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Molecular characterization of a multidrug-resistant/pandrug-resistant nosocomial polymicrobial infection with *Klebsiella pneumoniae*, *Providencia rettgeri*, and *Acinetobacter baumannii* from Rural Maharashtra, India

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The emergence of resistance against commonly used antibiotics has become a serious global concern. The rapid development of antibiotic resistance exhibited by *Enterobacteriaceae* has caused an increasing concern regarding untreatable bacterial infections. Here, we isolated four pathogens from a geriatric female patient who was hospitalized for a month with ventilator-associated pneumonia (VAP) and fever. The organisms isolated from the tracheal aspirates and urine included *Klebsiella pneumoniae*, pandrug-resistant *Providencia rettgeri*, and *Acinetobacter baumannii*. Resistome analysis indicated that the bacterial isolates from the polymicrobial infection were multiple-drug resitnat and pandrug resistant clones. Molecular characterization revealed presence of bla_{TEM-1} in *K. pneumonaie, P. rettgeri* and *A. baumannii*. The bla_{TEM-1} and bla_{NDM-1} genes were present in *P. rettgeri* and *A. baumannii,* whereas the bla_{TEM-1}, bla_{NDM-1} and bla_{OXA-23} traits were present in *A. baumannii* isolates. The patient has died due to the unavailability of effective antimicrobial treatment for this drug-resistant polymicrobial infection.

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Abbreviations: VAP, Ventilator Associated Pneumonia; ESBL, Extended Spectrum β-lactamase; MDR, Multiple Drug Resistant; XDR, Extensively Drug Resistant; PDR, PanDrug Resistant; CRE, Carbapenem-resistant Enterobacteriaceae; DPS, Delayed Premonition Syndrome

INTRODUCTION

Microorganisms that are primarily involved in antibiotic resistance are called the "ESKAPE" pathogens, and include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa* and *Enterobacter species*, capable of "escaping" from commonly used antibacterial treatments (Boucher *et al*., 2009). *A. baumannii* has emerged as a highly challenging pathogen due to its specific antibiotic resistance characteristics (Baucher *et al,*. 2009). Moreover, reports of extensively drug-resistant and pandrug-resistant *K. pneumoniae* (XDR-KP and PDR-KP) cases are increasing worldwide (Fiorellakrap *et al*., 2018). *K. pneumoniae* is the most clinically relevant *Klebsiella* species and is responsible for >70% of infections (Hansen *et al*., 1998). Antimicrobial resistance has become a global crisis because of escalating resistance coupled with diminished antibiotics in the developmental pipeline. A recent report estimates that by 2050, antimicrobial resistance-related mortality will be 10 000 000/year (de Kraker *et al*., 2016).

The rapid emergence of carbapenem-resistant *Enterobacteriaceae* (CRE) worldwide has led to the concern that these infections may be soon untreatable. Management of infections caused by *K. pneumoniae* has been complicated by antimicrobial resistance, especially that against carbapenems. Whole genome sequence analyses of six extensively drug resistant (XDR) enteric pathogens isolated at New Delhi revealed multiple mobile genetic elements that were physically linked to antibiotic resistance traits. Thus, these elements seem to be responsible for disseminating drug resistance among organisms through underlying mechanisms of horizontal gene transfer and resistance to commonly used antibiotics (Kumar *et al*., 2017). Resistance to carbapenems in *K. pneumonia* involves multiple mechanisms, including production of carbapenemases, such as KPC, NDM, VIM, and OXA-48-like (Johann *et al*., 2015).

A 10-year study at Nashik, India (Odsbu *et al*., 2018, Lokhande et al., 2019), revealed a significantly higher proportion of non-susceptible and extended-spectrum β-lactamase (ESBL)-producing isolates from inpatients than those from outpatients for both, *Escherichia coli* and *Klebsiella* spp. A higher proportion of non-susceptible isolates indicates a great need to focus on the optimal use of antibiotics to reduce the development of antibiotic resistance.

Diverse risk factors associated with multidrug-resistance (MDR) in A. baumannii and other *Enterobacteriaceae* members suggest that a separate outbreak investigation should be performed in each hospital setting. Development of innovative control strategies is needed to limit the spread of these pathogens (Falagas & Kopterides, 2006).

In this study, we aimed to elucidate the mechanisms underlying drug resistance exhibited by prevalent pathogens responsible for unresponsiveness to the treatment administered to the patient. *K. pneumoniae, P. rettgeri*, and *A. baumannii* were isolated from the urine and tracheal aspirate of the patient on admission to the Somani Hospital, Barshi, Maharashtra, India.

CASE PRESENTATION

A 64-year-old female patient was hospitalized in Barshi with altered behavior, history of fall, and intracranial hemorrhage; the patient was put on a ventilator. Earlier, for 4 weeks, she received treatment at the Neurology Centre in Solapur, Maharashtra, and upon stabiliza-

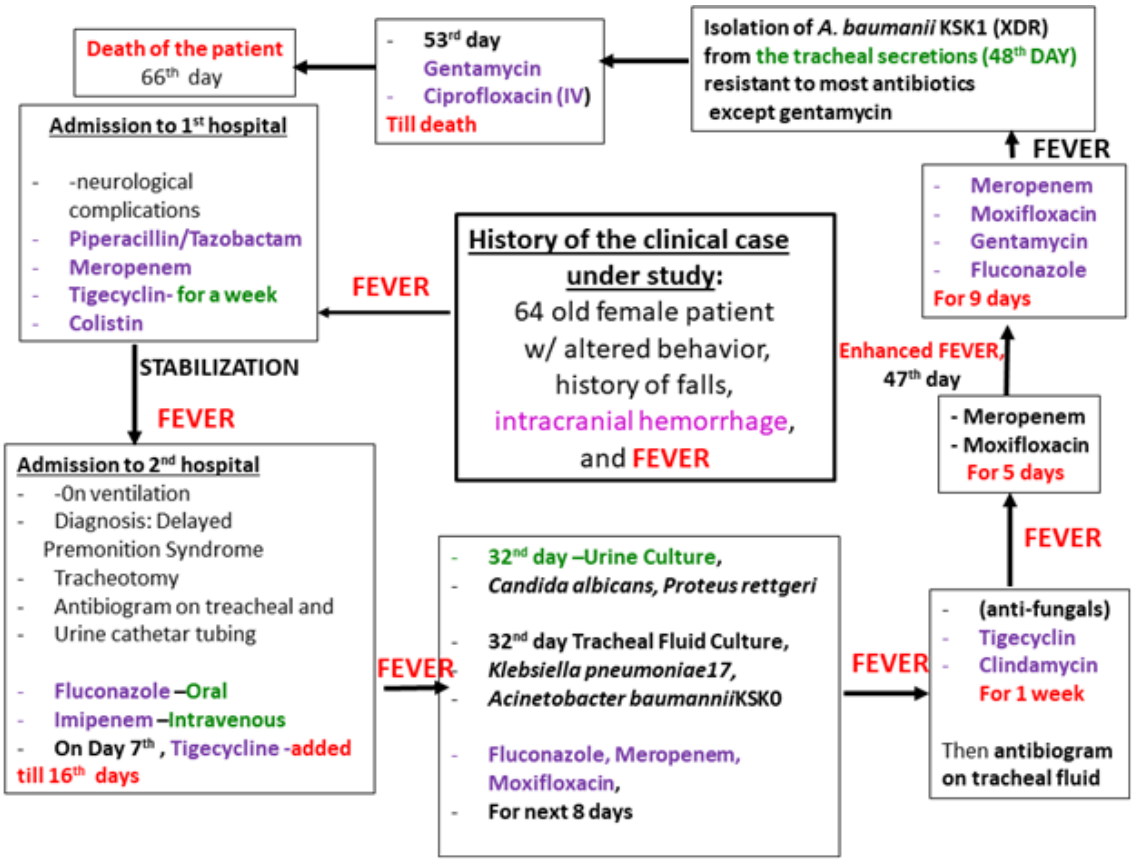

Figure 1. A 64 year old female patient was hospitalized twice at two different hospital.
Upon first admission, which was due to neurological complications, she was treated empirically for a month with Piperacillin/Tazobacta, Meropenem, Tigecyclin (a week) and Colistin. During the second hospitalization, culturing was performed for the tracheal fluid and one time culture was done from urine. Patient was found to have infected with yeast and three types of MDR, XDR and PDR bacterial pathogens.

tion, she was moved to the Dr. Yogesh Somani Hospital, Barshi. During treatment, the patient developed a ventilator-associated pneumonia (VAP) and was administered piperacillin/tazobactam, meropenem, tigecycline, and colistin without a culture and susceptibility testing (Fig. 1, first hospitalization).

On arrival at the Somani Hospital, the patient had a fever, was drowsy and arousable to the delayed premonition syndrome (DPS). She did not respond to verbal command, was on tracheostomy and discontinued from the ventilator use. She was treated with oral fluconazole and intravenous imipenem. Tigecycline was included in the treatment regimen without prior confirmation obtained by using an antibiogram or susceptibility testing. This therapy continued until the 16th day of hospitalization.

The patient was found to have bacteriuria and dead pus cells in the urine; therefore, urine culture and a susceptibility test was performed on the 32nd day of hospitalization; a mixed infection of *Candida albicans* and *P. rettgeri* was found. Based on susceptibility analysis, fluconazole, meropenem, and moxifloxacin was administered for the next 8 days. The fever continued and tigecycline was administered again for 7 days. On day 32, tracheal aspirate were tested for culture; *K. pneumoniae* and *A. baumannii* KSK0 were identified and isolated.

Based on the culture results and susceptibility analysis, treatment with meropenem and moxifloxacin was started. On day 47, she had high-grade fever, deteriorated CNS status, and was put on a ventilator support; the treatment was augmented and gentamicin was initiated in addition to meropenem and moxifloxacin.

On day 48, the culture and susceptibility analysis for the tracheal aspirate revealed *A. baumannii* KSK1, and gentamicin injections were administered along with the treatment on day 53, until the death of the patient on day 66. The cause of death was poly-microbial infection caused by resistant pathogens. Available antibiotics and treatment were insufficient.

MATERIALS AND METHODS

The isolates were collected during December 2017 at Barshi town in Maharashtra, India. Isolates were cultured on blood and MacConkey agars for purification. Well isolated, similar looking colonies were sub-cultured on trypticase soy agar and preserved in glycerol at -70°C for further analysis. Isolates were identified by the VITEK-2 (bioMérieux) system.

Susceptibility testing. The four selected isolates: *K. pneumoniae*, *A. baumannii* KSK0, and *A. baumannii* KSK1 from tracheal aspirates, and *P. rettgeri* from urine, were susceptibility tested using the VITEK-2 (bioMérieux) system. The panel covered a broad range of antibiotics to estimate resistance and guide the antibiotic therapy. The isolates were susceptibility tested by also using a reference broth microdilution minimum inhibitory concentrations (MIC) determination method, as described by the CLSI (M07, A10). Briefly, a serial two fold dilutions of the antibacterial agents were made in cation-adjusted Muller-Hinton broth (BD, USA) in the presence or absence of a fixed inhibitor concentration. Bacterial suspensions of 0.5 McFarland turbid-

Table 1. The -lactamase trait, primer sequence, amplicon size, annealing temperature and references are shown.

β-lactamase gene	Primer Sequence	Amplicon Size (bp)	Annealing temp (°C)	Annealing location	Reference
bla$_{CTX-M}$ group I	GACGATGTCACTGGCTGAGC	499	55	416–435	Lewis *et al.*, 2007
	AGCCG CCGACGCTAATACA			914–896	
bla$_{CTX-M}$ group II	GCGACCTGGTTAACTACAATCC	351	55	313–334	
	CGGTAGTATTGCCCTTAAGCC			663–643	
bla$_{CTX-M}$ group III	CGCTTTGCCATGTGCAGCACC	307	55	475–495	
	GCTCAGTACGATCGAGCC			781–764	
bla$_{CTX-M}$ group IV	GCTGGAGAAAAGCAGCGGAG	474	62	1857–1876	
	GTAAGCTGACGCAACGTCTG			2330–2311	
bla$_{TEM}$	CATTTCCGTGTCGCCCTTATTC	800	55	13–34	Dallenne *et al.*, 2010
	CGTTCATCCATAGTTGCCTGAC			812–791	
bla$_{SHV}$	AGCCGCTTGAGCAAATTAAAC	713	55	71–91	
	ATCCCGCAGATAAATCACCAC			783–763	
bla$_{NDM}$	GGTTTGGCGATCTGGTTTTC	621	55	133–153	Poirel *et al.*, 2011
	CGGAATGGCTCATCACGATC			734–754	
bla$_{OXA-48}$	GCGTGGTTAAGGATGAACAC	438	52	251–271	Canden *et al.*, 2015
	CATCAAGTTCAACCCAACCG			689–669	
bla$_{CMY-2}$	GCCGTTGCCGTTATCTAC	511	56	145–163	Mlynarcik Patrik *et al.*, 2016
	AATCTTTTTGTTCGTTCTGCG			656–635	
bla$_{OXA-23}$	GATCGGATTGGAGAACCAGA	501	53	NA	Woodford *et al.*, 2006
	ATTTCTGACCGCATTTCCAT			NA	
bla$_{OXA-24}$	GGTTAGTTGGCCCCCTTAAA	246	53	NA	
	AGTTGAGCGAAAAGGGGATT			NA	
bla$_{OXA-51}$	TAATGCTTTGATCGGCCTTG	353	53	NA	
	TGGATTGCACTTCATCTTGG			NA	

NA, not available

ity equivalents were prepared in sterile 0.85% Saline (NaCl) and were appropriately diluted to obtain a final cell density of 2–8×10^5 CFU/mL in the antibiotic containing medium. The plates were incubated for 18 h at 37°C. MICs were recorded as the lowest antibiotic concentration showing no visible growth of an organism. Categorical interpretations for all comparator agents were those found in the CLSI breakpoint tables (M100, S26). Quality control was performed using *Escherichia coli* ATCC 25922. All quality control MIC results were within acceptable ranges published in CLSI documents. The antibiotic panel included ceftazidime in combination with avibactam to detect the presence of KPC, Ambler Class C, and OXA-48 enzymes while, meropenem with EDTA was included to determine the presence of a metallo-β-lactamase (MBL) enzyme.

Genotype Determination. All of the isolates were tested for the presence of *bla*$_{CTX-M}$ variants, *bla*$_{TEM}$, bla$_{SHV}$, bla$_{KPC}$, bla$_{CMY-2}$, bla$_{NDM-1}$, and bla$_{OXA\ 23/24}$ genes by a PCR assay using specific primers (Table 1). The bacterial lysate was used as template DNA with a final reaction volume of 25 µl containing 10× buffer, 2.5 mM of dNTPs, 15 mM $MgCl_2$, 100 pM of each oligonucleotide primer, 1 U of Taq polymerase and 2 µl of bacterial lysate. PCR was carried out in a thermal cycler using specific annealing temperatures as shown in Table 1. Amplified products were resolved in an agarose gel. The band size for the specific amplified genes was compared with the control samples in the same run.

RESULTS AND DISCUSSION

A female patient was admitted at the Dr. Yogesh Somani Hospital, Barshi, MS, India after a treatment course for neurological complications. Over a longer period of stay in the hospital, the patient developed serial episodes of nosocomial infections. Four isolates: *K. pneumoniae* and *A. baumannii* KSK0 from the initial, *A. baumannii* KSK1 from the terminal tracheal aspirate sample, and *P. rettgeri* from the second to last urine sample were isolated subsequently. Upon antibiotic susceptibility testing using VITEK-2, as well as broth microdilution, *K. pneumoniae* was found to be susceptible to the first line treatment agents, including the third and fourth generation cephalosporins, carbapenems, and aminoglycosides. The given treatment appeared effective amid no recovery of *K. pneumoniae* in subsequent tracheal aspirate cultures.

P. rettgeri was isolated from the patient's urine. This was attributed to the prolonged catheterization and nosocomial infection. Susceptibility testing showed that, *P. rettgeri* was resistant to all of the tested antibacterial agents except to meropenem-EDTA. Susceptibility to the meropenem-EDTA combination exhibited an MBL expression. Moreover, intrinsic resistance of *P. rettgeri* to a few antibiotic classes further limited the treatment options. Notably, both *A. baumannii* isolates from the tracheal aspirates were multidrug-resistant, except for their

Table 2. Antibiotic susceptibility of pathogens isolated from the tracheal secretion and urine by VITEK-2

Antimicrobial agent	Symbol	*Klebsiella pneumoniae*		*Providencia rettgeri*		*Acinetobacter baumannii* KSK0		*Acinetobacter baumannii* KSK1	
		MIC	R/S	MIC	R/S	MIC	R/S	MIC	R/S
Amikacin	AK	≤2	S	≥64	R	≥64	R	≥64	R
Aztreonam	AT			32	R				
Cefepime	CPM	≤1	S	≥32	R	≥ 64	R	≥64	R
Cefoperazone-Sulbactam	C-S	≤8	S	≥64	R	≥64	R	≥64	R
Ceftazidime	CAZ			≥64	R			≥64	R
Ceftriaxone	CTR	≥64	R			≥64	R		
Cefuroxime	CXM	≥64	R	*	R	≥64	R	*	R
Ciprofloxacin	CIP	≤0.25	S	≥4	R	≥4	R	≥4	R
Colistin	CL					≤0.5	S		
Doxycycline	DO			*	R				
Ertapenem	ETP	≤0.5	S	*	R	≥8	R	*	R
Gentamicin	GEN	≤1	S	≥16	R	≥16	R	8	I
Imipenem	IPM	≤0.25	S	≥16	R	≥16	R	≥16	R
Levofloxacin	LE	*	S	≥8	R			≥8	R
Meropenem	MRP	≤0.25	S	≥16	R	≥16	R	≥16	R
Minocycline	MI			*	R			≤1	S
Moxifloxacin	MO	*	S	*	R				
Piperacillin-Tazabactam	PIT	≥128	R	≥128	R	≥128	R	≥128	R
Tigecycline	TIG			4.00	R	4.00	I		
Trimethoprim-Sulphomethoxazole	COT	≤20	S	≥320	R	≥320	R	≥320	R

MIC: minimum inhibitory concentration expressed in mg/L, S: susceptible, R: resistant, I: Intermediate, Interpretation of the drugs marked with (*) has been deduced based on phenotype of the isolate by VITEK-2. Hence MIC value cannot be reported.

intermediate resistance to gentamicin and tigecycline, and susceptibility to colistin (Table 2).

Resistotype and Genotyping studies

The VITEK-2 and broth microdilution MIC susceptibility results shown in Table 3 were validated by performing PCR amplifications for various β-lactamase traits. Results presented in Fig. 2 (a, b, c), and summarized in Table 4, show that the $bla_{\text{TEM-1}}$ variant was present in all four clinical isolates, whereas $bla_{\text{NDM-1}}$ was present only in *P. rettgeri*. Presence of $bla_{\text{NDM-1}}$ explains the susceptibility of *P. rettgeri* to meropenem in combination with the metal ion chelating agent EDTA. Interestingly, bla_{OXA} was found in *A. baumannii* KSK0 and *A. baumannii* KSK1, along with $bla_{\text{NDM-1}}$ isolated at different stages.

All clinical isolates were genetically ESBL positive, however, *A. baumannii* KSK0 and *A. baumannii* KSK1 had three β-lactamases, indicating evolution of a complicated drug resistance mechanism.

Virulent and resistant *K. pneumoniae* strains are a significant cause of hospital-acquired infections. Studies performed in Iran reported a high prevalence of resistance against several antibiotics, and the simultaneous presence

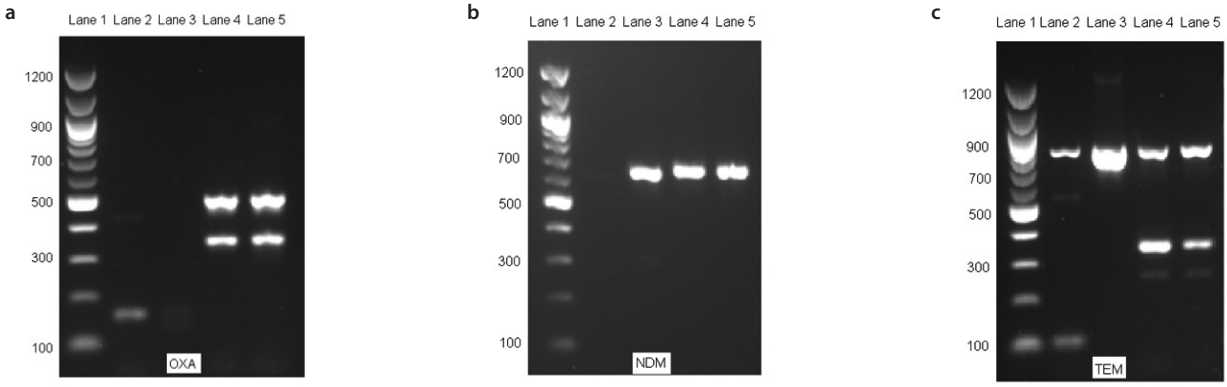

Figure 2. Amplification of *OXA* β-lactamase trait (a), *NDM* β-lactamase trait (b), and *TEM*-1 β-lactamse trait (c) among tracheal secretion and urine isolated from a patient with a polymicrobial infection.
Lane 1: DNA ladder, Land 2: *K. pneumoniae*, Lane 3: *P. rettgeri*, Lane 4: *A. baumannii* KSK0 and Lane 5: *A. baumannii* KSK1

Table 3. MIC established by broth double dilution method for the four clinical isolates

Antibacterial Agents	MIC (mg/L)			
	Klebsiella pneumoniae	*Acinetobacter baumannii* KSK0	*Providencia rettgeri*	*Acinetobacter baumannii* KSK1
Ceftazidime	0.12	>64	>64	>64
Ceftazidime+Clavulanic acid	0.12	>64	>64	>64
ceftazidime+avibactam	0.06	>64	>64	>64
Cefepime	<0.03	>64	>64	>64
Piperacillin+Tazobactam	1	>128	>128	>128
Meropenem	<0.03	>64	>64	>64
Meropenem+EDTA	<0.03	64	0.25	64
Imipenem	0.25	>64	>64	>64
Colistin	0.12	1	>32	1
Tigecycline	1	2	2	2
Amikacin	2	>256	>256	>256
Levofloxacin	0.06	16	>64	16
Trimethoprime-sulfamethoxazole	2	>64	>64	>64

MIC, minimum inhibitory concentration; ceftazidime with fixed clavulanic acid 4 mg/L, ceftazidime with fixed avibactam 4 mg/L, piperacillin with tazobactam 4 mg/L, EDTA, ethylenediaminetetraacetic acid at fixed 200 mg/L.

Table 4. Phenotypic and genotypic resistance mechanism for the four clinical isolates

Organism	Phenotype	Genotype
K. pneumoniae	WT	$bla_{\text{TEM-1}}$
A. baumannii KSK0	Class B+OXA	$bla_{\text{TEM-1}}$, $bla_{\text{NDM-1}}$, $bla_{\text{OXA-23}}$
P. rettgeri	Class B	$bla_{\text{TEM-1}}$, $bla_{\text{NDM-1}}$
A. baumannii KSK1	Class B+OXA	$bla_{\text{TEM-1}}$, $bla_{\text{NDM-1}}$, $bla_{\text{OXA-23}}$

WT, wild type

of certain virulence factors and MDR genes, contributing to a crucial public health issue (Ranjbar *et al*., 2019). Carbapenemases and ESBLs are responsible for pandrug-resistance (PDR) in *K. pneumoniae* clinical samples in Maharashtra, India (Lokhande *et al*., 2019). In their study, ESBL resistance was observed in 310 (88.57%) isolates and carbapenemase in 181 (51.71%) isolates; these were the primary mechanisms underlying antibiotic resistance. A total of 29 (8.28%) *K. pneumoniae* PDR isolates and 52 (14.85%) isolates susceptible to colistin alone were found. They found extreme drug resistance in 135 (38.57%) of the *K. pneumoniae* isolates.

The emergence and spread of MDR, ESBL producing carbapenem-resistant members of *Enterobacteriaceae* has become a worldwide health problem. A study in Bangkok, Thailand, revealed a unique prevalence of carbapenemase genes, where $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-232}}$ were predominant (Laolerd *et al*., 2018).

Providencia species are intrinsically resistant to colistin and tigecycline, making the treatment of MDR *Providencia* spp. challenging. Carbapenem-resistant *Providencia* spp. have been reported (Abdallah & Balshi, 2018). An outbreak of carbapenem-resistant *P. rettgeri,* involving 4 patients admitted to intensive care and high-care units at a tertiary hospital was reported. Their clinical and demographic characteristics were studied; experiments revealed that all *P. rettgeri* strains were resistant to carbapenems - imipenem, ertapenem, and meropenem (Tshisevhe *et al*., 2016). Our results are in line with these referred studies that the *P. rettgeri* strain isolated from urine of our patient was a PDR strain.

The MDR *A. baumannii* has been recognized as clinically significant. Numerous reports relay the spread of *A. baumannii* in hospital settings, leading to nosocomial outbreaks with increased mortality. However, many *Acinetobacter spp.* can also cause nosocomial infections. A review focused on the role of *Acinetobacter spp.* as nosocomial pathogens along with their persistence, antimicrobial resistance patterns, and epidemiology has been recently published (Almasaudi, 2018).

Antimicrobial resistance among *Acinetobacter* spp is a global threat (Clark *et al*., 2016). *A. baumannii* is a major cause of healthcare-associated infections. MDR *A. baumannii* is a rapidly emerging pathogen, especially in the intensive care units, causing infections including bacteremia, pneumonia/VAP, meningitis, urinary tract infection, central venous catheter-related infection, and wound infection. An optimal treatment for *A. baumannii* nosocomial infections has not been established (Clark *et al,* 2016). However, the antibiotics that are usually effective against *A. baumannii* infections include carbapenems, polymyxins E and B, sulbactam, piperacillin/tazobactam, tigecycline, and aminoglycosides. Carbapenems (imipenem, meropenem, doripenem) are the mainstay of *A. baumannii treatment*; however, carbapenem-resistant *Acinetobacter* strains have been recently reported. These bacteria commonly present resistance to multiple antimicrobial agents, including carbapenems and polymyxins; hence, they are considered the paradigm of MDR or PDR bacteria. The XDR *A. baumannii* KSK0 and *A. baumannii* KSK1 isolates were difficult to treat as their MIC values were much higher than the prescribed doses for these antibiotics.

The indiscriminate and widespread antibiotic use causes rise of the resistant *A. baumannii* strains. A study performed in Chennai, India, reported the frequency of MDR (71.23%) and XDR (39.72%) for *A. baumannii* iso-

lates (Girija & Priyadharsini, 2019). That study stated that periodical antibiotic surveillance is essential to curb the emergence of MDR and XDR *A. baumannii* in hospital environments, improving patient care using alternate drugs of choice or a combination therapy. A study in Algeria highlighted the high prevalence of imipenem-resistant A. baumannii in the Algiers hospitals, mediated by $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$, and $bla_{\text{NDM-1}}$ genes (Khorsi *et al.*, 2015). A study performed in Turkey revealed that the prominent genes responsible for carbapenem resistance in clinical *A. baumannii* strains were $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-23}}$, and the high prevalence of clones may constitute a threat for hospitalized patients (Direkel *et al.*, 2016).

In general, 95% of cases in rural India are treated empirically without culture and antibiogram reports. In spite of culturing facilities, treatment for a long duration and a polymicrobial infection can result in poor prognosis and outcomes. Similarly, the presented case study demonstrates the acquisition of nosocomial pathogens while undergoing treatment for neurological complications. The presence of MDR/XDR/PDR pathogen clones in the hospital environment makes the critical surgeries complicated, and the pathogens' elevated MICs add more difficulty to find a successful therapy.

CONCLUSION

A female geriatric patient was found to suffer a polymicrobial infection caused by *K. pneumoniae, P. rettgeri, A. baumnannii,* and *Candida albicans*. Co-existence of the ESBL traits: $bla_{\text{TEM-1}}$, $bla_{\text{NDM-1}}$, $bla_{\text{OXA-23,}}$ caused high MIC values posing difficulty to meet a desired dose of antibiotics and in turn led to fatality.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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