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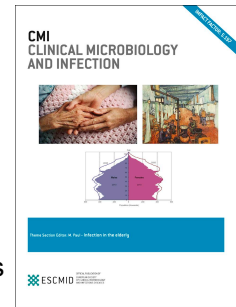
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Comparison of methods for detection of plasmid-mediated and chromosomally-encoded colistin resistance in Enterobacteriaceae

Aurélie Jayol, Patrice Nordmann, Philippe Lehours, Laurent Poirel, Véronique Dubois



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

3 Aurélie Jayol,^{1,2,3,4} Patrice Nordmann,^{3,4,5} Philippe Lehours,¹ Laurent Poirel,^{3,4} and
4 Véronique Dubois^{1,2*}

5 *¹Laboratory of Bacteriology, Bordeaux University Hospital, ²CNRS UMR5234, University of*
6 *Bordeaux, France, ³Emerging Antibiotic Resistance Unit, Medical and Molecular*
7 *Microbiology, Department of Medicine, University of Fribourg, Switzerland, ⁴INSERM*
8 *European Unit (LEA, IAME), Paris, France, and ⁵University of Lausanne and University*
9 *hospital Center, Lausanne, Switzerland*

10
11 **Keywords.** MCR-1, colistin, polymyxin B, resistance, susceptibility testing, Phoenix
12 automated system, Rapid Polymyxin NP test

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14 *Corresponding author. Véronique Dubois, MFP Laboratory CNRS UMR-5234. 146 rue Léo
15 Saignat, Batiment 3A, 33076 BORDEAUX Cedex, France. E-mail: [veronique.dubois@u-](mailto:veronique.dubois@u-bordeaux.fr)
16 [bordeaux.fr](mailto:veronique.dubois@u-bordeaux.fr)

ABSTRACT

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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

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

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

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

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

MATERIAL AND METHODS

75

76 **Bacterial strains.** This study was carried out using 123 non-duplicated clinical
77 isolates of various enterobacterial species. The collection included 40 colistin-susceptible and
78 83 colistin-resistant isolates. Out of the 83 colistin-resistant isolates, sixteen belonged to a
79 genus known to be naturally-resistant to colistin (*Morganella*, *Proteus*, *Providencia*, *Serratia*,
80 *Hafnia*), and 67 isolates belonged to the *Escherichia*, *Klebsiella*, *Enterobacter*, or *Salmonella*
81 genus with acquired resistance mechanisms to colistin. Identification of the isolates at the
82 species level was performed using the Microflex bench-top MALDI-TOF mass spectrometer
83 (Brücker, Champs-sur-Marne, France). Isolates were grown on Luria Bertani (LB)
84 (GibcoBRL, Cergy Pontoise, France) or Mueller Hinton (MH) (bioMérieux, Marcy-l'Etoile,
85 France) agar plates at $35\pm 2^\circ\text{C}$ for 18 h. The colistin-susceptible *E. coli* ATCC 25922 strain
86 was included in all experiments as quality control.

87

Susceptibility testing88 Reference antimicrobial susceptibility testing

89 The BMD method was performed according to the EUCAST/CLSI joined guidelines
90 (www.eucast.org). Briefly, BMD panels were prepared extemporaneously in 96-wells sterile
91 polystyrene microplates (Sarstedt, Nümbrecht, Germany). Dilutions of colistin (Sigma Aldrich,
92 St Louis, USA) ranging from 0.125 to 128 mg/l were made in cation-adjusted MH broth (Bio-
93 Rad, Marnes-la-Coquette, France), without addition of polysorbate 80 (Tween 80), and with a
94 final concentration of 5×10^5 CFU/ml of bacteria in each well. This procedure was performed
95 in triplicate in separate experiments and the Minimum Inhibitory Concentrations (MICs) were
96 read after 16 to 20 h of incubation at $35\pm 2^\circ\text{C}$ in ambient air. Results were interpreted
97 according to the EUCAST breakpoints [7], i.e. isolates with MICs of colistin ≤ 2 mg/l were
98 categorized as susceptible although those with MICs > 2 mg/l were resistant.

99 BD Phoenix automated system

100 Colistin susceptibility testing was assessed using the Phoenix automated system (BD Phoenix
101 100, BD Diagnostic systems, Le Pont de Claix, France), which performs automated BMD
102 method. The panel selected to perform this evaluation was the Gram-negative panel NMIC-
103 93, using the BMD method for colistin concentrations ranging from 0.5 to 4 mg/l in order to
104 cover the EUCAST breakpoints [7]. The bacterial suspension and the panel inoculation were
105 performed according to the manufacturer's guidelines. Panels were incubated up to 16 h at
106 $35\pm 2^\circ\text{C}$ under ambient air, and results were interpreted with the BD EpiCenter software.

107 Rapid Polymyxin NP test

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

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

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

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

134 RESULTS

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

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

144 Out of the 83 colistin-resistant enterobacterial isolates (MICs of colistin ranging from
145 4 to higher than 128 mg/l), the Phoenix system failed to detect colistin resistance for seven
146 *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

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

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

178 The *K. pneumoniae* resistant isolate and the seven *Enterobacter* spp. resistant isolates not
179 detected with the BD Phoenix system exhibited MIC values of colistin ranging from 16 to
180 higher than 128 mg/l and were identified as colistin resistant with the Rapid Polymyxin NP
181 test. During the determination of MICs by the BMD method, skipped wells (i.e. wells that
182 exhibit no growth although growth does occur at higher concentrations) were observed for
183 88% of those isolates (the *K. pneumoniae* isolate and six *Enterobacter* spp. isolates). This
184 observation suggests that the failure of the BD Phoenix system to detect colistin resistance in
185 those isolates could be related to a heteroresistance phenotype (defined by the presence of two
186 subpopulations exhibiting different susceptibilities to colistin) [14]. The skipped wells
187 observed during the MIC determination of those isolates by the BMD method are mainly for
188 dilutions comprised between 0.125 and 4 mg/l. The Phoenix panel used in this study
189 contained dilutions of colistin ranging from 0.5 to 4 mg/l. It is therefore likely that the failure
190 of detection of heteroresistance for those isolates was linked to the absence of testing at higher
191 colistin concentrations. The low sensitivity to detect colistin heteroresistance has already been
192 described for another automated system, i.e. the bioMérieux Vitek system [15].

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

195

CONCLUSION

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

204

205

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210

CONFLICT OF INTEREST

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

213

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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	<i>E. coli</i>	S	NA	0.25	≤0.5	No	-	No
2 to 15	<i>E. coli</i> (n= 14)	S	NA	0.12 to 0.5	≤0.5	No	-	No
16 to 26	<i>K. pneumoniae</i> (n=11)	S	NA	0.12 to 2	≤0.5	No	-	Yes, ME (n=1)
27 to 29	<i>K. oxytoca</i> (n=3)	S	NA	0.12 to 0.25	≤0.5	No	-	No
30 to 32	<i>E. cloacae</i> (n=3)	S	NA	0.12 to 0.25	≤0.5	No	-	No
33	<i>E. asburiae</i>	S	NA	0.12	≤0.5	No	-	No
34	<i>E. aerogenes</i>	S	NA	0.12	≤0.5	No	-	No
35 to 37	<i>C. freundii</i> (n=3)	S	NA	0.25	≤0.5	No	-	No
38 to 40	<i>C. koseri</i> (n=3)	S	NA	0.12 to 0.25	≤0.5	No	-	No
Isolates resistant to colistin								
41	<i>M. morgani</i>	R	Intrinsic	>128	>4	No	+	No
42-43	<i>P. mirabilis</i> (n=2)	R	Intrinsic	>128	>4	No	+	No
44	<i>P. vulgaris</i>	R	Intrinsic	>128	>4	No	+	No
45	<i>P. stuartii</i>	R	Intrinsic	>128	>4	No	+	No
46 to 48	<i>S. marcescens</i> (n=3)	R	Intrinsic	>128	>4	No	+	No
49 to 52	<i>H. alvei</i> (n=4)	R	Intrinsic	8 or 16	4 or >4	No	+	No
53 to 56	<i>H. paralvei</i> (n=4)	R	Intrinsic	8	4 or >4	No	+	No
57 to 68	<i>E. coli</i> (n= 11)	R	Plasmid-mediated <i>mcr-1</i> gene	4 or 8	4 or >4	No	+	No
69	<i>E. coli</i>	R	Plasmid-mediated <i>mcr-1</i> gene	64	>4	No	+	No
70	<i>E. coli</i>	R	Plasmid-mediated <i>mcr-2</i> gene	4	4	No	+	No
71	<i>K. oxytoca</i>	R	ISK <i>pn26</i> into <i>mgrB</i> promotor	64	>4	No	+	No
72	<i>E. coli</i>	R	Unknown	8	≤0.5	Yes, VME	-	Yes, VME
73	<i>E. coli</i>	R	Unknown	8	>4	No	+	No
74	<i>E. coli</i>	R	Unknown	4	4	No	+	No
75	<i>E. coli</i>	R	Unknown	16	>4	No	+	No
76	<i>K. pneumoniae</i>	R	PmrA G53C	64	>4	No	+	No
77-78	<i>K. pneumoniae</i> (n=2)	R	PmrA G53S	16 or 32	>4	No	+	No
79-80	<i>K. pneumoniae</i> (n=2)	R	PmrB T157P	16 or 32	>4	No	+	No
81	<i>K. pneumoniae</i>	R	PhoP D191Y	128	≤0.5	Yes, VME	+	No

82	<i>K. pneumoniae</i>	R	PhoQ R16C	128	>4	No	+	No
83	<i>K. pneumoniae</i>	R	MgrB N42Y et K43I	64	>4	No	+	No
84	<i>K. pneumoniae</i>	R	MgrB I45T	64	>4	No	+	No
85 to 87	<i>K. pneumoniae</i> (n=3)	R	MgrB truncated	64 or 128	>4	No	+	No
88	<i>K. pneumoniae</i>	R	Deletion of 11 nucleotides into <i>mgrB</i> gene	>128	>4	No	+	No
89	<i>K. pneumoniae</i>	R	<i>blaCTX-M-15/ISEcp1</i> into <i>mgrB</i>	64	>4	No	+	No
90	<i>K. pneumoniae</i>	R	IS5 into <i>mgrB</i> gene	64	>4	No	+	No
91	<i>K. pneumoniae</i>	R	IS102 into <i>mgrB</i> gene	>128	>4	No	+	No
92	<i>K. pneumoniae</i>	R	ISKpn14 into <i>mgrB</i> gene	32	>4	No	+	No
93	<i>K. pneumoniae</i>	R	ISKpn13 into <i>mgrB</i> gene	128	>4	No	+	No
94	<i>K. pneumoniae</i>	R	ISKpn26 into <i>mgrB</i> gene	64	>4	No	+	No
95	<i>K. pneumoniae</i>	R	IS903 into <i>mgrB</i> gene	128	>4	No	+	No
96	<i>K. pneumoniae</i>	R	IS903b into <i>mgrB</i> gene	64	>4	No	+	No
97	<i>K. pneumoniae</i>	R	IS5 into <i>mgrB</i> gene	128	>4	No	+	No
98	<i>K. pneumoniae</i>	R	IS10R into <i>mgrB</i> promotor	128	>4	No	+	No
99	<i>K. pneumoniae</i>	R	ISKpn14 into <i>mgrB</i> promotor	32	>4	No	+	No
100	<i>K. pneumoniae</i>	R	CrrB N141Y	>128	>4	No	+	No
101	<i>K. pneumoniae</i>	R	CrrB P151L	>128	>4	No	+	No
102	<i>K. pneumoniae</i>	R	CrrB G183V	>128	>4	No	+	No
103	<i>K. pneumoniae</i>	R	Plasmid mediated <i>mcr-1</i> gene	16	4	No	+	No
104	<i>K. pneumoniae</i>	R	Unknown	16	>4	No	+	No
105	<i>K. pneumoniae</i>	R	Unknown	64	>4	No	+	No
106	<i>K. pneumoniae</i>	R	Unknown	32	>4	No	+	No
107	<i>K. pneumoniae</i>	R	Unknown	>128	>4	No	+	No
108	<i>K. pneumoniae</i>	R	Unknown	64	>4	No	+	No
109	<i>K. pneumoniae</i>	R	Unknown	64	>4	No	+	No
110	<i>K. pneumoniae</i>	R	Unknown	32	>4	No	+	No
111	<i>E. cloacae</i>	R	Unknown	32	>4	No	+	No
112	<i>E. cloacae</i>	R	Unknown	>128	>4	No	+	No
113	<i>E. cloacae</i>	R	Unknown	32	>4	No	+	No
114	<i>E. cloacae</i>	R	Unknown	>128	1	Yes, VME	+	No
115	<i>E. cloacae</i>	R	Unknown	64	≤0.5	Yes, VME	+	No
116	<i>E. cloacae</i>	R	Unknown	>128	≤0.5	Yes, VME	+	No
117	<i>E. cloacae</i>	R	Unknown	16	≤0.5	Yes, VME	+	No

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

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

^aUnknown : no mutation in genes known to be involved in colistin resistance (*pmrA*, *pmrB*, *phoP*, *phoQ*, *mgrB* and *crrB* genes)

^bVME, very major error (false-susceptibility compared to the results obtained by broth microdilution reference method)

^cME, major error (false-resistance compared to the results obtained by broth microdilution reference method)