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Rapid detection of polymyxin-resistant *Enterobacteriaceae* from blood cultures

Aurélie Jayol,[1,2,3] Véronique Dubois,[3] Laurent Poirel,[1,2] and Patrice Nordmann[1,2,4*]

[1]Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, Department of Medicine, University of Fribourg, Switzerland, [2]INSERM European Unit (LEA Paris, France), University of Fribourg, Switzerland, [3]CNRS UMR5234, University of Bordeaux, France, and [4]University of Lausanne and University Hospital Center, Lausanne, Switzerland

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*Corresponding author. Medical and Molecular Microbiology, Department of Medicine, Faculty of Science, University of Fribourg, rue Albert Gockel 3, CH-1700 Fribourg, Switzerland. Phone: 41-26-300-9581. E-mail: patrice.nordmann@unifr.ch

Abstract

Enterobacterial strains resistant to polymyxins are increasingly reported worldwide. Conventional methods for detection of colistin-resistant isolates such as broth microdilution remain time-consuming (24 to 48 h), and methods such as disc diffusion and E-test are not reliable. Recently, the Rapid Polymyxin NP test was developed for a rapid identification of polymyxin-resistant *Enterobacteriaceae*. This test is based on the detection of glucose metabolism related to bacterial growth in the presence of a defined concentration of colistin (or polymyxin B). Formation of acid metabolites is evidenced by a color change of a pH indicator (red phenol) in less than 2h. In this study, the Polymyxin NP test has been evaluated to detect colistin-resistant *Enterobacteriaceae* directly from blood cultures. The test was performed with 73 blood culture sets (either spiked or clinical blood cultures) with various enterobacterial species. The test exhibited excellent discrimination between polymyxin-resistant and polymyxin-susceptible enterobacterial isolates, and results are obtained from blood cultures within 4 hours. It is easy to perform, and neither requires subculture nor centrifugation step. This test is rapid, specific, and sensitive, and allows early identification of polymyxin-resistant *Enterobacteriaceae* directly from blood cultures.

INTRODUCTION

The spread of carbapenemase-producing in *Enterobacteriaceae* has now been reported worldwide (1). Considering the paucity of effective antibiotics against those multidrug-resistant bacteria, it is of utmost importance to preserve the clinical efficacy of last-resort antibiotics such as polymyxins (colistin, polymyxin B) (2). However, polymyxin-resistant *Enterobacteriaceae* are being increasingly reported worldwide, either exhibiting chromosomal mutations (3) or plasmid-mediated *mcr-1* gene acquisition (4). Methods available to determine polymyxin susceptibility require isolation and growth of the bacteria from the infected samples, and the susceptibility testing methods add, at least, an additional delay of 18 hours to obtain the results (5). Moreover, routine methods used in the clinical microbiology laboratory (disk diffusion, E-test strips, and some automated systems such as the Vitek2 and the MicroScan) are not reliable to detect polymyxin-resistant isolates (5-9). Molecular tests are not effective to determine polymyxin resistance due to the variety of genes that may be altered or truncated as a source of chromosomal resistance.

Rapid identification and antimicrobial susceptibility testing are essential for guiding clinicians in the selection of the most appropriate treatment for patients with bloodstream

infections. Mortality rates from sepsis in intensive care units range from 20 to 60% worldwide. Inappropriate initial antimicrobial therapy for septic shock occurs in about 20% of patients, is associated with a five-fold reduction in survival (10), and is responsible for an increase length of hospital stay (11). This is largely due to the fact that its takes several days to obtain the susceptibility pattern of the bacteria responsible for septicemia. In Italy, a large hospital outbreak of colistin-resistant and carbapenemase (KPC)-producing *Klebsiella pneumoniae* isolates, correlating the local for increased colistin consumption, has been recently described (12). Therefore, there is a need for a rapid detection of polymyxin-resistant isolates from blood cultures.

Recently, a diagnostic test (Rapid Polymyxin NP test) based on the detection of the glucose metabolism related to bacterial growth in presence of colistin has been developed to rapidly identify (< 2h) polymyxin-resistant enterobacterial strains from colonies grown on solid media (13). This test yields values of sensitivity and specificity of 99.3% and 95.4% respectively. It had not yet been evaluated from clinical samples.

The aim of this study was to determine the ability of the Rapid Polymyxin NP test to detect polymyxin-resistant *Enterobacteriaceae* from blood cultures. First, we evaluated this test with blood cultures spiked with colistin-resistant enterobacterial strains exhibiting various

mechanism of resistance (intrinsic or acquired, chromosomal and plasmid-mediated). Then, the test was performed prospectively on clinical blood cultures positive for Gram-negative bacteria.

MATERIAL AND METHODS

Spiked blood cultures. The panel of strains used for spiking blood cultures included a single reference strain susceptible to colistin (*Escherichia coli* ATCC 25922), and 52 colistin-resistant enterobacterial strains (MICs of colistin ranging from 4 to higher than 128 µg/ml). Among the latter strains, five strains exhibited an intrinsic polymyxin resistance trait, being *Proteus* spp. (n = 2), *Providencia* spp. (n = 1), *Morganella* spp. (n = 1), and *Serratia* spp. (n = 1), and 47 strains presented an acquired resistance trait and belonged to various species; *E. coli* (n = 11), *Klebsiella* spp. (n = 31), *Enterobacter* spp. (n = 4), and *Hafnia alvei* (n = 1). Some of the strains had previously been characterized for their polymyxin resistance mechanisms content at the molecular level (13-19).

To spike the blood cultures, the colistin-resistant enterobacterial strains were subcultured onto Luria-Bertani agar overnight. Then, aerobic and anaerobic blood culture vials without charcoal (BacT/ALERT FA Plus and BacT/ALERT FN Plus, respectively [bioMérieux, Marcy-l'Etoile, France]) containing 10 ml of sterile human blood were inoculated with 10^3

CFU/ml of bacterial strain to test. This inoculum was prepared by diluting a 0.5 McFarland suspension (about 10^8 CFU/ml) in 0.9% NaCl. Then, blood culture vials were incubated until a positive blood culture was detected by the BacT/Alert 3D™ blood culture system. Using this protocol, the time for detecting positivity of the blood cultures ranged from 6 to 15 h and the final inoculum ranged from 10^7 to 10^9 CFU/ml, as previously published (20).

Clinical blood cultures. All sets of positive blood cultures recovered during the November 2015 – February 2016 period-of-time from non-duplicate patients, detected positive by the BacT/Alert 3D™ blood culture system (bioMérieux) and stained as Gram-negative were included. Both aerobic and anaerobic vials were tested. Species identification was carried out with the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) Biotyper System (Bruker Corporation, Bremen, Germany) after 3 h of incubation of the blood culture on chocolate agar. Positive blood cultures with non-enterobacterial isolates or with a mix of bacteria were excluded from the testing. Overall, a total of 20 monomicrobial blood cultures positive for enterobacterial isolates were tested (Table 1).

Colistin susceptibility testing. Colistin susceptibility testing was performed by determination of MICs using the broth microdilution method (BMD) according to the CLSI

guidelines (21, 22). The BMD method was carried out from colonies grown on chocolate agar after incubation of the positive blood culture. EUCAST guidelines (version 2015) were used for categorical agreement (23).

Detection of colistin resistance from positive blood cultures using the Rapid Polymyxin NP test. For each positive blood culture tested (spiked or clinical blood cultures), 50 µl of blood culture was mixed with 450 µl of sterile 0.9% NaCl (ten-fold dilution). The Rapid Polymyxin NP test was then performed as described with bacterial colonies (13). Briefly, 50 µl of the ten-fold dilution of the blood culture was mixed with i) 150 µl of colistin-free Polymyxin NP solution and ii) 150 µl of Polymyxin NP solution containing 5 µg/ml of colistin sulfate (Sigma Aldrich, Buchs, Switzerland) in a 96-well polystyrene plate (ref. 82.1582.001, Sarstedt, Nümbrecht, Germany) (Figure 1). The final concentration of colistin was 3.75 µg/ml in each well. In parallel, 50 µl of 0.9% NaCl was mixed with the Polymyxin NP solution to verify the absence of medium contamination. The plate was incubated at 35±2°C in ambient air during 4 hours and visual inspection was performed each hour to observe the color change. Test results were interpreted by technicians who were blinded to the sample identity. The test was considered as negative when the well containing the mix with colistin remained orange, and positive when it turned from orange to yellow (Figure 1). The

test was considered as non interpretable when the colistin-free well remained orange (absence of glucose metabolization) (Figure 1). *E. coli* ATCC 25922 and a *Morganella morganii* strain were used as negative and positive controls, respectively.

RESULTS AND DISCUSSION

To determine the performances of the Rapid Polymyxin NP test performed directly from blood cultures, 73 blood culture sets (aerobic and anaerobic vials) positive with enterobacterial strains were tested, including mono-microbial clinical samples and blood cultures spiked with colistin-resistant isolates presenting various mechanisms of resistance (intrinsic, acquired chromosomally-encoded, plasmid-mediated and unknown mechanisms). Results of the Rapid Polymyxin NP test were compared with the MICs determined by the BMD method taken as the gold standard.

The forty-three blood cultures spiked with enterobacterial strains resistant to colistin gave positive results in 2 to 4 hours (Table 1), except for a single colistin-resistant *E. coli* isolate that also gave a negative result by using the Rapid Polymyxin NP test performed from colonies [13]. The mechanism of colistin resistance of this single isolate remains unknown.

Twenty clinical positive blood culture sets detected by the BacT/Alert 3D^{TM} blood culture system were also tested. Among them, four were positive with colistin-resistant

enterobacterial isolates in 2 to 4 hours of incubation; three isolates belonged to species being naturally resistant to colistin (two *S. marcescens* and a single *Proteus vulgaris*) and a single *H. alvei* isolate showing acquired resistance to colistin. The Rapid Polymyxin NP test gave positive results for all those isolates (Table 1). Seventeen blood cultures grown with enterobacterial isolates belonging to genera naturally susceptible to colistin (*E. coli*, *Klebsiella*, *Enterobacter*, *Citrobacter*). None of them presented acquired resistance to colistin (MICs of colistin ranging from 0.125 to 0.5 μg/ml) and the Rapid Polymyxin NP test gave negative results. Positive blood cultures with non-fermenting bacterial isolates (*Acinetobacter* spp., *Pseudomonas* spp.) gave non-interpretable results (Figure 1).

A series of other conditions (variable blood culture dilutions or conditions of atmosphere incubation) were tested but gave less optimal results. Indeed, the test performed without blood culture dilution revealed that the excess of blood prevented the optimal reading of color change. We did not observe spontaneous lysis of the blood cultures that might possibly have interfered with the results of the test. Other blood culture dilutions (1/5 or 1/100) gave less rapid positive results (data not shown). Incubation under CO_2 gave false-positive results because of an excessive acidification of the Polymyxin NP solution, as

opposed to results obtained with the Rapid Polymyxin NP test performed with bacterial colonies.

This test performed from blood cultures may have the same limitation as the Rapid Polymyxin NP test performed from bacterial colonies (13), i.e the lack of detection of heteroresistant isolates with low level of MICs to polymyxins by BMD. This limitation has to be further explored.

CONCLUSION

The use of the Rapid Polymyxin NP test from positive blood cultures showed good performances for the detection of enterobacterial isolates resistant to colistin. It shortens the delay to obtain results to ca. 4 hours, versus 48 hours for the standard antibiotic susceptibility testing (24 hours for obtention of a bacterial culture and 24 additional hours for susceptibility testing). This test may be implemented in routine from blood cultures, best combined with a rapid identification technique such as the MADI-TOF technology. It is cost-effective since it avoids in particular the time-consuming steps of manual determination of MIC values (powder weighting, dilution…). As highlighted above, this test allows a rapid identification of polymyxin-resistant *Enterobacteriaceae* responsible for bacteremia, which would not be

possible with molecular techniques considering the complexity and the diversity of the polymyxin resistance mechanisms.

Early detection of colistin-resistant strains might contribute to an early initiation of appropriate antibiotic therapy for septic patients infected with multidrug-resistant *Enterobacteriaceae*. In countries where carbapenemase-producing enterobacterial isolates are endemic (e.g. Greece and Italy), implementation of the test could contribute to an early initiation of appropriate antibiotic therapy. It may preserve polymyxin as last resort antibiotherapy. In addition, implementation of contact isolation precautions for carriers of polymxin-resistant isolates could prevent the development of outbreaks with those multidrug-resistant isolates

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Figure legend

Figure. Strategy for identification of colistin-resistant *Enterobacteriaceae* from blood cultures using the Rapid Polymyxin NP test.

Table. Rapid Polymyxin NP test results for spiked blood cultures and clinical blood cultures

Strain	Species	Origin	Phenotype	Mechanism of resistance to colistin	MIC* colistin	Rapid Polymyxin NP test Aerobic vial	Rapid Polymyxin NP test Anaerobic vial
Clinical blood cultures							
B1	*E. coli*	France	S	NA	0.12	- (4h)	- (4h)
B2	*E. coli*	France	S	NA	0.12	- (4h)	- (4h)
B3	*E. coli*	France	S	NA	0.12	- (4h)	- (4h)
B4	*E. coli*	France	S	NA	0.12	- (4h)	- (4h)
B5	*E. coli*	France	S	NA	0.25	- (4h)	- (4h)
B6	*E. coli*	France	S	NA	0.5	- (4h)	- (4h)
B7	*E. coli*	France	S	NA	0.12	- (4h)	- (4h)
B8	*K. pneumoniae*	France	S	NA	0.12	- (4h)	- (4h)
B9	*K. pneumoniae*	France	S	NA	0.12	- (4h)	- (4h)
B10	*K. pneumoniae*	France	S	NA	0.12	- (4h)	- (4h)
B11	*K. pneumoniae*	France	S	NA	0.25	- (4h)	- (4h)
B12	*K. oxytoca*	France	S	NA	0.12	- (4h)	- (4h)
B13	*E. cloacae*	France	S	NA	0.25	- (4h)	- (4h)
B14	*E. cloacae*	France	S	NA	0.5	- (4h)	- (4h)
B15	*C. koseri*	France	S	NA	0.12	- (4h)	- (4h)
B16	*C. koseri*	France	S	NA	0.12	- (4h)	- (4h)
B17	*H. alvei*	France	R	Unknown	16	+ (3h)	+ (3h)
B18	*S. marcescens*	France	R	Intrinsic	128	+ (2h)	+ (3h)
B19	*S. marcescens*	France	R	Intrinsic	>128	+ (2h)	+ (3h)
B20	*P. vulgaris*	France	R	Intrinsic	>128	+ (2h)	+ (2h)
Spiked blood cultures							
FR-136	*E. coli*	ATCC25922	S	NA	0.25	- (4h)	- (4h)
FR-119	*E. coli*	France	R	Unknown	**8**	**- (4h)**	**- (4h)**
FR-120	*E. coli*	France	R	Unknown	16	+ (3h)	+ (3h)
FR-121	*E. coli*	France	R	Unknown	4	+ (3h)	+ (2h)
B21	*E. coli*	France	R	PmrB D152V	8	+ (4h)	+ (4h)
FR-93	*E. coli*	Switzerland	R	MCR-1 plasmid	4	+ (4h)	+ (3h)
FR-94	*E. coli*	South Africa	R	MCR -1 plasmid	8	+ (3h)	+ (3h)
FR-95	*E. coli*	South Africa	R	MCR -1 plasmid	8	+ (4h)	+ (3h)
FR-96	*E. coli*	South Africa	R	MCR -1 plasmid	8	+ (3h)	+ (3h)

FR-97	*E. coli*	South Africa	R	MCR -1 plasmid	16	+ (3h)	+ (3h)
FR-98	*E. coli*	South Africa	R	MCR -1 plasmid	8	+ (2h)	+ (2h)
FR-99	*E. coli*	South Africa	R	MCR-1 plasmid	16	+ (4h)	+ (3h)
FR-100	*K. pneumoniae*	France	R	Unknown	32	+ (3h)	+ (4h)
FR-101	*K. pneumoniae*	France	R	Unknown	64	+ (3h)	+ (3h)
FR-103	*K. pneumoniae*	France	R	Unknown	4	+ (3h)	+ (3h)
FR-104	*K. pneumoniae*	France	R	Unknown	64	+ (2h)	+ (3h)
FR-106	*K. pneumoniae*	France	R	Unknown	>128	+ (3h)	+ (3h)
FR-107	*K. pneumoniae*	Spain	R	Unknown	32	+ (2h)	+ (2h)
FR-108	*K. pneumoniae*	Spain	R	Unknown	32	+ (2h)	+ (2h)
FR-115	*K. pneumoniae*	Colombia	R	Unknown	64	+ (3h)	+ (3h)
FR-118	*K. pneumoniae*	Turkey	R	Unknown	4	+ (2h)	+ (2h)
FR-06	*K. pneumoniae*	Colombia	R	PmrA G53C	64	+ (2h)	+ (2h)
FR-10	*K. pneumoniae*	South Africa	R	PmrB T157P	16	+ (4h)	+ (2h)
FR-11	*K. pneumoniae*	Colombia	R	PmrB T157P	32	+ (4h)	+ (3h)
FR-16b	*K. pneumoniae*	South Africa	R	PhoP D191Y	128	+ (3h)	+ (3h)
FR-21	*K. pneumoniae*	France	R	MgrB N42Y et K43I	64	+ (3h)	+ (3h)
FR-22	*K. pneumoniae*	Angola	R	MgrB I45T	64	+ (3h)	+ (3h)
FR-25	*K. pneumoniae*	France	R	MgrB truncated	128	+ (4h)	+ (2h)
FR-32	*K. pneumoniae*	Switzerland	R	MgrB truncated	64	+ (4h)	+ (3h)
FR-36	*K. pneumoniae*	Colombia	R	MgrB truncated	64	+ (2h)	+ (3h)
FR-40	*K. pneumoniae*	France	R	$bla_{\text{CTX-M-15}}$/IS*Ecp1* into *mgrB* gene	64	+ (2h)	+ (2h)
FR-42	*K. pneumoniae*	France	R	IS*102* into *mgrB* gene	>128	+ (3h)	+ (3h)
FR-47	*K. pneumoniae*	Turkey	R	IS*903b* into *mgrB* gene	64	+ (2h)	+ (2h)
FR-48	*K. pneumoniae*	Spain	R	IS*903* into *mgrB* gene	128	+ (3h)	+ (3h)
FR-49	*K. pneumoniae*	France	R	IS*5* into *mgrB* gene	64	+ (2h)	+ (4h)
FR-52	*K. pneumoniae*	Turkey	R	IS*5* into *mgrB* gene	128	+ (2h)	+ (2h)
FR-54	*K. pneumoniae*	Colombia	R	IS*Kpn13* into *mgrB* gene	128	+ (2h)	+ (3h)
FR-56	*K. pneumoniae*	Spain	R	IS*Kpn26* into *mgrB* gene	64	+ (2h)	+ (2h)
FR-68	*K. pneumoniae*	Colombia	R	IS*Kpn14* into *mgrB* gene	32	+ (3h)	+ (3h)
FR-70	*K. pneumoniae*	Colombia	R	IS*10R* into *mgrB* promotor	128	+ (3h)	+ (3h)
FR-71	*K. pneumoniae*	Turkey	R	IS*Kpn14* into *mgrB* promotor	32	+ (2h)	+ (2h)
FR-89	*K. pneumoniae*	Colombia	R	Deletion of 11 bp into *mgrB*	>128	+ (3h)	+ (3h)
FR-92	*K. oxytoca*	Colombia	R	IS*Kpn26* into *mgrB* promoter	64	+ (2h)	+ (2h)
FR-122	*E. cloacae*	Colombia	R	Unknown	32	+ (3h)	+ (3h)

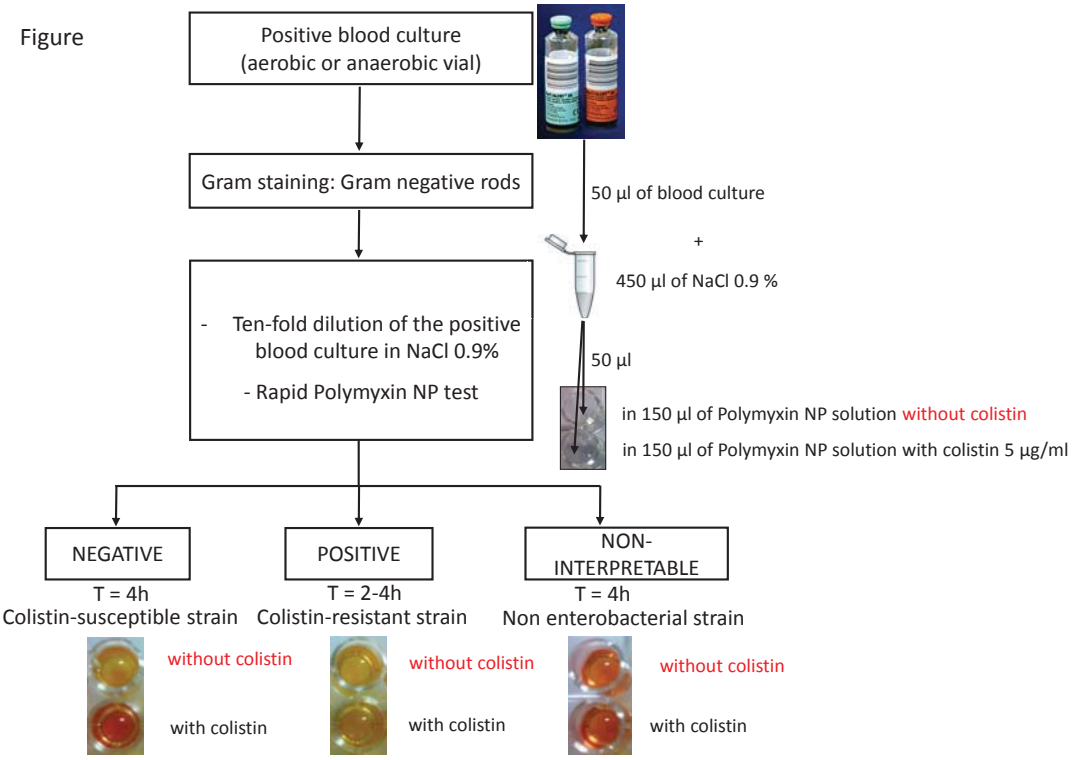
FR-123	*E. cloacae*	Colombia	R	Unknown	>128	+ (4h)	+ (4h)
FR-125	*E. cloacae*	France	R	Unknown	>128	+ (2h)	+ (2h)
FR-129	*E. cloacae*	France	R	Unknown	32	+ (3h)	+ (2h)
FR-135	*H. alvei*	France	R	Unknown	16	+ (2h)	+ (2h)
FR-01	*M. morganii*	France	R	Intrinsic	>128	+ (2h)	+ (2h)
FR-02	*P. mirabilis*	France	R	Intrinsic	>128	+ (3h)	+ (3h)
FR-03	*P. vulgaris*	France	R	Intrinsic	>128	+ (3h)	+ (2h)
FR-04	*P. stuartii*	France	R	Intrinsic	>128	+ (2h)	+ (3h)
FR-05	*S. marcescens*	France	R	Intrinsic	>128	+ (3h)	+ (3h)

Discrepant results obtained with the Rapid Polymyxin NP test compared to the broth dilution method are boldened. (-), negative; (+), positive

* MIC values of colistin, breakpoint values being susceptible ≤ 2 µg/ml and resistance >2 µg/ml for *Enterobacteriaceae* according to the EUCAST (23).

S, susceptible; R, resistant; NA, not applicable; CS, colistin.

Figure


Positive blood culture
(aerobic or anaerobic vial)
Gram staining: Gram negative rods
50 µl of blood culture
+
450 µl of NaCl 0.9 %
- Ten-fold dilution of the positive blood culture in NaCl 0.9%
- Rapid Polymyxin NP test
50 µl
in 150 µl of Polymyxin NP solution without colistin
in 150 µl of Polymyxin NP solution with colistin 5 µg/ml
NEGATIVE
T = 4h
Colistin-susceptible strain
POSITIVE
T = 2-4h
Colistin-resistant strain
NON-INTERPRETABLE
T = 4h
Non enterobacterial strain
without colistin
with colistin
without colistin
with colistin
without colistin
with colistin