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Original article

Proteus mirabilis – analysis of a concealed source of carbapenemases and development of a diagnostic algorithm for detection

Axel Hamprecht [1, 2, 3, *], Janko Sattler [2, 3], Janina Noster [1], Yvonne Stelzer [1], Frieder Fuchs [2, 4], Vivien Dorth [5], Sören G. Gatermann [6], Stephan Göttig [5]

[1)] *Institute for Medical Microbiology and Virology, University of Oldenburg and Klinikum Oldenburg, Oldenburg, Germany*
[2)] *Institute for Medical Microbiology, Immunology and Hygiene, University Hospital Cologne and Faculty of Medicine, University of Cologne, Cologne, Germany*
[3)] *DZIF (German Centre for Infection Research), Partner Site Bonn-Cologne, Cologne, Germany*
[4)] *Department of Microbiology and Hospital Hygiene, Bundeswehr Central Hospital Koblenz, Koblenz, Germany*
[5)] *Institute for Medical Microbiology and Infection Control, Hospital of Johann Wolfgang Goethe University, Frankfurt, Germany*
[6)] *German National Reference Centre for Multidrug-resistant Gram-negative Bacteria, Department of Medical Microbiology, Ruhr-University Bochum, Bochum, Germany*

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ABSTRACT

Objectives: To analyse carbapenemases in *Proteus mirabilis* and assess the performance of carbapenemase detection assays.
Methods: Eighty-one clinical *P. mirabilis* isolates with high-level resistance at least to ampicillin (>32 mg/L) or previous detection of carbapenemases were selected and investigated by three susceptibility testing methods (microdilution, automated susceptibility testing, and disk diffusion), six phenotypic carbapenemase assays (CARBA NP, modified carbapenemase inactivation method [CIM], modified zinc-supplemented CIM, simplified CIM, faropenem, and carbapenem-containing agar), two immunochromatographic assays, and whole-genome sequencing.
Results: Carbapenemases were detected in 43 of 81 isolates (OXA-48-like [$n = 13$]; OXA-23 [$n = 12$]; OXA-58 [$n = 12$]; New Delhi metallo-β-lactamase (NDM) [$n = 2$]; Verona integron–encoded metallo-β-lactamase (VIM) [$n = 2$]; Imipenemase (IMP) [$n = 1$]; Klebsiella pneumoniae carbapenemase (KPC) [$n = 1$]). Carbapenemase-producing *Proteus* were frequently susceptible to ertapenem (26/43; 60%), meropenem (28/43; 65%), ceftazidime (33/43; 77%), and some even to piperacillin-tazobactam (9/43; 21%). Sensitivity/specificity of phenotypic tests were 30% (CI: 17–46%)/89% (CI: 75–97%) for CARBA NP, 74% (CI: 60–85%)/82% (CI: 67–91%) for faropenem, 91% (CI: 78–97%)/82% (CI: 66–92%) for simplified CIM, and 93% (CI: 81–99%)/100% (CI: 91–100%) for modified zinc-supplemented CIM. An algorithm for improved detection was developed, which demonstrated sensitivity/specificity of 100% (CI: 92–100%)/100% (CI: 91–100%) on the 81 isolates, and 100% (CI: 29–100%)/100% (CI: 96–100%) in a prospective analysis of additional 91 isolates. Interestingly, several OXA-23-producing isolates belonged to the same clonal lineage reported previously from France.
Discussion: Current susceptibility testing methods and phenotypic tests frequently fail to detect carbapenemases in *P. mirabilis*, which could result in inadequate antibiotic treatment. In addition, the non-inclusion of $bla_{\text{OXA-23/OXA-58}}$ in many molecular carbapenemase assays further impedes their detection. Therefore, the prevalence of carbapenemases in *P. mirabilis* is likely underestimated. With the herein proposed algorithm, carbapenemase-producing *Proteus* can be easily identified. **Axel Hamprecht, Clin Microbiol Infect 2023;29:1198.e1–1198.e6**

Introduction

Proteus mirabilis belongs to the family Morganellaceae of the order Enterobacterales. It is the third most common species in urinary tract infections and a frequent cause of bloodstream infections

Abbreviations: CPE, carbapenemase-producing Enterobacterales; CPP, carbapenemase-producing *Proteus*.

* Corresponding author. Axel Hamprecht, University of Oldenburg, Ammerländer Heerstrasse 114-118, 26129 Oldenburg, Germany.
E-mail address: axel.hamprecht@uol.de (A. Hamprecht).

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[1–3]. *P. mirabilis* is usually susceptible to most β-lactams, including aminopenicillins, second/third-generation cephalosporins, ertapenem, and meropenem [4]. In contrast, minimal inhibitory concentrations (MICs) for imipenem are typically elevated.

Although there are ample data on *Escherichia coli, Klebsiella pneumoniae*, and other Enterobacterales [5–7], the knowledge about carbapenemases in *P. mirabilis* is limited. KPC, NDM, or VIM have been reported only in a few cases in this species. Carbapenemase-producing *Proteus* (CPP) isolates often have low MICs for meropenem/ertapenem, rendering detection difficult. In addition, confirmation tests such as immunochromatographic tests (ICTs) have shown false-negative results [8–10]. Furthermore, *Proteus* spp. frequently harbour the carbapenemases OXA-23 and OXA-58 that are common in *Acinetobacter* spp. but typically not found in Enterobacterales. Because most diagnostic assays focus on the big five carbapenemases (KPC, OXA-48-like, VIM, NDM, and IMP), OXA-23 and OXA-58 remain undetected. It is, therefore, very likely that carbapenemases in *Proteus* spp. are more frequently overlooked than in other species such as *K. pneumoniae*, where MICs are generally higher. Because of intrinsic resistance to polymyxins, tigecycline, and nitrofurantoin, the occurrence of carbapenemase-producing *P. mirabilis* is of particular concern.

Therefore, we analysed clinical *Proteus* isolates for carbapenemase production by phenotypic and molecular assays and developed an algorithm for improved detection of carbapenemases.

Methods

Study design and bacterial isolates

The study consisted of a retrospective analysis (part I) and a prospective analysis (part II). In part I, 81 clinical *P. mirabilis* isolates were included that were isolated between 2013 and 2021 at the institutes for medical microbiology of the university hospitals in Oldenburg, Cologne, and Frankfurt or from the German National Reference Centre for Multidrug-resistant gram-negative bacteria. Isolates were included if a carbapenemase or increased MICs for ertapenem or meropenem (>0.125 mg/L) were recorded or if they displayed a phenotype of an acquired β-lactamase, with high-level resistance at least to ampicillin (>32 mg/L). Isolates were analysed by susceptibility testing, phenotypic and immunochromatographic carbapenemase assays, and detection of antibiotic resistance genes by PCR and whole-genome sequencing (WGS). From these data, a diagnostic algorithm for improved carbapenemase detection was developed. In part II, this algorithm was prospectively evaluated on 91 *P. mirabilis* isolates with resistance to ampicillin, which were consecutively isolated as part of routine clinical diagnostics between January and July 2022 at the university hospital of Frankfurt.

Antimicrobial susceptibility testing

Isolates were tested for their susceptibility to 31 antibiotics. Three different methods were employed: disk diffusion (Oxoid, Wesel, Germany), Vitek2 automated susceptibility testing with the N223 card (bioMérieux, Nürtingen, Germany), and broth microdilution (BMD) using custom-manufactured plates (Merlin diagnostics, Bornheim, Germany) (Table S1). Results were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints v12.0.

Characterization of carbapenemases by PCR

All isolates were tested for the presence of bla_{KPC}, $bla_{OXA\text{-}48\text{-}like}$, bla_{VIM}, bla_{IMP}, bla_{NDM}, $bla_{OXA\text{-}23}$, and $bla_{OXA\text{-}58}$ as previously described [8,11].

WGS and bioinformatic analysis

All isolates from part I of the study were further analysed by WGS using short-read technology (Illumina, San Diego, USA) as previously reported [12,13]. Resistance gene and phylogenetic analysis were performed as described in the supplement.

Phenotypic and immunochromatographic carbapenemase detection tests

Carbapenemase production was investigated using six phenotypic assays. These included the faropenem disk test [14] and the CARBA NP test with Triton X-100 for bacterial lysis as previously described [15]. Three different versions of the carbapenemase inactivation method (CIM) were assessed, including the modified CIM as recommended by Clinical & Laboratory Standards Institute (CLSI) [16], the simplified CIM (sCIM) [17], and mzCIM, a modified version of zinc-supplemented CIM (zCIM) [18], supplementary data. Growth on carbapenem-containing screening plates (mSuperCARBA; Chromagar, Paris, France) was assessed as previously described [19].

Isolates producing OXA-48-like, KPC, VIM, NDM, or IMP were tested using the ICT RESIST-5 (Coris BioConcept, Gembloux, Belgium) and CARBA5 (NG Biotech, Guipry, France) as previously described [8].

Statistical analysis

The sensitivity and specificity of the tests were calculated using molecular characterization (PCR or WGS) as the reference standard. Violin plots of MIC distributions and inhibition zones were created using GraphPad Prism 8.1.

Ethics statement

All bacterial strains were isolated as part of routine microbiological diagnostics and stored in an anonymized database. No patient-related data were analysed. No ethical approval is necessary for this type of study according to the local ethics committees.

Results

Molecular characterization of Proteus spp.

Of 81 isolates analysed in part I of the study, 43 harboured carbapenemase genes, with $bla_{OXA\text{-}48\text{-}like}$ being the most frequent carbapenemase group ($n = 13$), followed by $bla_{OXA\text{-}23}$ and $bla_{OXA\text{-}58}$ ($n = 12$ each), bla_{NDM} and bla_{VIM} ($n = 2$ each), bla_{KPC} and bla_{IMP} ($n = 1$ each) (Table S2). Of these isolates, 4 (9%) additionally carried genes for AmpC β-lactamases (all bla_{CMY}-type) and 12 for extended-spectrum β-lactamases (ESBLs) (28%). Among carbapenemase-negative control isolates, AmpC and ESBL encoding genes were found in 13 of 38 (34%) isolates, respectively.

Phylogenetic analysis of carbapenemase-producing P. mirabilis isolates

To assess the genetic relatedness of the 81 *Proteus* isolates and to compare the isolates with other CPP, the genomes were compared by phylogenetic analysis (Fig. S1). Among OXA-23 producers, 11 isolates formed a cluster (Fig. S2(a)). These isolates are highly similar to isolates that have been reported from Belgium and France [20,21], indicating affiliation to the same clonal lineage. No obvious epidemiological link could be identified between the 11 OXA-23-producing isolates because they were recovered from different

areas and years. Three OXA-58 isolates (Carb-02, 04, 10) belonged to a second smaller cluster, whereas all other isolates were singletons. Comparison of *Proteus* isolates harbouring other carbapenemases revealed that they were mostly phylogenetically unrelated (Fig. S2(b)). Most isolates were singletons and occasionally two isolates belonged to the same lineage.

Antimicrobial susceptibility

When tested by BMD, carbapenem MICs of CPP were low. Isolates were susceptible to ertapenem (≤0.5 mg/L) in 26 of 43 isolates (60%) and meropenem (≤2 mg/L) in 28 of 43 (65%) (Table 1). When applying EUCAST screening breakpoints for carbapenemase detection, 9 of 43 isolates (21%) would be missed with the ertapenem MIC threshold (>0.125 mg/L) and 3 of 43 (7%) with meropenem (>0.125 mg/L). Discrimination between CPP and non-carbapenemase producers based on meropenem and ertapenem MICs was rather poor, because MIC distributions considerably overlapped (Fig. 1). When stratified by carbapenemase type, MIC distributions varied greatly (Fig. S3), which can be useful for presumptive identification of carbapenemases. Most CPP (33/43, 77%) were susceptible to ceftazidime, except those producing metallo-β-lactamases (0/5).

When comparing susceptibility by the different testing methods, the largest differences were observed for piperacillin-tazobactam. Interestingly, 9 of 43 (21%) were susceptible to piperacillin-tazobactam by BMD, 20 of 43 (47%) by Vitek2 compared with 6 of 43 (14%) by disk diffusion.

Similar to BMD, disk diffusion testing of carbapenems was not very discriminatory for differentiation between CPP and controls (Fig. S4). In contrast, an inhibition zone of temocillin (TEM) <14 mm was 100% specific (CI: 91–100%) for CPP and sensitivity was excellent for OXA-48-like and OXA-58 (25/25, 100%; CI: 86–100%), but modest among all carbapenemase producers (26/43, 60%; CI: 44–75%). All CPP were resistant to ampicillin-sulbactam, amoxicillin-clavulanate, and ticarcillin-clavulanate (TCC).

Performance of phenotypic and ICTs for carbapenemase detection

Sensitivity of phenotypic assays in CPP ranged from 30% (CI: 17–46%) for CARBA NP to 93% (CI: 81–99%) for mzCIM (Table 2). Specificity was highest for mzCIM (100%; CI: 91–100%) and lowest for sCIM (82%; CI: 66–92%). Both CARBA NP and sCIM resulted in a high number of indeterminate results (43% and 36%, respectively) (Table 2 and Fig. S5). The highest Youden index was recorded for mzCIM (0.93). Among the 19 isolates producing KPC, IMP, OXA-48-like, NDM, or VIM, carbapenemases were correctly detected in 18 isolates (95%; CI: 74–100%) by CARBA-5 and 17 (89%; CI: 67–99%) by RESIST-5 (Table S3). Both assays cannot detect OXA-23/OXA-58.

Development of an algorithm for the detection of carbapenemase production in Proteus spp.

For diagnostic laboratories that have not established phenotypic assays with high sensitivity for CPP, a new diagnostic algorithm was developed (Fig. 2).

If ampicillin-sulbactam and amoxicillin-clavulanate are resistant, disk diffusion for TCC and TEM is performed as a first step. If TCC is tested susceptible (≥20 mm), a carbapenemase can be excluded. If TEM is < 14 mm, a carbapenemase is proven (100% specificity) and the isolate likely produces OXA-58 or OXA-48-like. Isolates that are resistant to TCC but with TEM inhibition zones ≥14

Table 1
Comparison of susceptibility testing of P. mirabilis obtained by broth microdilution, Vitek2, and disk diffusion

	Ertapenem			Meropenem				Imipenem		Pip.-tazobactam	
	S (%)	R (%)	>Sc. BP (%)	S (%)	I (%)	R (%)	>Sc. BP (%)	I (%)	R (%)	S (%)	R (%)
Microdilution											
CPP ($n = 43$)	26 (60)	17 (40)	34 (79)	28 (65)	10 (23)	5 (12)	40 (93)	11 (26)	32 (74)	9 (21)	34 (79)
Controls ($n = 38$)	35 (92)	3 (8)	5 (13)	36 (95)	2 (5)	0	6 (16)	35 (92)	3 (8)	33 (87)	5 (13)
Vitek2											
CPP ($n = 43$)	28 (65)	15 (35)	n.a.	24 (56)	8 (19)	11 (26)	n.a.	12 (28)	31 (72)	20 (47)	23 (53)
Controls ($n = 38$)	32 (84)	6 (16)	n.a.	36 (95)	1 (3)	1 (3)	n.a.	28 (74)	10 (26)	35 (92)	3 (8)
Disk diffusion											
CPP ($n = 43$)	8 (19)	35 (81)	35 (81)	19 (44)	14 (33)	10 (23)	41 (95)	6 (14)	37 (86)	6 (14)	37 (86)
Controls ($n = 38$)	24 (63)	14 (37)	14 (37)	34 (89)	1 (3)	3 (8)	7 (18)	31 (83)	7 (18)	34 (89)	4 (11)

CPP, carbapenemase-producing *Proteus*; n.a., not available because of small calling range; >Sc. BP, above EUCAST screening breakpoint for further carbapenemase testing; S/R, susceptible/resistant according to EUCAST clinical breakpoints v12.

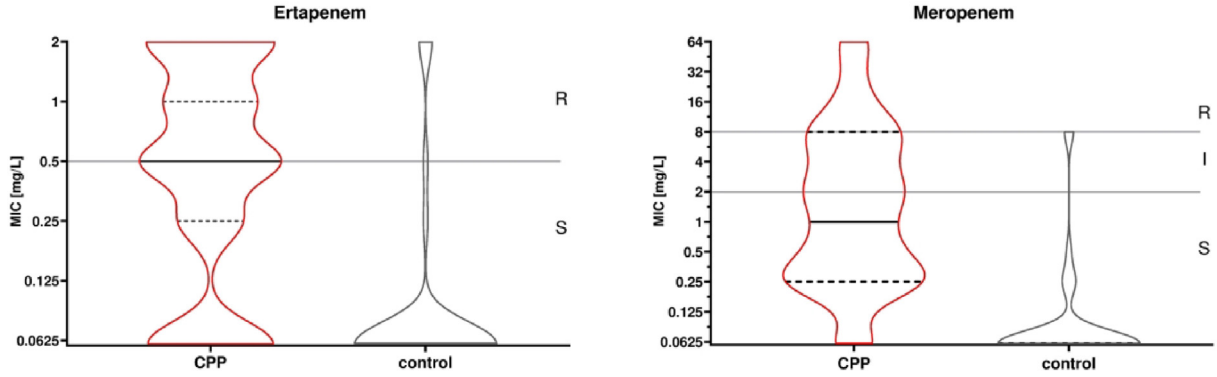

Fig. 1. MIC distributions by broth microdilution for 81 clinical *Proteus mirabilis* isolates. Violin plots of MIC distributions for all carbapenemase-producing *P. mirabilis* spp. (CPP) vs. controls for ertapenem (a) and meropenem (b).

Table 2
Performance of phenotypic carbapenemase confirmation tests for 81 P. mirabilis isolates

	Sensitivity % (95% CI)	Specificity % (95% CI)	Indeterminate % (95% CI)	Youden index
CARBA NP	30 (17–46)	89 (75–97)	43 (32–55)	0.19
Faropenem	74 (60–85)	82 (67–91)	—	0.5
mCIM	63 (47–77)	95 (82–99)	7 (3–15)	0.58
sCIM	91 (78–97)	82 (66–92)	36 (25–47)	0.73
Modified zCIM (mzCIM)	93 (81–99)	100 (91–100)	1 (0–7)	0.93
mSuperCarba[a]	70 (54–83)	83 (66–92)	—	0.53

For the calculation of sensitivity and specificity, indeterminate results were counted as negative; the percentage of indeterminate results for each test is provided for a better assessment of the overall performance.
mCIM, modified carbapenemase inactivation method; sCIM, simplified carbapenemase inactivation method; zCIM, zinc-supplemented CIM.
[a] Growth on mSuperCarba agar after 20 h incubation at 35 °C.

mm are further analysed using mzCIM. When evaluating this algorithm on the 81 isolates of part I of this study, sensitivity was 100% (CI: 92–100%) and specificity 100% (CI: 91–100%).

Prospective assessment of the algorithm

The newly developed algorithm was assessed prospectively on 91 ampicillin-resistant *P. mirabilis* isolates. Three CPP were detected (one NDM and two OXA-23); sensitivity of the algorithm was 100% (CI: 29–100%) and specificity 100% (CI: 96–100%). The positive predictive value for carbapenemase production was 8% (3/37) for resistance to ampicillin-sulbactam, 4% (3/67) for amoxicillin-clavulanate, and 25% (3/12) for TCC.

Discussion

The problem of carbapenemases in *P. mirabilis* is increasingly acknowledged [4]. Besides some case reports, a long-term OXA-48 outbreak has been described from Spain; in addition, a French OXA-23 cluster and a smaller KPC-producing *P. mirabilis* clade from the Czech Republic have been reported [20–23]. Phylogenetic comparison of isolates from our study and the literature showed that most of the German and French OXA-23-producing isolates belong to the same cluster, but the reason for the emergence of these isolates in geographically separated locations is currently unknown.

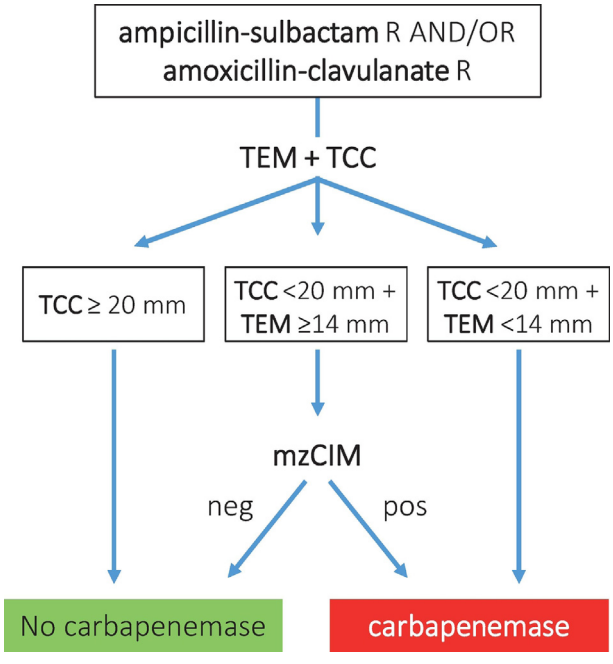

Fig. 2. Proposed algorithm for the phenotypic detection of carbapenemases in *P. mirabilis*. Ticarcillin-clavulanate (TCC) and temocillin (TEM) are tested in the first step, which will discriminate between most carbapenemase-positive and negative isolates. Isolates with TCC R and TEM <14 mm are carbapenemase-positive (presumptive OXA-48-like/OXA-58). Only those isolates with TCC R and TEM ≥14 mm have to be tested by mzCIM additionally, which will identify all other carbapenemases (OXA-23, MBL, KPC). mzCIM, modified zinc-supplemented carbapenemase inactivation method.

On the basis of our data, carbapenemases in *P. mirabilis* can be easily missed in routine diagnostics for several reasons. Meropenem and ertapenem MICs were in the susceptible range in 56%/65% of CPP as determined by Vitek2. The performance of phenotypic confirmation tests in *Proteus* has not been studied extensively, because most investigations included only a few isolates [10,15–17,24]. We demonstrate that the CLSI-recommended modified CIM and CARBA NP performed poorly, in line with previous results on CPP [15,25]. The ICTs RESIST-5/CARBA-5-targeting KPC, OXA-48-like, VIM, NDM, and IMP demonstrated a lower sensitivity for NDM and VIM than in other species. However, sensitivity of both assays for non-OXA-23/OXA-58 carbapenemases increased to 100% (19/19; CI: 82–100%) when the inoculum was harvested at the inhibition zone of an ertapenem disk, as previously reported [10]. Even by PCR, the detection of carbapenemases in *P. mirabilis* is problematic compared with other Enterobacterales, because most commercially available carbapenemase assays do not target $bla_{\text{OXA-23}}$/$bla_{\text{OXA-58}}$ [26], which are frequent in CPP. Furthermore, culture-based screening of patients is challenging, because most screening agars contain higher carbapenem concentrations and will impede the growth of CPP, as indicated within this study for mSuperCarba agar.

How could the detection of CPP be improved? Importantly, EUCAST screening breakpoints for carbapenemase detection in Enterobacterales do not seem suitable for *P. mirabilis* because 21% of CPP would be missed with the current ertapenem cut-off and 7% with meropenem [27]. When considering the ertapenem MIC distribution of *P. mirabilis* in the EUCAST database (Fig. S6), a species-specific screening breakpoint of 0.03 mg/L seems useful to improve sensitivity. In addition, piperacillin-tazobactam resistance is used as a criterion for isolates with a meropenem ZOI of 25–27 mm by EUCAST and others for carbapenemase detection [24,27]. However, many CPP are susceptible and therefore piperacillin-tazobactam resistance in diagnostic algorithms should be replaced by resistance to either TCC or ampicillin-sulbactam for *P. mirabilis*.

To improve detection of CPP, we developed a new algorithm, which can be performed in all laboratories at low cost. On the basis of the results of TEM and TCC, most isolates can be categorized as either carbapenemase negative or carbapenemase positive. The remaining isolates are further tested by mzCIM, which has a high sensitivity for OXA-23, KPC, and metallo-β-lactamases. Using this algorithm, sensitivity and specificity of 100% were achieved in both part I and II of this study. To shorten the time to result, mzCIM can also be directly performed in parallel to TCC/TEM disk testing.

As an alternative to the phenotypic algorithm or for confirmation purposes, isolates with resistance to TCC (alternatively ampicillin-sulbactam or amoxicillin-clavulanate) could be tested by a PCR that should include bla_{OXA-23} and bla_{OXA-58} in addition to the common targets bla_{KPC}, $bla_{OXA-48-like}$, bla_{VIM}, bla_{IMP}, and bla_{NDM}.

Given the stealthy phenotype of carbapenemase production in *P. mirabilis*, it can be hypothesized that this species serves as a concealed source of carbapenemase genes. However, to which extent *P. mirabilis* contributes to the dissemination of antimicrobial resistance genes (e.g. as an intermediate for bla_{OXA-48}, bla_{NDM}, or bla_{VIM} between different Enterobacterales or $bla_{OXA-23/58}$ between *Acinetobacter* spp.) has to be investigated in future studies.

Our study has some limitations, because the data and the developed algorithm are based on a collection of isolates from Germany. Non-class D carbapenemases were rare among our collection and the performance of the algorithm might change in countries with a different epidemiology. In the prospective part, the number of CPP was low ($n = 3$), and the CIs for sensitivity were, therefore, wide (CI: 29–100%); the performance of the algorithm should be assessed with more isolates in multicentre studies. Nevertheless, our study comprised a large collection of CPP, which have been extensively characterized by WGS and different susceptibility testing methods. This may provide useful data and new diagnostic tools to shed more light on the hidden reservoir of carbapenemases in *Proteus*.

Author contributions

Conceptualization: AH, SG, SGG. Data curation: AH, JS, SG, YS. Strain collection: AH, FF, SG, SGG. Formal analysis: AH, JN, JS, SG, YS, SGG. Funding acquisition: AH, SG. Investigation: AH, JN, JS, SG, YS, SGG, VD. Methodology: AH, JS, SG, SGG. Project administration: AH. Resources: AH, SG, SGG. Supervision: AH, SG. Writing—original draft: AH, SG. Writing—review and editing: AH, FF, JN, JS, SG, YS, SGG, VD.

Transparency declaration

AH has received speaker honoraria from BD, bioMérieux, and BeckmanCoulter, research support from bioMérieux and Coris (outside of the presented work), and served on the advisory board of bioMérieux. SGG has received speaker honoraria from bioMérieux, BeckmanCoulter, and ThermoFisher. All other authors declare no conflicts of interest. No external funding was received for this study.

Data availability

The complete nucleotide sequence assemblies of all isolates were deposited publicly in NCBI under BioProject no. PRJNA915754.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2023.05.032.

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