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Comparison of methods for detection of plasmid-mediated and chromosomally-encoded colistin resistance in Enterobacteriaceae

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ABSTRACT

Objectives: Because of the emergence of plasmid-mediated (*mcr-1* and *mcr-2* genes) and chromosomally-encoded colistin resistance, reliable methods for detecting colistin resistance/susceptibility in routine laboratories are required. We evaluated the respective performances of the BD Phoenix automated system, the newly-developed Rapid Polymyxin NP test and the broth microdilution (BMD) reference method to detect colistin resistance in Enterobacteriaceae, and particularly those producing MCR-1 and MCR-2.

Methods: Colistin susceptibility of 123 enterobacterial clinical isolates (40 colistin-susceptible and 83 colistin-resistant isolates) was tested with the Phoenix automated system, the Rapid Polymyxin NP test and the BMD method. Molecular mechanisms responsible for plasmid-mediated and chromosomally-encoded colistin resistance mechanisms were investigated by PCR and sequencing.

Results: Considering BMD as a reference method, the Phoenix system failed to detect ten colistin-resistant isolates (one *Escherichia coli*, one *Klebsiella pneumoniae*, seven *Enterobacter* spp., and one *Salmonella enterica*). The Rapid Polymyxin NP test failed to detect the same single *E. coli* isolate. Those two latter methods detected the sixteen *E. coli, K. pneumoniae* and *S. enterica* isolates producing the plasmid-encoded MCR-1 and MCR-2.

Conclusion: The Phoenix system and the Rapid Polymyxin NP test are reliable techniques for detecting plasmid-mediated MCR-1 and MCR-2-related colistin resistance. However, a high rate of false susceptibility was observed with the Phoenix system, indicating that susceptibility results obtained with that system should be confirmed by BMD method. By contrast, the Rapid Polymyxin NP test showed a good agreement with the BMD method and

48 results were obtained rapidly (within two hours). The BMD method should be performed if
49 MIC values are needed.

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INTRODUCTION

The increasing use of colistin in human medicine, and the recent discovery of plasmid-mediated polymyxin resistance [1–4], highlight the need for reliable methods for polymyxin susceptibility testing.

The Clinical Laboratory Standard Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recently gathered in a joint subcommittee, chose the broth microdilution (BMD) method as the reference method (www.eucast.org). It must be performed with sulfate salts of polymyxins (colistimethate used in human medicine shall not be used), with cation-adjusted Mueller-Hinton broth, without additive (in particular without polysorbate 80) and without treated polystyrene trays. Other methods such as, agar dilution, disk diffusion and gradient diffusion (E-test) have been ruled out. However, this gold standard BMD method is difficult to performed in routine laboratories since it requires qualified staff, is time-consuming, and requires manual preparation of antibiotic solutions [5].

Automated dilution methods such as those performed by the BD Phoenix system could be an alternative for the screening of colistin resistance for laboratories that cannot perform manual BMD. However, the performance of this automate for colistin susceptibility testing, especially its accuracy for the detection of isolates exhibiting a plasmid-mediated colistin resistance, have never been evaluated. Recently, a rapid colorimetric test, the Rapid Polymyxin NP test, has been developed for detecting polymyxin resistance in *Enterobacteriaceae* within 2 hours [6].

The objective of this study was to evaluate the performance of the BD Phoenix automated system to detect plasmid-mediated and chromosomally-encoded colistin resistance, using a collection of clinical enterobacterial isolates. We also aimed to compare their performances to those of the Rapid Polymyxin NP test and the BMD reference method.

MATERIAL AND METHODS

Bacterial strains. This study was carried out using 123 non-duplicated clinical isolates of various enterobacterial species. The collection included 40 colistin-susceptible and 83 colistin-resistant isolates. Out of the 83 colistin-resistant isolates, sixteen belonged to a genus known to be naturally-resistant to colistin (*Morganella*, *Proteus*, *Providencia*, *Serratia*, *Hafnia*), and 67 isolates belonged to the *Escherichia, Klebsiella*, *Enterobacter*, or *Salmonella* genus with acquired resistance mechanisms to colistin. Identification of the isolates at the species level was performed using the Microflex bench-top MALDI-TOF mass spectrometer (Brücker, Champs-sur-Marne, France). Isolates were grown on Luria Bertani (LB) (GibcoBRL, Cergy Pontoise, France) or Mueller Hinton (MH) (bioMérieux, Marcy-l'Etoile, France) agar plates at 35±2°C for 18 h. The colistin-susceptible *E. coli* ATCC 25922 strain was included in all experiments as quality control.

Susceptibility testing

Reference antimicrobial susceptibility testing

The BMD method was performed according to the EUCAST/CLSI joined guidelines (www.eucast.org). Briefly, BMD panels were prepared extemporaneously in 96-wells sterile polystyrene microplates (Sarstedt, Nümbrecht, Germany). Dilutions of colistin (Sigma Aldrich, St Louis, USA) ranging from 0.125 to 128 mg/l were made in cation-adjusted MH broth (Bio-Rad, Marnes-la-Coquette, France), without addition of polysorbate 80 (Tween 80), and with a final concentration of $5x10^5$ CFU/ml of bacteria in each well. This procedure was performed in triplicate in separate experiments and the Minimum Inhibitory Concentrations (MICs) were read after 16 to 20 h of incubation at 35±2°C in ambient air. Results were interpreted according to the EUCAST breakpoints [7], i.e. isolates with MICs of colistin ≤ 2 mg/l were categorized as susceptible although those with MICs > 2 mg/l were resistant.

BD Phoenix automated system

Colistin susceptibility testing was assessed using the Phoenix automated system (BD Phoenix 100, BD Diagnostic systems, Le Pont de Claix, France), which performs automated BMD method. The panel selected to perform this evaluation was the Gram-negative panel NMIC-93, using the BMD method for colistin concentrations ranging from 0.5 to 4 mg/l in order to cover the EUCAST breakpoints [7]. The bacterial suspension and the panel inoculation were performed according to the manufacturer's guidelines. Panels were incubated up to 16 h at 35±2°C under ambient air, and results were interpreted with the BD EpiCenter software.

Rapid Polymyxin NP test

The Rapid Polymyxin NP test is based on the detection of the glucose metabolism related to bacterial growth in presence of a fixed concentration of colistin (3.75 mg/l) in cation-adjusted MH broth medium [6]. Formation of acid metabolites consecutive to the glucose metabolism is evidenced by a color change (orange to yellow) of the pH indicator (red phenol). The test is positive (colistin resistance) if a strain grows in presence of colistin, whereas it is negative (colistin susceptibility) if a strain does not grow in presence of colistin. Results of the Rapid Polymyxin NP test were read at 2 h of incubation at 35±2°C in ambient air.

Molecular characterization of the colistin resistance. Molecular mechanisms responsible for plasmid-mediated (*mcr-1* and *mcr-2* genes) and chromosomally-encoded (*pmrA*, *pmrB*, *phoP*, *phoQ*, *mgrB*, and *crrB* alterations) colistin resistance were determined as described previously [1,2,8–12].

Results analysis. The results obtained with the BD Phoenix system and the Rapid Polymyxin NP test were compared to those obtained with the reference BMD method. Discrepancies were determined for each method in order to assess their performance to detect colistin resistance. For strains for which discrepant susceptibility results were obtained, the

isolates were retested with the three methods. Unsolved discrepancies were then maintained in the database for performance evaluation. Errors were ranked as follows: a very major error (VME) was defined when isolates were categorized as susceptible using the Phoenix system or the Rapid Polymyxin NP test but resistant by the BMD method (false-susceptible result), while a major error (ME) was defined when isolates were found resistant using the Phoenix system or the Rapid Polymyxin NP test, but were found susceptible by using the BMD method (false-resistant result). The number of resistant isolates, and the number of susceptible isolates were used as denominators for VME and ME calculations, respectively. Acceptance criteria that provide requirements, and specifications to evaluate performances of antimicrobial susceptibility test devices were those defined by the ISO standards (VME and ME must be ≤3%) [13].

RESULTS

The features of the 123 enterobacterial isolates included in this study to evaluate the performance of the BD Phoenix system and the Polymyxin NP test for determining colistin susceptibility are presented in the Table.

Fourty isolates defined as colistin-susceptible according to the results of the BMD method (MICs of colistin ranging from 0.12 to 2 µg/ml) were found susceptible by the BD Phoenix system (Table). While a single susceptible *K. pneumoniae* isolate with an MIC of colistin at 2 mg/l was found resistant using the Rapid Polymyxin NP test. The MIC value of colistin for this same isolate as determined by the BD Phoenix system was underestimated (MIC ≤ 0.5 mg/l) but the isolate was well categorized as susceptible.

Out of the 83 colistin-resistant enterobacterial isolates (MICs of colistin ranging from 4 to higher than 128 mg/l), the Phoenix system failed to detect colistin resistance for seven *Enterobacter* spp. isolates, a single *K. pneumoniae*, a single *S. enterica*, and a single *E. coli*

147 isolate, whereas the Rapid Polymyxin NP test only failed for detecting a single colistin-
148 resistant *E. coli* isolate. (Table). Identical results were obtained when those strains were
149 repeatedly tested with the Phoenix system and the Rapid Polymyxin NP test indicating a good
150 reproducibility of the methods.

151 Noteworthy, thirteen non clonally-related colistin-resistant *E. coli*, one *K. pneumoniae*,
152 and one *S. enterica* isolate possessing the plasmid-mediated *mcr-1* gene were tested (MICs of
153 colistin ranging from 4 to 64 mg/l using the BMD method) and all were identified as resistant
154 with the BD Phoenix system and the Rapid Polymyxin NP test. Similarly, the *E. coli* isolate
155 possessing the plasmid-mediated *mcr-2* gene (MIC = 4 mg/l) was detected by the two
156 methods.

157 **DISCUSSION**

158 Out of the 40 colistin-susceptible enterobacterial isolates, no ME (i.e. false resistance)
159 was found with the Phoenix system, and only a single susceptible *K. pneumoniae* isolate with
160 an MIC of colistin at 2 mg/l (therefore just below the EUCAST breakpoint value > 2 mg/l)
161 was falsely identified as colistin resistant with the Rapid Polymyxin NP test revealing a ME
162 rate of 2.5%.test

163 Out of the 83 colistin-resistant enterobacterial isolates, the BD Phoenix system and the
164 Rapid Polymyxin NP test showed excellent performances to detect the 13 isolates with
165 plasmid-mediated colistin resistance regardless of the level of resistance. However, ten VME
166 (i.e. false susceptibility) were found with the Phoenix system whereas a single VME was
167 found with the Rapid Polymyxin NP test (Table). A high VME rate of 12% was thus found
168 with the BD Phoenix system, whereas a low VME rate of 1.2% was found with the Rapid
169 Polymyxin NP test. The single colistin-resistant *E. coli* isolate that was not detected with the
170 BD Phoenix system and the Rapid Polymyxin NP test, presented a low level of resistance

(MIC of colistin at 8 mg/l). Its mechanism of colistin resistance remains unknown (neither chromosomally-encoded mutations in genes known to be involved in lipopolysaccharide modifications, i.e. *mgrB*, *pmrAB* and *phoPQ* genes, nor plasmid-mediated *mcr-1* and *mcr-2* genes were detected).

The *S. enterica* isolate identified as susceptible (MIC = 2 mg/l) with the BD Phoenix system presented a low level of colistin resistance (MIC = 4 mg/l) and its mechanism of resistance remains unknown (neither chromosomal mutations, nor plasmid-mediated resistance).

The *K. pneumoniae* resistant isolate and the seven *Enterobacter* spp. resistant isolates not detected with the BD Phoenix system exhibited MIC values of colistin ranging from 16 to higher than 128 mg/l and were identified as colistin resistant with the Rapid Polymyxin NP test. During the determination of MICs by the BMD method, skipped wells (i.e. wells that exhibit no growth although growth does occur at higher concentrations) were observed for 88% of those isolates (the *K. pneumoniae* isolate and six *Enterobacter* spp. isolates). This observation suggests that the failure of the BD Phoenix system to detect colistin resistance in those isolates could be related to a heteroresistance phenotype (defined by the presence of two subpopulations exhibiting different susceptibilities to colistin) [14]. The skipped wells observed during the MIC determination of those isolates by the BMD method are mainly for dilutions comprised between 0.125 and 4 mg/l. The Phoenix panel used in this study contained dilutions of colistin ranging from 0.5 to 4 mg/l. It is therefore likely that the failure of detection of heteroresistance for those isolates was linked to the absence of testing at higher colistin concentrations. The low sensitivity to detect colistin heteroresistance has already been described for another automated system, i.e. the bioMérieux Vitek system [15].

The limitation of our study could be the absence of testing of non-fermenting Gram negative rods in our collection.

CONCLUSION

This study shows that the BD Phoenix system and the Rapid Polymyxin NP test are reliable tools for detection of plasmid-mediated colistin resistance (*mcr-1* and *mcr-2* genes), which is currently a major concern. However, the BD Phoenix system is not reliable for detection of colistin heteroresistance in enterobacterial isolates. Thus, we recommend the determination of MICs by the BMD method when susceptible results are obtained and if clinical use is required. By contrast, the Rapid Polymyxin NP test showed a good agreement with the BMD method and results were obtained rapidly (within two hours), but BMD method should be performed if determination of MIC values is necessary.

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CONFLICT OF INTEREST

An international patent form has been filed on behalf of the University of Fribourg, Switzerland corresponding to the Rapid Polymyxin NP test.

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Table. MICs of colistin (mg/l) using the BMD method and the BD Phoenix system and results of the Rapid Polymyxin NP test.

Isolate	Species (number of isolates)	Phenotype	Mechanism of resistance to colistin[a]	BMD MIC colistin	Phoenix MIC colistin	Discrepancies[b]	Rapid Polymyxin NP test Result	Discrepancies[b,c]
Isolates susceptible to colistin								
ATCC25922	*E. coli*	S	NA	0.25	≤0.5	No	-	No
2 to 15	*E. coli* (n= 14)	S	NA	0.12 to 0.5	≤0.5	No	-	No
16 to 26	*K. pneumoniae* (n=11)	S	NA	0.12 to 2	≤0.5	No	-	**Yes, ME (n=1)**
27 to 29	*K. oxytoca* (n=3)	S	NA	0.12 to 0.25	≤0.5	No	-	No
30 to 32	*E. cloacae* (n=3)	S	NA	0.12 to 0.25	≤0.5	No	-	No
33	*E. asburiae*	S	NA	0.12	≤0.5	No	-	No
34	*E. aerogenes*	S	NA	0.12	≤0.5	No	-	No
35 to 37	*C. freundii* (n=3)	S	NA	0.25	≤0.5	No	-	No
38 to 40	*C. koseri* (n=3)	S	NA	0.12 to 0.25	≤0.5	No	-	No
Isolates resistant to colistin								
41	*M. morganii*	R	Intrinsic	>128	>4	No	+	No
42-43	*P. mirabilis* (n=2)	R	Intrinsic	>128	>4	No	+	No
44	*P. vulgaris*	R	Intrinsic	>128	>4	No	+	No
45	*P. stuartii*	R	Intrinsic	>128	>4	No	+	No
46 to 48	*S. marcescens* (n=3)	R	Intrinsic	>128	>4	No	+	No
49 to 52	*H. alvei* (n=4)	R	Intrinsic	8 or 16	4 or >4	No	+	No
53 to 56	*H. paralvei* (n=4)	R	Intrinsic	8	4 or >4	No	+	No
57 to 68	*E. coli* (n= 11)	R	Plasmid-mediated *mcr-1* gene	4 or 8	4 or >4	No	+	No
69	*E. coli*	R	Plasmid-mediated *mcr-1* gene	64	>4	No	+	No
70	*E. coli*	R	Plasmid-mediated *mcr-2* gene	4	4	No	+	No
71	*K. oxytoca*	R	IS*Kpn26* into *mgrB* promotor	64	>4	No	+	No
72	*E. coli*	R	Unknown	8	**≤0.5**	**Yes, VME**	-	**Yes, VME**
73	*E. coli*	R	Unknown	8	>4	No	+	No
74	*E. coli*	R	Unknown	4	4	No	+	No
75	*E. coli*	R	Unknown	16	>4	No	+	No
76	*K. pneumoniae*	R	PmrA G53C	64	>4	No	+	No
77-78	*K. pneumoniae* (n=2)	R	PmrA G53S	16 or 32	>4	No	+	No
79-80	*K. pneumoniae* (n=2)	R	PmrB T157P	16 or 32	>4	No	+	No
81	*K. pneumoniae*	R	PhoP D191Y	128	**≤0.5**	**Yes, VME**	+	No

82	*K. pneumoniae*	R	PhoQ R16C	128	>4	No	+	No
83	*K. pneumoniae*	R	MgrB N42Y et K43I	64	>4	No	+	No
84	*K. pneumoniae*	R	MgrB I45T	64	>4	No	+	No
85 to 87	*K. pneumoniae* (n=3)	R	MgrB truncated	64 or 128	>4	No	+	No
88	*K. pneumoniae*	R	Deletion of 11 nucleotides into *mgrB* gene	>128	>4	No	+	No
89	*K. pneumoniae*	R	bla*CTX-M-15*/IS*Ecp1* into *mgrB*	64	>4	No	+	No
90	*K. pneumoniae*	R	IS*5* into *mgrB* gene	64	>4	No	+	No
91	*K. pneumoniae*	R	IS*102* into *mgrB* gene	>128	>4	No	+	No
92	*K. pneumoniae*	R	IS*Kpn14* into *mgrB* gene	32	>4	No	+	No
93	*K. pneumoniae*	R	IS*Kpn13* into *mgrB* gene	128	>4	No	+	No
94	*K. pneumoniae*	R	IS*Kpn26* into *mgrB* gene	64	>4	No	+	No
95	*K. pneumoniae*	R	IS*903* into *mgrB* gene	128	>4	No	+	No
96	*K. pneumoniae*	R	IS*903b* into *mgrB* gene	64	>4	No	+	No
97	*K. pneumoniae*	R	IS*5* into *mgrB* gene	128	>4	No	+	No
98	*K. pneumoniae*	R	IS*10R* into *mgrB* promotor	128	>4	No	+	No
99	*K. pneumoniae*	R	IS*Kpn14* into *mgrB* promotor	32	>4	No	+	No
100	*K. pneumoniae*	R	CrrB N141Y	>128	>4	No	+	No
101	*K. pneumoniae*	R	CrrB P151L	>128	>4	No	+	No
102	*K. pneumoniae*	R	CrrB G183V	>128	>4	No	+	No
103	*K. pneumoniae*	R	Plasmid mediated *mcr-1* gene	16	4	No	+	No
104	*K. pneumoniae*	R	Unknown	16	>4	No	+	No
105	*K. pneumoniae*	R	Unknown	64	>4	No	+	No
106	*K. pneumoniae*	R	Unknown	32	>4	No	+	No
107	*K. pneumoniae*	R	Unknown	>128	>4	No	+	No
108	*K. pneumoniae*	R	Unknown	64	>4	No	+	No
109	*K. pneumoniae*	R	Unknown	64	>4	No	+	No
110	*K. pneumoniae*	R	Unknown	32	>4	No	+	No
111	*E. cloacae*	R	Unknown	32	>4	No	+	No
112	*E. cloacae*	R	Unknown	>128	>4	No	+	No
113	*E. cloacae*	R	Unknown	32	>4	No	+	No
114	*E. cloacae*	R	Unknown	>128	**1**	**Yes, VME**	+	No
115	*E. cloacae*	R	Unknown	64	**≤0.5**	**Yes, VME**	+	No
116	*E. cloacae*	R	Unknown	>128	**≤0.5**	**Yes, VME**	+	No
117	*E. cloacae*	R	Unknown	16	**≤0.5**	**Yes, VME**	+	No

118	*E. cloacae*	R	Unknown	>128	**≤0.5**	**Yes, VME**	+	No
119	*E. cloacae*	R	Unknown	>128	**≤0.5**	**Yes, VME**	+	No
120	*E. asburiae*	R	Unknown	>128	**≤0.5**	**Yes, VME**	+	No
121	*S. enterica*	R	Plasmid mediated *mcr-1* gene	16	>4	No	+	No
122	*S. enterica*	R	Unknown	4	**2**	**Yes, VME**	+	No
123	*S. enterica*	R	Unknown	4	>4	No	+	No

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

[a]Unknown : no mutation in genes known to be involved in colistin resistance (*pmrA*, *pmrB*, *phoP*, *phoQ*, *mgrB* and *crrB* genes)

[b]VME, very major error (false-susceptibility compared to the results obtained by broth microdilution reference method)

[c]ME, major error (false-resistance compared to the results obtained by broth microdilution reference method)