International Journal of Antimicrobial Agents ■■ (2018) ■■–■■

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Whole-genome sequencing enabling the detection of a colistin-resistant hypermutating *Citrobacter werkmanii* strain harbouring a novel metallo-β-lactamase VIM-48

S. Peter [a,b,*], D. Bezdan [c,d], P. Oberhettinger [a,b], W. Vogel [e], D. Dörfel [e,f], J. Dick [a,b], M. Marschal [a,b], J. Liese [a,b], C. Weidenmaier [a,b], I. Autenrieth [a,b], S. Ossowski [c,d], M. Willmann [a,b]

[a] *Institute of Medical Microbiology and Hygiene, University of Tübingen, Tübingen, Germany*
[b] *German Center for Infection Research (DZIF), partner site Tübingen, Tübingen, Germany*
[c] *Centre for Genomic Regulation (CRG), The Barcelona Institute of Science and Technology, Barcelona, Spain*
[d] *Universitat Pompeu Fabra (UPF), Barcelona, Spain*
[e] *Medical Center, Department of Hematology, Oncology, Immunology, Rheumatology & Pulmonology, University of Tübingen, Tübingen, Germany*
[f] *Clinical Collaboration Unit Translational Immunology, German Cancer Consortium (DKTK) and German Cancer Research Center (DKFZ), partner site Tübingen, Tübingen, Germany*

ARTICLE INFO

Article history:
Received 11 October 2017
Accepted 20 January 2018
Editor: S. Diene

Keywords:
Colistin resistance
Mutator phenotype
VIM
IMP
Next-generation sequencing

ABSTRACT

Citrobacter spp. harbouring metallo-β-lactamases (MBLs) have been reported from various countries and different sources, but their isolation from clinical specimens remains a rare event in Europe. MBL-harbouring Enterobacteriaceae are considered a major threat in infection control as therapeutic options are often limited to colistin. In this study, whole-genome sequencing was applied to characterise five clinical isolates of multidrug-resistant *Citrobacter werkmanii* obtained from rectal swabs. Four strains possessed a class 1 integron with a novel bla_{VIM-48} MBL resistance gene and the aminoglycoside acetyltransferase gene *aacA4*, whilst one isolate harboured a bla_{IMP-8} MBL. Resistance to colistin evolved in one strain isolated from a patient who had received colistin orally for 8 days. Genomic comparison of this strain with a colistin-susceptible pre-treatment isolate from the same patient revealed 66 single nucleotide polymorphisms (SNPs) and 26 indels, indicating the presence of a mutator phenotype. This was confirmed by the finding of a SNP in the *mutL* gene that led to a significantly truncated protein. Additionally, an amino acid change from glycine to serine at position 53 was observed in PmrA. Mutations in the *pmrA* gene have been previously described as mediating colistin resistance in different bacterial species and are the most likely reason for the susceptibility change observed. To the best of our knowledge, this is the first description of a colistin-resistant *Citrobacter* spp. isolated from a human sample. This study demonstrates the power of applying next-generation sequencing in a hospital setting to trace and understand evolving resistance at the level of individual patients.

1. Introduction

Increasing antimicrobial resistance in Gram-negative Enterobacteriaceae, when combined with the spread of carbapenemase-producing strains, represents a major global health concern [1]. Carbapenemases are enzymes capable of conferring resistance to almost all β-lactam antibiotics, including carbapenems, resulting in a severe lack of treatment options [1]. From a clinical perspective, KPC, NDM, VIM, IMP and OXA-48 types are the most important enzymes to date [1]. Metallo-β-lactamase (MBL) genes of the bla_{VIM} and bla_{IMP} types are often located on class 1 integrons, which can integrate several resistance genes [2] and thereby confer resistance to additional classes of antibiotics such as aminoglycosides and fluoroquinolones. Colistin given as monotherapy or in combination often remains the last valid therapeutic option against such multidrug-resistant Gram-negative bacteria [3]. The global spread of antimicrobial resistance has generally led clinicians to revive the application of colistin, which has a rapid bactericidal effect on Gram-negative bacterial cells through disruption of the outer cell membrane [4]. Although the exact mechanism is not fully understood, the interaction of positively charged colistin with the negatively charged lipid A component of the outer membrane lipopolysaccharide (LPS) appears to be crucial [4]. However, the rise

Present address: Institute of Medical Microbiology and Hygiene, Elfriede-Aulhorn-Str. 6, 72076 Tübingen, Germany.
* Corresponding author. Institute of Medical Microbiology and Hygiene, Elfriede-Aulhorn-Str. 6, Tübingen, 72076, Germany.
E-mail address: silke.peter@med.uni-tuebingen.de (S. Peter).

https://doi.org/10.1016/j.ijantimicag.2018.01.015
0924-8579/

in colistin usage has also been followed by increasing reports of colistin-resistant Gram-negative Enterobacteriaceae, in particular *Klebsiella pneumoniae* [5,6].

Citrobacter spp. colonise the human gastrointestinal tract, although they are also involved in a variety of opportunistic infections including sepsis, meningitis, urinary tract infection and brain abscesses [7,8]. *Citrobacter* spp. harbouring carbapenemases have been reported from different countries, e.g. Germany [9]. However, isolating these organisms continues to be rare, in particular in our hospital (a 1500-bed university hospital located in Southwest Germany), with rates of carbapenemase-producing Enterobacteriaceae being <1%.

Therefore, we set out to examine and characterise five carbapenemase-positive *Citrobacter* spp. strains recovered from rectal swabs of three haemato-oncology patients, including one colistin-resistant isolate. Whole-genome sequencing (WGS) was applied to characterise the molecular basis of the resistance phenotypes and to elucidate the molecular evolutionary trajectory in intrapatient samples of the colistin-resistant isolate.

2. Materials and methods

2.1. Characterisation of the strains

In the present study, five clinical isolates were recovered from rectal swabs of three patients with underlying haematological malignancies. The strains were recovered between August 2012 and March 2013 and represented the first bla_{VIM} carbapenemase-positive *Citrobacter* spp. strains detected in our hospital. Identification of the strains was performed using a matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) AXIMA Assurance system (bioMérieux SA, Marcy-l'Étoile, France; SARAMIS database v.4.09) and a microflex LT MALDI-TOF instrument (Bruker Daltonik GmbH, Bremen, Germany; MBT IVD Library.5627). In addition, biochemical-based identification using an API 20 E System (bioMérieux SA; apiweb™ v.5) and VITEK® GN ID card (bioMérieux SA) was applied. Antimicrobial susceptibility testing was initially performed with the VITEK®2 system (bioMérieux SA) and was confirmed by Etest (bioMérieux SA). Extended-spectrum β-lactamase (ESBL) and AmpC double-disk synergy tests (DD68C; Mast Diagnostica, Reinfeld, Germany) were performed according to the manufacturer's instructions. Carbapenemase activity was phenotypically determined for each isolate by the modified Carbapenem Inactivation Method performed as described previously [10]. Colistin susceptibility testing was performed by broth microdilution (MERLIN Diagnostika GmbH, Berlin, Germany) according to the manufacturer's instructions. Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.1_Breakpoint_Tables.pdf) or screening breakpoints for carbapenemase production (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Resistance_mechanisms/EUCAST_detection_of_resistance_mechanisms_170711.pdf). The study was conducted in accordance with the local ethics committee (Medical Faculty of the University of Tübingen, Tübingen, Germany).

2.2. Bacterial whole-genome sequencing

Bacterial DNA was extracted from overnight cultures using a QIAGEN Genomic-tip 100/G (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The sequencing library protocol was modified to obtain a broad distribution of DNA fragment sizes spanning 550–800 bp. The following Covaris settings were chosen: intensity, 5; duty cycles, 10%; cycles per burst, 200; time, 40 s; frequency, sweeping; temperature, 5–6 °C. The libraries were prepared using a TruSeq DNA LT Sample Prep Kit (Illumina Inc., San Diego, CA) with 24 different barcodes using the standard protocol modified only for the size selection as described above. Libraries were extracted in a broad band between 550 bp and 800 bp from a low-melting-point agarose gel prior to PCR amplification and with AMPure® beads (Agencourt® AMPure® XP; Beckman Coulter, Inc., Krefeld, Germany) in the second run. Barcoded libraries were analysed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and were quantified by real-time PCR. Normalised libraries were pooled to nine samples per run and were sequenced at 2 × 300-bp on a MiSeq platform (Illumina Inc.). Assembly of genome sequences was performed using the A5 pipeline with default settings [11]. Automated genome annotation was done using Prokka [12], supplemented by manual annotation using BLASTX (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

2.3. Core genome identification and phylogenetic analysis

To construct a phylogeny of the genus *Citrobacter*, 23 WGS data sets were downloaded from the National Center for Biotechnology Information (NCBI) genome database [*Citrobacter amalonaticus* Y19 (**CP011132_1**), *Citrobacter braakii* GTA-CB01 (**JRHK01000001.1**), *C. braakii* GTA-CB04 (**JRHL01000001.1**), *Citrobacter farmeri* GTC 1319 (**NZ_BBMX01000031.1**), *Citrobacter freundii* CFNIH1 (**NZ_CP007557_1**), *Citrobacter rodentium* ICC168 (**NC_013716_1**), *Citrobacter sedlakii* NBRC 105722 (**NZ_BBNB01000030.1**), *Citrobacter* sp. 30_2 (**ACDJ02000001.1**), *Citrobacter* sp. A1 (**AKTT01000001.1**), *Citrobacter* sp. CIP 55.13 (**CDHL01000001.1**), *Citrobacter* sp. JT3 (**JAQB01000001.1**), *Citrobacter* sp. KTE30 (**ASQJ01000002.1**), *Citrobacter* sp. KTE32 (**ASQL01000010.1**), *Citrobacter* sp. KTE151 (**ASQK01000005.1**), *Citrobacter* sp. L17 (**AMPE01000001.1**), *Citrobacter* sp. MGH 55 (**JMUK01000001.1**), *Citrobacter* sp. S-77 (**BAZH01000001.1**), *Citrobacter werkmanii* NBRC 105721 (**NZ_BBMW01000009.1**), *Citrobacter youngae* ATCC 29220 (**NZ_GG730308.1**), *Citrobacter koseri* ATCC BAA-895 (**CP000822.1**), *C. freundii* ATCC 8090 (**JMTA01000001.1**), *C. rodentium* ATCC 51459 (**JXUN01000001.1**), *C. amalonaticus* L8A (**JMQQ01000001.1**)] and *C. freundii* Tue1 [9]. progressiveMauve v.2.3.1 [13] was run to conduct a full alignment of 29 genomes using default settings. Locally collinear blocks with ≥1 kb in length were composed as core genome alignment, consisting of 2 629 153 bp. In addition, prophage regions were investigated using PHAST [14].

A maximum likelihood phylogenetic tree of the 29 WGS sequences was constructed by RAxML using the GTR model with GAMMA rates [15]. Branch support was determined by 1000 bootstrap replicates.

2.4. Determination of resistance gene content

All currently available bla_{VIM} genes were included in the analysis [bla_{VIM-1} (**Y18050.2**), bla_{VIM-2} (**AF191564.1**), bla_{VIM-3} (**AF300454.1**), bla_{VIM-4} (**AY135661.1**), bla_{VIM-5} (**AY144612.1**), bla_{VIM-6} (**AY165025.1**), bla_{VIM-7} (**AJ536835.1**), bla_{VIM-8} (**AY524987.1**), bla_{VIM-9} (**AY524988.1**), bla_{VIM-10} (**AY524989.1**), bla_{VIM-11} (**AY605049.2**), bla_{VIM-12} (**DQ143913.1**), bla_{VIM-13} (**DQ365886.1**), bla_{VIM-14} (**AY635904.1**), bla_{VIM-15} (**EU419745.1**), bla_{VIM-16} (**EU419746.1**), bla_{VIM-17} (**EU118148.2**), bla_{VIM-18} (**AM778091.1**), bla_{VIM-19} (**FJ822963.1**), bla_{VIM-20} (**GQ414736.1**), bla_{VIM-23} (**GQ242167.1**), bla_{VIM-24} (**HM855205.1**), bla_{VIM-25} (**HM750249.1**), bla_{VIM-26} (**FR748153.1**), bla_{VIM-27} (**HQ858608.1**), bla_{VIM-28} (**JF900599.1**), bla_{VIM-29} (**JX311308.1**), bla_{VIM-30} (**JN129451.1**), bla_{VIM-31} (**JN982330.1**), bla_{VIM-32} (**JN676230.2**), bla_{VIM-33} (**JX258134.1**), bla_{VIM-34} (**JX013656.1**), bla_{VIM-35}

Please cite this article in press as: S. Peter, et al., Whole-genome sequencing enabling the detection of a colistin-resistant hypermutating *Citrobacter werkmanii* strain harbouring a novel metallo-β-lactamase VIM-48, International Journal of Antimicrobial Agents (2018), doi: 10.1016/j.ijantimicag.2018.01.015