Colistin resistance in *Acinetobacter baumannii* is driven by multiple genomic traits:

Evaluating the role of IS*AbaI*-driven *eptA* overexpression among Indian isolates

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Running title: Characterization of colistin resistance among Indian isolates of *A. baumannii*

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Abstract

Colistin resistance in *Acinetobacter baumannii* is mediated by multiple mechanisms. Recently, mutations within *pmrAB* two component system and overexpression of *eptA* due to upstream insertion of *ISAbal* play a major role. To characterize colistin resistance mechanisms among the clinical isolates of *A. baumannii* in India. A total of 224 clinical isolates of *A. baumannii* collected from 2016 to 2019 were included in this study. Mutations within lipid A biosynthesis and *pmrAB* genes were characterized by Whole Genome Shotgun sequencing. Twenty eight complete genomes were further characterized for insertional inactivation of *lpx* genes and the association of *ISAbal-eptA* using hybrid assembly approach. Non-synonymous mutations like M12I in *pmrA*, A138T and A444V in *pmrB* and E117K in *lpxD* were identified. Four of the five colistin resistant *A. baumannii* isolates had insertion of *ISAbal* upstream *eptA*. No *mcr* genes were identified. Overall, the present study highlights the diversity of colistin resistance mechanisms in *A. baumannii*. *ISAbal*-driven *eptA* overexpression could be responsible for colistin resistance among Indian isolates of colistin resistant *A. baumannii*.

Introduction

Acinetobacter baumannii is a major nosocomial pathogen which is responsible for wide range of infections and has been reported as a public health problem globally [1, 2]. Multi-drug and extensively-drug resistant *A. baumannii* have been reported world-wide which results in the paucity of treatment options against *Acinetobacter* infections [3]. Carbapenems are considered to be the last-line antibiotics for treating *A. baumannii* infections and more than 80% resistance to carbapenem was reported [4]. More than 60% mortality rates have been reported for the most common carbapenem resistant *A. baumannii* (CRAB) infections like blood stream infections (BSI) and hospital-acquired pneumonia (HAP) [5].

Recently, World Health Organization (WHO) categorized CRAB as “Priority one” pathogen in the global list of antibiotic-resistant bacteria for the development of new antibiotics [3]. The most common antimicrobials considered for treating CRAB include colistin-based, tigecycline-based and sulbactam-based combinations [6]. Unfortunately, increased usage of colistin for treating *Acinetobacter* infection results in resistance to these last-line drugs [7].

In *A. baumannii* colistin resistance is mediated by multiple mechanisms [8]. This includes, (i) Loss of lipopolysaccharide (LPS) production, due to mutations in lipid A biosynthesis genes like *lpxA*, *lpxC* and *lpxD*. In addition, insertional inactivation of *lpxACD* genes due to insertion element, *ISAbal1* causes both loss of LPS and increased colistin resistance [9, 10]. (ii) Point mutations in *pmrA* and *pmrB* genes of *pmrAB* two component system (TCS) results in decreased membrane permeability leading to colistin resistance [11]. (iii) Recently, Gerson et al reported the presence of phosphoethanolamine (pEtN) transferase *eptA*, a homologue of *pmrC* and insertion of *ISAbal* upstream *eptA* results in overexpression and high

colistin resistance [3] and (iv) Colistin resistance due to plasmid mediated pEtN transferase, *mcr* genes [12].

In this study, a total of 224 clinical isolates of *A. baumannii* were characterized for their susceptibility profile and multiple resistance mechanism that contributes to colistin resistance. In addition, subset of complete genomes were characterized to investigate the inactivation of *lpxA* or *lpxC* by *ISAbal1* and to decipher the presence of *ISAbal1* upstream *eptA*.

Materials and methods

Bacterial isolates

A total of 1214 consecutive non-duplicate clinical isolates of *A. baumannii* collected during January 2016 to December 2019 from the routine cultures of clinical samples, blood (n=314) and endo-tracheal aspirate (ETA) (n=900) were included in this study. All the isolates were identified up to the species level as *Acinetobacter baumannii calcoaceticus* complex (*Acb* complex) using conventional biochemical methods [13]. MALDI-TOF was used to confirm at the species level as *Acinetobacter baumannii*. Further confirmation of *Acb* complex as *A. baumannii* was performed targeting chromosomally encoded *bla_{OXA-51}* like gene by PCR [14].

Anti-microbial susceptibility testing (AST)

AST was performed for all the isolates against different classes of antibiotics by Kirby Bauer disc diffusion (DD) method and interpreted according to Clinical Laboratory Standards Institute (CLSI) guidelines [15]. The antibiotics tested includes ceftazidime, cefepime, piperacillin/tazobactam, cefoperazone/Sulbactam, imipenem, meropenem, aztreonam, amikacin, netilmycin, tobramycin, levofloxacin, tetracycline, minocycline, tigecycline and trimethoprim-sulfamethoxazole.

Minimum inhibitory concentration (MIC) by broth micro dilution (BMD)

As recommended by CLSI 2017 guidelines, colistin MIC was determined for all the blood and ETA isolates using broth micro dilution (BMD) and interpreted accordingly [16]. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control (QC) strains. Also, *mcr-1* positive *E. coli* was used as an internal control. Two in-house quality controls, *Klebsiella pneumoniae* BA38416 with MIC of 0.5 µg/ml and *Klebsiella pneumoniae* BA25425 with 16 µg/ml were also included in every batch of testing.

Whole genome sequencing (WGS), assembly and annotation

A subset of 224 clinical isolates of *A. baumannii* (Blood = 117 & ETA = 107) were selected based on the susceptibility profile for further characterization by WGS. Genomic DNA was extracted using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and WGS was performed. In brief, short read sequencing was performed using IonTorrent™ Personal Genome Machine™ (PGM) (Life Technologies, Carlsbad, CA) with 400-bp read chemistry or Illumina MiSeq as per the manufacturer's instructions. Long read sequencing was performed using SQK-LSK108 Kit R9 version (Oxford Nanopore Technologies, Oxford, UK) using 1D sequencing method according to the manufacturer's protocol. To obtain complete genome, hybrid assembly was performed for a subset of 28 genomes (Blood = 21 and ETA = 7) using long reads from MinION and short reads from either IonTorrent or Illumina as described previously [17].

All the genomes were assembled and annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). Furthermore, downstream analysis was done using tools from Center for Genomic Epidemiology (CGE) server (<http://www.genomicepidemiology.org/>).

Antimicrobial resistance genes (ARG) were using ResFinder 3.0 database (<https://cge.cbs.dtu.dk/services/ResFinder/>) [18]. Sequence type of the isolates was assigned by MLST 2.0 tool using the Oxford scheme (*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD* genes) (<https://cge.cbs.dtu.dk/services/MLST/>) [19]. SNP based phylogenetic tree was constructed and meta-data like colistin susceptibility, chromosomal mutation profile and International Clones were added using iTOL (<https://itol.embl.de/>) [20].

Mutation analysis of *lpxACD* and *pmrAB* genes

In-silico mutation analysis of genes involved in lipid A biosynthesis pathway (*lpxA*, *lpxC* and *lpxD*) and *pmrAB* TCS (*pmrA* and *pmrB*) were determined in all the genomes using Blast analysis (<https://blast.ncbi.nlm.nih.gov>) and compared with the reference strain of *A. baumannii* ATCC 17978 (GenBank Accession Number CP000521). Other reference strains like *A. baumannii* AYE (GenBank Accession Number NC010410), *A. baumannii* ACICU (GenBank Accession Number NC010611) and ATCC 19606 (GenBank Accession Number CP046654) were included in the analysis to identify the genetic polymorphisms. Plasmid mediated colistin resistance determinant gene (*mcr*) were detected using Resfinder and by *in-silico* Blast analysis against the reference sequences of *mcr* genes reported so far [21]. In addition to the mutation analysis, all the 28 complete genomes were characterized manually for other colistin resistance mechanisms like insertional inactivation of *lpxA*, *lpxC* and *lpxD* genes due to insertion sequence, *ISAbal1* and over-expression of *pmrC* homologue, *eptA* due to upstream presence of *ISAbal*.

Nucleotide accession numbers

The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under BioProject numbers PRJNA603876, PRJNA604897, PRJNA610496 and PRJNA610503. The

complete genome project has been deposited at DDBJ/ENA/GenBank under accession numbers AB01 (CP040080), AB02 (CP035672), AB03 (CP050388), AB04 (CP040040), AB05 (CP040047), AB06 (CP040050), AB07 (CP035930), AB08 (CP038500), AB09 (CP038644), AB010 (CP040053), AB011 (CP040056), AB012 (CP040084), AB013 (CP040087), AB014 (CP050421), AB015 (CP050403), AB016 (CP040259), AB017 (CP050385), AB018 (CP050523), AB019 (CP050400), AB020 (CP050390), AB021 (CP050410), AB022 (CP050412), AB023 (CP050415), AB024 (CP050425), AB025 (CP050432), AB026 (CP051474), AB027 (CP050526) and AB028 (CP050401).

Results

Bacterial isolates and AST

All the study isolates (n=1214) were first identified as *Acb* complex and confirmed as *A. baumannii* by MALDI-TOF. *bla*_{OXA-51} like PCR re-confirmed *Acb* complex as *A. baumannii*. AST revealed that 99% (Blood, n=313 and ETA, n=899) of the study isolates were extensively drug resistant (XDR) and showed resistance to all the tested antibiotics. Of the remaining two isolates, one from ETA was pan-susceptible (AB01- Accession No. CP040080) while the other from blood was multi-drug resistant (MDR). The MDR (AB025 - Accession No. CP050432) isolate was resistant to cephalosporins, fluoroquinolones, aminoglycosides, tetracyclines, tigecycline and trimethoprim-sulfamethoxazole while susceptible to carbapenems.

Determination of colistin MIC by BMD

Among the tested isolates, 10.8% (n=34) from blood and 8.2% (n=74) from ETA were colistin resistant *A. baumannii* respectively. The colistin MIC range, MIC₅₀ and MIC₉₀ of the blood and ETA isolates were tabulated (Table 1).

Characterization of various colistin resistance mechanisms using WGS

A subset of 224 clinical isolates of *A. baumannii* was subjected to WGS based on the susceptibility profile. Of the 117 blood isolates, one isolate was MDR, 84 were carbapenem resistant-colistin susceptible *A. baumannii* (CR-ColSAB) and 32 were carbapenem -resistant-colistin resistant *A. baumannii* (CR-ColRAB). Similarly, among the 107 ETA, one was pan-susceptible, 46 were CR-ColSAB while 61 were CR-ColRAB.

Sequencing of *lpx* genes in both CR-ColSAB (n=131) and CR-ColRAB (n=93) revealed the presence of various amino acid substitutions. Within *lpxA*: Y131H, *lpxC*: C120R-P148S-F230Y-N287D and *lpxD*: Q4K-V63I-V93I-E117K-G166S-T287I-S299P substitutions were identified in comparison with the reference strain ATCC 17978. Three non-synonymous mutations Q4K, V63I and E117K within *lpxD* were identified in both CR-ColSAB and CR-ColRAB. Y131H, C120R and N287D substitutions were identified in all the three reference strains and all the CR-ColSAB and CR-ColRAB. Other amino-acid substitutions like P148S and F230Y in *lpxC*, V93I, G166S, T287I and S299P in *lpxD* were first described in this study. On the other hand, sequencing of *pmrAB* genes identified the following amino acid substitutions, *pmrA*: M12I and *pmrB*: A138T-L168S-A226T-V300E-G315A-P360Q-A444V. Single non-synonymous mutation, M12I in *pmrA* and A138T in *pmrB* was identified in all the CR-ColSAB and CR-ColRAB (Fig 1). None of the study isolates harbor any of the reported plasmid mediated colistin resistance determinant, *mcr-1* or its homologues.

Among the 28 complete genomes of *A. baumannii*, 21 were from blood of which 17 were CR-ColSAB while four were CR-ColRAB. Of the seven from ETA, five were CR-ColSAB, one was CR-ColRAB and one was pan-susceptible. Characterization of complete genomes for other colistin resistance mechanisms showed the presence of *ISAbal1* among two CR-ColSAB blood

isolates (AB016- Accession No. CP040259 & AB017- Accession No. CP050385), but no disruption of *lpxACD* observed. Atleast one copy of *eptA* was identified among 10 and 6 genomes of CR-ColSAB from blood and ETA respectively whereas more than one copy was present in 12 genomes (7 CR-ColSAB and 4 CR-ColRAB from blood while one CR-ColRAB from ETA). *ISAbal* was found in opposite orientation upstream *eptA* in two genomes of CR-ColRAB from blood (AB02- Accession No. CP035672 & AB05- Accession No. CP040047) and in one CR-ColRAB from ETA (AB03- Accession No. CP050388). *ISAbal* was present in direct orientation upstream *eptA* in one CR-ColRAB blood isolate (AB04- Accession No. CP040040). Two CR-ColSAB from blood have IS4 transposase but not upstream *eptA* (AB010- Accession No. CP040053 & AB011- Accession No. CP040056) whereas four CR-ColSAB from blood (AB08- Accession No. CP038500, AB09- Accession No. CP038644, AB025- Accession No. CP050432 and AB015- Accession No. CP050403) have other family transposases (Fig 2 - 3). In one CR-ColRAB (AB06- Accession No. CP040050), only amino-acid substitutions, Y131H in *lpxA*, C120R and N287D in *lpxC*, P148S and G315D in *lpxD* were identified while the other colistin resistance mechanisms were absent. The overall colistin resistance mechanisms characterized among the 28 hybrid genomes were described in (Table 2.)

MLST

MLST Finder revealed that both CR-ColSAB and CR-ColRAB were classified into International Clones (ICs) - IC1, IC2, IC7, IC8, CC862 and singletons. Majority of the CR-ColSAB isolates (n=90) (69%) belongs to IC2 with diverse sequence types like ST848, ST349, ST451, ST218 and ST195. Whereas IC2 was the only lineage with ST848 among the CR-ColRAB isolates (n=73) (78 %). Lineage specific non-synonymous mutations like Q4K, V63I and E117K within *lpxD* belongs to IC8, IC7 and IC2 respectively. Single non-synonymous

mutation, M12I in *pmrA*, A138T in *pmrB* and the combination of A138T-A444V within *pmrB* belongs to IC2.

Discussion

Colistin is considered as one of the few therapeutic options available against CRAB infections and due to increased usage, colistin resistance rate is gradually increasing globally and becoming a healthcare concern [22]. In the current study, 8% to 11% colistin resistance has been observed. Reports from other countries showed increased colistin resistance rates of 16.7% from Bulgaria, 19.1% from Spain and 27% from Greece [23 - 25]. Therefore, it is important to explore and understand the mechanisms that contribute to colistin resistance which is an utmost need for routine surveillance [3]. Several mutations and genetic polymorphisms have been reported within *lpxACD* and *pmrAB* genes by various studies using WGS which is in concurrence with the current study [9, 10].

In this study, clinical isolates of CR-ColRAB and CR-ColSAB were characterized for multiple colistin resistance mechanisms. Mutation analysis in *lpxACD* identified three non-synonymous mutations; Q4K, V63I and E117K within *lpxD* in both CR-ColRAB and CR-ColSAB which concurs with previous studies [26, 27]. Recent studies report that mutations in *pmrB* as the major contributor for colistin resistance in *A. baumannii* [24, 26, 28, 29]. The current study identified a non-synonymous mutation, A138T in *pmrB* while previous studies report other amino-acid substitutions together with A138T which is contrary to the current study [24, 26, 29]. In *pmrA*, a single non-synonymous mutation, M12I was first identified by Arroyo et al who also reported the association of M12I with colistin hetero-resistance [24, 26, 30]. In contrast, other studies reported G54E substitution within *pmrA* which was not identified in this study [29, 31]. Several studies reported the presence of mutations in *lpxD* along with *pmrB* genes [29]. In this

study, both CR-ColRAB and CR-ColSAB has *lpxD* mutation, E117K in combination with A138T-A444V mutation in *pmrB*. This observation indicates that mutations in *lpxD* alone and *pmrB* alone may not be sufficient to induce colistin resistance and support the presence of synergistic activity of mutations within these genes in promoting colistin resistance [26].

Two MLST schemes, PubMLST (Oxford) and Pasteur MLST are available for *A. baumannii* [32]. Earlier studies reported IC2/global clone 2 (GC2) as the predominant lineage associated with outbreaks [8]. This finding correlates with the current study and identified in both CR-ColRAB and CR-ColSAB In the present study, IC1/GC1 was identified among CR-ColSAB which is contrast to Snyman et al where GC1 was identified in both CR-ColSAB and CR-ColRAB [33]. Interestingly, lineage specific non-synonymous mutations, such as Q4K which belongs to IC8, V63I which belongs to IC7 and E117K-M12I-A138T which belongs to IC2 were observed in this study and such findings have not been previously reported.

Moffatt et al was the first to report on the role of *ISAbalI* which causes insertional inactivation of *lpxA* or *lpxC* and leads to loss of LPS production and colistin resistance [10]. Mutations in *lpxC* gene due to insertion of *ISAbalI* and increased colistin resistance were recently reported by Marta et al [34]. The current study revealed the presence of *ISAbalI* in two CR-ColSAB without disruption of *lpxA* or *lpxC* which is inconsistent with the previous studies.

eptA is a *pmrC* homologue with pEtN transferase activity [8]. In this study, *eptA* was present in both CR-ColSAB and CR-ColRAB and comparable with previous studies [3, 7 - 8, 35]. However, the presence of *ISAbalI* upstream *eptA* was identified only among the CR-ColRAB which results in overexpression of PetN transferase encoded either by *pmrC* or *eptA* and responsible for colistin resistance. Such findings are comparable with other studies whereas another study observed in both CR-ColSAB and CR-ColRAB [3, 7 - 8]. Presence of other family

transposases were noticed among the CR-ColSAB which could be novel and further studies are necessary. Other colistin resistance mechanisms like disruption of *eptA* by *ISAbal25* and increased expression of *eptA* due to the presence of *ISAbal* in reverse orientation upstream *eptA* were reported recently [3]. Though difference in the orientation of *ISAbal* with respect to *eptA* was identified in this study, no expression studies were done to confirm the same.

In 2016, plasmid mediated PetN transferase, *mcr-1* was identified from China that contributed to colistin resistance to *E. coli* [36]. Currently, nine *mcr* types (*mcr* 1-9) and approximately 56 *mcr* variants are available in the GenBank database [37]. Though recent studies reported the presence of *mcr-4.3* in *A. baumannii* isolated from pig feces, food sample and clinical strains, none of the current study isolates harbor *mcr* genes [37 - 39].

Though colistin-based combinations can be considered for treating CRAB infections, various controversies have been reported recently with the usage of polymyxins [40]. Two global organizations, CLSI and EUCAST revised the interpretive criteria for *in-vitro* polymyxin susceptibility testing and suggested to prefer non-polymyxin agents for treating *Acinetobacter* infections [41]. Such revision would effectively helpful in considering polymyxin as a treatment option in selected cases [41]. Further clinical trials to determine the efficacy of novel agents like cefiderocol and eravacycline against BSI and VAP are recommended [42]. None of the newly available drug combinations have clinical activity against CRAB infections except for the novel β -lactam- β -lactamase enhancer, cefepime-zidebactam [6, 42].

Conclusion

The current study provides characterization of multiple resistance mechanisms that could be responsible for colistin resistance among clinical isolates of *A. baumannii*. Previously reported non-synonymous mutations as well as other amino-acid substitutions that are not

described previously within *lpxD*, *pmrA* and *pmrB* genes were identified. Presence of *lpxD* mutation, E117K along with *pmrB* mutations A138T-A444V indicates the synergistic activity of mutations and results in colistin resistance. Recently, *mcr-4.3* on plasmids has been identified among clinical isolates of *A. baumannii* which is alarming. Two colistin susceptible isolates harbored *ISAbalI* and studies to understand the role of *ISAbalI* towards colistin resistance is essential. Though *pmrC* expression and addition of pEtN transferase to lipid A was regulated by *pmrAB* TCS, that alone doesn't considered as a sole contributor of colistin resistance. The presence of *eptA* was quite common and insertion of *ISAbalI* upstream *eptA* among colistin resistant isolates could be associated with colistin resistance. The exact resistance mechanism that contributes to colistin was not elucidated for one isolate and requires further investigation. Overall, the present study highlights the diversity of colistin resistance mechanisms described in *A. baumannii* and for complete understanding further extensive studies are necessary.

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Transparency declarations

None to declare

References

- 1 Casallas JC, Robayo-Amortegui H, Corredor-Rozo Z *et al.* Bacteremia by colistin-resistant *Acinetobacter baumannii* isolate: a case report. *J Med Case Rep* 2019;13:141.
- 2 Vijayakumar S, Rajenderan S, Laishram S *et al.* Biofilm formation and motility depend on the nature of the *Acinetobacter baumannii* clinical isolates. *Front Public Health* 2016; 24:105.
- 3 Gerson S, Betts JW, Lucaßen K *et al.* Investigation of novel *pmrB* and *eptA* mutations in isogenic *Acinetobacter baumannii* isolates associated with colistin resistance and increased virulence in vivo. *Antimicrob Agents Chemother* 2019;63:e01586-18.
- 4 Hsu LY, Apisarnthanarak A, Khan E *et al.* Carbapenem-resistant *Acinetobacter baumannii* and *Enterobacteriaceae* in south and southeast Asia. *Clin Microbiol Rev* 2017;30:1-22.
- 5 Wong D, Nielsen TB, Bonomo RA *et al.* Clinical and pathophysiological overview of *Acinetobacter* infections: a century of challenges. *Clin Microbiol Rev* 2017;30:409-47.
- 6 Piperaki ET, Tzouveleakis LS, Miriagou V *et al.* Carbapenem-resistant *Acinetobacter baumannii*: in pursuit of an effective treatment. *Clin Microbiol Infect* 2019;25:951-7.
- 7 Trebosc V, Gartenmann S, Tötzel M *et al.* Dissecting Colistin Resistance Mechanisms in Extensively Drug-Resistant *Acinetobacter baumannii* Clinical Isolates. *mBio*. 2019;10:e01083-19.
- 8 Potron A, Vuilleminot JB, Puja H *et al.* ISAbal-dependent overexpression of *eptA* in clinical strains of *Acinetobacter baumannii* resistant to colistin. *J Antimicrob Chemother* 2019; 74:2544-50.

312 9 Moffatt JH, Harper M, Harrison P *et al.* Colistin resistance in *Acinetobacter baumannii* is
313 mediated by complete loss of lipopolysaccharide production. *Antimicrob Agents Chemother*
314 2010 ;54:4971-7.

315 10 Moffatt JH, Harper M, Adler B *et al.* Insertion sequence ISAbal1 is involved in colistin
316 resistance and loss of lipopolysaccharide in *Acinetobacter baumannii*. *Antimicrob Agents*
317 *Chemother* 2011;55:3022-4.

318 11 Cai Y, Chai D, Wang R *et al.* Colistin resistance of *Acinetobacter baumannii*: clinical reports,
319 mechanisms and antimicrobial strategies. *J Antimicrob Chemother* 2012;67:1607-15.

320 12 Liu YY, Wang Y, Walsh TR *et al.* Emergence of plasmid-mediated colistin resistance
321 mechanism MCR-1 in animals and human beings in China: a microbiological and molecular
322 biological study. *Lancet Infect Dis* 2016;16:161-8.

323 13 Vijayakumar S, Biswas I, Veeraraghavan B *et al.* Accurate identification of clinically
324 important *Acinetobacter* spp.: an update. *Future Sci OA* 2019;5:FSO395.

325 14 Turton JF, Woodford N, Glover J *et al.* Identification of *Acinetobacter baumannii* by
326 detection of the blaOXA-51-like carbapenemase gene intrinsic to this species. *J Clin Microbiol*
327 2006;44:2974-6.

328 15 Clinical and Laboratory Standards Institute. (2019) Performance Standards for Antimicrobial
329 Susceptibility Testing: Twenty nine Informational Supplement M100-S29. CLSI, Wayne, PA,
330 USA

331 16 Clinical and Laboratory Standards Institute. (2017) Performance Standards for Antimicrobial
332 Susceptibility Testing: Twenty seven Informational Supplement M100-S27. CLSI, Wayne, PA,
333 USA

334 17 Vasudevan K, Ragupathi NK, Jacob JJ *et al.* Highly accurate-single chromosomal complete
335 genomes using IonTorrent and MinION sequencing of clinical pathogens. *Genomics*.
336 2020;112:545-51.

337 18 Zankari E, Hasman H, Cosentino S *et al.* Identification of acquired antimicrobial resistance
338 genes. *J Antimicrob Chemother* 2012;67:2640-44.

339 19 Larsen M.V, Cosentino S, Rasmussen S *et al.* Multilocus sequence typing of total-genome-
340 sequenced bacteria. *J Clin Microbiol* 2012;50: 1355-61.

341 20 Letunic I, Bork P. Interactive tree of life (iTOL) v3: an online tool for the display and
342 annotation of phylogenetic and other trees. *Nucleic acids research*. 2016;44:W242-5.

343 21 Partridge SR, Di Pilato V, Doi Y *et al.* Proposal for assignment of allele numbers for mobile
344 colistin resistance (*mcr*) genes. *J Antimicrob Chemother* 2018;73:2625-30.

345 22 Cafiso V, Stracquadanio S, Lo Verde F *et al.* Colistin resistant *A. baumannii*: genomic and
346 transcriptomic traits acquired under colistin therapy. *Front Microbiol* 2019;9:3195.

347 23 Nowak J, Zander E, Stefanik D *et al.* High incidence of pandrug-resistant *Acinetobacter*
348 *baumannii* isolates collected from patients with ventilator-associated pneumonia in Greece, Italy
349 and Spain as part of the MagicBullet clinical trial. *J Antimicrob Chemother* 2017;72:3277-82.

350 24 Mustapha MM, Li B, Pacey MP, Mettus RT *et al.* Phylogenomics of colistin-susceptible and
351 resistant XDR *Acinetobacter baumannii* *J Antimicrob Chemother* 2018;73:2952-9.

352 25 Abdulzahra AT, Khalil MA, Elkhatab WF. First report of colistin resistance among
353 carbapenem-resistant *Acinetobacter baumannii* isolates recovered from hospitalized patients in
354 Egypt. *New Microbes New Infect* 2018;26:53-8.

355 26 Nurtop E, Bayındır Bilman F, Menekse S *et al.* Promoters of Colistin Resistance in
356 *Acinetobacter baumannii* Infections *Microb Drug Resist* 2019;25:997-1002.

357 27 Haeili M, Kafshdouz M, Feizabadi MM. Molecular mechanisms of colistin resistance among
358 pandrug-resistant isolates of *Acinetobacter baumannii* with high case-fatality rate in intensive
359 care unit patients *Microb Drug Resist* 2018;24:1271-6.

360 28 Charretier Y, Diene SM, Baud D *et al.* Colistin heteroresistance and involvement of the
361 PmrAB regulatory system in *Acinetobacter baumannii*. *Antimicrob Agents Chemother*
362 2018;62:e00788-18.

363 29 Gerson S, Lucaßen K, Wille J *et al.* Diversity of amino acid substitutions in PmrCAB
364 associated with colistin resistance in clinical isolates of *Acinetobacter baumannii*. *Int J*
365 *Antimicrob Agents* 2020;55:105862.

366 30 Arroyo LA, Herrera CM, Fernandez L *et al.* The pmrCAB operon mediates polymyxin
367 resistance in *Acinetobacter baumannii* ATCC 17978 and clinical isolates through
368 phosphoethanolamine modification of lipid A. *Antimicrob Agents Chemother* 2011;55:3743-51.

369 31 Oikonomou O, Sarrou S, Papagiannitsis CC *et al.* Rapid dissemination of colistin and
370 carbapenem resistant *Acinetobacter baumannii* in Central Greece: mechanisms of resistance,
371 molecular identification and epidemiological data. *BMC Infect Dis* 2015;15:559.

32 Vijayakumar S, Mathur P, Kapil A *et al.* Molecular characterization & epidemiology of carbapenem-resistant *Acinetobacter baumannii* collected across India. *Indian J Med Res* 2019;149:240.

33 Snyman Y, Whitelaw AC, Reuter S *et al.* Clonal expansion of colistin-resistant *Acinetobacter baumannii* isolates in Cape Town, South Africa. *Int J Infect Dis* 2020;91:94-100.

34 Carretero-Ledesma M, García-Quintanilla M, Martín-Peña R *et al.* Phenotypic changes associated with Colistin resistance due to Lipopolysaccharide loss in *Acinetobacter baumannii*. *Virulence* 2018;9:930-42.

35 Lesho E, Yoon EJ, McGann P *et al.* Emergence of colistin-resistance in extremely drug-resistant *Acinetobacter baumannii* containing a novel *pmrCAB* operon during colistin therapy of wound infections. *J Infect Dis* 2013;208:1142-51.

36 Nhu NT, Riordan DW, Nhu TD *et al.* The induction and identification of novel Colistin resistance mutations in *Acinetobacter baumannii* and their implications. *Sci Rep* 2016;6:1-8.

37 Ma F, Shen C, Zheng X *et al.* Identification of a novel plasmid carrying *mcr-4.3* in an *Acinetobacter baumannii* strain in China. *Antimicrob Agents Chemother* 2019;63:e00133-19.

38 Martins-Sorenson N, Snestrud E, Xavier DE *et al.* A novel plasmid-encoded *mcr-4.3* gene in a colistin-resistant *Acinetobacter baumannii* clinical strain. *J Antimicrob Chemother* 2020;75:60-4.

39 Bitar I, Medvecky M, Gelbicova T *et al.* Complete Nucleotide Sequences of *mcr-4.3*-Carrying Plasmids in *Acinetobacter baumannii* Sequence Type 345 of Human and Food Origin from the Czech Republic, the First Case in Europe. *Antimicrob Agents Chemother* 2019;63:e01166-19.

40 Gurjar M. Colistin for lung infection: an update. J Intensive Care 2015;3:3.

41 Satlin MJ, Lewis JS, Weinstein MP *et al.* Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) position statements on polymyxin B and colistin clinical breakpoints. Clin Infect Dis 2020 Feb 13.

42 Bhagwat SS, Periasamy H, Takalkar SS *et al.* The novel β -lactam enhancer zidebactam augments the in vivo pharmacodynamic activity of cefepime in a neutropenic mouse lung *Acinetobacter baumannii* infection model. Antimicrob Agents Chemother 2019;63:e02146-18.

Figure Legends

Fig 1. Phylogenetic tree of clinical isolates of colistin resistant and colistin susceptible *A. baumannii*. The outer ring shows isolates belonging to different International Clones (ICs), the middle rings depicts chromosomal mutations in both colistin resistant and susceptible isolates and the stars in the inner ring represents the colistin susceptibility of the clinical isolates of *A. baumannii*

Fig 2: Graphical representation of genetic arrangement of *pmrC* homologue, *eptA* with upstream presence of *ISAbal* among complete genomes of colistin resistant *A. baumannii* (A, B, C and D). The direction of arrow represents the orientation, *eptA* are shown as red-dotted arrow, *ISAbal* as purple-dotted arrow, *pmrA* as blue arrow, *pmrB* as green arrow. The genetic arrangement of isolate E has *eptA* without *ISAbal*

413 **Fig 3:** Graphical representation of genetic arrangement of *pmrC* homologue, *eptA* among
 414 complete genomes of colistin susceptible *A. baumannii*. The genetic arrangement of: A. *eptA*
 415 without insertion element, B. *eptA* with IS26 transposase, C. *eptA* with IS6 like element, D & E.
 416 *eptA* with IS4 transposase, F. *eptA* with IS256 transposase and G. *eptA* with IS66 transposase.
 417 The direction of arrow represents the orientation. *eptA* are shown by red-dotted arrow, IS26, IS6,
 418 IS256 and IS66 transposases as light green arrow, *pmrA* as blue arrows and *pmrB* as green arrow

tree scale: 1

Figure

Figure

Figure

Table 1. MIC range, MIC₅₀ and MIC₉₀ of colistin for clinical isolates of *A. baumannii*

Colistin MIC (µg/ml)	Blood (n=314)	Respiratory (n=900)
MIC range	0.12 - 64	0.12 - 64
MIC ₅₀	0.5	1
MIC ₉₀	2	2

Table 2. Cumulative findings of various colistin resistance mechanisms among complete genomes of *A. baumannii* (n=28)

Isolate ID	Colistin susceptibility	Mutation profile					IS <i>AbaI-epiA</i>	Insertional inactivation of <i>lpxACD</i> by IS <i>AbaI1</i>	Complete <i>lpxACD</i> genes	Accession number
		<i>lpxA</i>	<i>lpxC</i>	<i>lpxD</i>	<i>pmrA</i>	<i>pmrB</i>				
AB01	Pan-susceptible	Y131H	C120R, N287D	Absent	Absent	Absent	Absent	Absent	Present	CP040080
AB02	Resistant	Y131H	C120R, N287D	E117K	Absent	A444V	Present	Absent	<i>lpxA</i> absent	CP035672
AB03	Resistant	Y131H	C120R, N287D, D159N	Q4K	Absent	Absent	Present	Absent	Present	CP050388
AB04	Resistant	Y131H	Absent	E117K	M12I	A138T, A444V	Present	Absent	<i>lpxC</i> absent	CP040040
AB05	Resistant	Y131H	C120R, N287D	E117K	M12I	A138T, A444V	Present	Absent	Present	CP040047
AB06	Resistant	Y131H	C120R, N287D, P148S	Absent	Absent	G315D	Absent	Absent	Present	CP040050
AB07	Susceptible	Y131H	C120R, N287D, P148S	E117K	Absent	A444V	Absent	Absent	<i>lpxA</i> absent	CP035930
AB08	Susceptible	Y131H	C120R, N287D	Q4K	Absent	Absent	Absent	Absent	Present	CP038500
AB09	Susceptible	Y131H	C120R, N287D	Absent	Absent	Absent	Absent	Absent	Present	CP038644
AB10	Susceptible	Y131H	C120R, N287D	Q4K	Absent	Absent	Absent	Absent	Present	CP040053
AB11	Susceptible	Y131H	C120R, N287D, D159N	Q4K	Absent	Absent	Absent	Absent	Present	CP040056
AB12	Susceptible	Y131H	C120R, N287D	E117K	Absent	A444V	Absent	Absent	Present	CP040084
AB13	Susceptible	Y131H	C120R, N287D, P148S	E117K	Absent	A444V	Absent	Absent	Present	CP040087
AB14	Susceptible	Y131H	C120R, N287D	Absent	Absent	Absent	Absent	Absent	Present	CP040259
AB05	Susceptible	Y131H	C120R, N287D	E117K	Absent	A444V	Absent	Absent	Present	CP050385
AB16	Susceptible	Y131H	C120R, N287D	V63I, G166S	Absent	Absent	Absent	IS <i>AbaI1</i> present without disruption	Present	CP050523

AB17	Susceptible	Y131H	C120R, N287D	Absent	Absent	Absent	Absent	<i>ISAbal1</i> present without disruption	Present	CP050400
AB18	Susceptible	Y131H	C120R, N287D	E117K	Absent	A444V	Absent	Absent	Present	CP050390
AB19	Susceptible	Y131H	C120R, N287D, P148S	E117K	Absent	A444V	Absent	Absent	Present	CP050410
AB20	Susceptible	Y131H	Absent	E117K	M12I	A138T, A444V	Absent	Absent	Present	CP050412
AB21	Susceptible	Y131H	C120R, N287D	E117K	M12I	A138T, A444V	Absent	Absent	Present	CP050415
AB22	Susceptible	Y131H	C120R, N287D, P148S	Absent	Absent	G315D	Absent	Absent	Present	CP050425
AB23	Susceptible	Y131H	C120R, N287D	Q4K	Absent	Absent	Absent	Absent	Present	CP050432
AB24	Susceptible	Y131H	C120R, N287D	Absent	Absent	Absent	Absent	Absent	Present	CP051474
AB25	Susceptible	Y131H	C120R, N287D	Q4K	Absent	Absent	Absent	Absent	Present	CP050526
AB26	Susceptible	Y131H	C120R, N287D	E117K	Absent	A444V	Absent	Absent	Present	CP050401
AB27	Susceptible	Y131H	C120R, N287D, P148S	E117K	Absent	A444V	Absent	Absent	Present	CP050421
AB28	Susceptible	Y131H	C120R, N287D	V63I, G166S	Absent	Absent	Absent	Absent	Present	CP050403