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Research Paper

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

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ABSTRACT

Acinetobacter baumannii is a nosocomial pathogen increasingly affecting the critically ill patients and represents a major public health challenge. Carbapenem-resistant *A. baumannii* (CRAB) is found to be associated with International Clones (ICs) and different classes of carbapenemases. The objective of the present study was to investigate the prevalence of carbapenem resistance genes, clonal relationship and genetic structure of clinical isolates of *A. baumannii*. In the present study, multi-locus sequence typing (MLST^{OX}) and analysis were carried out using Oxford scheme for 86 clinical isolates of CRAB along with 11 carbapenem sensitive *A. baumannii* (CSAB) collected over a period of two years (2014–2016) from two tertiary care hospitals of North India. We observed a high prevalence of the $bla_{\text{OXA-23}}$-like (97.7%) among the CRAB followed by $bla_{\text{NDM-1}}$ (29.1%) and bla_{OXA58}-like (3.5%). Forty-seven Sequence Types (STs) were represented by all 97 isolates, out of which, 28 (59.6%) were novel STs that were assigned to 41 isolates. STs 451 (13%), 447 (7%), 195 (6%) and 848 (5%) were the most common STs. The majority of CRAB isolates (44.3%) belonged to the CC92, followed by the CC447 (15.1%), CC109 (9.3%) and CC110 (3.4%), which corresponds to the IC2, 8, 1 and 7 respectively. Phylogenetic and recombination analysis suggested two major and one minor lineage in the population. Further linkage disequilibrium analysis suggested clonal nature of the population as recombination was noticed at a low frequency, which was not enough to split the clonal relationship. The knowledge of genetic structure of CRAB from this study will be invaluable to illustrate epidemiology, surveillance and understanding its global diversity.

1. Introduction

Acinetobacter baumannii is a nosocomial pathogen of clinical significance, which has been implicated in a wide spectrum of hospital-acquired infections, mostly among immunocompromised patients in intensive care units (Dijkshoorn et al., 2007; Howard et al., 2012; Peleg et al., 2008; Perez et al., 2007). Infections caused by carbapenem-resistant *A. baumannii* (CRAB) have become difficult to manage and are often associated with high morbidity and mortality (Doi et al., 2009; Peleg et al., 2007a). *A. baumannii* has the ability to acquire antibiotic resistance determinants, cause hospital outbreak leading to global dissemination of clones (Dijkshoorn et al., 2007). The emergence of such highly successful clones is leading to the increasing global occurrence of *A. baumannii* infections (Diancourt et al., 2010).

Carbapenems have good antibacterial activity against *A. baumannii*. Carbapenems are not the first choice antibiotic to treat *A. baumannii* infections, they are in fact one of the last resort treatments (Peleg et al., 2008). However, the emergence of resistance to carbapenem was first reported in 1993 (Paton et al., 1993) followed by similar reports from India and different parts of the world (Lee et al., 2007; Peleg et al., 2007b; Tiwari et al., 2012). Interestingly, several resistance islands have recently been identified among Multidrug-resistant (MDR) isolates of *A. baumannii*, which belong either to IC1or IC2 (Adams et al., 2008; Fournier et al., 2006; Nigro and Hall, 2012). Carbapenem resistance in

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A. baumannii is mainly mediated by the production of carbapenem-hydrolyzing enzymes (Peleg et al., 2008; Tiwari et al., 2012). Genes encoding a variety of carbapenem-hydrolyzing beta-lactamases have been detected in *A. baumannii* that belong to class B (bla_{IMP-1}, bla_{VIM-2}, bla_{SIM-1} & bla_{NDM}) and class D (bla_{OXA-23}-like, bla_{OXA-24}-like or bla_{OXA-40}-like, bla_{OXA-51}-like, bla_{OXA-58}-like, $bla_{OXA-143}$-like & $bla_{OXA-235}$-like) (Poirel et al., 2010; Poirel and Nordmann, 2006; Robledo et al., 2010; Robledo et al., 2011). The class D OXA-type carbapenemases are by far the most prevalent carbapenemases in *A. baumannii* (Poirel and Nordmann, 2006). The bla_{OXA-23}-like seems to be the most prevalent among CRAB isolates (Rafei et al., 2015). There is a report of newly found carbapenemase in *Acinetobacter* spp., which includes Tripoli metallo-beta-lactamase 1 (TMB-1) (Kayama et al., 2014). Therapy for infection due to CRAB often requires treatment by colistin methanesulfonate (CMS). However, there are reports on *A. baumannii* showing resistance to almost all the currently available antibiotics including colistin (Magiorakos et al., 2012; Nurtop et al., 2019).

Several molecular methods like Multi-locus sequence typing (MLST) (Bartual et al., 2005), Pulsed-field gel electrophoresis (PFGE) (Hamouda et al., 2010), rep-PCR (Grisold et al., 2010), multi-locus variable-number tandem-repeat (VNTR) analysis (MLVA) (Pourcel et al., 2011; Turton et al., 2009) have been used to type *A. baumannii* strains. MLST is a widely used method for typing of the pathogens, which is based on sequence analysis of loci from housekeeping genes. MLST has several advantages over the other typing methods. Apart from resolution, MLST provides unambiguous typing data to exchange and compare results among laboratories, which make it an ideal typing method for an epidemiological study (Bartual et al., 2005; Hamouda et al., 2010).

In this study, genetic and epidemiological relatedness of clinical isolates of CRAB & CSAB were carried out using $MLST^{OX}$ analysis. To the best of our knowledge, only two studies have investigated the molecular epidemiological characteristics of CRAB in India but these studies restricted to typing of a limited number of isolates (Rynga et al., 2013; Saranathan et al., 2015). However, a large number of clinical isolates of *A. baumannii* are included in this study from two hospitals of North India. We also studied the prevalence of different carbapenemases and clonal nature of the population under study.

2. Materials and methods

2.1. A. baumannii isolate collection

A total of 161 isolates of *A. baumannii* were collected and the study was carried out with those that were found to be carbapenem resistant ($n = 86$) and susceptible ($n = 11$). A total of 75 isolates of *A. baumannii* (94% carbapenem resistant) were consecutively isolated from March to June 2014. In addition, 7 & 15 isolates were included from the year 2013 and 2016 respectively. These isolates were recovered from the patients admitted to Nehru Hospital, PGIMER, and Government Medical College & Hospital Chandigarh, India. We have included only one isolate per patient in this study. These isolates were recovered from blood ($n = 55$), cerebrospinal fluid (CSF) ($n = 20$), body fluid ($n = 14$), pus ($n = 4$) and respiratory specimens (n = 4) (S1 Table). Patient's informed consent was not required as it was a part of routine clinical testing. These isolates were identified as *A. baumannii* by using MALDI-TOF MS followed by PCR of *gyrB* & bla_{OXA-51}-like genes (Brown et al., 2005; Higgins et al., 2007). Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as per user manual and used for subsequent tests.

2.2. Antimicrobial susceptibility testing

All isolates were tested by Kirby Bauer Disk diffusion method for imipenem, meropenem, doripenem (Becton Dickinson, Franklin Lakes, New Jersey, USA), chloramphenicol, amikacin, ceftazidime, cefepime, cefotaxime, piperacillin/tazobactam, ciprofloxacin & gentamicin (HiMedia Laboratories GmbH, Germany) using updated breakpoints of the Clinical and Laboratory Standards Institute (CLSI) (http://www.clsi.org). MICs for imipenem, meropenem and doripenem (Sigma Aldrich, St. Louis, Missouri, USA) were also determined for *A. baumannii* isolates by agar dilution method on Mueller-Hinton agar according to the CLSI guidelines. CRAB isolate was defined as an isolate, which was resistant to both imipenem and meropenem (i.e., MIC ≥ 8 μg/ml) (Chen et al., 2017).

2.3. PCR based screening for carbapenemase genes

Intrinsic carbapenemase encoding gene in *A. baumannii* bla_{OXA-51}-like was detected using previously standardized PCR primer (Higgins et al., 2007). Major groups of beta-lactamases were screened using PCR, which confer resistance to carbapenems. These included bla_{OXA-23}-like, bla_{OXA-24}-like (40-type), bla_{OXA-58}-like, class-B beta-lactamases, i.e. bla_{IMP-1}, bla_{SIM-1}, bla_{NDM-1} and class-C beta-lactamases, i.e. bla_{ampC}. The primers used in the screening of the carbapenemase gene are listed in the S2 Table. PCR was carried out using PCR master mix (Sigma Aldrich, St. Louis, Missouri, United States) in the 'Veriti' thermal cycler (Applied Biosystems, Foster City, California, USA).

2.4. Multi-locus sequence typing

All isolates were subjected to MLST by PCR amplification and sequencing of seven housekeeping genes (*gltA, gdhB, gyrB, recA, gpi, cpn60,* and *rpoD*) as described by Oxford scheme introduced by Bartual et al., 2005 (Bartual et al., 2005). PCR amplification was carried out in 25 μl reaction mixture volume containing 2 mM $MgCl_2$, 20 mM Tris-HCl, 50 mM KCl, 250 μM of each dNTP, 0.5 μM of each primer and 2 U of Taq polymerase (Sigma Aldrich, St. Louis, Missouri, United States). The amplified product was resolved on 1.2% agarose gel and purified by using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Nucleotide sequences were determined using sequencing primers with the BigDye Terminator ready reaction mix, version 3.1 (Applied Biosystems, Waltham, MA.) as per standard sequencing conditions in accordance with the manufacturer's protocol. The reaction products were further separated and detected on an ABI PRISM genetic analyzer 3100 (Applied Biosystems, Waltham, MA). The sequences from both strands of each of seven loci of the same isolate were aligned separately, trimmed to the desired length and edited using SeqMan II program from the Lasergene software package (DNASTAR, Inc., Madison, WI). Sequence types (ST), allele profiles and clonal complexes were assigned by PubMLST database (www.pubmlst.org/abaumannii/). New allele numbers and STs were assigned to unique alleles and STs from this study.

2.5. Genetic diversity and selection analysis

The DnaSP Version 5.10 (Librado and Rozas, 2009) was used for assessing sequence diversity by using concatenating sequences of the seven MLST housekeeping gene loci to calculate the number of polymorphic sites, nucleotide diversity (π) as the average number of nucleotide differences per site between two sequences and average GC content. The π index is determined for each of the MLST loci for identifying the loci having the greatest diversity. The average non-synonymous/synonymous substitution rate ratios (dN/dS) were calculated by using MEGA version 6.06 (Tamura et al., 2013) to infer about the positive and purifying selection.

2.6. Population structure analysis

The eBURSTv3 (Feil et al., 2004) was used to assign the STs generated in this study to the clonal complex. The eBURSTv3 is based on a model of bacterial evolution, in which a particular ancestor founder ST

undergoes diversification to produce a subset of closely related STs and available at http://eburst.mlst.net/. Global optimal eBURST (goeBURST) (Francisco et al., 2009) was used to cluster the STs generated in this study with the existing STs in the global MLST database at single locus variants limitation, which generate the minimal spanning tree to visualize the possible evolutionary relationship between STs. The maximum likelihood (ML) method was used to generate the phylogenetic trees of strains or the STs with 1000 bootstrap replications. The split network of the STs was generated by the neighbor-net methods (Bryant and Moulton, 2004) using SplitsTree4 (Huson and Bryant, 2005).

2.7. Recombination and linkage disequilibrium analysis

The pairwise homoplasy index (*phi)* test (Bruen et al., 2006) implemented in SplitTree4 (Huson and Bryant, 2005) was used to detect the possibility of recombination among seven individual loci, all STs and STs belonging to different lineages. The *phi* test is a simple statistical test based on the pattern of nucleotide polymorphisms in the sequence alignment. If it is not consistent with the model of clonal population structure, the *phi* test gives the signal for recombination (*p*-value < .05). LDhat program (Auton and McVean, 2007) used in Recombination Detection Program (RDP) v.4.66 (Martin et al., 2015) was implemented to calculate mean per site ρ/θ ratio based on the concatenated sequences of seven loci with 1,000,000 MCMC updates. The parameters ρ and θ symbolized the rate of recombination and mutation respectively. Linkage disequilibrium from allelic data was analyzed by calculating the standardized index of association (I_A^S) using LIAN v3.7 (Haubold and Hudson, 2000) (http://adenine.biz.fh-weihenstephan.de/lian_3.1/). The I_A^S is a measure of clonality among the population and calculated using the Monte Carlo methods to test the null hypothesis of complete linkage equilibrium with 10,000 iterations on the allelic profile. For completely clonal population structure, alleles would be in completely disequilibrium and value for $I_A^S > 0$. SplitTree4 (Huson and Bryant, 2005) and the neighbor-net method (Bryant and Moulton, 2004) were used to generate a split network of seven individual loci.

3. Results

3.1. Antimicrobial susceptibility testing

Bacterial isolates were identified as *A. baumannii* using MALDI-TOF-MS, $bla_{\text{OXA-51}}$-like and *gyrB* PCR after initial collection in the clinical bacteriology laboratory. Among them 86 isolates were found to be resistant to three carbapenems including imipenem, meropenem, and doripenem as MIC was ≥16 μg/ml for all three carbapenems. Along with the three carbapenems, all 86 CRAB were also resistant to cefepime, cefotaxime, ceftazidime ciprofloxacin, and piperacillin-tazobactam.

3.2. Screening for carbapenemase genes

All 86 CRAB and 11 CSAB isolates (S1 Table) were screened for the presence of beta-lactamases genes. The major carbapenemase gene $bla_{\text{OXA-23}}$-like was detected in 84/86 CRAB isolates (97.7%). Interestingly, 25 isolates (29.1%) and 3 isolates (3.5%) were positive for the $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-58}}$-like respectively (Table 1). Other beta-lactamase genes including $bla_{\text{SIM-1}}$, $bla_{\text{IMP-1}}$ and $bla_{\text{OXA-24}}$-like were undetectable in all the isolates under study (Table 1).

3.3. MLST and population structure analysis

All 97 (86 CRAB and 11 CSAB) isolates were subjected to typing using Oxford Bartual scheme of MLST. 47 STs were identified among 97 isolates under study. The most common ST was ST 451 represented by 13 isolates. Among the 47 STs identified, we reported 18 novel STs, which were submitted to the PubMLST database and assigned new STs (S1 Table). STs 451 (13.4%), 447 (7.2%), 195 (6.1%) and 848 (5.1%) were most common STs in this study. Four new alleles (*cpn*60–89, *gpi* 282, *rpoD* 117 & *rpoD* 118) were also assigned to 3 isolates of this study, which were submitted to PubMLST (http://pubmlst.org/abaumannii/).

Table 1
Prevalence of β-lactamases among CRAB and CSAB isolates.

S. No.	β-Lactamase genes	No. of isolates	Percentage
1	$bla_{\text{OXA-58}}$-like	03	3.5% (CRAB)
2	$bla_{\text{OXA-23}}$-like	84	97.7% (CRAB)
3	$bla_{\text{OXA-24}}$-like	0	0
4	$bla_{\text{SIM-1}}$	0	0
5	$bla_{\text{IMP-1}}$	0	0
6	$bla_{\text{NDM-1}}$	25	29.1% (CRAB)

A total of 47 STs were identified among 97 isolates, which were subjected to eBURST analysis and assigned into five clonal complexes (CC), three doubletons and thirteen singletons. CC1 contained 13 STs (42 isolates) and is largest CC with ST 451 founder ST, followed by the CC2 with 6 STs (13 isolates) with founder ST 447. CC3 and CC5 comprised of 3 STs with 8, 3 and 6 isolates respectively. To determine the clonal relationships of the STs obtained in this study, together with the 1489 STs in the global PubMLST *A. baumannii* database were clustered using the goeBURST (Fig. 1A). The resultant minimal spanning tree revealed that the 13 STs (represented by 43 isolates) are linked to the CC92 (Fig. 1B). CC92 is the largest CC among all the STs and represents the IC2. Three STs from this study ST 441, 491 and 231 comprising of eight isolates were linked to CC109, which belongs to the IC1. The 6 STs from this study ST 447, 585, 20, 391, 1389 and 1390 comprising 13 isolates from this study were linked to CC447, which represents IC8. Three STs with single isolates each from this study were linked to CC110, which represents IC 7. STs were also linked to the international clones and their respective clonal complex by Oxford and Pasteur MLST scheme (Table 3).

Majority of the CRAB isolates were linked to CC92 (45%) and CC447 (13%), which represented IC2 and IC8 respectively as reported previously (Diancourt et al., 2010). There were few isolates, which were linked to CC109 and CC110 and represented IC1 and IC7. None of the CSAB was linked to any clonal complex except ST 585, which was a part of CC442 (IC 8). STs and clonal complex for $bla_{\text{OXA-58}}$-like and $bla_{\text{NDM-1}}$ were respectively highlighted in Table 2.

3.4. Genetic diversity and selection analysis

The sequence alignment of seven loci showed no insertion-deletion events. The length of the concatenated sequence of all seven loci is 2895 bp. The average GC content, the nucleotide diversity (π), the number of polymorphic sites and ratio of non-synonymous (d_N) to synonymous (d_S) substitutions were determined for each MLST locus, and for the concatenated sequence of all seven loci of 97 isolates under study, and its mentioned in Table 4.The d_N/d_S values ranging from the 0.000 (*gltA*) to 0.028425 (*gpi*) indicate that all seven MLST loci exhibit purifying selection. The number of polymorphic sites varied from 9 for *cpn60* to 79 for the *gpi* and that for the concatenated sequence was 160. *Gpi* is the most polymorphic locus among the seven loci. The number of alleles ranged from 9 (*gltA*, *recA,* and *cpn60*) to 24 (*gpi*). The nucleotide diversity index (π) was 0.01452 for concatenated sequences and ranged from 0.00155 (*gltA*) to 0.007893 (*gpi*) at different loci, suggesting the *gpi* locus is most diverse as compared to others.

3.5. Phylogenetic analysis

The concatenated sequences of all the 43 STs were used to build the phylogenetic tree by using the Maximum likelihood method and split

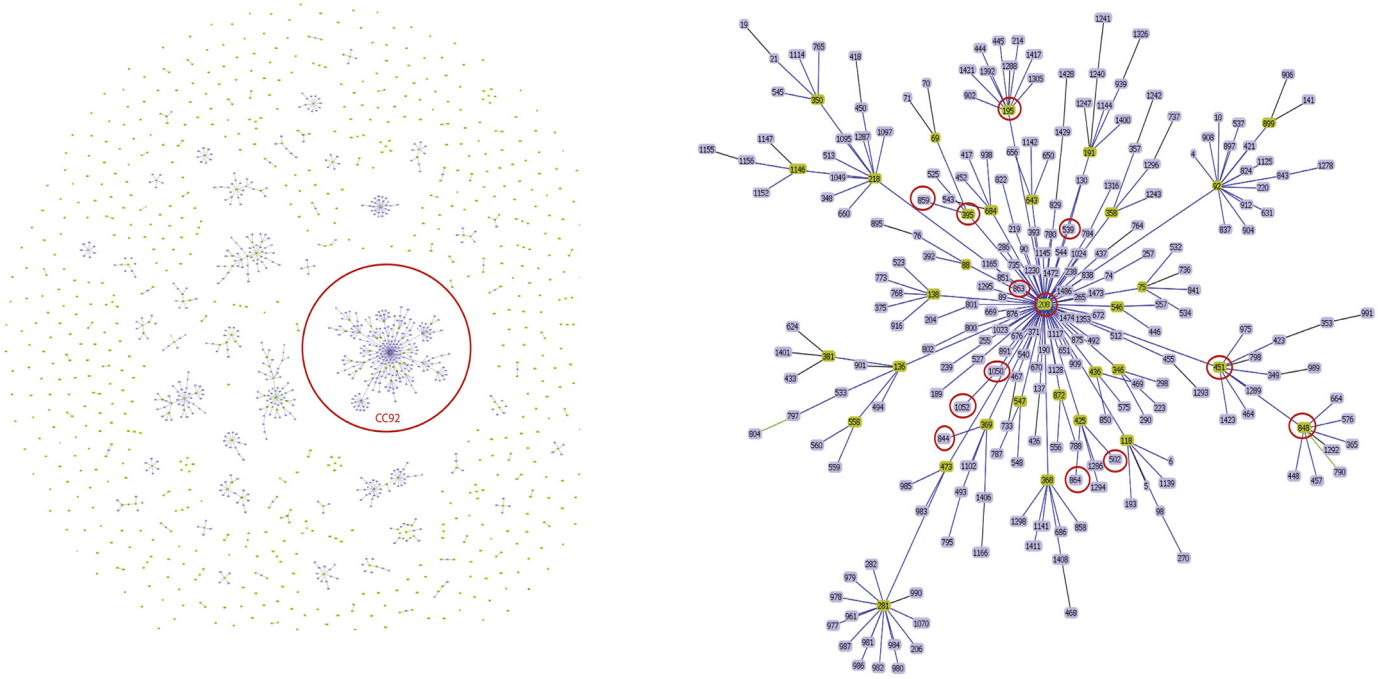

Fig. 1. Population snapshot. A) goeBURST analysis of ST 1489 present in the PubMLST database. The largest CC 92 is marked as red colored circles. B) Snapshot of CC 92. STs marked as red-colored circles are from the present study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
STs positive for the $bla_{\text{OXA-58}}$-like and $bla_{\text{NDM-1}}$ and respective clonal complex.

Resistance gene	STs	Clonal complex
$bla_{\text{OXA-58}}$-like	930	
	1089	
$bla_{\text{NDM-1}}$	1053	2
	441.	1
	862	
	391	8
	865	
	539	2
	451	2
	930	
	1089	

network analysis. The phylogenetic tree revealed that there were two major and one minor but distinct lineage, termed as L1, L2 & L3. The branches of the three lineages showed low bootstrap values suggesting the possible existence of homologous recombination (Fig. 2A). In addition, the split graph of 47 STs also revealed that three distinct subgroups corresponded to that of L1 to L3 (Fig. 2B).

3.6. Recombination and linkage disequilibrium analysis

Phi test is an efficient statistical test for the recombination. The *P*-value determined by the *Phi* test for 47 STs (whole population) and individual lineage were < 0.05 except L3, which had *P*-value of 0.2402. The split network analysis of 47 STs (Fig. 2B) showed lineage L1 and L2 as a parallelogram-shaped very complex network structure, which was absent in the L3. L1 and L2 displayed a very complex interconnecting network structure, however, merely visualized intersections were observed between L1 and L2 with L3. Further split network graphs of the seven individual loci revealed that the *gyrB* and *gpi* showed a network like parallelogram structures (S1Fig.), however, the split graphs of *gltA, gdhB, recA, cpn60* and *rpoD* genes were tree-like structures.

The mean per-site recombination/mutation ratio (ρ/θ) values were 13.35, 4.98, 0.95 and 0.20 for the whole population, L1, L2, and L3 respectively (Table 5). Standard association of index (I_A^S) showed the homologous recombination by calculating the linkage disequilibrium among loci. Analysis of 47 STs understudy yielded I_A^S of 0.2838 (P = 1.00E-04) and 0.2433 (P = 1.00E-04), 0.3688 (P = 1.00E-04) and 0.2805 (P = 7.00E-04) for individual lineages L1, L2 and L3 respectively.

4. Discussion

Carbapenem-resistant *A. baumannii* (CRAB) has been deemed a critical-priority pathogen by the World Health Organization (Tacconelli et al., 2018). This study revealed that $bla_{\text{OXA-23}}$-like is the major mechanism responsible for the carbapenem resistance phenotype as has been described in earlier studies (Fu et al., 2010; He et al., 2011; Ji et al., 2013; Wang et al., 2013). First time in India, we have detected three isolates positive for $bla_{\text{OXA-58}}$-like. $bla_{\text{OXA-58}}$-like is a plasmid-borne gene first traced in France in 2003 (Poirel et al., 2005) and later spread worldwide (Cameranesi et al., 2018; Ramoul et al., 2016).

Table 3
STs linked to the International Clones along with respective clonal complex assigned by Oxford and Pasteur MLST scheme.

STs	IC	CC Oxford	CC Pasteur
208,863,539,1050,395,859,195,1050,1052,844,864,502,451,848	2	92	2
441, 491, 231	1	109	1
447, 585, 20, 391, 1389, 1390	8	447	157
993,1387,1388	7	110	25

Table 4
Nucleotide and allelic diversity among seven MLST loci.

Locus	Length (bp)	Average G + C content (%)	No. of alleles	No. of polymorphic sites	Average dN/dS ratio	Nucleotide diversity(π)
gltA	484	41.1	9	8	0	0.00155
gyrB	457	41.6	14	22	0.014189	0.01000
gdhB	344	36.6	11	21	0.070168	0.01418
recA	371	43.6	9	11	0	0.00665
cpn60	421	37.1	9	9	0.000368	0.00392
gpi	305	41.4	24	79	0.028425	0.007893
rpoD	513	39.1	12	10	0.006438	0.00336
All (47STs)	2895	40	46	160	1.752635	0.01452

Although there are serval reports of bla_{NDM-1} alone positive *A. baumannii* from India (Pragasam et al., 2016; Rahman et al., 2018), however, our study revealed an increasing incidence of CRAB isolates with bla_{NDM-1} gene, which were also positive for bla_{OXA-23}-like or bla_{OXA-58}-like. There are reports of coexistence of bla_{OXA-23}-like or bla_{OXA-58}-like with $bla_{NDM-1,}$ synergistically which gave a very broad resistance profile (Krizova et al., 2012; Ramoul et al., 2016). However, we did not observe the high level of resistance profile in isolates of bla_{OXA-23}-like or bla_{OXA-58}-like with bla_{NDM-1} positive isolates. This may be due to the lack of promotor for bla_{OXA-23}-like or bla_{OXA-58}-like or involvement of some other genetic regulatory mechanism (Hadjadj et al., 2018). The co-occurrence of bla_{NDM-1} with active bla_{OXA-23}-like or bla_{OXA-58}-like in CRAB was a matter of concern as this will limit future therapeutic options (Zhou et al., 2015). Although the co-occurrence of bla_{NDM-1} and bla_{OXA-23}-like was earlier reported from India, this is the first report of the co-occurrence of bla_{NDM-1} with bla_{OXA-58}-like from India (Karthikeyan et al., 2010; Rahman et al., 2018). It was noticed that three STs of bla_{NDM-1} harboring strains were found belonging to CC 2 but no similarity was seen in bla_{OXA-58}-like. Interestingly, ST 1089 was shared by both of the bla_{OXA-58}-like & bla_{NDM-1} (Table 2). The other class-D beta-lactamase, the bla_{OXA-24}-like gene was first identified in 1997, followed by several reports of the outbreak of *A. baumannii* harboring bla_{OXA-24}-like gene (Bou et al., 2000) but none of the *A. baumannii* isolates was found to harbor bla_{OXA-24}-like in this study. This study also showed the absence of bla_{VIM-2} andbla_{IMP-1} in all the isolates under study.

Currently, WGS and SNPs analysis methods are considered as the most discriminative typing methods but MLST is still very much accepted and employed due to its inter-laboratory reproducibility. MLST revealed diversity in the population under study both by number and novelty as 47 STs were represented by all 97 isolates, out of which 28 (59.6%) were novel STs that were assigned to 41 isolates. Majority of the CRAB isolates under study belonged to IC2. There have been many reports of bla_{OXA-23}-like belonging to globally disseminated IC2 (Chen et al., 2017; Pagano et al., 2015; Potron et al., 2015; Zarrilli et al., 2012; Zarrilli et al., 2013). IC2 has already been designated as a pandemic lineage in Asia, North America and Europe (Adams-Haduch et al., 2011; Asai et al., 2011; Tan et al., 2013). Interestingly, in the South or Latin America, the predominant *A. baumannii* lineages reported so far were different from those reported in rest of the world, as the most prevalent ICs are IC5 ($CC79^{Pas}$) and IC7 ($CC25^{Pas}$) (Higgins et al., 2010; Opazo-Capurro et al., 2019). There are recent reports that suggested an increased prevalence of IC2 in South America (Levy-Blitchtein et al., 2018; Vasconcellos et al., 2017). Apart from IC2, isolates from our study also belonged to IC1, IC7, and IC8. There are reports of IC2 isolates from India but this is the first time that we described the prevalence of IC1, IC7, and IC8 in Indian hospital settings. This underpins the hospital-wide spread of the international clones of CRAB. Presence of isolates belonging to IC8 and IC7 suggested the dissemination of CRAB, which differs from dominated IC1–3 in the Indian hospitals. We would also like to highlight that the majority of the STs, which were positive for the bla_{NDM-1} belonged to the IC2 (Table 2).

The phylogenetic analysis using ML phylogenetic tree and split network analysis revealed three diverse lineages, majority of isolates belonged to lineage L1 along with the prevalence of the minor lineage L2 and L3 among the isolates from Indian hospitals. Further recombination analysis using *phi* test and split network analysis suggested significant evidence of recombination across the whole population level and within lineages L1 and L2. The split network analysis of the whole population indicated that inter-lineage recombination between the L1 and L2 lineages indicating an ongoing evolution. The split tree analysis on the individual MLST loci *gyrB* and *gpi* displayed intergenic recombination had occurred during the evolutionary history of these genes. However, *gltA, gdhB, recA, cpn60,* and *rpoD* gene loci do not have a sign of intergenic recombination, which suggested that the descent of these genes were clonal. The mean per-site recombination/mutation ratio (ρ/θ) values suggested that the recombination events occurred more frequently in the whole population level and point mutations were more prominent in L2 and L3. Linkage equilibrium analysis suggested that although the recombination played an important role in generating the genotypic diversity that was not enough to break down the clonal relationship, which indicated the clonal nature of the population. This suggested that the natural barriers were presented to prevent gene flow across lineages.

5. Conclusion

This is the first study of its kind to describe the epidemiology and genetic recombination of a large number of CRAB isolates from India. The bla_{OXA-23}-like and bla_{NDM-1} are observed as major carbapenem resistance determinants in the CRAB isolates. Isolates of this study were found to be representative of the global diversity of the clinically important CRAB, where maximum isolates are linked to the international spread clones. More studies with isolates from different parts of India

Table 5
Results of recombination tests among the whole population and different lineages of population.

Population(n)	*Phi* test	Recombination analysis					Linkage disequilibrium	
		Theta/site	Rho/site	Rho/theta	LB 95%	UB 95%	I_A^S	P value
All (n = 47STs)	0.0	1.015E-02	0.135529	13.357	6.909E-02	0.293E-03	0.2838	< 1.00E-04
L1 (n = 29STs)	0.0	1.013E-02	5.053E-02	4.989	2.913E-02	9.467E-02	0.2433	< 1.00E-04
L2 (n = 12STs)	1.356E-9	5.494E-03	5.497E-03	0.9573	3.75E-03	7.96E-03	0.3688	< 1.00E-04
L3 (n = 6STs)	0.2402	5.698E-03	1.182E-03	0.2074	7.712E-04	1.573E-03	0.2805	7.0E-04

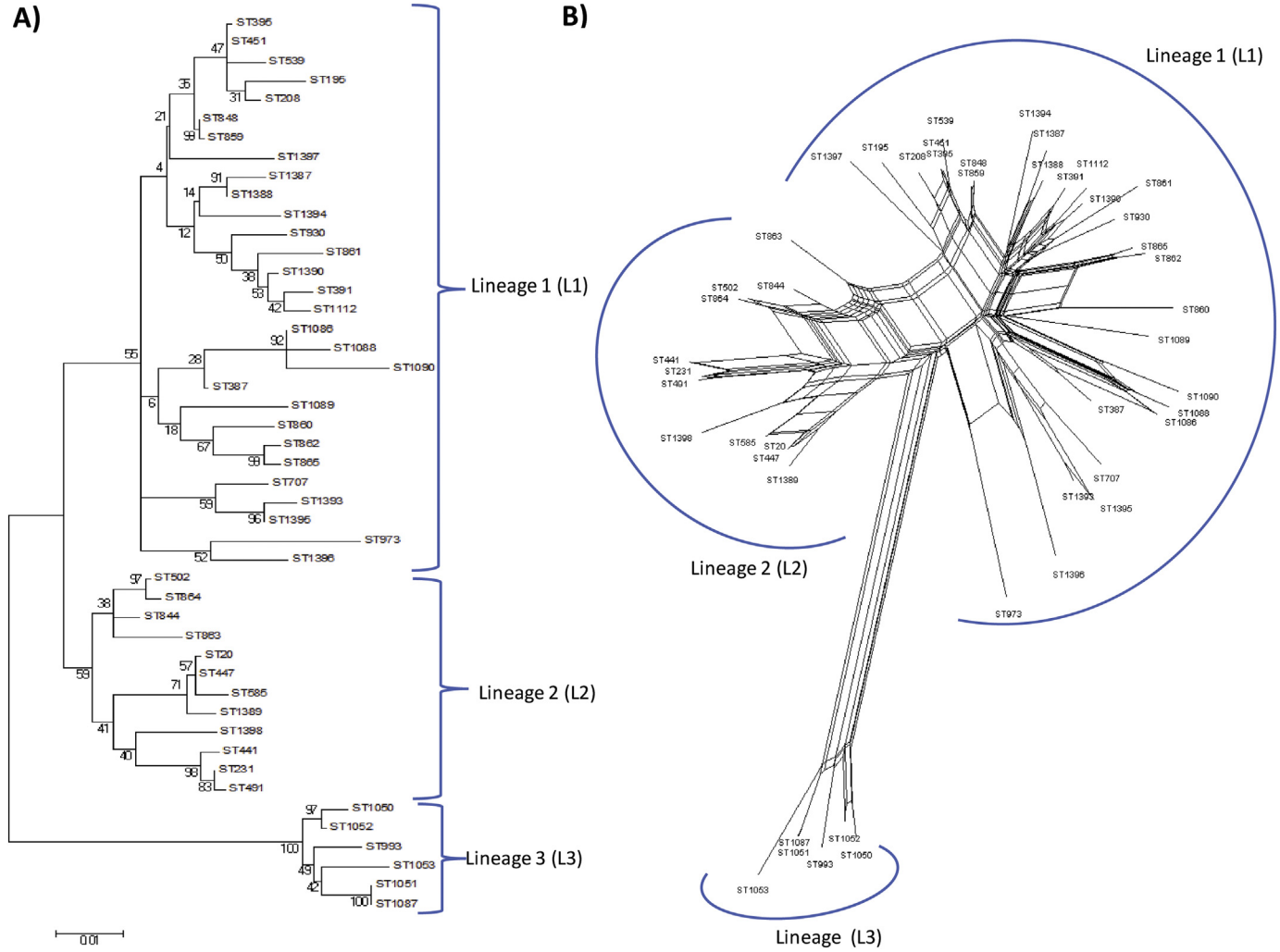

Fig. 2. Phylogenetic analysis. A) Phylogenetic tree generated from concatenated sequences of the MLST allelic loci with maximum likelihood method using MEGA 6.1. Three lineages L1, L2, and L3 are marked. B) Split network analysis of 47 STs from the present study and based on the concatenated sequences of seven MLST loci. The numbering in the figure refers to ST. Three subgroups corresponding to lineage L1, L2 and L3 are marked.

are recommended to understand the nationwide epidemiology of this bug. Further, Whole Genome Sequencing of this collection of CRAB isolates will give major insights into the genetic diversity, resistome, mobile genetic elements and virulence determinants of the CRAB from India.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.103986.

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