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Genotyping of *Acinetobacter baumannii* isolates from a tertiary care hospital in Cochin, South India

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Abstract

Acinetobacter baumannii poses a significant challenge in healthcare settings across the globe, with isolates exhibiting carbapenem resistance at unprecedented rates. Here, we characterized a collection of *A. baumannii* isolates (*n*=64) recovered during the period September 2020 – November 2021 at a teaching hospital in Cochin, South India. The species identity of the isolates was confirmed with $bla_{\text{OXA-51-like}}$ PCR. The major carbapenemase determinants identified were $bla_{\text{OXA-23-like}}$ (45, 70.3%) and $bla_{\text{NDM-1}}$ (31, 48.4%); co-occurrence of these genes was also observed in 27 (42.2%) isolates. Other resistance genes identified included bla_{PER} (34, 53.1%), *aac(6')-Ib-cr* (42, 65.6%), *qnrS* (25, 39.1%), *sul1* (32, 50%), *sul2* (33, 51.6%), *strA/strB* (36, 56.3%), *aphA1-Iab* (35, 54.7%) and *tetB* (32, 50%). Mapping PCR revealed the insertion element, IS*AbaI* upstream of $bla_{\text{OXA-23-like}}$ in all isolates possessing this gene. Concerning disinfectant resistance, all isolates carried the quaternary ammonium compound (QAC) resistance gene, *qacEΔ1*. Minimal inhibitory concentration (MIC) of benzalkonium chloride was high among the isolates and ranged from 8 to 128 µg ml^{-1}. However, low MICs were observed for chlorhexidine and triclosan, with the majority (54, 80.6%) of isolates showing an MIC of 2 µg ml^{-1} for chlorhexidine and all isolates exhibiting MICs of ≤0.125 µg ml^{-1} for triclosan. Further, all isolates were strong biofilm-producers, as assessed by the crystal violet-based microtitre plate assay. The *Apa*I-pulsed-field gel electrophoresis (PFGE) revealed the multi-clonal nature of the isolates, with 16 clusters and 16 unique pulsotypes identified at a cut-off of 80%. In short, this study provides useful data on the molecular features of *A. baumannii* from this region, which could be helpful to assess the local epidemiology of this pathogen and also to devise infection control strategies.

DATA SUMMARY

The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files.

INTRODUCTION

Acinetobacter baumannii has emerged as an important nosocomial pathogen that mainly infects critically ill patients. However, cases of community-acquired infections which are usually associated with preexisting conditions such as old age, diabetes, cancer, obstructive pulmonary disorders, alcoholism etc. have also been reported [1]. The Centres for Disease Control and Prevention (CDC) has included carbapenem-resistant *Acinetobacter* in the category of 'urgent threat', thus calling for increased surveillance and prevention activities to manage this pathogen [2]. Infections caused by *A. baumannii* include pneumonia, bacteremia, urinary tract infections, meningitis, skin/wound infections etc. Closely related and phenotypically indistinguishable species of

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Keywords: *Acinetobacter baumannii*; carbapenem resistance; disinfectant resistance; biofilm formation; pulsed-field gel electrophoresis.
Abbreviations: Acb, *Acinetobacter calcoaceticus-baumannii*; ARGs, antibiotic resistance genes; ATCC, american type culture collection; BSA, bovine serum albumin; CFU, colony-forming unit; CLSI, Clinical and Laboratory Standards Institute; CRAB, carbapenem-resistant *Acinetobacter baumannii*; ESBL, extended-spectrum beta-lactamase; IS, insertion sequence; KARSAP, Kerala Antimicrobial Resistance Strategic Action Plan; MDR, multidrug-resistant; MIC, minimal inhibitory concentration; OD, optical density; PBP, penicillin-binding protein; PFGE, pulsed-field gel electrophoresis; QAC, quaternary ammonium compound; TBE, tris-borate-EDTA; TE, tris-EDTA; TSA, tryptic soy agar; UPGMA, unweighted pair group method with arithmetic mean; UTI, urinary tract infection; XDR, extensively drug-resistant.
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A supplementary table is available with the online version of this article.

Acinetobacter have been clustered into what is called *Acinetobacter calcoaceticus-baumannii* (Acb) complex. The Acb complex comprises five pathogenic species namely *A. baumannii*, *A. nosocomialis*, *A. pittii*, *A. seifertii* and *A. dijkshoorniae* as well as the non-pathogenic *A. calcoaceticus*. The epidemiological success of *A. baumannii* is attributed mainly to its ability to persist in the environment and to acquire resistance to various drugs including carbapenems. Resistance to desiccation, disinfection and oxidative stress, as well as the ability to form biofilms are the hallmarks of this pathogen that enable it to thrive successfully in the apparently harsh healthcare environments [3].

Acinetobacter baumannii has acquired resistance to virtually all antibiotics including fluoroquinolones, cephalosporins, aminoglycosides and carbapenems. Incidence of carbapenem-resistant *A. baumannii* (CRAB) has increased alarmingly in the past decade, threatening one of the last resort treatment options for this pathogen. A recent multicentre surveillance study from India documented carbapenem susceptibility rate as low as ~12% for *A. baumannii* isolates [4]. Intense usage of carbapenems has been shown to facilitate the persistence of CRAB isolates in intensive care units, causing sustained outbreaks [5]. Various intrinsic and acquired mechanisms have been known to confer carbapenem resistance to *A. baumanni* which include production of carbapenemases, increased expression of efflux pumps, alterations in porins and modifications in penicillin-binding proteins (PBPs) [6]. Genes encoding diverse carbapenem-hydrolysing β-lactamases belonging to class A (bla_{KPC} and bla_{GES}), class B (bla_{NDM}, bla_{SIM}, bla_{IMP} and bla_{VIM}) and class D ($bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24\text{-}like}$, $bla_{OXA\text{-}40\text{-}like}$, $bla_{OXA\text{-}51\text{-}like}$, $bla_{OXA\text{-}58\text{-}like}$ and $bla_{OXA\text{-}143\text{-}like}$) have been reported in *A. baumannii* [7, 8]. However, class D enzymes such as OXA-23 have been by far the most common carbapenemases among CRAB isolates from many countries including India [9–11]. The intrinsic $bla_{OXA\text{-}51}$ gene codes for a weak carbapenemase and is often employed as a marker for the species identification of *A. baumannii* [7]. The expression and mobilization of OXA enzymes are known to be regulated by certain insertion sequences (IS) such as the IS*AbaI* associated with bla_{OXA} genes in *Acinetobacter* [6]. Owing to the increased prevalence of carbapenem resistance, limited treatment options are available for managing severe CRAB infections. This mainly includes mono- and combination therapy involving colistin, tigecycline, carbapenem, rifampicin, amikacin, sulbactam and minocycline. However, toxicity, suboptimal pharmacokinetics and/or increasing resistance, associated with one or more of these agents pose significant challenges to effectively managing the infection.

Understanding the molecular epidemiology of *Acinetobacter* is vital to monitor its spread and devise containment strategies in hospitals and long term care facilities. In this study, we characterized a collection of isolates of *A. baumannii* from a tertiary care hospital in the city of Cochin, India with respect to their antibiotic resistance features and genetic diversity.

METHODS

Study setting and isolates

This study analysed a total of 64 isolates of *A. baumannii* collected as part of the routine clinical testing at the Government Medical College, Ernakulam, India during the period September 2020–November 2021. The study was approved by the Institutional Ethical Committee of Government Medical College Ernakulam vide ref. no. IEC40/2020 dated 16 November 2020. The isolates were recovered from cases of pneumonia (*n*=17), urinary tract infection (UTI) (*n*=16), surgical site infection (*n*=7), diabetic foot (*n*=7), sepsis (*n*=7), COPD exacerbation (*n*=2), abscess (*n*=2), pleural effusion (*n*=1), biliary peritonitis (*n*=1), cellulitis (*n*=1), burn infection (*n*=1), gangrene (*n*=1) and pyoderma (*n*=1). The isolates were identified as *A. baumannii* by BD Phoenix M50 automated system (BD Diagnostics, USA) and later confirmed with a PCR for $bla_{OXA\text{-}51\text{-}like}$ gene, which is intrinsic to this species [12].

Antibiotic susceptibility testing

Phenotypic susceptibility of the isolates towards selected antibiotics (amikacin, cefepime, ceftriaxone, ceftazidime, ciprofloxacin, gentamicin, imipenem, levofloxacin, meropenem, piperacillin-tazobactam, tetracycline and trimethoprim-sulfamethoxazole) was determined using the ID/AST combo panel, NMIC/ID55 in BD Phoenix M5O automated system. The panel consists of an ID side containing wells with dried substrates for bacterial identification and an AST side containing wells having varying concentrations of antibiotics. Briefly, bacterial colonies from pure cultures (grown on trypticase soy agar- TSA) were transferred to the BD Phoenix ID broth and the inoculum density was adjusted to 0.5 McFarland using BD PhoenixSpec nephelometer. Twenty five microlitres of the adjusted ID broth suspension was then transferred to the BD Phoenix AST broth with the AST indicator which is a resazurin-based dye. The suspensions (ID broth inoculum and AST broth inoculum) were then poured to the corresponding fill ports in the panel. Panels were sealed and loaded into the instrument for incubation at 35 °C for around 16 h. BD Phoenix system is connected to the data management software BD EpiCenter (version V7.22/V6.41A) which analyses test results and generates reports. EpiCenter implements CLSI breakpoints and provides sensitive/resistant/intermediate (S/I/R) interpretations based on the MIC of the tested antibiotics.

PCR screening for antibiotic resistance genes

Genomic DNA was extracted from all the cultures using DNeasy Blood and Tissue kit (Qiagen, Germany) following the manufacturer's instructions and used for subsequent PCR analyses. All PCRs were carried out in a final volume of 25 µl containing

1X Jumpstart RedTaq Ready Mix (Sigma-Aldrich, USA), primers in the required concentrations and 2 µl of the extracted DNA. Screening for the four groups of bla_{OXA} carbapenemase genes ($bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24\text{-}like}$, $bla_{OXA\text{-}51\text{-}like}$ and $bla_{OXA\text{-}58\text{-}like}$) was performed using a previously described multiplex PCR [12]. The isolates were also screened for other carbapenemase genes (bla_{KPC}, bla_{IMP}, bla_{VIM}, $bla_{SIM\text{-}1}$ and $bla_{NDM\text{-}1}$), various extended-spectrum β-lactamase (ESBL) genes (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$ and bla_{PER}), fluoroquinolone resistance genes (*qnrA, qnrB, qnrS, aac(6′)-Ib-cr, qepA, oqxA* and *oqxB*), aminoglycoside resistance genes (*strA, strB,* and *aphA1-Iab*), sulphonamide resistance genes (*sul1* and *sul2*) and tetracycline resistance genes (*tetA* and *tetB*) using primers and PCR conditions described elsewhere [13–17] (Table S1, available in the online version of this article).

Detection and mapping the position of IS*AbaI*

Presence of the insertion sequence, IS*AbaI* was investigated in all the isolates as described previously [18]. In order to map the position of IS*AbaI* in relation to $bla_{OXA\text{-}51}$ and $bla_{OXA\text{-}23}$ genes, two independent PCRs were carried out using the following combinations of primer pairs: (a) IS*AbaI*-forward primer (IS*AbaI*F) and $bla_{OXA\text{-}51\text{-}like}$ reverse primer (OXA-51-likeR); and (b) IS*AbaI*F and $bla_{OXA\text{-}23\text{-}like}$ reverse primer (OXA-23-likeR) [19].

Determination of MICs of disinfectants

Minimal inhibitory concentrations (MICs) of three disinfectants namely benzalkonium chloride, chlorhexidine and triclosan were determined using agar dilution method according to CLSI guidelines [20]. Stock solutions of 5120 µg ml^{-1} of the disinfectants (all purchased from Sigma, USA) were prepared in appropriate solvents: benzalkonium choloride in water, chlorhexidine in dimethyl formamide and triclosan in ethanol. Suspensions of pure bacterial cultures were prepared in 3 ml of 0.85% saline by using overnight colonies from TSA plates, and the preparation was adjusted to a turbidity equivalent to that of 0.5 McFarland standard. The suspensions were further ten-fold diluted using 0.85% saline, and 2 µl (an inoculum of ~10^4 c.f.u. per spot) was spotted on Mueller-Hinton agar plates containing disinfectants at various concentrations ranging from 0.125 to 512 µg ml^{-1}. The reference strain *Escherichia coli* ATCC25922 was used as quality control. Plates were incubated at 37 °C for 24 h and MIC was recorded as the lowest concentration of the disinfectant that completely inhibited bacterial growth.

Screening for disinfectant resistance genes

All the isolates were screened for disinfectant resistance genes such as *qacE, qacEΔ1, qacG* and *qacH* using previously described PCRs [21].

Screening for biofilm formation

Biofilm-forming ability of the isolates was assessed using crystal violet assay as previously described by Naves *et al.* with slight modifications [22]. Briefly, 20 µl of the each overnight culture of *A. baumannii* was inoculated to 96-well microtitre plate containing 180 µl of brain heart infusion broth with 2% glucose. The experiment was performed in triplicates and uninoculated wells served as negative control. The plate was incubated at 37 °C for 24 h without agitation. After incubation, the medium was carefully removed and the wells were rinsed with 0.85% saline to remove the unbound bacteria. Methanol was added to the well and incubated for 20 min to fix the biofilm. Plate was then air-dried, 200 µl of 0.1% crystal violet solution was added to the wells and incubated for 20 min. The residual dye was removed and the wells were rinsed with distilled water. After drying, the bound crystal violet was re-solubilized in 200 µl of 33% (v/v) glacial acetic acid for 15 min. The optical density was measured at 585 nm using the microplate reader. The biofilm-forming ability was evaluated by comparing the average OD value (OD_T) for each tested strain with the cut-off OD (OD_C), which is the average OD of the negative control plus three times the standard deviation of the ODs of negative control wells. Isolates with $OD_T > 4\,OD_C$ were considered as strong biofilm-producers.

Molecular subtyping by pulsed-field gel electrophoresis

The genetic relatedness among the isolates was assessed by *ApaI*-pulsed-field gel electrophoresis (PFGE) [23]. Pure cultures of *A. baumannii* were inoculated on TSA plates and incubated at 37 °C overnight. A loopful of the bacteria was taken from the agar surface and suspended in 3 ml of cell suspension buffer (100 mM Tris, 100 mM EDTA, pH 8). Cell suspensions were adjusted to an OD_{610} of 0.8–1 (~10^9 cells ml^{-1}). Two hundred microlitres of the cell suspensions were mixed with 10 µl of proteinase K (20 mg ml^{-1}) in 1.5 ml microcentrifuge tubes. It was then mixed with 200 µl of molten megabase agarose (Bio-Rad, Germany) prepared in TE buffer (10 mM Tris, 1 mM EDTA, pH 8). The agarose-cell suspension was immediately dispensed into the wells of reusable plug moulds (Bio-Rad, Germany) and the plugs were allowed to solidify for 10 min. Plugs were then transferred to a 50 ml polypropylene tubes containing 5 ml cell lysis buffer (50 mM Tris, 50 mM EDTA, 1% sarcosine, pH 8) and 25 µl of proteinase K (20 mg ml^{-1}). Lysis was carried out at 55 °C in a shaking incubator for 2 h with vigorous agitation (180 r.p.m.). After lysis, buffer was removed carefully and the plugs were washed twice in sterile distilled water and thrice in TE buffer at 55 °C in a shaking incubator. Plugs were stored in fresh TE buffer at 4 °C until use.

A slice from each plug was cut using a sterile coverslip and subjected to a pre-digestion incubation in 200 µl 1X restriction buffer (NEB, Germany) for 15 min at 25 °C . This restriction buffer was replaced with 200 µl of fresh restriction buffer containing 30 U

of *ApaI* (NEB, Germany) and 100 µg ml^{-1} bovine serum albumin (BSA). The tubes were incubated at 25 °C for 2 h. The restriction mixture was then replaced with 200 µl of 0.5 X TBE buffer (10X TBE has 0.9 M Tris, 0.9 M boric acid and 20 mM EDTA, pH 8.3). Plugs were loaded into 1% agarose gel (Megabase agarose, Bio-Rad) prepared in 0.5 X TBE buffer. Plugs of Salmonella serotype Braenderup H9812 digested with *XbaI* (50 U per plug, NEB) were used as molecular markers. Electrophoresis was performed in a CHEF-Mapper XA system (Bio-Rad, United States) with 0.5 X TBE as running buffer. Electrophoretic conditions included a voltage of 6 V cm^{-1}, pulse time ranging from 5 to 20 s, an included angle of 120° and a total run time of 19 h. The gels were stained in ethidium bromide (1 µg ml^{-1}) and visualized under UV illumination. Gel images were exported to Bionumerics software package 7.6.3 (Applied Maths, Belgium) and cluster analysis was performed by Unweighted Pair Group Method with Arithmetic mean (UPGMA) and Dice coefficient with a tolerance setting of 1.5%. Genetic similarity of the isolates was assessed at a cut-off of 80%.

RESULTS

Antibiotic susceptibility of the isolates

The identity of the isolates (*n*=64) as *A. baumannii* was confirmed by a positive PCR for $bla_{\text{OXA-51-like}}$ gene. Percentage of resistance among the isolates towards different antibiotics was as follows: amikacin (41, 64.1%), cefepime (45, 70.3%), ceftriaxone (55, 85.9%), ceftazidime (45, 70.3%), ciprofloxacin (43, 67.2%), gentamicin (39, 60.9%), imipenem (38, 59.4%), levofloxacin (39, 60.9%), meropenem (40, 62.5%), piperacillin-tazobactam (43, 67.2%), tetracycline (38, 59.4%) and trimethoprim-sulfamethoxazole (37, 57.8%). Table 1 shows the various resistance patterns observed among the isolates.

Prevalence of antibiotic resistance genes

Among the carbapenemase genes screened, the intrinsic $bla_{\text{OXA-51-like}}$ was present in all the isolates (*n*=64), and $bla_{\text{OXA-23-like}}$ and $bla_{\text{NDM-1}}$ were detected in 45 (70.3%) and 31 (48.4%) isolates respectively. The ESBL gene, bla_{PER} was identified in 34 (53.1%) isolates. Other resistance genes detected were *qnrS* (25, 39.1%) and *aac(6')-Ib-cr* (42, 65.6%) coding for fluoroquinolone resistance; *sul1* (32, 50%) and *sul2* (33, 51.6%) conferring resistance to sulphonamides; *strA* (36, 56.3%), *strB* (36, 56.3%) and *aphA1-Iab* (35, 54.7%) conferring resistance to aminoglycosides; and the tetracycline resistance gene, *tetB* (32, 50%). A master chart detailing the phenotypic and genotypic resistance features of the isolates is provided as Table 2.

IS*AbaI mapping*

All isolates were positive for the insertion element, IS*AbaI*. Mapping PCRs revealed the position of IS*AbaI* relative to $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-51}}$ genes. All the 45 isolates that were positive for both $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-51}}$, gave amplification with a product size of approximately 1.6 kb in a PCR using the forward primer for IS*AbaI* and the reverse primer for $bla_{\text{OXA-23}}$. This indicated that, in these isolates, the IS element is associated with, and upstream of $bla_{\text{OXA-23}}$. Concerning the association of IS*AbaI* with $bla_{\text{OXA-51}}$, only 23 (51%) out of the 45 gave a band (of around 1.2 kb) in a PCR involving forward primer for IS*AbaI* and the reverse primer for $bla_{\text{OXA-51}}$. This revealed the presence of IS*AbaI* upstream of $bla_{\text{OXA-51}}$ in those isolates. However, none of the isolates with $bla_{\text{OXA-51}}$ as the sole bla_{OXA} gene had IS*AbaI* upstream of it.

Incidence of disinfectant resistance

In general, MIC of benzalkonium chloride was high and ranged from 8 to 128 µg ml^{-1}. Of all isolates, 24 (37.5%), 18 (28.1%) and 21 (32.8%) had MICs of 128, 64 and 32 µg ml^{-1} respectively for benzalkonium chloride; a single isolate had an MIC of 8 µg ml^{-1}. In the case of chlorhexidine, the majority (53, 82.8%) of isolates showed an MIC of 2 µg ml^{-1}; whereas, six (9.4%) and five (7.8%) isolates had MICs of 1.0 and 0.5 µg ml^{-1} respectively. Invariably, all the isolates exhibited a low MIC (≤0.125 µg ml^{-1}) of triclosan. Among the disinfectant resistance genes screened, *qacEΔ1* was the only one detected and was present in all the isolates.

Biofilm formation

In this study, the biofilm-forming capacities of the isolates were assessed by crystal violet assay using a 96-well plate. The OD_{585} of the test isolates (OD_T) ranged from 0.563 to 3.481 (Fig. 1) and the cut-off OD (OD_C) was determined to be 0.12. The OD_T of all isolates were >4OD_C, indicating that they are strong biofilm producers.

PFGE genotyping

PFGE revealed considerable heterogeneity among the isolates (Fig. 2). At 80% similarity cut-off, 48 out of the 64 isolates formed 16 clusters, with each cluster having two or more isolates. The rest of the isolates (*n*=16) had unique banding patterns and therefore did not form part of any cluster. The two largest clusters had only six isolates each; all of them were positive for $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-23-like}}$ and $bla_{\text{NDM-1}}$ genes. Moreover, one of these clusters contained isolates recovered in a 1 month period in 2021, indicating a possible cross-transmission. Isolates with the carbapenemase genes, $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-23-like}}$ were distributed over 12 and 23 clusters respectively.

Table 1. Antibiotic resistance patterns observed among the isolates of *A. baumannii* in the present study

Sl no.	Phenotypic resistance pattern	no. of resistant isolates (%)
1	AK-CPM-CTR-CAZ-CIP-GEN-IP-LE-MP-PTZ-TET-SXT	19 (29.7%)
2	CTR	8 (12.5%)
3	AK-CPM-CTR-CAZ-CIP-GEN-IP-LE-MP-PTZ-TET	7 (10.9%)
4	CPM-CAZ-CIP-GEN-IP-LE-MP-PTZ-SXT	3 (4.7%)
5	TET	1 (1.6%)
6	AK-CPM-CTR-CAZ-CIP-GEN-IP-LE-MP-PTZ- SXT	1 (1.6%)
7	AK-CPM-CTR-CAZ-GEN-LE-PTZ-SXT	1 (1.6%)
8	AK-CPM-CTR-CAZ-CIP-IP-MP-PTZ	1 (1.6%)
9	CPM-CTR-CAZ-LE-TET-SXT	1 (1.6%)
10	AK-CPM-CTR-CAZ-CIP -IP-MP-PTZ-TET-SXT	1 (1.6%)
11	AK-CTR-CAZ-GEN-SXT	1 (1.6%)
12	CPM-CTR-CAZ-CIP-GEN	1 (1.6%)
13	AK-CPM-CTR-CIP-IP-LE-MP-PTZ-TET-SXT	1 (1.6%)
14	AK-CPM-CTR-CIP-GEN-PTZ	1 (1.6%)
15	AK-CPM-CTR-CAZ-CIP-IP-LE-MP-PTZ-TET-SXT	1 (1.6%)
16	CAZ-GEN-MP-SXT	1 (1.6%)
17	CTR-PTZ-SXT	1 (1.6%)
18	CPM-CTR	1 (1.6%)
19	CTR-TET-SXT	1 (1.6%)
20	AK-CPM-CTR-CAZ-CIP-GEN-IP-MP-PTZ-TET	1 (1.6%)
21	AK-CTR-CAZ-CIP-GEN-LE-SXT	1 (1.6%)
22	CTR-TET	1 (1.6%)
23	AK-CPM-CTR-CAZ-CIP-LE-MP-PTZ-TET-SXT	1 (1.6%)
24	AK-CPM-CTR-CAZ-CIP-GEN-LE-PTZ-TET-SXT	1 (1.6%)
25	AK-CPM-CTR-CAZ-GEN-IP-LE-MP-PTZ-TET	1 (1.6%)
26	CTR-CIP	1 (1.6%)
27	AK-CPM-CAZ-CIP-GEN-IP-LE-MP-PTZ-SXT	1 (1.6%)

AK, Amikacin; CAZ, Ceftazidime; CIP, Ciprofloxacin; CPM, Cefepime; CTR, Ceftriaxone; GEN, Gentamicin; IP, Imipenem; LE, Levofloxacin; MP, Meropenem; PTZ, Piperacillin-Tazobactam; SXT, Trimethoprim-Sulfamethoxazole; TE, Tetracycline.

DISCUSSION

This study provides the first data on the comprehensive molecular characterization of a collection of *Acinetobacter baumannii* isolates from the southern state of Kerala, India. Kerala was the first Indian state to implement an AMR containment plan called the Kerala Antimicrobial Resistance Strategic Action Plan (KARSAP) in 2018, and the state's first antibiogram report has recently been published [24]. In the present study, phenotypic carbapenem resistance was nearly 63%, and multidrug-resistant (MDR) phenotype (non-susceptibility to ≥1 agent in ≥3 antimicrobial categories) was observed in 76.5% of the isolates. Further, 36% of the isolates belonged to the category of extensively drug-resistant (XDR) (non-susceptibility to ≥1 agent in all but ≤2 antimicrobial categories) bacteria. The classification of isolates as XDR/MDR was made according to the criteria laid down by Magiorakos *et al.* [25]. As we have analysed only a fewer number of isolates, our findings cannot be generalized and extrapolated to other settings in the region. It is noteworthy that, in our study, 53% of the isolates from cases of pneumonia were resistant to all the antibiotics tested. In the case of isolates implicated in UTI, 56% were resistant to eight or more antibiotics. Overall, almost 30% of the isolates

Table 2. Master chart showing the phenotypic and genotypic features of the isolates

Isolate information					Resistance phenotype*										Resistance genes*												
Sl no	ID	Sample type	Infection	Date of collection	AK	CPM	CTR	CAZ	CIP	GEN	IP	LE	MP	PTZ	TET	SXT	bla_{NDM}	$bla_{OXA-51-like}$	$bla_{OXA-23-like}$	bla_{NDM-1}	*qnrS*	*aac(6')-Ib-cr*	*Sul1*	*Sul2*	*strA*	*strB*	*aphA1-1AB*	*tetB*	*qacEΔ1*
1	SP25	Sputum	Pneumonia	25-02-2021																									
2	U304	Urine	Urinary tract infection	09-04-2021																									
3	U312	Urine	Urinary tract infection	10-04-2021																									
4	P231	Pus	Non healing ulcer, diabetic foot	25-04-2021																									
5	P115	Pus	Surgical site infection	18-03-2021																									
6	SP95	Sputum	Pneumonia	28-04-2021																									
7	P235	Pus	Burns	30-04-2021																									
8	SP102	Sputum	Pneumonia	13-05-2021																									
9	SP108	Sputum	Pneumonia	27-05-2021																									
10	U490	Urine	Urinary tract infection	06-06-2021																									
11	U439	Urine	Urinary tract infection	19-05-2021																									
12	SP119	Sputum	Pneumonia	09-06-2021																									
13	SP120	Sputum	Pneumonia	15-06-2021																									
14	SP123	Sputum	Pneumonia	23-06-2021																									
15	U1327	Urine	Urinary tract infection	06-09-2020																									
16	U1355	Urine	Urinary tract infection	06-10-2020																									
17	P512	Pus	Abscess thigh, diabetic	26-10-2020																									
18	P513	Pus	Surgical site infection	23-10-2020																									
19	P514	Pus	Surgical site infection	24-10-2020																									
20	BAC824	Blood	Pneumonia	05-11-2020																									
21	SP690	Sputum	Pneumonia	06-11-2020																									
22	U1400	Urine	Urinary tract infection	06-11-2020																									
23	P517	Pus	Surgical site infection	11-11-2020																									
24	SP693	Sputum	Pneumonia	10-11-2020																									
25	BAC841	Blood	Blood stream infection	09-11-2020																									
26	SP696	Sputum	Pneumonia	14-11-2020																									
27	SP697	Sputum	Pneumonia	14-11-2020																									
28	U1435	Urine	Urinary tract infection	23-11-2020																									
29	U1477	Urine	Urinary tract infection	15-12-2020																									
30	U1475	Urine	Urinary tract infection	14-12-2020																									
31	U601	Urine	Urinary tract infection	11-07-2021																									
32	SP156	Sputum	Pneumonia	02-08-2021																									
33	SP137	Sputum	Sepsis	15-07-2021																									
34	BAC548	Blood	Blood stream infection	06-08-2021																									
35	P297	Pus	Diabetic foot	12-07-2021																									
36	P344	Pus	Diabetic foot	10-08-2021																									
37	P347	Pus	diabetic foot	14-08-2021																									
38	SP202	Sputum	Pneumonia	27-08-2021																									
39	BAC667	Blood	Sepsis	03-09-2021																									
40	OF43	Pleural Fluid	Pleural effusion	24-08-2021																									
41	P395	Pus	Soft tissue abscess on knee	02-09-2021																									
42	P411	Pus	Cellulitis	04-09-2021																									
43	BAC733	Blood	Sepsis	08-09-2021																									
44	P428	Pus	Recurrent pyoderma	12-09-2021																									
45	SP243	Sputum	COPD-exacerbation	17-09-2021																									
46	U1116	Urine	Urinary tract infection	25-09-2021																									
47	U1122	Urine	Urinary tract infection	27-09-2021																									
48	OF52	Bilious Aspiration	Biliary peritonitis	27-09-2021																									
49	SP254	Sputum	Pneumonia	27-09-2021																									
50	U1162	Urine	Urinary tract infection	30-09-2021																									
51	SP261	Sputum	Pneumonia	03-10-2021																									
52	U1188	Urine	Acute exacerbation of COPD	04-10-2021																									
53	P525	Pus	Diabetic foot, septic shock	08-10-2021																									
54	U1258	Urine	Urinary tract infection	09-10-2021																									
55	SP276	Sputum	Pneumonia	11-10-2021																									
56	BAC962	Blood	Sepsis	15-10-2021																									
57	P556	Pus	Surgical wound infection	16-10-2021																									
58	P575	Pus	Surgical wound infection	19-10-2021																									
59	U1332	Urine	Urinary tract infection	14-10-2021																									
60	P599	Pus	Diabetic foot	26-10-2021																									
61	P620	Pus	Diabetic ulcer	26-10-2021																									
62	P64	Pus	Surgical wound infection	02-11-2021																									
63	P700	Pus	Fournier's gangrene	13-11-2021																									
64	BAC1135	Blood	Sepsis	13-11-2021																									

*Darker and lighter shades indicate the presence and absence of the respective resistance phenotype/genes respectively.
AK, Amikacin; CAZ, Ceftazidime; CIP, Ciprofloxacin; CPM, Cefepime; CTR, Ceftriaxone; GEN, Gentamicin; IP, Imipenem; LE, Levofloxacin; MP, Meropenem; PTZ, Piperacillin-Tazobactam; SXT, Trimethoprim-Sulfamethoxazole; TE, Tetracycline.

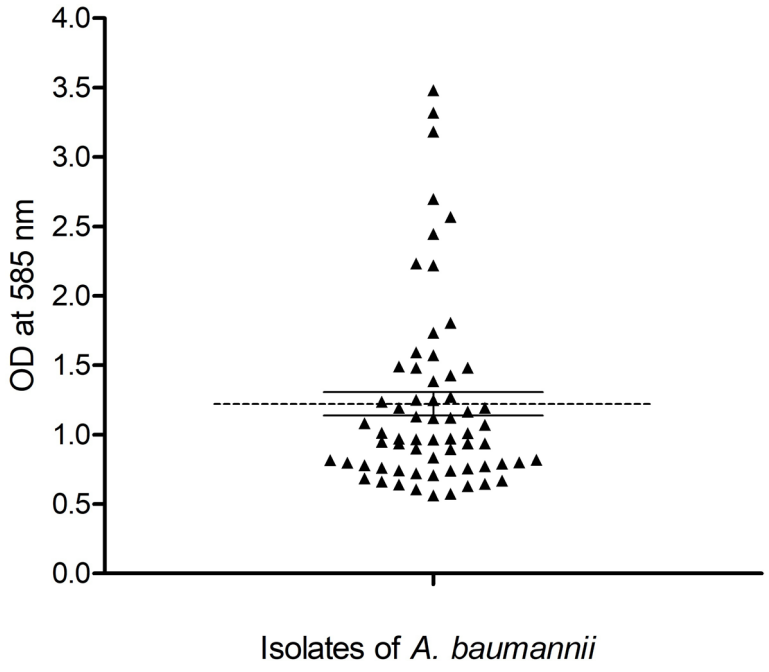
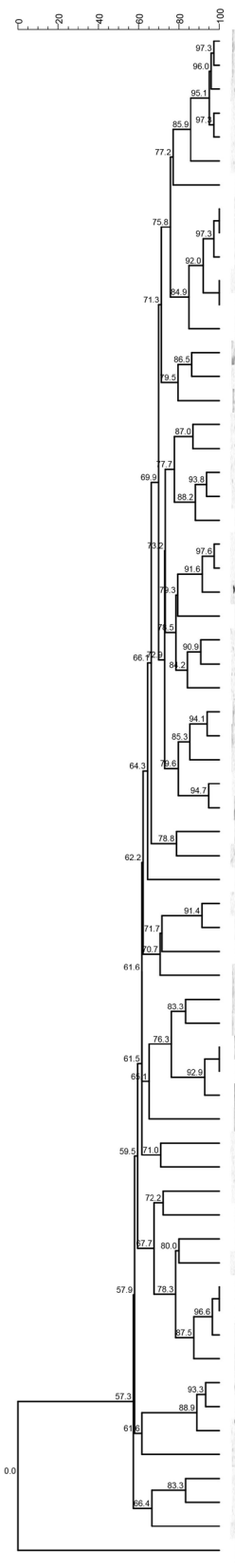


Fig. 1. Scatter plot showing the biofilm-forming capacities of the *A. baumannii* (*n*=64) isolates as determined by crystal violet assay. Each symbol represents the average OD_{585} of triplicate wells of individual isolates. The data is presented as mean±SEM, with the dashed line indicating the mean OD_{585} of all isolates.

showed resistance to all the 12 antibiotics tested. A similar 1 year-long study in 2017 at a major teaching hospital in Kerala reported a carbapenem resistance of 41% among the isolates of *Acinetobacter baumannii* implicated in lower respiratory tract infections [26]. One of the earlier studies from India which screened *A. baumannii* isolates from eight major hospitals during 2014–2017 reported 100% resistance to imipenem and meropenem [27]. According to the National Antimicrobial Resistance Surveillance Network, imipenem resistance was 62% among a large collection of *A. baumannii* isolates from major hospitals across India during January–December 2020 [28]. Also, more than 50% of those isolates exhibited resistance to all the tested drugs, except minocycline to which resistance was 26%.

All our isolates carried $bla_{\text{OXA-51-like}}$, which is intrinsic to *A. baumannii* and codes for a weak carbapenemase. Carbapenem MICs for *A. baumannii* isolates which overexpress $bla_{\text{OXA-51-like}}$ due to the insertion of IS*AbaI* have been found to be similar to those for isolates producing acquired carbapenemases [29]. While it is expected that the association of IS*AbaI* with $bla_{\text{OXA-51-like}}$ confers resistance to carbapenems, carbapenem-susceptible isolates having IS*AbaI*/$bla_{\text{OXA-51}}$ genotype have also been described [30]. In our study, out of the 15 isolates with $bla_{\text{OXA-51-like}}$ as the sole carbapenemase determinant, only two exhibited phenotypic carbapenem resistance. However, they did not have IS*AbaI* upstream of $bla_{\text{OXA-51-like}}$, suggesting the presence of non-carbapenemase mediated resistance in those isolates. The major genetic determinant of carbapenem resistance among our isolates was the $bla_{\text{OXA-23-like}}$ gene followed by $bla_{\text{NDM-1}}$. A similar trend has been reported among CRAB isolates in many previous studies from India [9–11, 31–33]. OXA-23 group was the first group of carbapenem-hydrolysing oxacillinases to be identified in *A. baumannii* and the genes encoding these enzymes are mostly plasmid-borne [7]. OXA-23 has disseminated worldwide and is found predominantly in isolates from the USA, India and South Korea [34]. The expression of $bla_{\text{OXA-23-like}}$ is largely regulated by IS*Aba1* or IS*Aba4* found upstream of this gene by providing promoter sequences. The data on the high frequency of IS*AbaI*/$bla_{\text{OXA-23}}$ structure among our isolates align with previous reports from many parts of the world including India [27, 35, 36].

The relatively higher proportion (48.4%) of the metallo-β-lactamase gene, $bla_{\text{NDM-1}}$ in the present study is a cause of concern. We also observed the co-occurrence of $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-23-like}}$ in 27 (42.2%) isolates; notably, these isolates showed a broad resistance profile, with 74% of them being resistant to no less than 10 out of the 12 antibiotics tested. Isolates co-harbouring these genes have been reported sporadically from different countries including India [11, 37–40]. NDM-1 was first reported in 2008 in a *Klebsiella pneumoniae* isolate from India; however, NDM-1-positive *Acinetobacter baumannii* isolates from an Indian hospital in 2005 have been retrospectively identified as the earliest known NDM-1-carrying bacteria [41]. Since then, this gene has been acquired by various Gram-negative pathogens and has spread globally. A recent study has shown that the global dissemination of this gene is driven mainly by transposon jumps, with plasmid horizontal transfers playing more of a role in local transmission [42].



Isolate ID	Beta-lactamse genes	Age/Sex	Infection	Date of Collection
P115	PER, OXA-51-like, OXA-23 like, NDM-1	38/F	surgical site infection	18-03-2021
SP95	OXA-51-like, OXA-23 like, NDM-1	69/F	pneumonia	28-04-2021
U601	OXA-51-like, OXA-23 like, NDM-1	52/F	UTI	11-07-2021
U1475	PER, OXA-51-like, OXA-23 like, NDM-1	20/F	UTI	14-12-2020
SP156	OXA-51-like, OXA-23 like, NDM-1	68/M	pneumonia	02-08-2021
P344	OXA-51-like, OXA-23 like, NDM-1	60/M	diabetic foot	10-08-2021
SP254	OXA-51-like	56/M	pneumonia	27-09-2021
OF43	OXA-51-like, OXA-23 like, NDM-1	73/M	pleural effusion	24-08-2021
P411	OXA-51-like, OXA-23 like, NDM-1	78/M	cellulitis	04-09-2021
BAC733	OXA-51-like, OXA-23 like, NDM-1	86/M	sepsis	08-09-2021
SP202	OXA-51-like, OXA-23 like, NDM-1	72/F	pneumonia	27-08-2021
SP261	OXA-51-like, OXA-23 like, NDM-1	73/F	pneumonia	03-10-2021
OF52	OXA-51-like, OXA-23 like, NDM-1	33/F	biliary peritonitis	27-09-2021
U490	PER, OXA-51-like, OXA-23 like	56/F	UTI	06-06-2021
P620	PER, OXA-51-like, OXA-23 like	74/F	diabetic foot	26-10-2021
SP25	PER, OXA-51-like	65/M	pneumonia	25-02-2021
P525	OXA-51-like, OXA-23 like, NDM-1	70/M	diabetic foot	08-10-2021
U1258	OXA-51-like, NDM-1	67/M	UTI	09-10-2021
SP276	OXA-51-like, NDM-1	68/M	pneumonia	11-10-2021
P64	PER, OXA-51-like, NDM-1	27/F	surgical site infection	02-11-2021
U1116	PER, OXA-51-like, NDM-1	62/M	UTI	25-09-2021
SP137	OXA-51-like, OXA-23 like	newborn/M	sepsis	15-07-2021
BAC667	OXA-51-like, OXA-23 like	78/F	sepsis	03-09-2021
SP123	PER, OXA-51-like, OXA-23 like	51/M	pneumonia	23-06-2021
SP690	PER, OXA-51-like, OXA-23 like	22/F	pneumonia	06-11-2020
BAC962	OXA-51-like, OXA-23 like, NDM-1	61/F	sepsis	15-10-2021
P599	PER, OXA-51-like, OXA-23 like, NDM-1	55/F	diabetic fot	26-10-2021
U1332	PER, OXA-51-like, OXA-23 like, NDM-1	53/F	UTI	14-10-2021
U304	PER, OXA-51-like, OXA-23 like	30/F	UTI	09-04-2021
SP108	PER, OXA-51-like, OXA-23 like	19/F	pneumonia	23-05-2021
U439	PER, OXA-51-like, OXA-23 like	29/F	UTI	19-05-2021
U1435	PER, OXA-51-like, OXA-23 like	30/F	UTI	23-11-2020
U1327	PER, OXA-51-like, OXA-23 like	25/F	UTI	06-09-2020
BAC824	OXA-51-like, OXA-23 like, NDM-1	63/F	pneumonia	05-11-2020
SP120	PER, OXA-51-like, OXA-23 like	57/F	pneumonia	15-06-2021
U1122	OXA-51-like, OXA-23 like	33/F	UTI	27-09-2021
P297	OXA-51-like	60/M	diabetic foot	12-07-2021
P395	OXA-51-like	26/M	soft tissue abscess	02-09-2021
SP696	PER, OXA-51-like, OXA-23 like	65/M	pneumonia	14-11-2020
U312	PER, OXA-51-like, OXA-23 like	25/F	UTI	10-04-2021
P231	PER, OXA-51-like, OXA-23 like, NDM-1	59/F	diabetic foot	25-04-2021
P235	PER, OXA-51-like, OXA-23 like, NDM-1	63/F	burn	30-04-2021
P512	OXA-51-like, OXA-23 like, NDM-1	43/M	thigh abscess	26-10-2020
P517	PER, OXA-51-like, OXA-23 like, NDM-1	59/M	surgical site infection	11-11-2020
P513	OXA-51-like, OXA-23 like, NDM-1	79/M	surgical site infection	23-10-2020
U1355	PER, OXA-51-like	24/F	UTI	06-10-2020
BAC548	OXA-51-like	75/M	sepsis	06-08-2021
SP119	PER, OXA-51-like	57/F	pneumonia	09-06-2021
U1477	OXA-51-like, OXA-23 like	34/F	UTI	15-12-2020
U1188	OXA-51-like, OXA-23 like	51/F	COPD exacerbation	04-10-2021
U1162	OXA-51-like	24/F	UTI	30-09-2021
P575	PER, OXA-51-like	25/F	surgical site infection	19-10-2021
SP693	PER, OXA-51-like	65/F	pneumonia	10-11-2020
BAC841	PER, OXA-51-like	73/M	sepsis	09-11-2020
U1400	PER, OXA-51-like	20/F	UTI	06-11-2020
P514	PER, OXA-51-like	55/F	surgical site infection	24-10-2020
P428	OXA-51-like	38/M	recurrent pyoderma	12-09-2021
SP243	PER, OXA-51-like, OXA-23 like, NDM-1	65/M	COPD exacerbation	17-09-2021
P347	PER, OXA-51-like	54/M	diabetic foot	14-08-2021
SP102	PER, OXA-51-like, OXA-23 like, NDM-1	32/M	pneumonia	13-05-2021
P700	PER, OXA-51-like, OXA-23 like, NDM-1	76/M	fournier's gangrene	13-11-2021
BAC1135	PER, OXA-51-like, OXA-23 like, NDM-1	60/M	sepsis	13-11-2021
P556	OXA-51-like, OXA-23 like, NDM-1	64/F	surgical site infection	16-10-2021
SP697	OXA-51-like, OXA-23 like	75/M	pneumonia	14-11-2020

Fig. 2. *Apa*I-PFGE dendrogram of *A. baumanni* isolates analysed in the present study.

In line with the previous studies from India, the predominant ESBL gene identified among our isolates was bla_{PER-1}, with an incidence rate of 53% [10, 27]. However, in contrast to those studies, none of our isolates carried other ESBL genes such as bla_{TEM}. Apparently, PER is the most common ESBL encountered in *A. baumannii* and PER-1-producing strains have been reported from many parts of the world including the USA, Europe, Asia and the Middle East [43]. Recently, it has been shown that PER-like β-lactamases contribute significantly to reduced susceptibility to cefiderocol, a novel siderophore cephalosporin with broad spectrum activity against a variety of Gram-negative pathogens [44].

Besides carbapenemase-encoding genes, various other resistance genes were also identified among our isolates. This included genes conferring resistance to fluoroquinolones (*qnrS* and *aac(6′)-Ib-cr*), aminoglycosides (*strA, strB* and *aphA1-Iab*), sulphonamides (*sul1* and *sul2*) and tetracycline (*tetB*). A recent whole genome-based study on *A. baumannii* isolates (*n*=47) from India revealed the presence of as many as 79 types of ARGs of intrinsic and acquired nature [45]. Similar to our observations, high prevalence of *sul2* and *tetB* among *A. baumannii* has been reported in previous studies from India [32, 45], Pakistan [46] and Algeria [47]. Besides conferring resistance to tetracycline and doxycycline, the *tetB* gene mediates resistance to minocycline, a widely employed drug for CRAB infections with the ability to overcome most of the resistance mechanisms affecting other tetracyclines including tigecycline [48]. The streptomycin resistance genes, *strA*/*strB* have been frequently found in *A. baumannii* and it has been shown that a group of highly-related conjugative plasmids harbouring *sul2* and *strA*/*strB* are widely distributed in *A. baumannii* clones including the globally disseminated global clone 1 (GC1) [49]. The *aphA1-Iab* gene, also known as *aph(3′)-Ic* and *aphA7* encodes an aminoglycoside-O-phosphotransferase and has sporadically been reported in *A. baumannii* isolates; however, reports are scanty on the prevalence of this gene in *Acinetobacter* species from India[50, 51]. Emergence of plasmid-mediated quinolone resistance (PMQR) is a cause of serious concern as fluoroquinolones are one of the safer and widely employed treatment options for serious Gram-negative infections. As observed for the isolates from our study, high prevalence *aac(6′)-Ib-cr*, and co-occurrence of this gene with *qnrS* has been reported in *A. baumannii* isolates from a recent study at a teaching hospital in South India [52]. However, our isolates did not harbour other PMQR determinants such as *oqxAB* or *qepA* genes.

In addition to being resistant to multiple antibiotics, the isolates from this study showed reduced susceptibility to the quaternary ammonium compound (QAC), benzalkonium chloride, with 98% of the isolates exhibiting MICs in the range of 32–128 μg ml^{-1}. QACs are disinfectants with little toxicity and high microbicidal activity over a wide range of pH, and are extensively used in clinical and industrial settings [21]. Unlike antibiotics, there are no standard breakpoints determined for disinfectants to categorize the isolates as susceptible/intermediate/resistant. A study by Rajamohan *et al.* reported higher MICs (30–120 μg ml^{-1}) of benzalkonium chloride for the majority of *A. baumannii* isolates analysed; however, Babaei *et al.* from Malaysia reported MICs as low as 0.02–0.2 μg ml^{-1} [53, 54]. It is noteworthy here that the in-use concentration of benzalkonium chloride in clinical settings is usually well in excess of these MICs, with disinfectant solutions containing at least 500 μg ml^{-1} of the active compound [55]. However, monitoring disinfectant and antiseptic resistance is crucial for devising infection control strategies for a pathogen like *A. baumannii* which is known to have environmental reservoirs in hospital and can infect critically ill patients.

Among the various QAC resistance genes, *qacE* and its deletion mutant *qacEΔ1,* which mediate resistance by an efflux transporter, have been frequently encountered in Gram-negative bacteria including *A. baumannii* [56]. Interestingly, all isolates from the present study carried *qacEΔ1*; previous studies have reported similar prevalence rates for this gene in *A. baumannii* [57, 58]. However, concerning the effect of *qacE*/*qacEΔ1* on the MICs of QACs, different studies have shown varying results. While studies by Babaei *et al.,* Kucken *et al.,* and Nor A'shimi *et al.* showed no correlation between the presence of *qacE*/*qacEΔ1* and increased MICs to benzalkonium chloride, Liu *et al.* reported that the carriage of *qacE* (but not *qacEΔ1*) was significantly associated with higher MIC (64 μg ml^{-1}) [54, 55, 59]. Regarding chlorhexidine and triclosan, low MICs were observed among our isolates, indicating the potent antimicrobial activity of these agents. Low prevalence (~3%) of triclosan resistance in *A. baumannii,* with the majority of isolates showing MIC of <1 μg ml^{-1}, has been reported in previous studies involving large collection of isolates [60, 61].

We also assessed the biofilm-forming ability of the isolates employing the microtitre plate assay and found that all isolates belonged to the category of strong biofilm-formers. This has serious implications for infection control as biofilm-residing bacteria are often recalcitrant to antibiotic treatments and can cause persistent or recurring infections. Previous studies have shown that biofilm formation rate is higher in *A. baumannii* compared to other *Acinetobacter* species [62, 63]. Biofilm is a major virulence determinant of *A. baumannii* and is mainly regulated by the abaI/abaR quorum sensing system [64]. Also, many factors such as the biofilm-associated protein (BAP), the exopolysaccharide poly-N-acetyl glucosamine (PNAG), the extended-spectrum β-lactamse PER-1, Chaperone-Usher secretion system (CUS), and the outermembrane protein A (OmpA) have been shown to contribute to biofilm formation in *A. baumannii* [65]. However, we have not investigated the genetic basis of biofilm formation in our isolates.

The molecular subtyping of the isolates was performed by PFGE which revealed high genetic heterogeneity among the isolates (*n*=64), with no more than six isolates forming clusters at 80% cut-off. This clearly indicates the multi-clonal nature of *A. baumannii* isolates circulated in the hospital during the study period. Apparently, the type of infection or the antibiogram of the isolates did not influence PFGE clustering; however, 50% of the isolates from the two largest clusters were resistant to all the antibiotics tested. Also, the presence or absence of the major carbapenemase and ESBL determinants (bla_{OXA-23}, bla_{NDM-1} and bla_{PER-1}) was not associated with PFGE patterns. Nevertheless, in some instances, isolates recovered within a few days apart from

each other and with identical ARG profile were found to be clustered, possibly indicating clonal dissemination of the strains. A study by Jain *et al.* has demonstrated significant cross transmission of *A. baumannii* strains between the patients and the ICU environment at an Indian hospital by employing PFGE [66]. *Apa*I-PFGE is considered the gold standard for studying the local epidemiology of *A. baumannii* and has been widely used in outbreak investigations [67]. However, the discriminatory power of PFGE to accurately distinguish *A. baumannii* isolates with close genetic backgrounds has been found low, with other approaches such as multi-locus sequence typing (MLST_oxford) and core-genome MLST showing better resolution capabilities [68].

In conclusion, this study demonstrates a diverse population of clinical *A. baumannii* from this region, with $bla_{\text{OXA-23}}$ and $bla_{\text{NDM-1}}$ as major carbapenemase determinants. The high proportion of isolates exhibiting MDR phenotype, reduced disinfectant susceptibility and strong biofilm-forming ability is a cause for concern. Continuous surveillance and stringent infection control measures are key to manage this pathogen in nosocomial settings.

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Author contributions
V.R.: Investigation, methodology, writing-original draft; A.V.: Investigation, methodology, writing-review & editing; A.P.M.: Investigation, methodology; J.L.: Conceptualization, supervision, resources; G.K.S.: Conceptualization, supervision, project administration, resources, writing-review & editing.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study was approved by the institutional ethical committee of Government Medical College, Ernakulam vide ref. no. IEC40/2020 dated 16 November 2020.

References

1. Dexter C, Murray GL, Paulsen IT, Peleg AY. Community-acquired *Acinetobacter baumannii*: clinical characteristics, epidemiology and pathogenesis. *Expert Rev Anti Infect Ther* 2015;13:567–573.
2. CDC. *Antibiotic Resistance Threats in the United States, 2019*. Atlanta, GA: U.S. Department of Health and Human Services, CDC; 2019.
3. Harding CM, Hennon SW, Feldman MF. Uncovering the mechanisms of *Acinetobacter baumannii* virulence. *Nat Rev Microbiol* 2018;16:91–102.
4. Indian Council of Medical Research (ICMR). Antimicrobial Resistance Research and Surveillance Network Annual Report. Division of Epidemiology and Communicable Disease. ICMR. NewDelhi; 2021. https://iamrsn.icmr.org.in/index.php/resources/amr-icmr-data
5. Sharma S, Das A, Banerjee T, Barman H, Yadav G, *et al*. Adaptations of carbapenem resistant *Acinetobacter baumannii* (CRAB) in the hospital environment causing sustained outbreak. *J Med Microbiol* 2021;70:001345.
6. Kyriakidis I, Vasileiou E, Pana ZD, Tragiannidis A. *Acinetobacter baumannii* antibiotic resistance mechanisms. *Pathogens* 2021;10:1–31.
7. Evans BA, Amyes SGB. OXA β-lactamases. *Clin Microbiol Rev* 2014;27:241–263.
8. Nguyen M, Joshi SG. Carbapenem resistance in *Acinetobacter baumannii*, and their importance in hospital-acquired infections: a scientific review. *J Appl Microbiol* 2021;131:2715–2738.
9. Vijayakumar S, Gopi R, Gunasekaran P, Bharathy M, Walia K, *et al*. Molecular characterization of invasive carbapenem-resistant *Acinetobacter baumannii* from a tertiary care hospital in South India. *Infect Dis Ther* 2016;5:379–387.
10. Vijayakumar S, Mathur P, Kapil A, Das BK, Ray P, *et al*. Molecular characterization & epidemiology of carbapenem-resistant *Acinetobacter baumannii* collected across India. *Indian J Med Res* 2019;149:240–246.
11. Kumar S, Patil PP, Singhal L, Ray P, Patil PB, *et al*. Molecular epidemiology of carbapenem-resistant *Acinetobacter baumannii* isolates reveals the emergence of $bla_{\text{OXA-23}}$ and $bla_{\text{NDM-1}}$ encoding international clones in India. *Infect Genet Evol* 2019;75:103986.
12. Woodford N, Ellington MJ, Coelho JM, Turton JF, Ward ME, *et al*. Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. *Int J Antimicrob Agents* 2006;27:351–353.
13. Lee K, Yum JH, Yong D, Lee HM, Kim HD, *et al*. Novel acquired metallo-beta-lactamase gene, bla(SIM-1), in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. *Antimicrob Agents Chemother* 2005;49:4485–4491.
14. Dallenne C, Da Costa A, Decré D, Favier C, Arlet G. Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in Enterobacteriaceae. *J Antimicrob Chemother* 2010;65:490–495.
15. Kaase M, Szabados F, Wassill L, Gatermann SG. Detection of carbapenemases in Enterobacteriaceae by a commercial multiplex PCR. *J Clin Microbiol* 2012;50:3115–3118.
16. Wajid M, Awan AB, Saleemi MK, Weinreich J, Schierack P, *et al*. Multiple drug resistance and virulence profiling of *Salmonella enterica* serovars typhimurium and enteritidis from poultry farms of Faisalabad, Pakistan. *Microb Drug Resist* 2019;25:133–142.
17. Sivaraman GK, Rajan V, Vijayan A, Elangovan R, Prendiville A, *et al*. Antibiotic resistance profiles and molecular characteristics of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from shrimp aquaculture farms in Kerala, India. *Front Microbiol* 2021;12:622891.
18. Segal H, Garny S, Elisha BG. Is IS(ABA-1) customized for *Acinetobacter*? *FEMS Microbiol Lett* 2005;243:425–429.
19. Turton JF, Ward ME, Woodford N, Kaufmann ME, Pike R, *et al*. The role of ISAba1 in expression of OXA carbapenemase genes in *Acinetobacter baumannii*. *FEMS Microbiol Lett* 2006;258:72–77.
20. Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Susceptibility Testing*. 30th. edn. Wayne, USA: CLSI Supplement M100; 2020.
21. Zou L, Meng J, McDermott PF, Wang F, Yang Q, *et al*. Presence of disinfectant resistance genes in *Escherichia coli* isolated from retail meats in the USA. *J Antimicrob Chemother* 2014;69:2644–2649.
22. Naves P, del Prado G, Huelves L, Gracia M, Ruiz V, *et al*. Correlation between virulence factors and in vitro biofilm formation by *Escherichia coli* strains. *Microb Pathog* 2008;45:86–91.

23. Seifert H, Dolzani L, Bressan R, van der Reijden T, van Strijen B, *et al*. Standardization and interlaboratory reproducibility assessment of pulsed-field gel electrophoresis-generated fingerprints of *Acinetobacter baumannii*. *J Clin Microbiol* 2005;43:4328–4335.
24. Singh S, Charani E, Devi S, Sharma A, Edathadathil F, *et al*. A road-map for addressing antimicrobial resistance in low- and middle-income countries: lessons learnt from the public private participation and co-designed antimicrobial stewardship programme in the State of Kerala, India. *Antimicrob Resist Infect Control* 2021;10:32.
25. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, *et al*. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* 2012;18:268–281.
26. Thomas B, Sukumaran P, Seema M M, George D. Antimicrobial resistance of *Acinetobacter baumannii* infection of lower respiratory tract and mortality – a cross-sectional study from a tertiary care teaching hospital in Kerala. *IJIRM* 2020;3:183–187.
27. Vijayakumar S, Anandan S, Ms DP, Kanthan K, Vijayabaskar S, *et al*. Insertion sequences and sequence types profile of clinical isolates of carbapenem-resistant *A. baumannii* collected across India over four year period. *J Infect Public Health* 2020;13:1022–1028.
28. National Centre for Disease Control. National Antimicrobial Resistance Surveillance Network (NARS-Net India). Annual Report; 2021. https://ncdc.gov.in/showfile.php?lid=811
29. A. Evans B, Hamouda A, G.B. Amyes S. The rise of carbapenem-resistant *Acinetobacter baumannii*. *Curr Pharm Des* 2013;19:223–238.
30. Pagano M, Martins AF, Machado ABMP, Barin J, Barth AL. Carbapenem-susceptible *Acinetobacter baumannii* carrying the ISAba1 upstream blaOXA-51-like gene in Porto Alegre, southern Brazil. *Epidemiol Infect* 2013;141:330–333.
31. Saranathan R, Vasanth V, Vasanth T, Shabareesh PRV, Shashikala P, *et al*. Emergence of carbapenem non-susceptible multidrug resistant *Acinetobacter baumannii* strains of clonal complexes 103(B) and 92(B) harboring OXA-type carbapenemases and metallo-β-lactamases in Southern India. *Microbiol Immunol* 2015;59:277–284.
32. Vijayakumar S, Jacob JJ, Vasudevan K, Mathur P, Ray P, *et al*. Genomic characterization of mobile genetic elements associated with carbapenem resistance of *Acinetobacter baumannii* from India. *Front Microbiol* 2022;13:869653.
33. Rose S, Shamanna V, Underwood A, Nagaraj G, Prasanna A, *et al*. Molecular dissection of carbapenem-resistant *Acinetobacter baumannii* circulating in Indian hospitals using Whole Genome Sequencing. *bioRxiv* 2021. DOI: 10.1101/2021.07.30.454432.
34. Ramirez MS, Bonomo RA, Tolmasky ME. Carbapenemases: transforming *Acinetobacter baumannii* into a yet more dangerous menace. *Biomolecules* 2020;10:720.
35. Agoba EE, Govinden U, Peer AKC, Osei Sekyere J, Essack SY. ISAba1 regulated OXA-23 carbapenem resistance in *Acinetobacter baumannii* strains in Durban, South Africa. *Microb Drug Resist* 2018;24:1289–1295.
36. Jun SH, Lee DE, Hwang HR, Kim N, Kwon KT, *et al*. Clonal evolution and antimicrobial resistance of *Acinetobacter baumannii* isolates from Korean hospitals over the last decade. *Infect Genet Evol* 2023;108:105404.
37. Nakazawa Y, Ii R, Tamura T, Hoshina T, Tamura K, *et al*. A case of NDM-1-producing *Acinetobacter baumannii* transferred from India to Japan. *J Infect Chemother* 2013;19:330–332.
38. Ramoul A, Loucif L, Bakour S, Amiri S, Dekhil M, *et al*. Co-occurrence of blaNDM-1 with blaOXA-23 or blaOXA-58 in clinical multidrug-resistant *Acinetobacter baumannii* isolates in Algeria. *J Glob Antimicrob Resist* 2016;6:136–141.
39. Miltgen G, Bour M, Allyn J, Allou N, Vedani T, *et al*. Molecular and epidemiological investigation of a colistin-resistant OXA-23-/NDM-1-producing *Acinetobacter baumannii* outbreak in the Southwest Indian Ocean Area. *Int J Antimicrob Agents* 2021;58:106402.
40. Zhao F, Liu H, Yao Y, Zhang L, Zhou Z, *et al*. Description of a rare pyomelanin-producing carbapenem-resistant *Acinetobacter baumannii* strain coharboring chromosomal OXA-23 and NDM-1. *Microbiol Spectr* 2022;10:e02144–22.
41. Jones LS, Toleman MA, Weeks JL, Howe RA, Walsh TR, *et al*. Plasmid carriage of bla NDM-1 in clinical *Acinetobacter baumannii* isolates from India. *Antimicrob Agents Chemother* 2014;58:4211–4213.
42. Acman M, Wang R, van Dorp L, Shaw LP, Wang Q, *et al*. Role of mobile genetic elements in the global dissemination of the carbapenem resistance gene bla_{NDM}. *Nat Commun* 2022;13:1131.
43. Potron A, Poirel L, Nordmann P. Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: mechanisms and epidemiology. *Int J Antimicrob Agents* 2015;45:568–585.
44. Poirel L, Sadek M, Nordmann P. Contribution of PER-Type and NDM-Type β-lactamases to cefiderocol resistance in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2021;65:e00877–21.
45. Kumkar SN, Kamble EE, Chavan NS, Dhotre DP, Pardesi KR. Diversity of resistant determinants, virulence factors, and mobile genetic elements in *Acinetobacter baumannii* from India: a comprehensive in silico genome analysis. *Front Cell Infect Microbiol* 2022;12:1130394.
46. Khurshid M, Rasool MH, Siddique MH, Azeem F, Naeem M, *et al*. Molecular mechanisms of antibiotic co-resistance among carbapenem resistant *Acinetobacter baumannii*. *J Infect Dev Ctries* 2019;13:899–905.
47. Khorsi K, Messai Y, Hamidi M, Ammari H, Bakour R. High prevalence of multidrug-resistance in *Acinetobacter baumannii* and dissemination of carbapenemase-encoding genes blaOXA-23-like, blaOXA-24-like and blaNDM-1 in Algiers hospitals. *Asian Pac J Trop Med* 2015;8:438–446.
48. Lashinsky JN, Henig O, Pogue JM, Kaye KS. Minocycline for the treatment of multidrug and extensively drug-resistant *A. baumannii*: a review. *Infect Dis Ther* 2017;6:199–211.
49. Hamidian M, Ambrose SJ, Hall RM. A large conjugative *Acinetobacter baumannii* plasmid carrying the sul2 sulphonamide and strAB streptomycin resistance genes. *Plasmid* 2016;87–88:43–50.
50. Cherubini S, Perilli M, Segatore B, Fazii P, Parruti G, *et al*. Whole-genome sequencing of ST2 *A. baumannii* causing bloodstream infections in COVID-19 patients. *Antibiotics* 2022;11:955.
51. Higgins PG, Hagen RM, Kreikemeyer B, Warnke P, Podbielski A, *et al*. Molecular epidemiology of carbapenem-resistant *Acinetobacter baumannii* isolates from Northern Africa and the Middle East. *Antibiotics* 2021;10:291.
52. Venkataramana GP, Lalitha AKV, Mariappan S, Sekar U. Plasmid-mediated fluoroquinolone resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. *J Lab Physicians* 2022;14:271–277.
53. Rajamohan G, Srinivasan VB, Gebreyes WA. Novel role of *Acinetobacter baumannii* RND efflux transporters in mediating decreased susceptibility to biocides. *J Antimicrob Chemother* 2010;65:228–232.
54. Babaei MR, Sulong A, Hamat RA, Nordin SA, Neela VK. Extremely high prevalence of antiseptic resistant quaternary ammonium compound E gene among clinical isolates of multiple drug resistant *Acinetobacter baumannii* in Malaysia. *Ann Clin Microbiol Antimicrob* 2015;14:1–5.
55. Kücken D, Feucht HH, Kaulfers PM. Association of qacE and qacE-Delta1 with multiple resistance to antibiotics and antiseptics in clinical isolates of Gram-negative bacteria. *FEMS Microbiol Lett* 2000;183:95–98.
56. Vijayakumar R, Sandle T. A review on biocide reduced susceptibility due to plasmid-borne antiseptic-resistant genes-special notes on pharmaceutical environmental isolates. *J Appl Microbiol* 2019;126:1011–1022.
57. Boost MV, Chan J, Shi G, Cho P. Effect of multipurpose solutions against *Acinetobacter* carrying QAC genes. *Optom Vis Sci* 2014;91:272–277.
58. Liu WJ, Fu L, Huang M, Zhang JP, Wu Y, *et al*. Frequency of antiseptic resistance genes and reduced susceptibility to biocides

in carbapenem-resistant *Acinetobacter baumannii*. *J Med Microbiol* 2017;66:13–17.

59. Nor A'shimi MH, Alattraqchi AG, Mohd Rani F, A Rahman NI, Ismail S, *et al*. Biocide susceptibilities and biofilm-forming capacities of *Acinetobacter baumannii* clinical isolates from Malaysia. *J Infect Dev Ctries* 2019;13:626–633.
60. Chen Y, Pi B, Zhou H, Yu Y, Li L. Triclosan resistance in clinical isolates of *Acinetobacter baumannii*. *J Med Microbiol* 2009;58:1086–1091.
61. Yu K, Zhang Y, Xu W, Zhang X, Xu Y, *et al*. Hyper-expression of the efflux pump gene adeB was found in *Acinetobacter baumannii* with decreased triclosan susceptibility. *J Glob Antimicrob Resist* 2020;22:367–373.
62. Martí S, Rodríguez-Baño J, Catel-Ferreira M, Jouenne T, Vila J, *et al*. Biofilm formation at the solid-liquid and air-liquid interfaces by *Acinetobacter* species. *BMC Res Notes* 2011;4:2–5.
63. Karunasagar A, Maiti B, Shekar M, Shenoy M S, Karunasagar I. Prevalence of OXA-type carbapenemase genes and genetic heterogeneity in clinical isolates of *Acinetobacter* spp. from Mangalore, India. *Microbiol Immunol* 2011;55:239–246.
64. Niu C, Clemmer KM, Bonomo RA, Rather PN. Isolation and characterization of an autoinducer synthase from *Acinetobacter baumannii*. *J Bacteriol* 2008;190:3386–3392.
65. Roy S, Chowdhury G, Mukhopadhyay AK, Dutta S, Basu S. Convergence of biofilm formation and antibiotic resistance in *Acinetobacter baumannii* infection. *Front Med* 2022;9:793615.
66. Jain M, Sharma A, Sen MK, Rani V, Gaind R, *et al*. Phenotypic and molecular characterization of *Acinetobacter baumannii* isolates causing lower respiratory infections among ICU patients. *Microb Pathog* 2019;128:75–81.
67. Zarrilli R, Pournaras S, Giannouli M, Tsakris A. Global evolution of multidrug-resistant *Acinetobacter baumannii* clonal lineages. *Int J Antimicrob Agents* 2013;41:11–19.
68. Li T, Yang Y, Yan R, Lan P, Liu H, *et al*. Comparing core-genome MLST with PFGE and MLST for cluster analysis of carbapenem-resistant *Acinetobacter baumannii*. *J Glob Antimicrob Resist* 2022;30:148–151.

Peer review history

VERSION 3

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000662.v3.1

Danielle Allen; Queen's University Belfast, School of Biological Sciences, UNITED KINGDOM, Belfast

Date report received: 26 October 2023
Recommendation: Accept

Comments: I am pleased to tell you that your article has now been accepted for publication in Access Microbiology. The work presented is clear and the arguments well formed. The manuscript is well written and contributes to the literature. Thank you for addressing all reviewers comments satisfactorily and in a timely manner.

Author response to reviewers to Version 2

Response to the comments (Revision 2)

Editor

1. Thank you for addressing all reviewers comments satisfactorily and in a timely manner. The revised manuscript was sent for review and further amendments are required before this manuscript can be accepted for publication. I agree with the reviewer regarding the biofilm formation section and think a plot showing the results for the whole isolate cohort should be included. I will be pleased to consider a revised manuscript along with your response to the reviewer.

Thank you for your time and effort in reviewing our manuscript. Following the reviewer's suggestion, a scatter plot showing the distribution of OD measurements pertaining to the biofilm experiment is now included in the manuscript.

Reviewer 2

1. I would like to commend the authors for their efforts in implementing my suggestions in the manuscript and respond to my comments. I really think this is a valuable contribution to the field. However, I still have concerns with the respect to the biofilm formation section. I agree that it reads much clearer now, but mentioning a range of values does not really show the results for the whole isolate cohort. I would like to insist in showing this represented in any form of plot (either a bar chart or a heat map would be appropriate).

Thank you! Your insights and suggestions have been immensely helpful in improving the quality of our work. We have now included a scatter plot in the manuscript to represent the biofilm production capacities of the isolates. Also, we identified minor errors in the OD values and have corrected them.

VERSION 2

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000662.v2.2

Danielle Allen; Queen's University Belfast, School of Biological Sciences, UNITED KINGDOM, Belfast

Date report received: 18 October 2023
Recommendation: Minor Amendment

Comments: Thank you for addressing all reviewers comments satisfactorily and in a timely manner. The revised manuscript was sent for review and further amendments are required before this manuscript can be accepted for publication. I agree with the reviewer regarding the biofilm formation section and think a plot showing the results for the whole isolate cohort should be included. I will be pleased to consider a revised manuscript along with your response to the reviewer.

Reviewer 1 recommendation and comments

https://doi.org/10.1099/acmi.0.000662.v2.1

Anonymous.

Date report received: 18 October 2023
Recommendation: Major Revision

Comments: I would like to commend the authors for their efforts in implementing my suggestions in the manuscript and respond to my comments. I really think this is a valuable contribution to the field. However, I still have concerns with the respect to the biofilm formation section. I agree that it reads much clearer now, but mentioning a range of values does not really show the results for the whole isolate cohort. I would like to insist in showing this represented in any form of plot (either a bar chart or a heat map would be appropriate).

Please rate the manuscript for methodological rigour
Very good

Please rate the quality of the presentation and structure of the manuscript
Very good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Author response to reviewers to Version 1

Reviewer 1

1. What is the study type?

This is a retrospective observational study on the molecular epidemiology of drug-resistant *Acinetobacter baumannii*isolates carried out at a single tertiary care centre for a period of 15 months from September 2020 to November 2021.

2. What are the study guidelines/checklists used to ensure validity?

In the present study, the identity and antibiogram of the isolates were determined using BD Phoenix M50. This is a well validated automated system and is widely employed in clinical laboratories across the globe. This system has an advanced data management software namely BD EpiCenter™(version V7.22/V6.41A) which implements CLSI breakpoints and provides sensitive/resistant/intermediate (S/I/R) interpretations based on the MIC of the tested antibiotics. To ensure the validity of susceptibility testing, quality control was performed along with the test isolates using the reference strain, *Escherichia coli*ATCC 25922. Concerning the PCR experiments, previously characterized strains from our laboratory served as positive controls for various resistance genes.

3. Authors should mention valid statements considering ethics in human research

This study was approved by the Institutional Ethical Committee of Government Medical College Ernakulam vide ref. no. IEC40/2020 dated 16.11.2020. This information has now been incorporated in the manuscript (L94-96)

4. Why authors used 64 isolates with bla$_{OXA-51}$?

bla$_{OXA-51}$was sought in the isolates to confirm their identity as *A. baumannii*as this carbapenemase gene is a species-specific locus.

5. What are the reference guidelines for susceptibility testing? How can the authors interpret the antibiogram to individual antibiotics?

The present study employed BD Phoenix M50 automated system for susceptibility testing. It uses broth microdilution for determining MICs of antibiotics and is equipped with an automated reading and interpretation technology for reporting susceptibility patterns. BD uses a rule based software tool called BDXpert which is in compliance with CLSI breakpoints and rules. The BDXpert system is updated regularly to incorporate the corrections or breakpoint changes brought about by international committees such as CLSI. After the run, the system generates a lab report for each isolate with information on the species identity and antibiotic susceptibility pattern.

6. What are the definitions for MDR and XDR if any?

We followed the criteria suggested by Magiorakos et al., 2012 for categorizing the isolates as MDR or XDR. An isolate is considered MDR if it is non-susceptible to at least 1 agent in≥3 antimicrobial categories (aminoglycosides, antipseudomonal carbapenems, antipsseudomonal fluoroquinolones, antipseudomonal penicillins + beta-lactamase inhibitors, extended-spectrum cephalosporins, folate pathway inhibitors, penicillins + beta-lactamase inhibitors, polymixins and tetracyclines). XDR is defined as non-susceptibility to at least 1 agent in all but two or fewer antimicrobial categories mentioned above.

7. Why authors do not confirm CR via conventional methods?

We have determined carbapenem resistance in our isolates based on the MIC results generated by BD Phoenix M50 system. Isolates of *A. baumannii*with MIC≥8μg/ml of imipenem or meropenem is regarded as carbapenem-resistant (CLSI). As MIC by broth microdilution is a recommended method for the reliable detection of carbapenem resistance, we did not employ other conventional methods. Also, the isolates were tested for major carbapenem resistance determinants by PCR.

8. What is the reference for MIC of disinfectants? Why authors do not use quality control in such experiment?

In contrast to antibiotics for which reference limits are well defined by the CLSI to categorize the isolates as susceptible or resistant, there are no standard breakpoints/guidelines available for disinfectants. Thus we compared our results with those from the available reports to gain an insight on the susceptibility profile of our isolates towards the tested disinfectants. The reference *strain E. coli*ATCC25922 was used as quality control. This information has now been included in the manuscript (L150-151)

9. Discussion is too long

Though the discussion is a bit lengthy, we believe it maintains a logical and coherent flow and making changes could disrupt the overall narrative and clarity. However, we are open to discussing any major concerns the reviewer may have regarding the discussion section.

10. Where are limitations of the study?

The major limitation of the study is the less number of isolates analysed (L274-275). Also, more valuable information on the epidemiology of this pathogen in the study setting would have been obtained if environmental samples from the hospital wards and ICUs had been screened. Nevertheless, our findings provide the first data on the molecular features of clinical *A. baumannii*isolates from this region and would be beneficial to clinicians and various stakeholders to support the efforts to improve antibiotic use.

Reviewer 2

1. L100-104: the authors used an automated system to determine the antibiotic susceptibility profile of the isolate collection. In all honesty, I am not familiar with this method, as might be the case of many potential readers. Could you please clarify in this paragraph the culture media used, if it follows the EUCAST or CLSI guidelines and the criteria for determining an isolate sensitive or resistant to a certain antibiotic?

BD Phoenix M50 automated system performs bacterial identification and susceptibility testing using cartridge-based broth microdilution method. In the present study, we employed NMIC-ID55, an ID/AST combo panel designed for Gram negative bacterial species. The panel consists of an ID side containing wells with dried substrates for bacterial identification and an AST side containing wells having varying concentrations of antibiotics. Briefly, bacterial colonies from pure cultures (grown on trypticase soy agar) were transferred to the BD Phoenix ID broth and the inoculum density was adjusted to 0.5 McFarland using BD PhoenixSpec nephelometer. Twenty five microliters of the adjusted ID broth suspension was then transferred to the

BD Phoenix AST broth with the AST indicator which is a resazurin-based dye. The suspensions (ID broth inoculum and AST broth inoculum) were then poured to the corresponding fill ports in the panel. Panels were sealed and loaded into the instrument for incubation at 35°C for around 16 h. BD Phoenix system is connected to BD EpiCenter™(version V7.22/V6.41A), the data management software to analyze test results and generate reports. EpiCenterimplements CLSI breakpoints and provides sensitive/resistant/intermediate (S/I/R) interpretations based on the MIC of the tested antibiotics. We have now included this information in the manuscript (L106-119)

2. L139-140: different genes are targeted to find disinfectant resistance determinants using previously established protocols in E. coli. Can the authors clarify if the PCR primers used will still work efficiently to detect those genes in A. baumannii?

In the present study, we screened the isolates for disinfectant resistance genes such as *qacE, qacEΔ1, qacF, qacG, sugE(c)*and *sugE(p),*all ofwhich confer efflux-mediated resistance to quaternary ammonium compounds (QACs). However, *qacEΔ1*was the only one detected among our isolates. Among the *qac*genes, *qacE*and its attenuated form *qacEΔ1*are widely distributed in Enterobacteriaceae, *Pseudomonas*sp, *Aeromonas*sp, *Acinetobacter*sp and *Vibrio*sp. (Vijayakumar and Sandle, 2019; Liu et al., 2017). In order to ensure that the primers (originally designed by Zou et al., 2014 based on *E. coli*sequences) will detect the respective genes in *A. baumannii,*a PrimerBLAST analysis was performed. This predicted PCR products with various *A. baumannii*genomes in the NCBI for all the *qac*gene primers, but not for the *sugE*primers. So we have now removed the information with respect to *sugE*from the manuscript (L155-156)

3. L146-147: In my experience working with A. baumannii, it forms stronger biofilms when grown in a shaking incubator. This might just be a lab-to-lab variability of the assay, but I strongly suggest to remove the "…to favour biofilm formation" statement from this sentence, as it is not really necessary and may add some controversy.

This has now been corrected (L163).

4. L152-158: the typical biofilm formation value from a crystal violet assay is given as the raw OD value of the well minus the value of the uninoculated well, but here the authors give a more complex normalisation criterion comparing the normalised OD value (after subtracting that of the uninoculated well) to the OD value of the uninoculated well. Can the authors provide any reference for this or justify the criteria? Also, as all the isolates are considered strong biofilm formers (L234), I see unnecessary to give a definition for non-biofilm producers, weak producers… The biofilm section needs to be simplified and a bar chart with the results described in L232-234 needs to be shown.

We thank the reviewer for pointing this out. The biofilm formation capacity was evaluated according to the calculation given by Sun et al., 2018. Following the comment, we have now re-assessed the biofilm-forming ability based on the more acceptable criterion suggested by the reviewer (L169-172). The mean OD (OD_T) from three independent replicates of each test isolate was compared to the cut-off OD (OD_C), which is equal to the average OD of the negative control plus three times the standard deviation of the ODs of negative control. All isolates still fell into category of strong biofilm formers, with $OD_T > 4OD_C$(L252-253). Also, as suggested by the reviewer, we have removed the criteria for defining no-biofilm and weak biofilm formers. However, as all isolates belong to a single category -strong biofilm producers- and the data is comprehensively presented in the text, we believe a bar diagram will not significantly enhance the message. We would appreciate the reviewer's thought on this and we are open for further discussions.

5. L145, L161: For different experiment, A. baumanniiis grown using different media formulations (brain heart infusion, tryptic soy, plus that used for AST (see comment above). The use of Mueller-Hinton (L134) would be justified as per the CLSI guidelines, but I struggle to understand the changes in media formulation, rather than running all experiments with one standard culture media. Could the authors justify this? Also, it is mentioned in L145 that brain heart infusion broth is supplemented with glucose. However, the majority of A. baumanniistrains cannot grow on hexoses as glucose (PMID: 32989034). What would be the reason for this supplementation?

We used TSA and BHI as culture media for PFGE and biofilm experiments respectively. Both are general purpose, non-selective media suitable for a wide variety of bacteria to grow. PFGE requires a pure culture and either of these media would serve the purpose. Concerning the biofilm experiment, BHI is the most commonly used medium as it supports maximum growth and biofilm formation. Many previous studies including those involving *A. baumannii*document increased biofilm formation in media with higher glucose concentrations and suggest the role of glucose as a specific inducer of extracellular polysaccharide (EPS), a major component of biofilm (Nucleo et al., 2009).

6. Table 2: I think this table would be made more comprehensive by adding the SI numbers from Table 1 as appropriate and including the resistance levels to the different disinfectants. Please take this as a suggestion for improvement to be made at the authors' discretion, the information is correctly given as it is.

While we agree with the reviewer that the table is quite detailed, we wish to retain it in the existing format and content for reasons of clarity and consistency.

7. L216-220: Please reword this paragraph or try representing it with a Venn diagram. It is a bit hard to picture the matching between the ISAbaIelement and the bla$_{OXA}$genes.

We have now elaborated this section to bring more clarity (L230-239): Mapping PCRs revealed the position of IS*AbaI*relative to *bla*$_{OXA-23}$and *bla*$_{OXA-51}$genes. All the 45 isolates that were positive for *bla*$_{OXA-23}$and *bla*$_{OXA-51}$gave amplification with a product size of approximately 1.6 kb in a PCR using the forward primer for IS*AbaI*and the reverse primer for *bla*$_{OXA-23.}$ This indicated that, in these isolates, the IS element is upstream of *bla*$_{OXA-23.}$Concerning the position of IS*AbaI*relative to *bla*$_{OXA-51}$, only 23 (51%) out of the 45 gave a band (of around 1.2 kb) in a PCR involving forward primer for IS*AbaI*and the reverse primer for *bla*$_{OXA-51.}$ This revealed the presence of IS*AbaI*upstream of bla$_{OXA-51}$in those isolates. However, none of the isolates with bla$_{OXA-51}$as the sole *bla*$_{OXA}$gene had IS*AbaI*upstream of it.

8. L228: is the qacEΔ1 gene specific to any disinfectant or is it a broad-spectrum resistance gene?

*qacE*and its attenuated form *qacEΔ1*mediate resistance by a proton pump, and both confer bacterial resistance to quaternary ammonium disinfectants (e.g. benzalkonium chloride), biguanide compounds (e.g. chlorhexidine) and hydrazones.

9. L309-311: "This mainly included genes conferring resistance….” As these genes were specifically targeted, the author could only find those. Please remove "mainly".

It is corrected (L330)

10. L363-367: The systems mentioned here are indeed involved in biofilm formation in A. baumannii, but except for the quorum sensing system (please include the original reference for this, not a review), the rest of the systems mentioned are not regulators. Please correct.

It is corrected and a new reference is included (L384-389)

11. L52: Please correct the acronym for the Acinetobacter calcoaceticus-baumanniicomplex and use only one.

It is corrected (L52)

12. L74: change "and also" for "including".

It is changed (L74)

13. L75-76, L194, L206: in these lines there is a mention to the blaOXA-51 gene as an A. baumanniimarker, but no bibliographic reference is given. Please include an appropriate reference.

A reference is now included.

14. L76-78: Please include a reference

A reference is now included (L78)

15. L83: change "challenge" to "challenges"

It is corrected (L83)

16. L86-87: "Here, in this study…" sounds a bit redundant. Please choose "here" or "in this study".

It is corrected (L86-87)

17. For referring to β-lactamases, please use the Greek symbol instead of the spelling "beta" throughout the manuscript.

It has been changed.

References

1. Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T., Monnet, D.L., 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18, 268–281. https://doi.org/10.1111/j.1469-0691.2011.03570.x

2. Vijayakumar, R., Sandle, T., 2019. A review on biocide reduced susceptibility due to plasmid-borne antiseptic-resistant genes—special notes on pharmaceutical environmental isolates. J. Appl. Microbiol. 126, 1011–1022. https://doi.org/10.1111/jam.14118

3. Liu, W.J., Fu, L., Huang, M., Zhang, J.P., Wu, Y., Zhou, Y.S., Zeng, J., Wang, G.X., 2017. Frequency of antiseptic resistance genes and reduced susceptibility to biocides in carbapenem-resistant *Acinetobacter baumannii*. J. Med. Microbiol. 66, 13–17. https://doi.org/10.1099/jmm.0.000403

4. Sun, Y., Hu, X., Guo, D., Shi, C., Zhang, C., Peng, X., Yang, H. and Xia, X., 2019. Disinfectant resistance profiles and biofilm formation capacity of *Escherichia coli*isolated from retail chicken. Microb Drug Res. 25, 703-711.

5. Nucleo, E., Steffanoni, L., Fugazza, G., Migliavacca, R., Giacobone, E., Navarra, A., Pagani, L. and Landini, P., 2009. Growth in glucose-based medium and exposure to subinhibitory concentrations of imipenem induce biofilm formation in a multidrug-resistant clinical isolate of *Acinetobacter baumannii*. BMC microbiol. 9, 1-14.

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VERSION 1

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000662.v1.5

Danielle Allen; Queen's University Belfast, School of Biological Sciences, UNITED KINGDOM, Belfast

Date report received: 06 September 2023
Recommendation: Major Revision

Comments: Thank you for submitting your manuscript for publication in Access Microbiology. It has been examined by expert reviewers who have concluded that the work is of potential interest to the readership of Access Microbiology. However, based on the comments received, it is clear that a major revision of this manuscript will be required before a decision can be made on its publication. I will be pleased to consider a revised manuscript along with a document including a point by point response to each of the reviewers comments. Your revised manuscript may be returned to one or more of the original reviewers, along with your itemised response to the reviewers' comments.

Reviewer 2 recommendation and comments

https://doi.org/10.1099/acmi.0.000662.v1.4

Anonymous.

Date report received: 05 September 2023
Recommendation: Major Revision

Comments: In Rajan, et al., the authors describe a basic genotypic and phenotypic characterisation of an A. baumannii isolate collection from a hospital in South India. Formally, the text is very well written and the descriptions, as well as the figures, give a clear picture of the findings. Please find below some points that need clarification, suggestions and minor amendments. Major comments L100-104: the authors used an automated system to determine the antibiotic susceptibility profile of the isolate collection. In all honesty, I am not familiar with this method, as might be the case of many potential readers. Could you please clarify in this paragraph the culture media used, if it follows the EUCAST or CLSI guidelines and the criteria for determining an isolate sensitive or resistant to a certain antibiotic? L139-140: different genes are targeted to find disinfectant resistance determinants using previously established protocols in E. coli. Can the authors clarify if the PCR primers used will still work efficiently to detect those genes in A. baumannii? L146-147: In my experience working with A. baumannii, it forms stronger biofilms when grown in a shaking incubator. This might just be a lab-to-lab variability of the assay, but I strongly suggest to remove the "…to favour biofilm formation" statement from this sentence, as it is not really necessary and may add some controversy. L152-158: the typical biofilm formation value from a crystal violet assay is given as the raw OD value of the well minus the value of the uninoculated well, but here the authors give a more complex normalisation criterion comparing the normalised OD value (after subtracting that of the uninoculated well) to the OD value of the uninoculated well. Can the authors provide any reference

for this or justify the criteria? Also, as all the isolates are considered strong biofilm formers (L234), I see unnecessary to give a definition for non-biofilm producers, weak producers… The biofilm section needs to be simplified and a bar chart with the results described in L232-234 needs to be shown. L145, L161: For different experiment, A. baumannii is grown using different media formulations (brain heart infusion, tryptic soy, plus that used for AST (see comment above). The use of Mueller-Hinton (L134) would be justified as per the CLSI guidelines, but I struggle to understand the changes in media formulation, rather than running all experiments with one standard culture media. Could the authors justify this? Also, it is mentioned in L145 that brain heart infusion broth is supplemented with glucose. However, the majority of A. baumannii strains cannot grow on hexoses as glucose (PMID: 32989034). What would be the reason for this supplementation? Table 2: I think this table would be made more comprehensive by adding the SI numbers from Table 1 as appropriate and including the resistance levels to the different disinfectants. Please take this as a suggestion for improvement to be made at the authors' discretion, the information is correctly given as it is. L216-220: Please reword this paragraph or try representing it with a Venn diagram. It is a bit hard to picture the matching between the ISAbaI element and the blaOXA genes. L228: is the qacΔ1 gene specific to any disinfectant or is it a broad-spectrum resistance gene? L309-311: "This mainly included genes conferring resistance…". As these genes were specifically targeted, the author could only find those. Please remove "mainly". L363-367: The systems mentioned here are indeed involved in biofilm formation in A. baumannii, but except for the quorum sensing system (please include the original reference for this, not a review), the rest of the systems mentioned are not regulators. Please correct. Minor ammendments L52: Please correct the acronym for the Acinetobacter calcoaceticus-baumannii complex and use only one. L74: change "and also" for "including". L75-76, L194, L206: in these lines there is a mention to the blaOXA-51 gene as an A. baumannii marker, but no bibliographic reference is given. Please include an appropriate reference. L76-78: Please include a reference L83: change "challenge" to "challenges" L86-87: "Here, in this study…" sounds a bit redundant. Please choose "here" or "in this study". For referring to β-lactamases, please use the Greek symbol instead of the spelling "beta" throughout the manuscript. I hope the authors find these comments helpful for the improvement of the manuscript.

Please rate the manuscript for methodological rigour
Very good

Please rate the quality of the presentation and structure of the manuscript
Very good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 1 recommendation and comments

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Date report received: 03 September 2023
Recommendation: Minor Amendment

Comments: This is an interesting paper investigating epidemiology of MDR A. baumannii clinical isolates. Some issues are raised to authors for addressing. -What is the study type? -What are study guidelines/checklist used in the study to ensure validity? -Authors should mention valid statement considering ethics in human research -Why authors used 64 isolates with blaOXA-51? -What is the Reference guidelines for susceptibility testing? How can authors interpret the antibiogram to individual antibiotics? -What are definitions for MDR and XDR if any? -Why authors do not confirm CR via conventional methods? -What is the

ref. for MIC of disinfectants? Why authors do not use quality control in such experiment? -Discussion is too long. -Where are limitations of this study?

Please rate the manuscript for methodological rigour
Very good

Please rate the quality of the presentation and structure of the manuscript
Satisfactory

To what extent are the conclusions supported by the data?
Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
No: Authors should mention valid statement considering ethics in human research

SciScore report

https://doi.org/10.1099/acmi.0.000662.v1.1

iThenticate report

https://doi.org/10.1099/acmi.0.000662.v1.2