A rare occurrence of multidrug-resistant environmental *Acinetobacter baumannii* strains from the soil of Mangaluru, India

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Abstract

Over the last decade, *Acinetobacter baumannii* has emerged as one of the main causes of infections acquired in the hospital setting. Outbreaks associated with this pathogen are caused mainly due to contamination and transmission in hospital territories. However, the natural habitats of *A. baumannii* of clinical significance still remain unclear. In this study, we highlight the isolation and identification of multidrug-resistant environmental strains of *A. baumannii* from the soil of Mangaluru city. All the recovered isolates were biofilm formers, and two isolates were multidrug-resistant and showed resistance to fluoroquinolone, aminoglycosides, sulfonamide, tetracycline, and carbapenems. In addition, they exhibited protease activity, and produced phospholipase C and siderophore. To the best of our knowledge, this is the first study to isolate and identify drug-resistant strains of *A. baumannii* from the soil.

Keywords  *Acinetobacter baumannii* · Nosocomial pathogen · Multidrug resistance · Soil · Virulence factors

Introduction

*Acinetobacter baumannii* is recognized to be an important opportunistic nosocomial pathogen causing infections such as secondary meningitis, bacteremia, urinary tract infection, surgical site infection, wound or burn infection, and ventilator-associated pneumonia (McConnell et al. 2013). During the last 10 years, bacteria belonging to the genus *Acinetobacter* have emerged as the major cause of hospital-acquired infections and approximately 80% of these infections are caused by *A. baumannii* (Manchanda et al. 2010). *A. baumannii* is considered to be an ESKAPE pathogen, a group of multidrug-resistant pathogens responsible for nosocomial infections (Rice 2008). Possession of various virulence factors by *A. baumannii* makes it a potential pathogen. It has one of the most sophisticated nosocomial weapons among the *Acinetobacter* species (Wong et al. 2017). The members of the genus *Acinetobacter* have been reported to be saprophytes of water, soil, food, and sewage as per Bergey’s Manual of Systematic Bacteriology (Garrity et al. 2005). The saprophytic nature of the species of *Acinetobacter* has been cited in various research articles dealing with clinical strains of *Acinetobacter*. Such reports stating the ubiquitous presence of clinically important species of *Acinetobacter*, including *A. baumannii* in natural environments, such as soil and water, are now considered to be misconceptions (Towner 2009). This is because of the fact that the natural habitats of the clinically relevant strains of drug-resistant *Acinetobacter* spp. remain undefined. The likely dissemination of the clinical strains found in the environment into and/or out of the hospital environment remains unexplained (Hrenovic et al. 2014). Although the presence of clinically important multidrug-resistant strains of *A. baumannii* in the...
environment is very rare, in this investigation, we report the isolation and identification of environmental strains of *A. baumannii* from the soil. The isolated strains were assessed for their resistance pattern and virulence attributes.

**Materials and methods**

**Isolation of *A. baumannii* from the environmental samples**

Soil and water samples collected from sites away from hospitals in and around Mangaluru were used for the isolation of *A. baumannii*. The samples were collected for a period of 6 months from September 2019 to March 2020. 1 g of soil or 1 ml of water sample was inoculated into 5 ml of Dijkshoorn enrichment medium taken in a sterile test tube and incubated at 37 °C overnight. A loopful of inoculum from the enrichment medium was streaked onto Leeds Acinetobacter agar (HiMedia, India), a selective medium, and incubated for 24 h. A single colony from the selective medium was used to inoculate the nutrient agar for carrying out staining and various biochemical tests including oxidation/fermentation test, oxidase test, catalase test, and indole test for the presumptive identification of *A. baumannii* (McFaddin 1980). The tentatively identified *A. baumannii* isolates were further confirmed by PCR using species specific primers *bla* OXA-51 and 16S-23S rRNA ITS (Table 1). The primer for the 16S rRNA-23S rRNA ITS region was designed by us for which the sequence of *A. baumannii* was retrieved from NCBI. Using primer3 program, the primer was designed and the primer blast was performed to ensure 100% similarity. Based on the G + C content (40–55%), primers were picked and selected for the study. Matrix-associated laser desorption/ionization time of flight mass spectroscopy (MALDI-TOF MS) (Vitek MS, bioMerieux Inc., France) was used for the bacterial identification. Additionally, the PCR products were purified using QIAquick PCR purification kit (Qiagen, Germany) and sequenced using 16 s rRNA primers for the confirmation of bacteria by Sanger sequencing in a reference laboratory (Eurofins Genomics India Pvt Ltd., Bengaluru, India).

**Antibiotic susceptibility testing**

Environmental *A. baumannii* isolates were investigated for their antibiotic susceptibility by the Kirby–Bauer disc diffusion method according to the CLSI guidelines (CLSI 2017). Antibiotics such as cefepime (50 mcg), ceftazidime (30 mcg), ciprofloxacin (5 mcg), amikacin (30 mcg), gentamicin (10 mcg), co-trimoxazole (25 mcg), doxycycline (30 mcg), tetracycline (30 mcg), tigecycline (15 mcg), imipenem (10 mcg), and meropenem (10 mcg) were used. The measured zones of inhibition were compared with the standard interpretive chart of CLSI, and designated as sensitive, intermediate, and resistant. Susceptibility to colistin was carried out by broth dilution method as described in the CLSI guidelines. Briefly, serial twofold dilutions of colistin were prepared in the medium (500 μl) ranging from 0.125 to 256 μg/ml. Based on the turbidity, the isolates were categorized into susceptible (≤ 2 μg/ml) and resistant (≥ 4 μg/ml) as per the current CLSI colistin breakpoints.

**Virulence assays**

The biofilm assay was carried out according to the method of O’Toole with minor modifications as described in our previous study (Premanath et al. 2019). Biofilm formation of the isolates was quantified by computing the absorbance at 600 nm for different time intervals of 24 h, 48 h, 72 h, 96 h, and 120 h. To carry out phospholipase C assay, *A. baumannii* isolates were spot inoculated on egg-yolk nutrient agar plates and incubated for 24 h at 37 °C. Phospholipase C activity (Pz) was expressed in terms of the ratio between the diameters of the colony to the diameter of zone of precipitation (mm). The isolates were classified as negative (−) with Pz of 1.0, 0.99–0.9 as weak (+), 0.89–0.8 as mild (++), 0.79–0.7 as relatively strong (+++), and < 0.69 as very strong (++++) (Elleboudy et al. 2016). Twitching motility in *A. baumannii* was investigated by the previously described method of Semmler et al. (1999). Positive twitchers were identified as those with a zone of > 10 mm around the site of inoculation. To investigate swarming motility, 10 μl of freshly grown cultures of *A. baumannii* were inoculated at the center of the petriplate containing 0.45%

| Table 1 | Primers used in this study |
|---------|----------------------------|
| **Gene** | **Sequence (5′–3′)** | **Product length (bp)** | **References** |
| *bla*OXA-51 | F:TAATGCTTTGATCGGCCTTG R:TGGATTGCACTTCATCTTTG | 353 | (Karunasagar et al. 2011) |
| 16S-23S rRNA ITS | F:CATTATCACGGTAATTAGTG R:AGAGCAGTCGTGCACCTTAA | 208 | This study |
| 16S- rRNA | F:AGAGTTTGATCCCTGTGCAG R:TACGGTACCTTGTTACGACTT | 1500 | (Chulhong et al. 2010) |
Luria Bertani agar. The plates were incubated at 37 °C for 24 h. Swarvers were defined as those that showed a zone of > 20 mm around the site of inoculation (Bart et al. 2011). Chrome azurol S assay for the quantification of siderophore was carried out according to the method of Arora et al. (2017). Protease assay was performed using the method of Rarely et al. (2002). Clear zones were measured to determine the protease activity and less than 10 mm was considered as a negative result.

**Results**

A total of 60 soil and 22 water samples were collected during the study period. The appearance of pale pink-colored colonies on Leeds Acinetobacter agar was observed in all the 60 soil and 18 water samples indicating the presence of *Acinetobacter* spp (Fig. S1). The colonies developed were oxidation positive and fermentation negative, oxidase negative, catalase positive, and indole negative consisting of Gram-negative, non-motile cocacobacilli. Molecular confirmation of the 78 isolates by blaOXA-51 and 16S-23S rRNA ITS identified only 5 strains to be of *A. baumannii* (Fig. S2). MALDI-TOF MS results also confirmed these 5 isolates as *A. baumannii*. The obtained raw nucleotide sequences from these 5 strains were analyzed using blast programs, blastp and blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). ORF finder was used to determine the presence of ORF (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). A web-based java program, molecular tool kit (http://www.vivo.colostate.edu/molkit), was employed to obtain the amino acid sequences from the DNA. BLAST analysis of the sequence data confirmed the isolates as *A. baumannii*. Partial sequences of 16 s rRNA were deposited in GenBank with the accession numbers OM900032, OM980781, OM981133, OM981139, and OM981158. The antibiotic profile of the five isolates indicated the presence of two multidrug strains (PS12 and PS31). PS12 was resistant to fluoroquinolone (ciprofloxacin), aminoglycosides (amikacin and gentamicin), sulfonamide (co-trimaxazole), tetracycline (doxycycline), and carbapenems (imipenem and meropenem). It was susceptible only to the glycyclcline class of antibiotic, tigecycline. Whereas, PS31 was resistant to ciprofloxacin, tetracycline, and imipenem. All the five isolates were found to be sensitive to colistin. The antibiotic susceptibility of the isolates is depicted in Table 2.

| Antibiotics       | PS12 | PS16 | PS21 | PS31 | PS37 |
|-------------------|------|------|------|------|------|
| Ciprofloxacin     | R    | R    | S    | R    | S    |
| Amikacin          | R    | S    | S    | S    | S    |
| Gentamicin        | R    | S    | S    | S    | S    |
| Sulfamethoxazole  | R    | S    | S    | I    | I    |
| Trimethoprim      |      |      |      |      |      |
| Tetracycline      | I    | R    | R    | R    | I    |
| Doxycycline       | R    | S    | S    | S    | S    |
| Tigecycline       | S    | S    | S    | S    |
| Imipenem          | R    | S    | S    | R    |
| Meropenem         | R    | S    | I    | S    |
| Colistin          | S    | S    | S    | S    |

R: Resistant, I: Intermediate, S: Sensitive

with one isolate displaying 67% activity. Protease activity was exhibited by all the isolates with more than 20 mm zone around the well. The difference in the virulence attributes of the isolates is depicted in Table 3.

**Discussion**

Infections with *A. baumannii* are associated with significant morbidity, mortality, and increased medical costs. It is known to be persistent in the clinical setting. The transfer of this pathogen from patient to patient leading to an outbreak is well recognized (Naas et al. 2006). However, the presence of an extra-hospital reservoir of *A. baumannii* and its role in causing specific infections is still controversial. Reports related to the occurrence of these clinically important bacteria in nature and in natural habitats outside the hospital are scarce (Towner 2009). Moreover, the misconception of the ubiquitous nature of *A. baumannii* can also be attributed to the difficulties confronted during the precise identification of this organism (Peleg et al. 2008). The current investigation was undertaken due to the fact that non-clinical isolates can offer a vital clue in providing important insights into the evolution of multidrug-resistant nature of *A. baumannii*. In this report, we discuss the isolation and identification of drug-resistant *A. baumannii* from soil collected from sites away from the vicinity of hospitals.

During the last decade, there have been some reports of isolation of *A. baumannii* from environmental sources such as soil contaminated with petroleum hydrocarbons from countries like India and France (Sarma et al. 2004), from surfaces such as tables in parks and game consoles in South Korea (Choi et al. 2012), from agricultural soil and pig slurry in the UK (Byrne-Bailey et al. 2009), from vegetables collected in groceries, supermarkets and in private

Table 2: Antimicrobial susceptibility pattern of the environmental *A. baumannii* isolates to the antibiotics used in the study
gardens (Berlau et al. 1999), from aquaculture environments of Southeast Asia (Huys et al. 2007), and from veterinary hospitals (Endimiani et al. 2011). The environmental isolates used in the present study were retrieved from the soil using Dijkshoorn enrichment medium, which helps in the recovery of a greater number of *Acinetobacter* spp. Two specific primers were employed for the molecular identification of *A. baumannii*. *bla*OXA-51 was selected, because it is intrinsically found in the chromosome, and its G + C content closely matches with that of *A. baumannii* and has been reported to be used for species identification (Turton et al. 2006; Karunasagar et al. 2011). The ITS region was chosen, because it has been suggested to be a good candidate for species identification (Cheng et al. 2005).

Further characterization of these isolates was performed principally for two reasons. First, the pathogen is typically not found in the environment (Peleg et al. 2008). Second, the majority of the identified strains have a clinical origin (Bennahmod et al. 2019; El-Badawy et al. 2019). *A. baumannii* is one of the notorious bacteria that has developed resistance to a number of commercially available drugs. Not only resistance but also infections by *A. baumannii* have increased the mortality rate worldwide from 5 to 54% in patients under intensive care (Asif et al. 2018). The multidrug-resistant nature of clinical strains of *A. baumannii* is well known and there are several reports to date concerning the isolation of drug-resistant *A. baumannii* from patients and the hospital environment (El-Kazzaz et al. 2020; Yadav et al. 2020). In the present study, the confirmed five isolates of *A. baumannii* showed a variable susceptibility pattern to antibiotics. Two isolates were found to be multidrug-resistant and three isolates were found to be resistant. The isolation of MDR strains in our investigation corroborates with the findings from a study by Hrenovic et al. (2014) carried out in Paleosol from Croatia, where an isolated environmental isolate of *A. baumannii* resembled clinical strains. To the best of our knowledge, there are no reports stating the elaboration of virulence factors by the environmental isolates. In our study, all five isolates were biofilm formers, swimmers, siderophore, protease, and phospholipase C producers. Although the virulence in the environmental isolates was less in comparison to the clinical ones, the possession of virulence is a hallmark in pathogenic strains. Additionally, multidrug-resistant nature of the two strains is indicative of serious infections if the isolates disseminate from soil to the hospital environment. The presence of drug-resistant *A. baumannii* strains in the environment is indicative of an alternative natural reservoir.

### Conclusion

Although the occurrence of clinically relevant multidrug-resistant *A. baumannii* strains in the environment is extremely unusual, we describe the isolation and identification of environmental *A. baumannii* strains from the soil. The elaboration of virulence factors in the recovered isolates is indicative of its pathogenicity and an important characteristic feature of pathogenic strains. Even though the strains were less virulent, the multidrug resistance nature indicates the risk of severe illnesses if the isolates disseminate from the soil to the hospital environment. The existence of drug-resistant *A. baumannii* strains in the environment suggests the possibility of a new natural reservoir. Further studies are required to delineate the genetic similarities or variation of environmental strains with the hospital isolates.

### Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s00203-022-03035-0.

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### Declarations

**Conflict of interest** The authors declare that there is no known conflict of interest associated with this publication.
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