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Evaluation of resazurin-based rapid test to detect colistin resistance in *Acinetobacter baumannii* isolates

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Abstract
Acinetobacter baumannii primarily causes colonization, yet it can be an opportunistic pathogen associated with hospital-acquired infections. Many countries report rapid spread of carbapenem-resistant *Acinetobacter baumannii* (CRAb) which limits treatment options, with colistin frequently being the last line treatment option. The aim of our study was to evaluate a recently developed rapid method, namely the Rapid ResaPolymyxin test, for detection of colistin resistance (ColR) in *Acinetobacter baumannii*. This test was used for rapid screening of colistin resistance in a clinical setting where there is endemicity of CRAb isolates. A total of 82 *A. baumannii* clinical isolates were included in the evaluation. The majority of them were resistant to carbapenems (75/82, 91.5%). A total of 37 isolates (45.1%) were resistant to colistin, all being resistant to carbapenems. None of the ColR isolates carried the plasmid-mediated *mcr-1* to *-5* genes. The Rapid ResaPolymyxin NP test reached a 95.1% categorical agreement with results of reference broth microdilution method, with 93.3% sensitivity and specificity, and positive and negative predictive values being respectively at 92.3% and 97.7%. The Rapid ResaPolymyxin NP test performed well on our collection of clinical and surveillance CRAb isolates from the Central Slovenia region. The test is inexpensive and easy to integrate into laboratory workflow. The main value of the test is rapid categorization of susceptibility and resistance which has important implications with respect to the treatment strategy as well as the infection control measures.

Keywords *Acinetobacter baumannii* · Colistin resistance · Rapid diagnostic test · Susceptibility testing

Introduction

Acinetobacter baumannii primarily causes colonization but may be an opportunistic pathogen associated with hospital-acquired infections, particularly among intensive care unit (ICU) patients with prolonged hospitalizations and long-term exposure to broad-spectrum antibiotics. ICUs are usually primarily affected, but secondary spreads to general wards and even long-term care facilities are also frequent [1].

While *A. baumannii* is intrinsically resistant to many antibiotic agents, it can also rapidly develop or acquire resistance to virtually all antibiotics [2, 3]. Particularly worrisome is the rapid spread of carbapenem-resistant *A. baumannii* (CRAb). Overall, higher percentages of CRAb are reported from south-eastern and southern Europe compared with northern and western Europe [4]. Since 2014, invasive *Acinetobacter* sp. isolates are included into systematic EARS-Net surveillance following the 2012–2013 pilot phase. In 2017, the population-weighted EU/EEA mean percentage for carbapenem resistance was 33.4% (range 0.0–96.2%) and even more worrying, the population-weighted EU/EEA mean percentage for combined resistance to fluoroquinolones, aminoglycosides, and carbapenems was 28.4% (range 0.0–84.3%) [5]. Treatment options for infections caused by those multidrug-resistant

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bacteria are limited, with colistin being the most important last-resort option. Data on colistin resistance among CRA are scarce. In 2015 in Europe, resistance to colistin was observed in 4.1% of carbapenem-resistant *Acinetobacter* sp. isolates and 3.9% of carbapenem-susceptible invasive *Acinetobacter* sp. isolates as reported to EARS-Net. As stated in the report, these data have to be interpreted with caution as the number of isolates included was relatively small and colistin susceptibility testing was not performed routinely. It should also be noted that testing is technically challenging and that testing procedure recommendations have recently been changed [4–6]. Nevertheless, increasingly frequent reports of colistin-resistant (ColR) CRAb are worrying [4].

In Slovenia, national data show a persistent decrease in susceptibility to carbapenems in *A. baumannii* clinical isolates, from 89.0% in 2011 to 66.7% in 2017 [7, 8]. Initially, the main reasons for the spread of CRAb were transfers of patients from foreign hospitals colonized and/or infected with CRAb followed by a subsequent spread of these clones in Slovenian hospitals. One of the most affected hospitals is the tertiary teaching hospital University Medical Centre Ljubljana with CRAb prevalence among clinical isolates increasing from 20.6% in 2011 to 62.0% in 2017 [9]. Until 2015, the prevalence of ColR among clinical CRAb isolates in this hospital was under 5%, but it increased to 7.8% in 2016, followed by a significant spike in 2017 (17.5%) followed by a decrease to 7.1% in 2018 ColR (Pirs M, unpublished data). The increase in ColR prevalence was partially due to the implementation of new recommendations with regard to the susceptibility testing with the integration of the broth microdilution (BMD) into routine practice [6], and mainly due to the nosocomial spread of some ColR CRAb clones (Pirs M, unpublished data).

Recently, the first rapid test for detection of colistin resistance in *Enterobacteriaceae*, namely the Rapid Polymyxin NP test, was developed [10]. This test, being based on detection of glucose metabolization, cannot be applied to non-fermenters such as *A. baumannii*. Therefore, and more recently, another test was developed for rapid detection of colistin resistance in *Pseudomonas aeruginosa* and *A. baumannii*, namely the Rapid ResaPolymyxin NP test, based on the resazurin reagent reduction which enables visual detection of viable bacteria as observed by its color change [11]. The aim of our study was to evaluate this new method for rapid screening of colistin resistance from a collection of *A. baumannii* isolates recovered in a clinical setting known to be endemic for CRAb, and among which ColR CRAb may occur.

Materials and methods

Bacterial strains A total of 82 *A. baumannii* isolates were recovered from patients at the tertiary teaching hospital University Medical Centre Ljubljana (UMCL) in 2017 and 2018 and included in this study. Identification at the species level was performed using the matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometer (Microflex LT, Brucker Daltonics, Bremen, Germany). Approximately half of the isolates (42/82, 51.2%) were from clinical samples, the others being from rectal swab surveillance samples (40/82, 48.8%).

Additionally, 20 *Enterobacteriaceae* isolates from our laboratory strain collection were also tested using the Rapid ResaPolymyxin NP test: 10 *Escherichia coli* (4 ColR), 6 *Klebsiella pneumoniae* (4 ColR), 4 *Enterobacter cloacae* (3 ColR), being all negative for the *mcr-1* to *-5* genes. Seven *mcr-1*-positive *E. coli* isolates (MIC of colistin at 4 mg/L) were also tested for the sake of comparison [12].

Control strains The colistin-susceptible *E. coli* ATCC 25922 and the colistin-resistant (*mcr-1*-positive) *E. coli* NCTC 13846 reference strains were used as positive and negative controls, respectively.

Antimicrobial susceptibility testing Antimicrobial susceptibility to imipenem, aminoglycosides, trimethoprim/sulfamethoxazole, and fluoroquinolones was determined using the disk diffusion method. Susceptibility to colistin was determined in duplicate using the in-house broth microdilution method [6]. Colistin (sulfate salt; Sigma-Aldrich, France) was used in a serial two-fold dilution ranging from 0.12 to 128 mg/L; non-treated polystyrene 96-microtiter plates were used. Results of susceptibility testing were interpreted according to the valid EUCAST breakpoints (resistance being at > 2 mg/L) [13].

Isolation of DNA Genomic DNA was isolated using the Instant Gene Matrix (Bio-Rad Laboratories, Hercules, USA) following the manufacturer's instructions.

Detection of *mcr* genes Detection of *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* was performed on isolates with colistin MIC ≥ 2 mg/L using the multiplex PCR as previously described [14].

Carbapenemase gene detection CRAb were screened for the $bla_{\text{OXA-23/-40/-58}}$ carbapenemase genes with a multiplex PCR, as previously described [15].

Rapid ResaPolymyxin NP test The test was performed as described previously [10]. Briefly, we used the Mueller-Hinton broth (MHB) (BBL, Becton Dickinson, Sparks, USA), with or without colistin sulfate (sulfate salt; Sigma-Aldrich, France), at a final concentration of 3.75 mg/L. The bacterial suspension was prepared at the 3.5 McFarland standard from overnight bacterial colonies grown on a blood agar inoculated into two

parallel wells, with and without colistin, in a non-treated polystyrene 96-microtiter plate (Sarstedt, Nümbrecht, Germany). After 3 h of incubation at 35 °C in ambient air, 22 μL of the 10% resazurin dye PrestoBlue (Thermo Fisher Scientific, Waltham, USA) was added to each well, and the contents of each well were mixed by pipetting up and down. The plate was again incubated at 35 °C in ambient air and visually inspected every 15 min during the first hour, followed by inspections at 2 and 3 h.

Negative controls using MHB, with and without colistin, without the addition of a bacterial suspension were used in every test batch to check for potential medium contamination. Positive and negative control reference strains were also included in every test batch.

The test was considered positive if the well contents were purple/pink in color, indicating a viable polymyxin-resistant isolate, and negative if the well contents were blue, indicating the isolate was no longer viable, and thus polymyxin susceptible. The result of the test for a bacterial isolate was valid if the well without colistin remained blue. The test results were valid if (i) both negative control wells without a bacterial suspension remained blue (no MHB contamination), (ii) the wells with a bacterial suspension without colistin turned from blue to purple/pink (growth control), (iii) the colistin-susceptible reference bacterial suspension in MHB with colistin remained blue, and (iv) the colistin-resistant reference bacterial suspension in MHB with colistin turned from blue to purple/pink.

To evaluate the Rapid ResaPolymyxin NP test performance, MICs of colistin were determined with isolates recovered from the same plate which was used for the Rapid ResaPolymyxin NP test.

Results analysis The results obtained with the Rapid ResaPolymyxin NP test were compared with the reference BMD method. Categorical agreement (CA), very major errors (VME, false-susceptible results), and major errors (ME, false-resistant results) were calculated as described elsewhere [16, 17].

Results

A total of 82 *A. baumannii* clinical isolates were tested, among which 37 (45.1%) were found resistant to colistin according to MIC determinations (Fig. 1). None of the ColR isolates carried any *mcr-1* to *-5* gene. The majority of *A. baumannii* isolates were carbapenem resistant (75/82, 91.5%), and carried the $bla_{\text{OXA-23}}$ (64/75, 85.3%) or $bla_{\text{OXA-40}}$ (11/75, 14.7%) carbapenemase genes. All ColR isolates were resistant to carbapenems and fluoroquinolones, most of them being resistant to aminoglycosides (24/37, 65% to gentamicin; 34/37, 8.1% to amikacin).

The Rapid ResaPolymyxin NP test reached a 95.1% categorical agreement with results of BMD. It detected all but one ColR *A. baumannii* with MIC at 32 mg/L (1.2% very major error). Three isolates with colistin MICs 2 mg/L were false positives (3.7% major error). The sensitivity of the Rapid ResaPolymyxin NP test was 93.3% (CI 85.84–99.93%), the specificity 93.33% (CI 81.73–98.60%), with a 92.31% positive predictive value (CI 80.06–97.29%) and a 97.67% negative predictive value (CI 85.85–99.66%). Overall, the performances of the Rapid ResaPolymyxin NP test were excellent. They were even better with *Enterobacteriaceae* isolates, since a 100% categorical agreement and successful detection of both all ColR isolates was obtained, regardless of the resistance mechanisms.

Discussion

We have evaluated the performance of the novel method for rapid detection of colistin resistance among *Acinetobacter* spp. using a collection of CRAb recovered in a single hospital [10]. In the past 7 years, Slovenian prevalence of CRAb

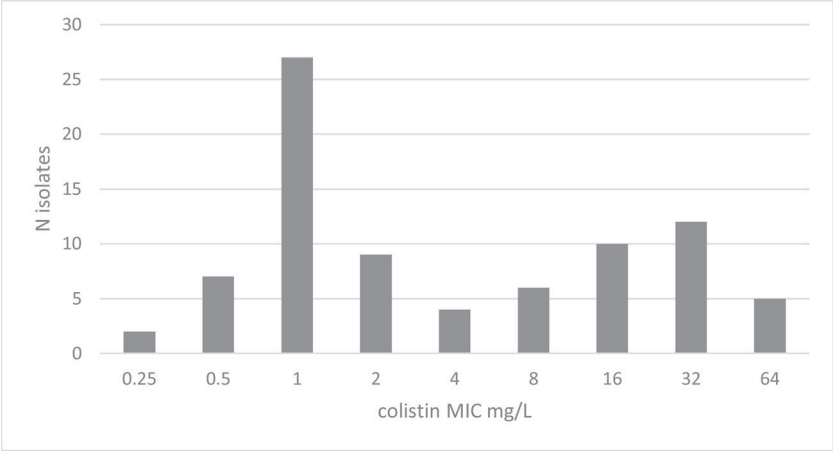

Fig. 1 Distribution of colistin MICs of *Acinetobacter baumannii* isolates included in the study

among the total number of *A. baumannii* isolated increased from 11.0% in 2011 to 33.3% in 2017 [7, 8]. Active infection control measures help reducing the spread of CRAb on affected wards; however, continuing influx of colonized patients particularly from foreign hospitals remains a problem. One of the most affected hospitals is UMCL with ongoing spread of multiple CRAb clones with two different carbapenemases (OXA-23 or OXA-40) [9]. An increase in CRAb prevalence led to an increased use of colistin, and it is challenging to face out such phenomenon since detection methods are not always adapted, being either inaccurate, fastidious, or time-consuming. By using the Rapid ResaPolymyxin NP test, rapid and accurate detection of colistin resistance was possible and very few major or very major errors were observed. Three isolates were false positives (3.7% ME), but in two cases, the patients had ColR CRAb isolates from subsequent samples taken a few days after the original isolate with a false positive Rapid ResaPolymyxin NP test (in the third case, no subsequent samples were taken). Since the inoculum for the ResaPolymyxin NP is higher than the inoculum used for BMD, it might be speculated that resistant subpopulation undetected by the BMD method could be detected by using the Rapid ResaPolymyxin NP test. This test is inexpensive and easy to integrate into laboratory workflow. Even though this test does not precisely determine the MIC of colistin of the isolates tested, its main value is rapid categorization between susceptibility and resistance, which has important implications with respect to the treatment strategy and/or rapid implementation of isolation measures.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflicts of interests.

Ethical approval The study was approved by a Medical Ethics Committee of the Republic of Slovenia (ref: 0120-709/2017/6).

Informed consent Not needed.

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