Insights into the complete genomes of carbapenem-resistant *Acinetobacter baumannii* harbouring bla\(_{\text{OXA-23}}\), bla\(_{\text{OXA-420}}\) and bla\(_{\text{NDM-1}}\) genes using a hybrid-assembly approach

Saranya Vijayakumar\(^1\), Chand Wattal\(^2\), Oberoi J.K.\(^2\), Sanjay Bhattacharya\(^3\), Karthick Vasudevan\(^1\), Shalini Anandan\(^1\), Kamini Walia\(^4\) and Balaji Veeraraghavan\(^1,\)*

**Abstract**

Carbapenem resistance in *Acinetobacter baumannii* is due to bla\(_{\text{OXA-23}}\) which is endemic in India. Recently, the sporadic presence of bla\(_{\text{OXA-58}}\) as well as the occurrence of dual carbapenemases were observed. The mobility as well as the dissemination of these resistance genes were mainly mediated by various mobile genetic elements. The present study was aimed at characterizing the genetic arrangement of bla\(_{\text{OXA-23}}, \) bla\(_{\text{NDM-1}}\) and bla\(_{\text{OXA-58}}\) identified in two complete genomes of carbapenem-resistant *A. baumannii* (CRAB). Complete genomes obtained using a hybrid-assembly approach revealed the accurate arrangement of Tn2006 with bla\(_{\text{OXA-23}}\), ISAba125 with bla\(_{\text{NDM-1}}\) and ISAba3 with bla\(_{\text{OXA-58}}\). In addition, the association of IntI1 integrase with the bla\(_{\text{CARB-2}}\) gene and several virulence factors required for type-IV pili assembly, motility and biofilm formation have been identified. The current study provided deeper insight into the complete characterization of insertion sequences and transposons associated with the carbapenem-resistant genes using short reads of IonTorrent PGM and long reads of MinION in *A. baumannii*.

**INTRODUCTION**

*Acinetobacter baumannii* is a nosocomial pathogen of particular concern due to the global occurrence of multi-drug-resistant (MDR) and pan-drug-resistant (PDR) strains [1]. *A. baumannii* is a leading cause of various health-care-associated infections like blood-stream infections, ventilator-associated pneumonia, urinary tract infections and wound infections [2]. *A. baumannii* has acquired resistance to an array of antimicrobial agents belonging to different classes of antibiotics like cephalosporins, carbapenems, aminoglycosides, fluoroquinolones, chloramphenicol and tetracyclines [3]. Carbanpemems belong to the β-lactam class of antimicrobials [4]. According to Ambler’s system of classification, β-lactamases are classified into four classes: A, B, C and D [4]. Further, classes A, C and D are termed as serine β-lactamases whereas class B is called metallo-β-lactamases [4].

Carbanpemems are the last resort drugs to treat *A. baumannii* [4]. Therefore, the development of resistance to carbapenem is highly concerning [4]. Carbapenem resistance in *A. baumannii* is most predominantly mediated by oxacilllinases (OXAs) like bla\(_{\text{OXA-23}}\), like, bla\(_{\text{OXA-420}}\) like and metallo-β-lactamases (MBLs) like bla\(_{\text{NDM-1}}\), like, bla\(_{\text{VIM-1}}\) like, and bla\(_{\text{IMPA}}\) like [5]. The sporadic presence of other carbapenemases like bla\(_{\text{KPC}}\) like and bla\(_{\text{GES}}\) like have also been reported [6, 7]. A recent study by Nordmann et al. reported region-wise carbapenem non-susceptibility rates in *A. baumannii*, which have ranges as follows: (i) Asia-Pacific – 55–91%; Europe – 58–85%; North America – 32–50%; and Latin America – 53–90% [8]. In India, results from recent studies reported 40–75% carbapenem resistance in *A. baumannii* [9]. bla\(_{\text{OXA-23}}\) like is the most commonly reported carbapenemase followed by bla\(_{\text{NDM-1}}\) like in *A. baumannii* strains [10]. In *A. baumannii* carbapenem-resistance genes can be disseminated by mobile genetic elements (MGEs) like insertion sequences, integrons and transposons [11].

Next-generation sequencing (NGS) has facilitated the complete characterization of whole-genome sequences from microbial species [12]. In addition, whole-genome sequencing (WGS) helps in studying the dynamics and genomic evolution of bacterial pathogens [13]. In this study, hybrid assembly...
was performed to obtain complete genomes using long reads from MinION and short reads from IonTorrent. With this, the main objective of this current study is (i) to identify genes encoding carbapenem resistance and (ii) to decipher the genetic arrangement of insertion sequences and integrons, which are involved in the mobilization and dissemination of resistance genes among CRAB.

**METHODS**

**Bacterial isolates and identification**
Two clinical isolates, ACN21 from Sir Ganga Ram hospital, Delhi NCR and CIAT758 from Tata Medical Center, Kolkata collected as a part of Indian Council of Medical Research (ICMR) surveillance study in the year 2018 were included in this study. Both isolates were identified as *A. baumannii-calcoaceticus* complex (*Acb* complex) using standard biochemical methods. *bla*OXA-51* PCR was performed to confirm both isolates as *A. baumannii* [14].

**Anti-microbial susceptibility testing (AST)**
ACN21 and CIAT758 were subjected to AST to determine the MIC of imipenem and meropenem using broth microdilution (BMD) and interpreted as per The Clinical and Laboratory Standards Institute (CLSI) guidelines [15].

**Multiplex PCR for anti-microbial resistance (AMR) genes**
To determine the presence of AMR genes, multiplex PCR for class A, B and D carbapenemases was performed [5]. Known positive control for appropriate genes were included.

**Next-generation sequencing**

**Short-read sequencing using IonTorrent PGM**
DNA was isolated from pure cultures using Qiagen DNA Mini Kit (QIAGEN, Hilden, Germany). Whole-genome shotgun sequencing was performed using Ion Torrent PGM TM platform with 400 bp chemistry (Life Technologies, Carlsbad, CA, USA) as per the manufacturer’s instructions.

**Long-read sequencing using MinIon**
Long-read sequencing was performed using MinIon Oxford Nanopore sequencing. DNA library preparation was constructed using SQK-LSK108 Kit R9 version (Oxford Nanopore Technologies, Oxford, UK) using the 1D sequencing method for sequencing of genomes according to the manufacturer’s protocol (https://nanoporetech.com/resource-centre/protocols). FLO-MIN106 R9 flow cell in a MinION Mk 1B sequencer was utilized for sequencing. The sequencing was conducted up to 48 h and raw fast5 files were generated using MinKNOW software ver. 1.15.1.

**De novo assembly of genomes**
**Short-read assembly**
AssemblerSPAdes version 5.0.0.0 embedded in Torrent Suite Server v.5.0.3 was employed for short-read error correction and assembly. Subsequently, the quality metrics of resulting fragmented genome with multiple contigs were analysed.

**Long-read assembly**

**Reads processing and base calling**
The raw fast5 files generated from sequencing were subjected to basecalling using ONT Albacore Sequencing Pipeline Software version 2.0.2 (https://nanoporetech.com/about-us/news/new-basecaller-now-performs-raw-basecalling-improved-sequencing-accuracy). Further, the genomes were separated according to the barcodes and resulting fastq files were merged for subsequent analysis.

**Canu assembly and polishing**
Low-quality reads often lead to mis-assemblies and frame-shifted INDELs. The low-quality reads were removed using Nanofilt v2.5 (https://github.com/wdecoster/nanofilt). The filtered fastq files are then error corrected and trimmed with canu v1.7 [16] to further increase the quality of reads. Further, the trimmed reads were assembled using canu assembly option incorporated in canu v1.7. The long-read assembly generally contains a significant amount of errors and INDELS. Hence, long-read assemblies were polished using Nanopolish version 0.10.1 (https://github.com/jts/nanopolish) to reduce the inconsistency of the assembly. Initially, the genomes were split into fragments 50kb each and individually polished in parallel. Then the polished fragments were merged to obtain complete genome assembly.

**Hybrid assembly and quality assessment**
Hybrid assembly incorporating both IonTorrent and MinION reads was performed using Unicycler (v0.4.6) [17]. Initially, the short reads were error corrected with different k-mers using SPAdes [18] to filter out low-quality reads. In addition, long-read assembly and polishing was performed using Miniasm v0.3 [19] and Racon v1.3.3 [20], respectively. Further, both long-read and short-read assemblies were bridged to generate genome assembly. Finally, repeated short-read polishing steps with Pilon [21] were performed to generate a complete and accurate genome assembly. The assembly statistics and other quality metrics were analysed using Quast [22] to verify the quality, completeness and contiguity of the genomes.

**Genome annotation**
Genome annotation was performed using NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (www.ncbi.nlm.nih.gov/genome/annotation_prok/) and the PATRIC database (http://www.patricbrc.org) [23]. Antimicrobial resistance genes were identified using ResFinder v3.0 (https://cge.cbs.dtu.dk/services/ResFinder/) [24]. Virulence factors were detected using the Virulence Factors Database, VFDB [25]. Insertion sequences and the presence of prophage-related sequences were screened with IS Finder (https://www-is.biotoul.fr/blast.php) and the PHAST tool, respectively [26, 27]. Genomic Island was detected using Island Viewer 4 with the SIGI-HMM algorithm [28]. Sequence types of
Table 1. Genomic features and resistance genes identified in A. baumannii strains ACN21 and CIAT758

| Strain name | ACN21  | CIAT758 |
|-------------|--------|---------|
| Genomic size | 3827138 bp | 4017696 bp |
| Total coding sequences | 3822 | 4040 |
| Total pseudo genes | 183 | 277 |
| GC content | 38.88 | 39.02 |

Antimicrobial resistance genes

| Gene | Location | Gene | Location |
|------|----------|------|----------|
| NDM-1* | Chromosome | OXA-68 | Chromosome |
| ADC-25 | Chromosome | OXA-23* | Chromosome |
| OXA-94 | Chromosome | ADC-25 | Chromosome |
| mph(E) | Chromosome | OXA-58* | Plasmid 1 |
| msr(E) | Chromosome | msr(E) | Plasmid 1 |
| Sul1 | Plasmid 2 | mph(E) | Plasmid 1 |
| armA | Plasmid 2 | tet(39) | Plasmid 1 |
| CARB-2* | Plasmid 2 | aac(3)-IId | Plasmid 1 |
| OXA-420 | Plasmid 2 | Sul1 | Plasmid 2 |
| mph(E) | Plasmid 2 | aac(3)-IId | Plasmid 2 |
| msr(E) | Plasmid 2 | PER-7 | Plasmid 2 |

*Genetic arrangement of these resistance genes were shown in Figs 1 and 2.

the isolates were identified with MLST 2.0 tool (multi-locus sequence typing) [29].

RESULTS AND DISCUSSION

Both isolates were confirmed as A. baumannii by the presence of intrinsic, bla_{OXA-23}*-like gene. Both phenotypically and genotypically, ACN21 and CIAT758 were confirmed as carbapenem resistant. However, they differ only in the type of carbapenem resistance gene they harbour. The MIC of imipenem and meropenem were determined as 128 µg ml^{-1} and 256 µg ml^{-1} for ACN21, respectively, whereas for CIAT758 the MICs for both were 128 µg ml^{-1}. Multiplex PCR revealed the presence of bla_{NDM} like and bla_{OXA-23} like in ACN21 and bla_{OXA-23} like in CIAT758.

Hybrid assembly of ACN21 revealed the presence of one chromosome of size 3827138 bp with eight plasmids ranging from 116047–5734 bp size whereas CIAT758 had a single chromosome with 4017696 bp size and three plasmids of 78125, 47417 and 29128 bp size. The genome features of the isolates were mentioned in Table 1.

Ion Torrent short-read assembly produced accurate genome with multiple fragments. In contrast, Minlon Nanopore long-read assembly generated a single chromosomal contig but with higher frame-shifted INDELS (>30%) even after repeated long-read polishing that makes downstream analysis difficult. Further, the repeated polishing with short reads generated a highly accurate single chromosomal contig with reduced frame-shifted INDELS (<10%), which enabled accurate analysis of the AMR and other MGEs.

Long-read-sequencing technologies such as Pacific Biosciences (PacBio) and Oxford Nanopore will be helpful in generating longer reads, which in turn will allow the gaps to be covered [30]. Minlon only reads may be inclined to errors, however that can be efficiently overcome by combining with short-read sequencers like IonTorrent and Illumina [30].

The genome of ACN21 has been deposited in GenBank with accession number CP038644 for chromosome and CP038645–CP038652 for plasmids 1–8. For CIAT758, the chromosome and three plasmids were deposited with accession numbers CP038500 and CP038501, CP038502, CP038503, respectively.

Resistance determinants

Multiple resistant genes conferring resistance to various antimicrobial classes were identified in both ACN21 and CIAT758 genomes. The genes that may contribute resistance to aminoglycosides, beta lactams, macrolides, lincosamide streptogramin B, sulphonamide and tetracycline are listed in Table 1.

In A. baumannii, carbapenem resistance is predominantly due to acquired class-D bla_{OXA-23} followed by other oxacillinases and metallo-beta lactamas [1]. The genome ACN21 was devoid of bla_{OXA-23}, while the presence of bla_{NDM} and bla_{OXA-23} could be encoding for carbapenem resistance. In ACN21, bla_{NDM} resides in chromosome and bla_{OXA-23} in plasmids whereas previous studies reported the co-presence of bla_{NDM} and bla_{OXA-23} on the same plasmid [31–34]. In CIAT758, bla_{OXA-58} was carried on the plasmid (Table 1). Presence of bla_{NDM} and bla_{OXA-58} in six CRAB isolates was previously reported by Principe et al. [35]. El Bannah et al. reported that 86% of carbapenem resistance could be due to the presence of either bla_{OXA-23} alone or bla_{OXA-23} together with bla_{KPC} [36]. However, in this study bla_{OXA-23}, bla_{OXA-58} and bla_{NDM} was present, but bla_{KPC} was not observed.

Mobile genetic elements

The main characteristic feature of A. baumannii is its ability to acquire, retain and disseminate multiple resistance mechanisms by combining resistance genes with an array of MGEs like insertion sequences, resistance islands and bacteriophages, which mediate the exchange of genetic material, rearrange bacterial genomes and provides an endless source of genetic adaptability [11]. Increased resistance to carbapenems due to upstream insertion of bla_{OXA-51} and bla_{OXA-23} with ISAbra1, bla_{OXA-58} with ISAbra3 and bla_{NDM} with ISAbra125 were reported from previous studies [37–39]. In this study, IS Finder analysis revealed the presence of various insertion sequences belonging to IS family IS1, IS3, IS4, IS5 and IS30 in both ACN21 and CIAT758 genomes. Phage analysis identified three phage regions (one intact, one incomplete and one
questionable) in the ACN21 genome and one intact phage region in CIAT758 genome. Further analysis using Island viewer revealed the presence of six and nine genomic islands in ACN21 and CIAT758 genomes, respectively.

Genetic context of resistance genes

**A. baumannii strain ACN21 bla**\textsubscript{OXA-420}

Resolvase IS26 Resolvase HP ISNcY DCP Transposase IS66 IS66 IS3 Transposase ISAab3 OXA-420 ISAab3

**A. baumannii strain ACN21 bla**\textsubscript{CARB-2}

Transposase DCP IS3 Recombinase IS6 IS1 OXA-58 IS1

**A. baumannii strain ACN21 bla**\textsubscript{NDM-1}

Fig. 1. Graphical representation of genetic arrangement of bla\textsubscript{OXA-420}, bla\textsubscript{CARB-2} and bla\textsubscript{NDM-1} in A. baumannii strain ACN21. The direction of arrows indicates the orientation of ORFs.

was first reported in a plasmid from P. aeruginosa in 1991 by Huovinen and Jacoby [42] and known as *Pseudomonas*-specific enzyme-1 (*PSE-1*). Variants of the bla\textsubscript{CARB} gene, bla\textsubscript{CARB} - 4, 5, 8 and 10 have been reported earlier in A. baumannii. Kamolvit et al. reported the presence of the bla\textsubscript{CARB-2} gene in A. pittii with upstream presence of truncated class 1 integrase gene (intI1) [43]. In the ACN21 genome, though the bla\textsubscript{CARB-2} gene has upstream presence of intI1, it is not truncated (Fig. 1).

The ACN21 genome has the bla\textsubscript{NDM-1} gene with downstream ISAba125, upstream ble-MBL gene and bracketed by two copies of ISAba125. However, an additional insertion sequence, ISAba14 was identified upstream ATP binding protein thereby forming a Tn125-like composite transposon, which could be a novel finding. This is similar to the report documented by Poirel et al. and Jones et al. [44, 45]. The genetic arrangement of bla\textsubscript{NDM-1} gene is shown in Fig. 1.
**A. baumannii strain CIAT758 bla\textsubscript{OXA-23}**

The CIAT758 genome harbours \textit{bla}_{OXA-23} on the chromosome and bracketed by two copies of the IS4 family insertion element, IS\textit{Aba1} (Fig. 2). A similar scenario was documented by several other studies [1, 5, 46].

In addition, CIAT758 harbours the \textit{bla}_{OXA-58} gene with two copies of IS\textit{Aba3} with one copy present downstream being truncated. Downstream of IS\textit{Aba3} possess another insertion element, IS1008 belonging to IS6 family transposase (Fig. 2). Chen et al. reported the presence of \textit{bla}_{OXA-58} with truncated IS\textit{Aba3} and IS1008, which provides two independent promoters for the transcription of \textit{bla}_{OXA-58} gene [47]. Further, the deletion of promoters provided by IS1008 results in decreased transcription of the \textit{bla}_{OXA-58} gene and was not noticed in this genome [47].

**Virulence factors**

Several virulence factors required for pathogenesis have been identified in \textit{A. baumannii} [12]. Both the sequenced genomes harbour genes required for assembly of type-IV pili (\textit{pilB} and \textit{pilF}), for twitching motility (\textit{pilT} and \textit{pilU}), surface polysaccharides (\textit{pgaA/B/C/D}), which is involved in the synthesis of poly β-(1-6)-N-acetyl glucosamine for biofilm formation and protects the bacteria against innate host defenses [48, 49], outer membrane protein (OmpA) involved in pathogenesis and signal processing [50], phospholipase C, an important factor in cellular damage [51] and phospholipase D, important for human serum resistance and epithelial cell invasion, siderophores (\textit{bauA} and \textit{basD}), encodes for acinetobactin transport and biosynthesis, respectively [11]. Additionally, ACN21 possess the \textit{csuE} gene, which belongs to the \textit{CsuA/ BABCDE} chaperone usher complex responsible for pili production and biofilm formation [52] whereas CIAT758 possesses the biofilm-associated protein (Bap) required for the development of mature biofilm structures [53].

**Multi-locus sequence typing (MLST)**

Currently, in \textit{A. baumannii}, epidemiological characterization of clinical isolates has been described with eight international clonal lineages (IC-1 to IC-8). Among these, IC-1, IC-2 and IC-3 were the most common clonal lineages reported from various countries [54]. The majority of outbreaks due to CRAB were reported to be associated with isolates belonging to the IC-2 lineage [10].

\textit{In silico} analysis of MLST using Oxford scheme identified ACN21 with ST1089. Uwingabiye et al. reported the presence of ST1089 and also mentioned that this ST was first reported in India in 2015 from PGIMER, Chandigarh [55]. Unlike other STs, ST195, which was widely reported from Asian countries and European Nations, ST1089 is very rare. While in CIAT758, ST585 was identified and belongs to International Clone 8 (IC-8). ST585 is the single loci variant (SLV) of ST391, which was first reported in India by Rynga et al. [56]. Another study from Egypt showed that the study isolates belonging to ST391 carries \textit{bla}_{OXA-23} alone or \textit{bla}_{NDM} alone or \textit{bla}_{OXA-23}
with \( \text{bla}_{\text{OXA-23}} \), which is in contrast to the current study where CIAT758 belongs to ST589 and carries \( \text{bla}_{\text{OXA-23}} \) with \( \text{bla}_{\text{OXA-58}} \) [36]. Such findings indicate that despite diverse sequence types, \( \text{bla}_{\text{OXA-23}} \) is the major contributor of carbapenem resistance in \( \text{A. baumannii} \) worldwide. As discussed by El Bannah et al., certain STs are endemic in particular countries whereas others belong to a worldwide clonal complex and disseminate globally [36]. Similarly, a previous study by the same authors reported ST208 as the endemic sequence type, which is a SLV of globally disseminated ST92 [57].

**Novel findings using hybrid assembly**

In \( \text{A. baumannii} \), MGEs like insertion sequences and integrons play a major role in the dissemination of anti-microbial resistance genes. Short reads from Ion Torrent sequencing results in a greater number of contigs, which leads to difficulty in deciphering the appropriate arrangement of MGEs. Hybrid assembly provides the complete genome, which is helpful in identifying the association of insertion sequences/integrons with resistance genes. In ACN21, the association of \( \text{IntI1} \) integrase with the \( \text{bla}_{\text{CARR-2}} \) gene was observed in the complete genome using a hybrid-assembly approach, whereas this association was completely missing in short-read genome assembly. Similarly, in the CIAT758 genome the presence of insertion element, \( \text{ISAba1} \), was observed in duplicates, whereas the hybrid genome assembly approach enabled the complete and accurate structural arrangement to be revealed.

**Limitations of the study**

Only two complete genomes of CRAB were characterized in this study. Characterization of the clinical isolates of more complete genomes of \( \text{A. baumannii} \) with different variants of the above mentioned resistance genes will be helpful in the comprehensive understanding of the variations in their genetic arrangement.

**Future recommendations**

Whole-genome sequencing of \( \text{A. baumannii} \) using the hybrid-assembly approach in the upcoming studies are necessary. Such studies will be helpful to gain thorough knowledge regarding various antibiotic resistance genes and the role of respective MGEs that are involved in the dissemination of the same.

**CONCLUSION**

In this study, complete genomes of two CRAB, ACN21 and CIAT758 were characterized using the hybrid-assembly approach. This study deciphered the genetic arrangement of \( \text{bla}_{\text{OXA-23}} \) with Tn006, \( \text{bla}_{\text{NDM-1}} \) with a Tn125-like transposon, \( \text{bla}_{\text{OXA-23}} \) with \( \text{ISAba3} \) and \( \text{bla}_{\text{CARR-2}} \) with class 1 integron. A novel Tn125-like transposon carrying \( \text{bla}_{\text{NDM-1}} \) was identified in the complete genome of ACN21. One limitation with this study is that only two complete genomes were characterized. However, such findings using complete genomes will be helpful in studying the genetic backbone of significant resistance genes and also in exploration of associated novel MGEs.

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**Author contributions**

B.V., S.A., S.V., K.V., C.W., J.K.O., S.B. and K.W. contributed to the design of the study; S.V. collected and analyzed the data; K.V. performed bioinformatic analysis; B.V., S.A. and S.V. wrote the manuscript. The manuscript was revised, read and approved by all authors.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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