessed in the presence of the efflux pump inhibitor CCCP. Fifteen isolates that demonstrated a high level of rifampicin resistance at MIC $\geq$16 μg/mL were included in the study (Table 3). SK059 and SK067 were positive to efflux pump phenotype expressing a 4-fold reduction in rifampicin MIC in presence of CCCP.

3.6. Phylogenetic relatedness and sequence types of A. baumannii clinical isolates

The phylogenetic tree aligned with the pan-genome matrix reveals the evolutionary diversity of isolates and their distribution (Fig. 6). Comparatively, of the 29 sequences assessed in this study, 19 (66%), 6 (21%), 3(10%), and 1 (3%) were ST02, ST025, ST164, and ST016, respectively. Approximately 58% of the pan-genomic matrix was dominated by the accessory genome indicating a progressive evolution of the organisms.

3.7. Antimicrobial resistance genes in CRAB clinical isolates

Antibiotic resistance of the isolates may be associated with the presence of different classes of antibiotic-resistant genes. Here, a total of 34 AMR genes were identified among the 10 isolates, that were sequenced in this study. The $bla_{\text{OXA-23}}$ which confer resistance to carbapenems was expressed in 9/10 (90%) of the isolates. Other β-lactam resistant related genes including the $bla_{\text{ADC-25}}$, $bla_{\text{OXA-66}}$, and $bla_{\text{PER-7}}$ were also detected with a percentage distribution of 50–70%. About 50% of the isolates harbored the *arr-3* gene while only 1 isolate TR131 was found with the *arr-2* gene resulting in 60% rifampicin-resistant mediating genes. Other widely distributed AMR genes identified include the putative nucleotidyltransferasenzyme encoding genes for aminoglycoside acyltransferases (AAC family), aminoglycoside adenyltransferases (ANT family), aminoglycoside phosphotransferases, (APH family) and the nucleotidyltransferase. Enzymes encoding genes prevalent among the isolates include *aph (6)-Id* (80%) *armA* (80%), *aph (3′)-Ia* (50%), *aadA1* (50%) and, *aac (6′)-Ib* (40%), as shown in Fig. 4. Also, *tet(B)* was carried by 60% of the isolates while *tet39* was only present in one out of 10 (10%) of the isolates. The *cmlA1* and *catB8* genes were predicted to be responsible for chloramphenicol resistance and were present in 60% (6/10) and 40% (4/10) of the sequenced genomes, respectively. The *aac (6′)-Ib-cr*, a bifunctional gene was predicted among (4/10) 40% of the isolates and may be responsible for ciprofloxacin and aminoglycoside-resistant. Although the isolates harbored similar AMR genes, the TR131 was identified with 8 different and unique AMR genes including the *aac(3)-IId, ant(2″)-Ia, aph(3′)-VI,* $bla_{\text{OXA-70}}$, $bla_{\text{OXA-58}}$, $bla_{\text{VEB-1}}$, $bla_{\text{NDM-1}}$, and *arr-2*. Additionally, the $bla_{\text{OXA-91}}$ was predicted in ST002 (Fig. 7).

3.8. Plasmid types

The presence of 11 plasmids which might have facilitated the spread of antimicrobial-resistant genes in *A. baumannii* clinical isolates were identified. The highly prevalent plasmids include repAci3, repAci7, repAci8, pS30-1, and repM-ci9. A distribution rate of 50–100% of these plasmids were detected among the 9 isolates. Notably, only SK024, ST002 and TR131 were found harboring p1ABSDF, RepApAB49, and p3ABAYE0002, respectively. Almost all the isolates harbored at least 5 different plasmids while TR125

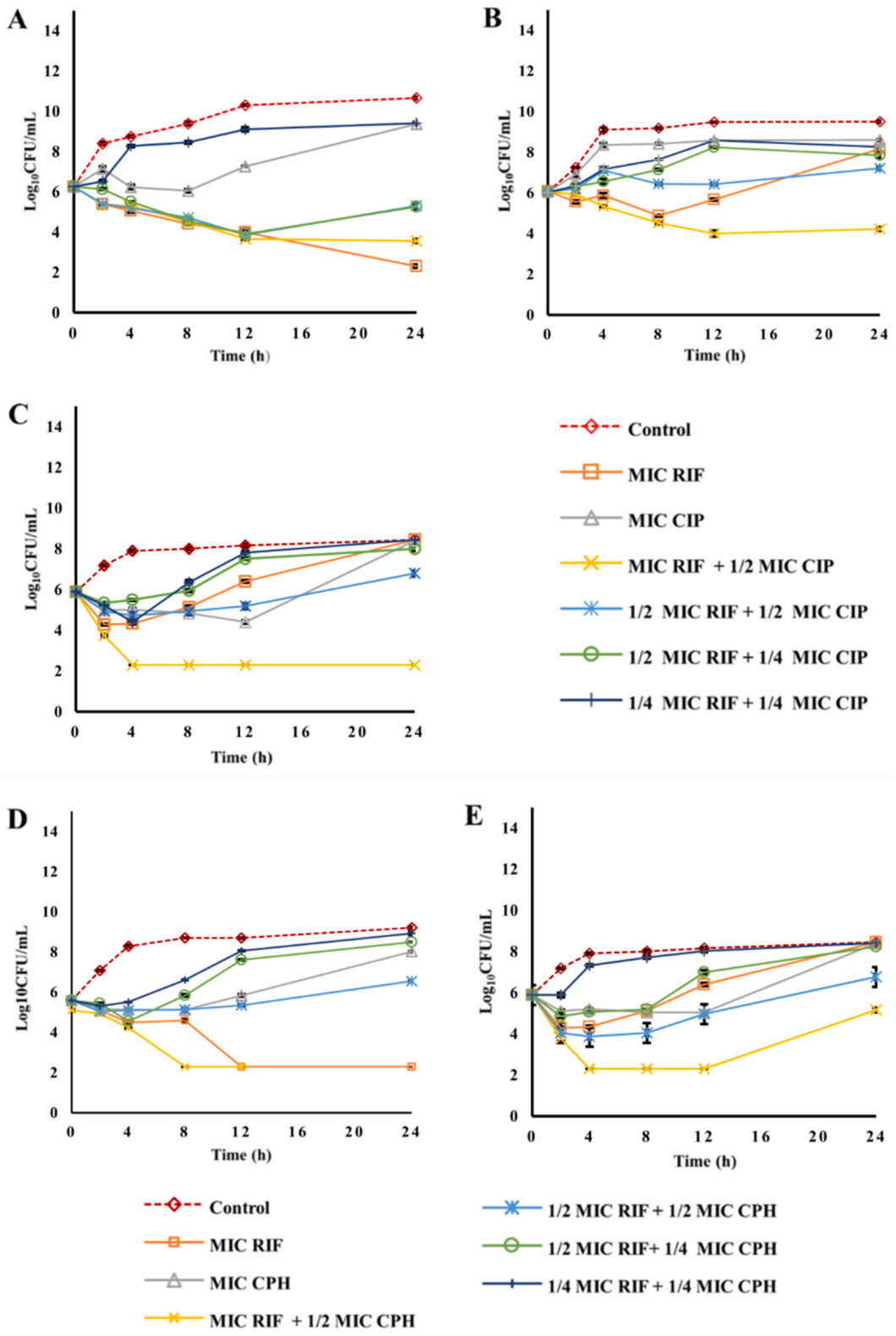

Fig. 3. Time killing curve of rifampicin plus ciprofloxacin or chloramphenicol against CRAB clinical isolates. (A) rifampicin combination with ciprofloxacin against TR023, (B) rifampicin combination with ciprofloxacin against TR123, (C) rifampicin combination with ciprofloxacin against TR131 (D) rifampicin combination with Chloramphenicol against TR023 (E) rifampicin combination with Chloramphenicol against TR131. RIF, rifampicin chloramphenicol, CPH; CIP, ciprofloxacin; MIC, Minimum inhibitory concentration.

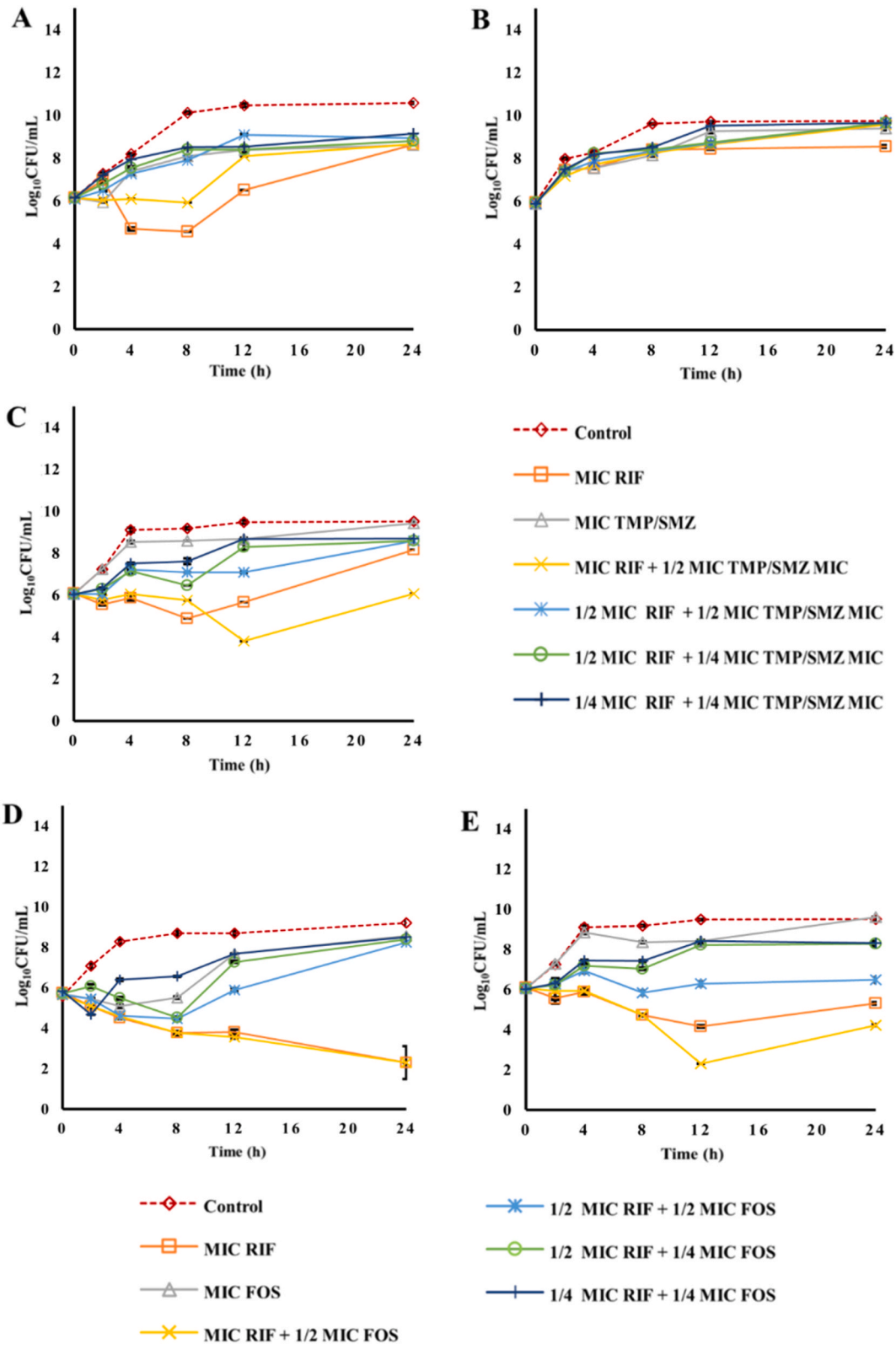

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Fig. 4. Time killing curve of rifampicin plus trimethoprim-sulfamethoxazole, or fosfomycin against CRAB clinical isolates. (A) rifampicin combination with trimethoprim-sulfamethoxazole against SK059, (B) rifampicin combination with trimethoprim-sulfamethoxazole against SK009, (C) rifampicin combination with trimethoprim-sulfamethoxazole against TR123 (D) rifampicin combination with fosfomycin against TR023 (E) rifampicin combination with fosfomycin against TR123 RIF, rifampicin; TMP/SMZ, trimethoprim-sulfamethoxazole and fosfomycin, FOS; MIC, minimum inhibitory concentretion

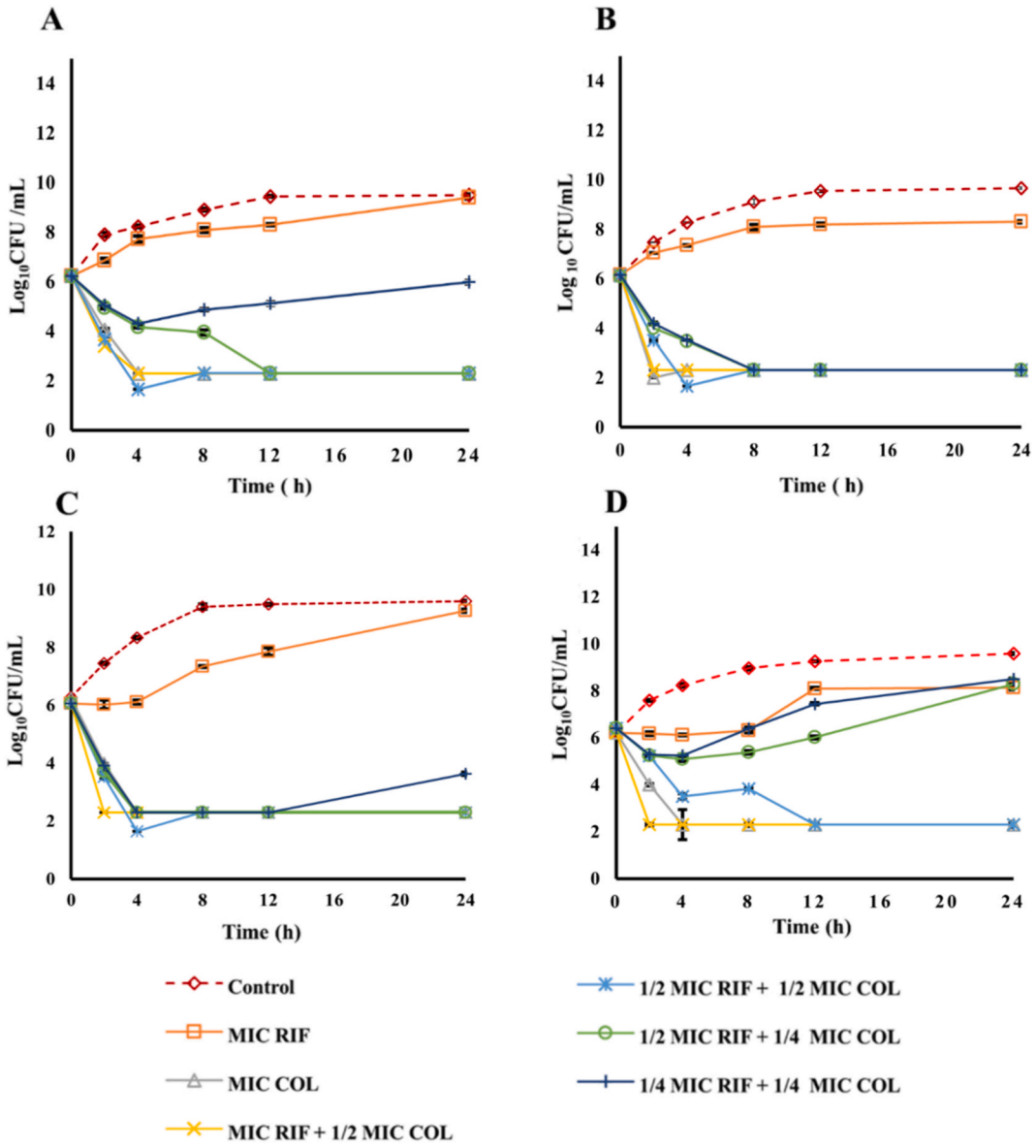

Fig. 5. Time killing kinetic of rifampicin combination with colistin against (A) TR082 (B) TR069 (C) PT046, and (D) SK059. RIF, rifampicin; COL, colistin.

harbored 3 plasmids. Comparatively, based on data retrieved from the previous genomic analysis and those of the current study repAci7, repAci3, and pS30-1 were highly prevalent among the 29 isolates with a percentage distribution of approximately 92% (Table 4).

3.9. Virulent factor genes

The ability to acquire virulent genes was then investigated and 65 putative virulent mediating genes were expressed in the isolates. About 71% of the genes were present in all isolates and 29% were not expressed by all isolates. A repertoire of genes coding for capsular

Table 3
The effect of carbonyl cyanide chlorophenyl hydrazone on rifampicin MIC of *Acinetobacter baumannii* clinical isolates.

Isolates ID	ST	MIC (μg/mL)		
		RIF	RIF + CCCP	Fold reduction
SK009	2	256	128	2
SK015	2	64	32	2
SK024	2	16	16	–
SK056	2	64	16	4
SK059	2	64	32	2
SK065	2	32	32	–
SK067	2	64	16	4
TR009	2	16	16	
TR069	25	32	32	–
TR082	25	32	32	–
TR125	25	64	64	–
PA033	25	32	32	–
ST004	2	64	64	–
PT004	25	32	16	2
PT046	25	32	32	2

ST, sequence type; RIF, rifampicin; CCCP, carbonyl cyanide chlorophenyl hydrazone (efflux pump inhibitor); MIC, minimum inhibitory concentration.

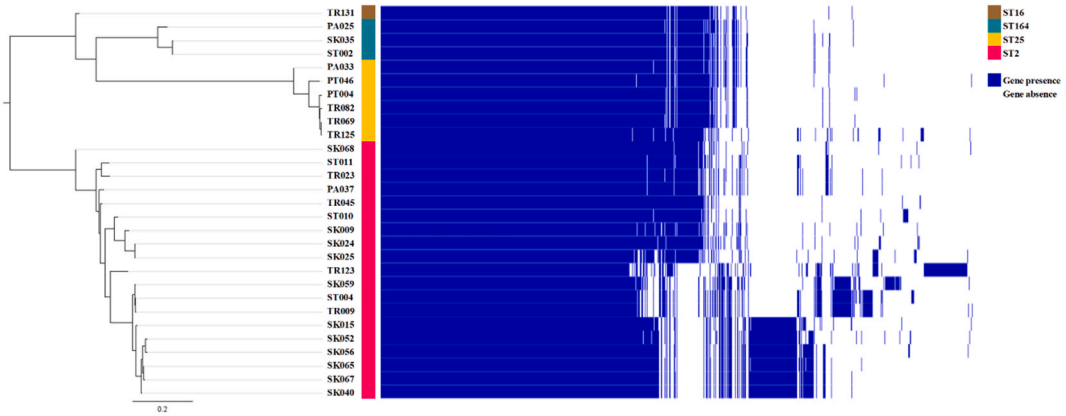

Fig. 6. A comparative phylogenetic tree against the pan-genome matrix of 29 *Acinetobacter baumannii* clinical isolates. Nineteen isolates were assessed by Chukamnerd et al., 2022 and 10 others were sequenced in this study. ST16; sequence type 16; ST164, sequence type 164; ST25, sequence type 25; ST2, sequence type 2.

polysaccharides *ACICU_00071–80, ACICU_00086–89, ACICU_00091–92*, and *pgi* were observed. The genes, *ACICU_00091 – 92* and *pgi* occurred in all the isolates, 80% of the isolates expressed *ACICU_00074 and ACICU_00087*. In addition, *ACICU_00071 and ACICU_00086* were detected in exactly 70% (7/10) of the isolates whereas 60% of the isolates harbored *ACICU_00071–73, ACICU_00075–77, ACICU_00080*, and *ACICU_00088–89*. The genes *ACICU_00078* and *ACICU_00079* were present in only SK024 (Fig. 7). In addition, a total of 16 biofilm-associated genes - were also revealed. The *AdeFGH* RND (resistant nodulation division) which are mostly multidrug efflux pump genes were present in all the isolates. Research has shown the roles of adeFGH efflux pump in chloramphenicol resistant, adeIJK in β-lactams, chloramphenicol, tetracycline, fluoroquinolones, rifampicin, trimethoprim resistant [41] However the role of adeFGH has not been described in *A. baumannii* clinical isolates in connection with rifampicin resistant.The *abaI* and *abaR* were identified in 100% and 60% of the isolates, respectively. Another biofilm-associated gene identified is the *bap* (biofilm-associated protein) which was present in 8 isolates except for ST002 and TR125. Other biofilm-associated genes including chaperone-usher pathway assembled fimbriae encoded genes (*csuA – csuE*) and a surface polysaccharide β-(1–6)-poly-N-acetyl-D-glucosamine (PNAG) *pgaA – pgaD* were equally predicted in all the isolates. A well-established iron uptake system composed of 21 siderophore acinetobactin encoding genes including *barA, barB, basA – basD, basF – basJ, bauA–bauF, bfmRS, entE,* and *hemO* were present in almost all the isolates. However, TR125 and TR131 do not express the *bauA* and *hemO* siderophore encoding genes, respectively. Other virulent genes responsible for immune evasion and lipopolysaccharide synthesis, (*lpsB, lpxA – lpxD, lpxL, and lpxM*), synthesis of degradative enzymes (*plc, and plcD*), cell adhesion and immune evasion (*ompA*) and penicillin-binding protein (*pbpG*) responsible for serum resistance were present in 100% of the isolates (Fig. 8).

3.10. Screening for rifampicin resistance due to chromosomal mutation in the rpoB gene

The *rpoB* gene sequence (NZ_CP015121.1) of *A. baumannii* obtained from the NCBI database was aligned to the genome of the

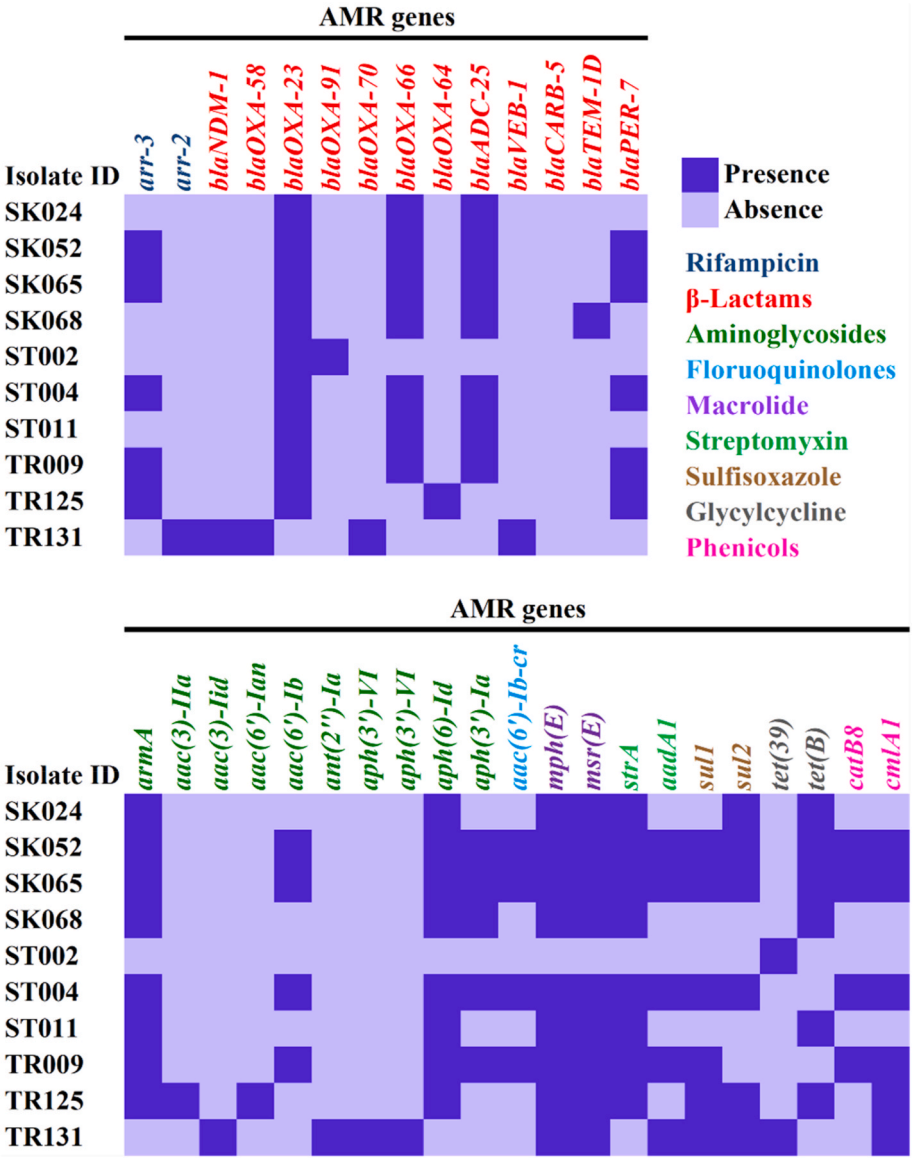

Fig. 7. Distribution of antimicrobial resistance (AMR) genes in some ICU patients infected with *A. baumannii* clinical isolates.

isolates. Single nucleotide polymorphism was detected in 5 out of 29 isolates. Amino acid substitutions were predicted at location H535Q, S521F, and R788H and may be deleterious against SK024, SK025, SK009, TR131, and TR123 due to the alteration in protein functions. Several changes were predicted in TR131, and this may probably be responsible for rifampicin resistance of this isolate (Table 5).

4. Discussion

Carbapenem-resistant *A. baumannii* (CRAB) burdens global health and standard medical practices with the widespread of multidrug-resistant phenotypes. The evolutionary variation of pathogens by cargoes harbored by mobile genetic elements (MGEs) aggravate the problem through the dissemination of resistance variants that hamper the use of conventional antibiotics [42]. Combination therapy with rifampicin was proposed as a potential strategy to revive the activity of inactive antibiotics. This study further used both phenotypic and genotypic analysis to investigate the resistance determinants in rifampicin-resistant CRAB clinical isolates obtained from hospitals in Southern Thailand. The studied isolates demonstrated multidrug-resistant to various classes of antibiotics including rifampicin. This is in support of a previous study, which revealed that rifampicin-resistant clinical isolates are molecular biomarkers for MDR detection [43]. Twenty-nine isolates utilized in this study demonstrated resistance to almost all classes of antibiotics except for the glycylcyclines. Similarly, an increased susceptibility rate of CRAB clinical isolates to tigecycline and minocycline

Table 4
The distribution of plasmids among 10 *A. baumannii* clinical isolates.

Isolate ID	ST	Predicted plasmids											
		p1ABSDF	repAci1	repAci2	repAci3	repAci7	repAci8	repM-Aci9	repM-Aci90	RepApAB49	pABTJ2	p3ABAYE0002	pS30-1
SK024	2	+	+	+	+	+	–	–	–	–	–	–	+
SK052	2	–	–	–	+	+	+	+	–	–	–	–	+
SK065	2	–	–	–	+	+	+	+	–	–	–	–	+
TR009	2	–	–	–	+	+	+	+	–	–	–	–	+
ST004	2	–	–	–	+	+	+	–	+	–	–	–	+
ST002	2	–	–	–	–	–	–	–	–	+	–	–	–
ST011	2	–	+	+	+	+	–	–	–	–	–	–	+
SK068	2	–	+	+	+	+	–	–	–	–	+	–	+
TR125	25	–	–	–	+	+	–	–	–	–	–	–	+
TR131	16	–	+	–	–	–	+	+	–	–	+	+	–

+, plasmid present; -, plasmid absent.

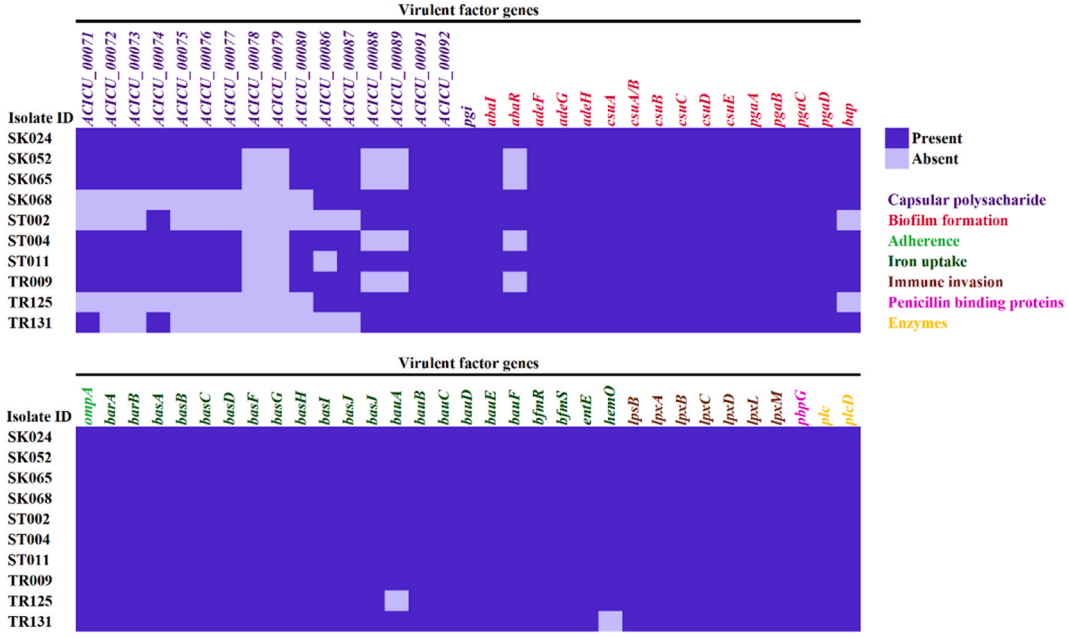

Fig. 8. Distribution of virulence factor genes among *A. baumannii* clinical isolates.

has been reported [42,44]. However, the glycylglycine resistance genes are being disseminated and threatening as variants of the *tetB* (tetracycline efflux pump regulatory gene) and *tet39* genes may emerge. Further analysis was performed with 20 isolates that have demonstrated resistance to almost all the classes of antibiotics. Among the 20 tested isolates, 5 were resistant to colistin at MIC of 4 μg/mL while others were intermediate. In a previous study, an increase in colistin resistance rate of 15% was reported in Thailand [45]. However, in this study, a 25% colistin-resistant rate was exhibited by the isolates. Additionally, the time-kill for isolates PT046, TR069, andTR082 confirms the synergistic study while inconsistency in the time-kill and checker board assay was identified in SK059. A similar case have been reported in a previous research were inconsistency was demonstrated between the time-kill dynamics and the synergism study of certain bacteria isolates [46]. Previous studies have reported a >90% resistance rate of *A. baumannii* to ciprofloxacin and gentamycin [45,47], and this tallies to the finding of the current study with over 80% resistance rate to the antibiotics. Notably, the limited synergistic activity of rifampicin with other antibiotics may be due to the diverse mechanisms of resistance discovered among isolates. Although, a bactericidal killing of some isolates was achieved when rifampicin was combined with minocycline/tigecycline in the time-kill kinetic, most of the antibiotic's combinations were addictive. The MICs of rifampicin against these resistant isolates were further investigated in the presence of the efflux pump inhibitor CCCP..Two isolates SK059 and SK065 expressed a 4-fold reduction in MIC. We suspect that the overexpression of efflux pumps genes might confer resistance to rifampicin among *A. baumannii* clinical isolates [48,49].

Our findings indicate that the WGS results validated the phenotypic analysis. This was demonstrated by the isolate's observable phenotypes and antibiotics resistant mechanisms. The genotypic analysis revealed the different resistant patterns of the isolates. Diverse resistant determinant was predominant among isolates despite their sequence types and may be linked to the strain specificity demonstrated by each of the isolates to various antibiotic treatments. The result also revealed that ST2 is the widest disseminated global clone and indicates a progression in the rise of more variants in the accessory genome replicating a previous study by Chukamnerd et al. (2022). The genotypic analysis affirmed the phenotype and further demonstrates that the isolates were MDR.

A recent study evinced that multidrug resistance due to the acquired AMR gene was mainly conferred by five different plasmids that were identified except for the $bla_{\text{OXA-23}}$ gene [50]. Interestingly, these genes can also mediate resistance to all the choice treatment options recently used against *Acinetobacter* spp. including amikacin, gentamicin, tetracycline, and ciprofloxacin monotherapies. The $bla_{\text{OXA-23,}}$ a class D carbapenemase was found in almost all the sequenced isolates except for TR131 and in association with $bla_{\text{OXA-66,}}$ an Acinetobacter-derived cephalosporinase $bla_{\text{ADC-25}}$ (70%) and $bla_{\text{PER-7}}$ (50%). These genes were recently described among the highly prevalent AMR genes in CRAB isolates [51]. Several aminoglycoside-resistant genes including *aph (6)-Id, armA, aph (3′)-Ia,* and *aadA1* were increasingly spread among isolates. In addition, the presence of several aminoglycoside-resistant genes may be the reason for the high level of aminoglycoside-resistant among isolates from the antimicrobial susceptibility test conducted. The genotypic annlysis validates the results obtained from the susceptibility test conducted for aminoglycosides. Another study also reported the distribution of these aminoglycoside-modifying enzyme variants [52]. Our finding revealed that rifampicin resistance among the isolates was majorly due to the spread of *arr-3* gene which may be plasmid-mediated, a chromosomal mutation in the *rpoB* gene, or an expression of efflux pump. Many *rpoB* mutations at locations H532 and S521 have been previously reported [48]. There is a limited number of studies addressing rifampicin resistance in *A. baumannii* due to efflux pumps mediated resistance.

Generally, the transfer of Mobile genetic elements comprises all mechanisms of resistance including the downregulation or

Table 5
Chromosomal mutation in *rpoB* gene with altered protein function.

Isolate ID	Nucleotide/ protein	Molecular mechanisms *P*-values ≤0.05												
		Loss of Allosteric site at R538	Gain of Methylation at K536	Altered Disordered interface	Altered Metal binding	Altered Ordered interface	Loss of Relative solvent accessibility	Altered *Trans*-membrane protein	Loss of Ubiquitylation at K785	Loss of ADP-ribosylation at R788	Altered Stability	Loss of Proteolytic cleavage at D790	Loss of Methylation at K785	Loss of Allosteric site at R788
SK024	T1605A/ H535Q	+	+	–	–	–	–	–	–	–	–	–	–	–
SK025	T1605A/ H535Q	+	+	–	–	–	–	–	–	–	–	–	–	–
TR131	G2363A/ R788H	–	–	+	+	+	+	+	+	+	+	+	+	+
TR123	T1605A/ H535Q	+	+	–	–	–	–	–	–	–	–	–	–	–

Occurrence of specific change/mechanism (+). Absence of Change/mechanisms (+). All alignments showed 99.5% identity.

overexpression of some virulent factor genes. This study also found 65 different virulent genes predicted with the ability to increase the pathogenicity of the isolates. Among the virulent genes identified, the absence of abaR regulatory systems in isolates SK052, SK065, TR009, and ST004 may have a detrimental effect by promoting quorum sensing and enhancing biofilm formation. In addition, the formation of biofilm is often associated with elevated pathogenicity of species and increased tolerance to harsh environmental conditions leading to colonization and the manifestation of chronic diseases [53]. A study recently reported that the presence of a biofilm-forming gene in an isolate was associated with the upregulation of the *abaI* gene in the biofilm-forming stage [54]. The implication of excessive expression of virulence genes may lead to the dysregulation and function of some cellular features including the AdeFGH efflux system, ACUCI_00071–92 which encodes the capsular polysaccharides. The expression level of the gene in all the isolates may also result in carbapenem resistance. The role of capsular polysaccharide, OmpA, and RND AdeFGH efflux systems modifications in the increased pathogenicity of *A. baumannii* was reported previously [50].

The presence of 11 different plasmids indicated that rifampicin-resistant CRAB clinical isolates can accumulate MGEs, especially plasmids, that increase the chance of antimicrobial resistance. Notably, the repAci7, repAci3, and repAci1-like plasmids identified in this study might carry the $bla_{\text{OXA-23}}$ gene as previously described [42]. This is also in concordance with previous findings that *A. baumannii* clinical isolates easily attract MGEs leading to a high rate of resistance to antimicrobial agents [55]. Based on the high MICs obtained from the antimicrobial susceptibility test, plasmids spread of resistant genes may have led to MDR in the isolates. The variability of the plasmids might also be the cause of the increased resistance to antibiotics of various classes. Recently, several mobile genetic elements were also identified in *A. baumannii* clinical isolates obtained from patients in the intensive care units of hospitals in Thailand [56]. Thus, the outcome of this research showed consistency between the phenotypic and genotypic analysis which highlights the benefits of this research.

The integration of phenotypic and genotypic analyses yields valuable insights into the molecular changes associated with isolates, offering clinicians a comprehensive understanding for tailored treatment strategies. This study not only aids in resolving the specificity issues of individual isolates but also equips clinicians with informed decision-making capabilities for treatment selection. The practical application of research outcomes stands as a testament and rationale for further investigations. Moreover, deciphering effective antibiotic combinations and resistance mechanisms may unveil potential strategies for advancing treatment and drug discovery.

Unfortunately, our failure to investigate the level of expression of the virulent genes presents a limitation to the study. Hence, it is advisable to further investigate the level of expression of the virulent factor genes. In addition, more research is needed to determine the activities of various efflux pumps implicated in *A. baumannii* infection and characterize efflux pumps associated with rifampicin resistance.

5. Conclusion

The phenotypic analysis of rifampicin-resistant CRAB clinical isolates obtained from southern Thailand showed that the isolates exhibited different responses to antibiotics treatments which may be attributed to the diversity of their genome. We recommend that the treatment of antibiotics resistance clinical isolates should be specified based on the genomic makeup of the individual isolate. Furthermore, rifampicin adjunctive therapy with Colistin, tigecycline, and minocycline may serve as the choice treatment options of some rifampicin-resistant CRAB clinical isolates based on the genome.

Ethical approval

This study was approved by the Human Research Ethics Committee (HREC), Faculty of Medicine, Prince of Songkla University (Reference Number: 64-284-14-1). Also, the researchers were granted permission to extract the data from the database with waiver of consent because of the observational nature of the study. All data were fully anonymized before the researcher accessed and analyzed.

Data availability

This Whole Genome Shotgun project of 10 CRAB clinical isolates have been deposited at the DDBJ/ENA/GenBank NCBI with the BioProject ID: PRJNA940623 and the BioSample numbers: SAMN33575613 (ST002), SAMN33575614 (ST004), SAMN33575615 (ST011), SAMN33575616 (TR009), SAMN33575617 (TR125), SAMN33575618 (TR131), SAMN33575619 (SK024), SAMN33575620 (SK052), SAMN33575621 (SK065), and SAMN33575622 (SK068). The genome accessions JAREUR000000000, JAREUQ000000000, JAREYB000000000, JAREUP000000000, JAREUO000000000, JAREYA000000000, JAREUN000000000, JAREUM000000000, JAREUL000000000, and JAREUK000000000 were assigned for ST002, ST004, ST011, TR009, TR125, TR131, SK024, SK052, SK065, and SK068, respectively.

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CRediT authorship contribution statement

Lois Chinwe Nwabor: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Arnon Chukamnerd:** Writing – review & editing, Visualization, Supervision, Software, Methodology, Formal analysis, Data curation. **Ozioma Forstinus Nwabor:** Writing – review & editing, Validation, Supervision, Data curation, Conceptualization. **Komwit Surachat:** Writing – review & editing, Software, Resources. **Rattanaruji Pomwised:** Writing – review & editing, Resources. **Kongpop Jeenkeawpiam:** Writing – review & editing, Software. **Sarunyou Chusri:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Assoc. Prof. Dr. Sarunyou Chusri, M.D. reports financial support was provided by The National Science, Research, and Innovation Fund (NSRF) and Prince of Songkla University, Thailand (Grant No. MED6505096f). Lois Chinwe Nwabor reports a relationship with Prince of Songkla University Faculty of Medicine that includes: funding grants.

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References

[1] R.E. Nelson, M.L. Schweizer, E.N. Perencevich, et al., Costs and mortality associated with multidrug-resistant healthcare-associated Acinetobacter infections, Infect. Control Hosp. Epidemiol. 37 (10) (2016) 1212–1218.
[2] J.M. Colquhoun, P.N. Rather, Insights into mechanisms of biofilm formation in Acinetobacter baumannii and implications for uropathogenesis, Front. Cell. Infect. Microbiol. 10 (2020) 253.
[3] A. Kiddee, K. Assawatheptawee, A. Na-Udom, et al., Risk factors for extended-spectrum β-lactamase-producing Enterobacteriaceae carriage in patients admitted to intensive care unit in a tertiary Care Hospital in Thailand, Microb. Drug Resist. 25 (8) (2019) 1182–1190.
[4] B. Cai, R. Echols, G. Magee, et al., Prevalence of carbapenem-resistant Gram-negative infections in the United States predominated by Acinetobacter baumannii and Pseudomonas aeruginosa, in: Open Forum Infectious Diseases, Oxford University Press, 2017.
[5] M. Tilahun, A. Gedefie, H. Bisetegn, et al., Emergence of high prevalence of extended-spectrum beta-lactamase and carbapenemase producing acinetobacter species and Pseudomonas aeruginosa among hospitalized patients at dessie comprehensive specialized hospital, north-east Ethiopia, Infect. Drug Resist. 15 (2022) 895.
[6] Y. Duman, C. Kuzucu, Y. Ersoy, et al., The Effect of sodium dichloroisocyanurate dihydrate to prevent the environmental transmission of multidrug-resistant Acinetobacter Baumannii in hospital settings, Fresenius Environ. Bull. 29 (2020) 7191–7197.
[7] K. rangel Tc, S.G. De-Simmone, Acinetobacter Baumannii Infection in Times of Covid 19 Pandemic, Mdpi antibiotics, 2021.
[8] L.K. Logan, S. Gandra, A. Trett, et al., Acinetobacter baumannii resistance trends in children in the United States, 1999–2012, Journal of the Pediatric Infectious Diseases Society 8 (2) (2019) 136–142.
[9] S.S. Kadri, J. Adjemian, Y.L. Lai, et al., Difficult-to-treat resistance in gram-negative bacteremia at 173 US hospitals: retrospective cohort analysis of prevalence, predictors, and outcome of resistance to all first-line agents, Clin. Infect. Dis. 67 (12) (2018) 1803–1814.
[10] A. Chukamnerd, K. Singkhamanan, V. Chongsuvivatwong, et al., Whole-genome analysis of carbapenem-resistant Acinetobacter baumannii from clinical isolates in Southern Thailand, Comput. Struct. Biotechnol. J. 20 (2022) 545–558.
[11] A.Y. Peleg, D.C. Hooper, Hospital-acquired infections due to gram-negative bacteria, N. Engl. J. Med. 362 (19) (2010) 1804–1813.
[12] D.M. Livermore, Current epidemiology and growing resistance of gram-negative pathogens, Kor. J. Intern. Med. 27 (2) (2012) 128.
[13] S. Ebrahimi, B. Sisakhtpour, A. Mirzaei, et al., Efficacy of isolated bacteriophage against biofilm embedded colistin-resistant Acinetobacter baumannii, Gene Reports 22 (2021) 100984.
[14] S.M. Ibrahim, E.M. Ibrahim, O.A. Ibrahim, et al., Molecular detection of carbapenemase genes in extensive drug resistant acinetobacter baumannii clinical isolates from ICU patients, khartoum, Open J. Med. Microbiol. 12 (1) (2022) 38–48.
[15] C. Bartal, K.V. Rolston, L. Nesher, Carbapenem-resistant Acinetobacter baumannii: colonization, infection and current treatment options, Infect. Dis. Ther. (2022) 1–12.
[16] W. Ni, Y. Han, J. Zhao, et al., Tigecycline treatment experience against multidrug-resistant Acinetobacter baumannii infections: a systematic review and meta-analysis, Int. J. Antimicrob. Agents 47 (2) (2016) 107–116.
[17] G.C. Leite, M.S. Oliveira, L.V. Perdigao-Neto, et al., Antimicrobial combinations against pan-resistant Acinetobacter baumannii isolates with different resistance mechanisms, PLoS One 11 (3) (2016) e0151270.
[18] J. Teo, T.-P. Lim, L.-Y. Hsu, et al., Extensively drug-resistant Acinetobacter baumannii in a Thai hospital: a molecular epidemiologic analysis and identification of bactericidal Polymyxin B-based combinations, Antimicrob. Resist. Infect. Control 4 (1) (2015) 1–7.
[19] Y. Bai, B. Liu, T. Wang, et al., In vitro activities of combinations of rifampin with other antimicrobials against multidrug-resistant Acinetobacter baumannii, Antimicrob. Agents Chemother. 59 (3) (2015) 1466–1471.
[20] A.C. Lois Chinwe Nwabor, Ozioma Forstinus Nwabor, Rattanaruji Pomwised, Supayang P. Voravuthikunchai, Sarunyou Chusri, Rifampicin enhanced carbapenem activity with improved antibacterial effects and eradicates established acinetobacter baumannii biofilms *pharmaceuticals* 16 (4) (2023) 477.
[21] P. Vos, G. Garrity, D. Jones, et al., Bergey's Manual of Systematic Bacteriology: Volume 3: the Firmicutes, vol. 3, Springer Science & Business Media, 2011.
[22] M. MarÝ-Almirall, C. Cosgaya, P.G. Higgins, et al., MALDI-TOF/MS identification of species from the Acinetobacter baumannii (Ab) group revisited: inclusion of the novel A. áseifertii and A. ádijkshoorniae species, Clin. Microbiol. Infection 23 (3) (2017) 210. e1–e210. e9.
[23] M. Petrova, P. Petrov, A new method for manual measurements of inhibition zones with the Bauer-Kirby disk susceptibility test, Mathematics and Education in Mathematics 50 (2021) 185–190.
[24] P. Wayne, Clinical and Laboratory Standards Institute, Performance standards for antimicrobial susceptibility testing, 2011.
[25] P. Wayne, Clinical and Laboratory Standards Institute: Performance Standards for Antimicrobial Susceptibility Testing: 20th Informational Supplement. *CLSI Document M100-S20*, 2010.
[26] U. Singkham-In, T. Chatsuwan, In vitro activities of carbapenems in combination with amikacin, colistin, or fosfomycin against carbapenem-resistant Acinetobacter baumannii clinical isolates, Diagn. Microbiol. Infect. Dis. 91 (2) (2018) 169–174.

[27] C.R. Bonapace, J.A. Bosso, L.V. Friedrich, et al., Comparison of methods of interpretation of checkerboard synergy testing, Diagn. Microbiol. Infect. Dis. 44 (4) (2002) 363–366.
[28] S. Leelasupasri, W. Santimaleeworagun, T. Jitwasinkul, Antimicrobial susceptibility among colistin, sulbactam, and fosfomycin and a synergism study of colistin in combination with sulbactam or fosfomycin against clinical isolates of carbapenem-resistant Acinetobacter baumannii, Journal of pathogens (2018) 2018.
[29] U. Singkham-In, T. Chatsuwan, Synergism of imipenem with fosfomycin associated with the active cell wall recycling and heteroresistance in Acinetobacter calcoaceticus-baumannii complex, Sci. Rep. 12 (1) (2022) 1–18.
[30] A. Bankevich, S. Nurk, D. Antipov, et al., SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing, J. Comput. Biol. 19 (5) (2012) 455–477.
[31] G.C. Park, J.A. Choi, S.J. Jang, et al., In vitro interactions of antibiotic combinations of colistin, tigecycline, and doripenem against extensively drug-resistant and multidrug-resistant Acinetobacter baumannii, Annals of laboratory medicine 36 (2) (2016) 124.
[32] M. Seppey, M. Manni, E.M. Zdobnov, BUSCO: assessing genome assembly and annotation completeness, Gene prediction: methods and protocols (2019) 227–245.
[33] T. Seemann, Prokka: rapid prokaryotic genome annotation, Bioinformatics 30 (14) (2014) 2068–2069.
[34] E. Zankari, H. Hasman, S. Cosentino, et al., Identification of acquired antimicrobial resistance genes, J. Antimicrob. Chemother. 67 (11) (2012) 2640–2644.
[35] A. Carattoli, E. Zankari, A. García-Fernández, et al., In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing, Antimicrob. Agents Chemother. 58 (7) (2014) 3895–3903.
[36] K.A. Jolley, J.E. Bray, M.C. Maiden, Open-access Bacterial Population Genomics: BIGSdb Software, the PubMLST. Org Website and Their Applications, vol. 3, Wellcome open research, 2018.
[37] L. Chen, J. Yang, J. Yu, et al., VFDB: a reference database for bacterial virulence factors, Nucleic Acids Res. 33 (suppl_1) (2005) D325–D328.
[38] A. Chukamnerd, K. Singkhamanan, V. Chongsuvivatwong, et al., Whole-genome analysis of carbapenem-resistant Acinetobacter baumannii from clinical isolates in Southern Thailand, Comput. Struct. Biotechnol. J. 20 (2022) 545–558.
[39] A.J. Page, C.A. Cummins, M. Hunt, et al., Roary: rapid large-scale prokaryote pan genome analysis, Bioinformatics 31 (22) (2015) 3691–3693.
[40] J. Hadfield, N.J. Croucher, R.J. Goater, et al., Phandango: an interactive viewer for bacterial population genomics, Bioinformatics 34 (2) (2018) 292–293.
[41] C. Xu, S. Bilya, W. Xu, adeABC efflux gene in Acinetobacter baumannii, New microbes and new infections 30 (2019) 100549.
[42] B.P. Brito, J. Koong, A. Wozniak, et al., Genomic analysis of carbapenem-resistant acinetobacter baumannii strains recovered from Chilean hospitals reveals lineages specific to south America and multiple routes for acquisition of antibiotic resistance genes, Microbiol. Spectr. 10 (5) (2022) e02463, 22.
[43] O. Zewdie, R. Dabsu, E. Kifle, et al., Rifampicin-resistant multidrug-resistant tuberculosis cases in selected hospitals in Western Oromia, Ethiopia: cross-sectional retrospective study, Infect. Drug Resist. (2020) 3699–3705.
[44] Y.G. Ju, H.J. Lee, H.S. Yim, et al., In vitro synergistic antimicrobial activity of a combination of meropenem, colistin, tigecycline, rifampin, and ceftolozane/tazobactam against carbapenem-resistant Acinetobacter baumannii, Sci. Rep. 12 (1) (2022) 7541.
[45] P.A. Hassan, A.K. Khider, Correlation of biofilm formation and antibiotic resistance among clinical and soil isolates of Acinetobacter baumannii in Iraq, Acta Microbiol. Immunol. Hung. (2019) 1–10.
[46] P. Terbtothakun, O.F. Nwabor, T. Siriyong, et al., Synergistic antibacterial effects of meropenem in combination with aminoglycosides against carbapenem-resistant Escherichia coli harboring bla NDM-1 and bla NDM-5, Antibiotics 10 (8) (2021) 1023.
[47] S. Srisakul, D.L. Wannigama, P.G. Higgins, et al., Overcoming addition of phosphoethanolamine to lipid A mediated colistin resistance in Acinetobacter baumannii clinical isolates with colistin–sulbactam combination therapy, Sci. Rep. 12 (1) (2022) 11390.
[48] M. Giannouli, A. Di Popolo, E. Durante-Mangoni, et al., Molecular epidemiology and mechanisms of rifampicin resistance in Acinetobacter baumannii isolates from Italy, Int. J. Antimicrob. Agents 39 (1) (2012) 58–63.
[49] L. Xing, P.A. Barnie, Z. Su, et al., Development of Efflux Pumps and Inhibitors (EPIs) in A. Baumanii, 2014.
[50] M. Douraghi, J.J. Kenyon, P. Aris, et al., Accumulation of antibiotic resistance genes in carbapenem-resistant Acinetobacter baumannii isolates belonging to lineage 2, global clone 1, from outbreaks in 2012–2013 at a Tehran burns hospital, mSphere 5 (2) (2020) e00164, 20.
[51] S. Cherubini, M. Perilli, B. Segatore, et al., Whole-genome sequencing of ST2 A. baumannii causing bloodstream infections in COVID-19 patients, Antibiotics 11 (7) (2022) 955.
[52] T. Wongsuk, S. Boonsilp, A. Homkaew, et al., Whole genome sequence of pan drug-resistant clinical isolate of Acinetobacter baumannii ST1890, PLoS One 17 (3) (2022) e0264374.
[53] C. Dexter, G.L. Murray, I.T. Paulsen, et al., Community-acquired Acinetobacter baumannii: clinical characteristics, epidemiology and pathogenesis, Expert Rev. Anti-infect. Ther. 13 (5) (2015) 567–573.
[54] Z. Li, Z. Ding, Y. Liu, et al., Phenotypic and genotypic characteristics of biofilm formation in clinical isolates of Acinetobacter baumannii, Infect. Drug Resist. (2021) 2613–2624.
[55] S.R. Partridge, Analysis of antibiotic resistance regions in Gram-negative bacteria, FEMS Microbiol. Rev. 35 (5) (2011) 820–855.
[56] P. Chopjitt, T. Wongsurawat, P. Jenjaroenpun, et al., Complete genome sequences of four extensively drug-resistant Acinetobacter baumannii isolates from Thailand, Microbiology Resource Announcements 9 (40) (2020) e00949, 20.