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Verona integron-encoded metallo-β-lactamase (VIM) and Vietnam extended-spectrum β-lactamase (VEB) producing *Pseudomonas balearica* from a clinical specimen

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Abstract

Pseudomonas balearica, a saprophyte found in marshy and marine habitats, is not routinely differentiated from *P. aeruginosa* and *P. stutzeri* using automated systems and hence has not been reported from clinical samples. This study describes the identification of *P. balearica* using MALDI-TOF-MS and 16S rDNA sequence from a patient admitted to an intensive care unit (I.C.U.). The isolate was found to be Verona integron-mediated Metallo-β-lactamase (V.I.M.), and Vietnam extended-spectrum β-lactamase (V.E.B.) producer and resistant to Ceftriaxone, Imipenem, and Tobramycin. *P. balearica* can be a source for horizontal transfer of bla_{VEB} and bla_{VIM}. Its pathogenesis has yet to be understood.

Keywords: Emerging pathogen, β-lacamases, *Pseudomonas balearica*, *Pseudomonas stutzeri.*

Introduction

With the advent of new techniques and their implementation in the diagnosis of clinical specimens, many new species have been identified, while previously known species have either been reclassified or have been isolated from unusual habitats. *Pseudomonas* species other than *P. aeruginosa* were commonly considered as

saprophytes, but recently these have been considered as opportunistic human pathogens, and outbreaks have also been reported from such species [1, 2].

P. balearica is mainly found in marine and freshwater, salt marshes, and wastewater [3-4]. It is characteristically tolerant to higher salt concentrations (8.5% NaCl) and was recognized and reclassified as a separate species of *Pseudomonas* due to its ability of denitrification [3, 5]. In this study, *P. balearica* was isolated from the tracheal aspirate of a patient with ventilator-associated pneumonia (V.A.P.). This is the first report describing the isolation of *P. balearica* from a clinical specimen.

Case Report

The specimen was obtained on June 2017 from a patient aged 52 years admitted to the Medical ICU-23 of Jinnah Post Graduate Medical Centre (JPMC), Karachi, Pakistan, diagnosed with Guillain-Barre Syndrome (G.B.S.) and had been referred from a hospital for mechanical ventilation. On the 7^{th} day of admission, the patient developed Ventilator-Associated Pneumonia (V.A.P.) with high fever, leucopenia (2900WBCs/mm^2)**,** tachypnoea, increased or purulent secretions, haemoptysis, and bronchospasm. Ceftriaxone was given after sample collection, but no improvement was observed. Considering susceptibility testing results, Ceftriaxone was replaced with Ciprofloxacin. The patient improved and was discharged from the I.C.U. The improved parameters included normalization of the WBC count, temperature, and the absence of other indicators of V.A.P.

The pathogen was isolated from the tracheal aspirate and initially identified as *P. stutzeri* by the conventional methods using the API 20NE strip (Biomerieux, Marcy I'Etoile France). While identification through BD Phoenix™ Automated Microbiology System (instrument version 5.15A, software version 6.01A/V5.15A) [Becton Dickinson, Oxford, U.K.] presented the pathogen as *P. aeruginosa*. Consequently, further biochemical tests such as the ability to utilize xylose and to tolerate high salt concentration (Table 1) were carried out which indicated the necessity to investigate the strain through more sophisticated techniques including

sequencing 16S rDNA and by Matrix-Assisted Laser Desorption Ionization–Time-of-Flight mass spectrometry [MALDI-TOF-MS] (Microflex, Bruker Daltonics, Bremen, Germany) and sequencing. These techniques identified the isolated strain as *P. balearica.* The cut-off scores (MALDI-TOF-MS) for identification was ≥1.7 for genus level and ≥2 for species level, while a score of <1.5 was disregarded. The details of the Biotyper threshold for the log score was interpreted and recorded as recommended by the manufacturer protocols.

The antimicrobial susceptibility of *P. balearica* to the most common antipseudomonal drugs was assayed by the disc diffusion and microtitre broth dilution methods, and automated B.D. Phoenix (version 5.15A) at the Clinical Centre for Microbiology U.C.L., London, using CLSI breakpoints for minimum inhibitory concentration (M.I.C.) interpretive standards (μg/mL) for other Non-Enterobacteriaceae [7]. The antimicrobial susceptibility of imipenem and meropenem was confirmed by the determination of M.I.C.s using Etest strip (BioMérieux, Lyon, France, and M.I.C. Evaluator, Oxoid, Basingstoke, U.K.). The data showed that *P. balearica* was resistant to imipenem, Ceftriaxone, and tobramycin and sensitive to ceftazidime, cefepime, and antipseudomonal penicillin. Results of automated B.D. Phoenix system for antimicrobial susceptibility was similar to the M.I.C.s by microtitre broth dilution and disc diffusion method (Table 2).

The detection of carbapenemases, Modified Hodge test (M.H.T.), Rapidec Carba NP (RCNP; Biomerieux, Marcy-l'Etoile, France), and ethylenediaminetetraacetic acid (EDTA) double-disc synergy test with imipenem and meropenem were employed. Rosco kit test for *Klebsiella pneumoniae* carbapenemase (KPC)/ Metallo-β-lactamase (M.B.L.) for *P. aeruginosa* and *Acinetobacter* species (ROSCO's Diagnostic, Tassstrup, Denmark) was also performed. Positive control strains for phenotypic and genotypic tests included *P. aeruginosa* NCTC 13437 (for V.I.M. and V.E.B.), *Klebsiella pneumoniae* NCTC 13443 (for NDM-1), and *Escherichia coli* NCTC 13476 (for IMP-types). The carbapenem hydrolyzing and other β-lactamases encoding genes were also investigated by the check-MDR CT103XL DNA microarray (Check-Points

Health B.V., Wageningen, Netherlands) and by conventional PCR using the primers as listed in Table 3.

The results of phenotypic tests for ESBL (double disc diffusion), carbapenemases M.H.T. and M.B.L.s (EDTA double-disc synergy and Rosco kit) were negative. In contrast, the Rapedic CARBA NP phenotypic test was positive for carbapenemase production. Genotypic analysis revealed the presence of bla_{VIM} and bla_{VEB}. The results of microarray were also the same as manual PCR, showing that the strain was positive for V.E.B. and V.I.M.

Amplicon and its size were measured with positive and negative controls of bla_{VIM} (Supplementary material Figure1). The location of bla_{VIM} and bla_{VEB} on the plasmid was determined by extracting plasmid using GeneJET plasmid miniprep kit (Thermo Scientific™ #K0502) according to the manufacturer's instructions. The PCR mixture and conditions were kept the same as for the amplification of V.I.M. and V.E.B. The VIM PCR product was sequenced and submitted to GenBank with an accession number of KY798549, whereas the V.E.B. was not sequenced.

To study conjugation mediated transfer of bla_{VIM} and $bla_{VEB,}$ the protocol given previously was followed using *P. balearica* as a donor and *azi*^{r} carrying *E. coli* J53 as a recipient [9]. The resultant transconjugants were selected on Mueller- Hinton (M.H.) agar containing sodium azide (100µg mL^{-1}) and imipenem (1µg mL^{-1}). The presence of the resistant markers in transconjugants was further confirmed by PCR as described above.

Discussion

Previously, *P. balearica* was included in a diverse group of *P. stutzeri*; however, heterogeneity within the group rendered further investigations to update the classification [6]. Indeed, *P. balearica* was separated from this group when sufficient distinguishing features were explored and supported by (a) multilocus sequence with phylogenetic analysis. This species was first separated from *P. stutzeri* after 16S rRNA gene sequence analysis in 1996 by Bennasar et al. [3]. However, the routine

protocols in diagnostic laboratories still report it as *P. stutzeri* because the semi-automated or API and automated systems cannot differentiate between the two species.

Moreover, the results of antimicrobial susceptibility testing indicate the need for updating surveillance programmes to curtail the spread of this pathogen. Contrary to the present results for susceptibility to ceftazidime, aztreonam, and cefepime, Laudy et al. reported that all the VEB-9 positive isolates were resistant to these antibiotics [10].

The presence of V.I.M. and V.E.B. β-lactamases which are found in *P. aeruginosa* strains from the same unit needs further analysis, as it suggests *P. balearica* may be a potential reservoir for outbreaks of highly resistant strains in I.C.U.s and a potential source for the horizontal transfer of antimicrobial resistance genes to other pathogenic bacteria. These findings support the conclusion that the resistance markers are plasmid-borne.

Conclusion

This case revealed that *Pseudomonas* species should identified by molecular assays and the prevalence of *P. balearica* needs to be determined. *P. balearica* can be a potential source of V.E.B. and M.B.L. (V.I.M.) by horizontal gene transfer. However, further investigations are required to understand the etiology and pathogenesis of *P. balearica*.

Disclaimer: This case was identified in Ph.D. research work and is part of the thesis.

Conflict of interest: The authors do not declare any conflict of interest.

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Table 1: Characteristics of *Pseudomonas balearica* and *P. stutzeri* has given by Bennasar et al. [3] and characteristics of *P. balearica* isolated in present study.

Characteristic	*P. baleanca*	*P. stutzeri*	*P. balearica* (present study)
Hydrolysis of:			
Gelatin	Negative	Negative	Negative
Starch	Positive	Positive	Positive
Utilization of:			
Maltose	Positive	Positive	Positive
Xylose	Positive	Negative	Positive
Malate	Positive	Positive	Positive
Mannitol	Negative	+/-	Negative
Denitrification	Positive	Positive	Positive
Growth at:			
42 °C	Positive	+/-	Positive
46 °C	Positive	+/-	Positive
Growth in 8.5% NaCl	Positive	Negative	Positive
Oxidase	Positive	Positive	Positive

Table 2: Minimum Inhibitory Concentration (MICs) of antimicrobial agents as determined by BD Phoenix and broth dilution method

Antimicrobial agent	BD Phoenix	Broth dilution method	Interpretation *
Amikacin	4	4	Sensitive
Gentamicin	4	4	Sensitive
Tobramycin	>4	8	Resistant
Aztreonam	<1	1	Sensitive
Cefepime	<1	1	Sensitive
Ceftazidime	2	2	Sensitive
Ceftriaxone	>4	>32	Resistant
Ciprofloxacin	0.25	0.25	Sensitive
Colistin	1	1	Sensitive
Imipenem	>8	>16	Resistant
Meropenem	8	8	Intermediate
Piperacillin	8	8	Sensitive
Piperacillin-tazobactam	8/4	8/4	Sensitive

*According to the CLSI broth dilution method criteria, 2016

Table 3: Primers used in this study

Resistant determinant	**Primer Sequence**	**Gene**	**Size (bp)**	**Reference**
VIM	F- GGTGTTTGGTCGCATATCGC R- CCATTCAGCCAGATCGGCATC	*bla*$_{VIM}$	503	[8]
IMP	F- GGAATAGAGTGGCTTAATTC R- CAACCAGTTTTGCCTTACC	*bla*$_{IMP}$	327	
NDM	F- CACCTCATGTTTGAATTCGCC R- CTCTGTCACATCGAAATCGC	*bla*$_{NDM}$	984	
VEB	F- GTTAGCGGTAATTTAACCAGATAG R- CGGTTTGGGCTATGGGCAG	*bla*$_{VEB}$	1070	

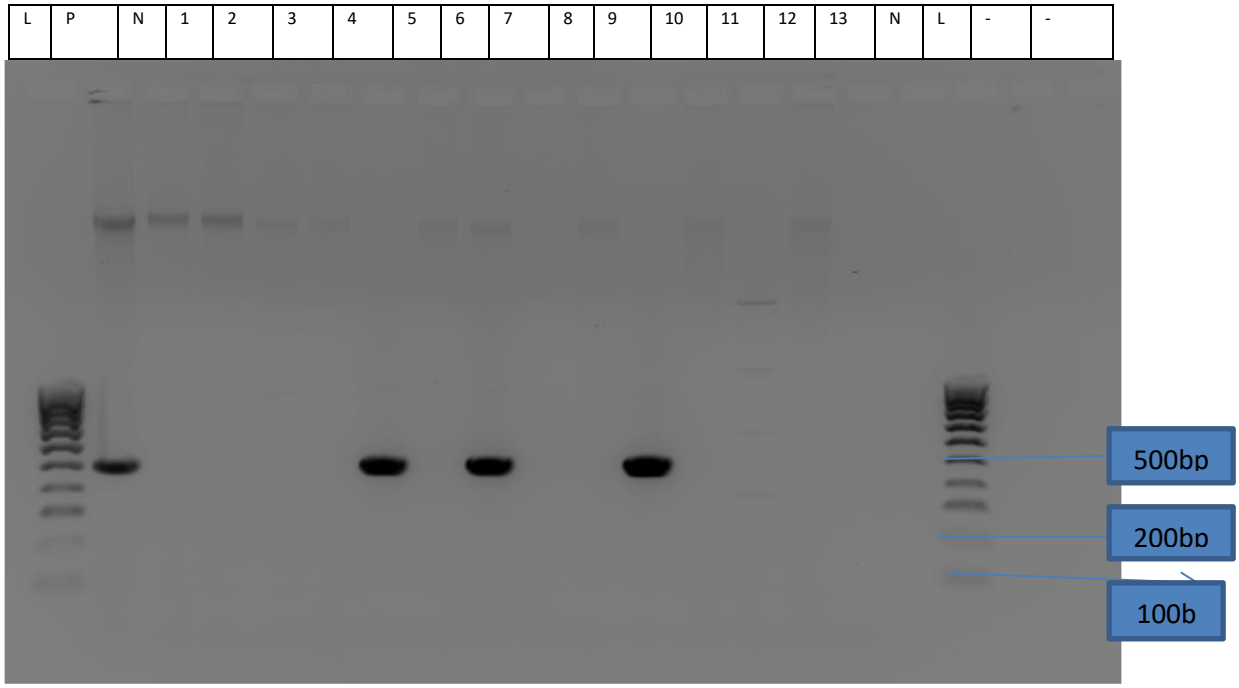

Fig:1 PCR amplified product of *bla*$_{VIM}$ (503 bp). L, DNA Ladder 50 bp, N, Negative control, P Positive Control (*P. aeruginosa*, NCTC 13437), VIM positive samples #4 (*P. balearica*), 6 and 9 of *P. aeruginosa*, while sample #1, 2, 3 5, 7, 8, 10, 11, 12 and 13 were negative for VIM of *P. aeruginosa*