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A resazurin reduction-based assay for rapid detection of polymyxin resistance in *Acinetobacter baumannii* and *Pseudomonas aeruginosa*

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Running head. Rapid diagnostic of polymyxin resistance in non fermenters

Keywords. Susceptibility testing, rapid diagnostic test, colistin, polymyxin B

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A rapid test was developed for identification of polymyxin resistance in non-fermenting bacteria. This test detects viable cells after growth in a medium containing a defined concentration of colistin. The principle of this test is based on the visual detection of the reduction of the resazurin reagent, a viability colorant, observed by its color change (blue to purple or pink). Its evaluation was performed by using 92 colistin-resistant and colistin-susceptible *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates. Sensitivity and specificity were respectively found to be 100 and 95% by comparison with the standard broth microdilution method. The Rapid ResaPolymyxin *Acinetobacter* / *Pseudomonas* NP test is inexpensive, easy-to-perform, highly sensitive and specific, and can be completed in 4 hours. It could be useful in countries facing endemic spread of colistin-resistant non fermenters.

INTRODUCTION

The ESKAPE is a group of bacterial species retrieved in clinical settings and able to acquire multidrug resistance. *Acinetobacter baumannii* and *Pseudomonas aeruginosa* belonging to this group are carbapenem-resistant isolates (1) and infections due to these multidrug-resistant (MDR) species are increasingly reported in healthcare facilities and may lead to fatal outcomes due to limited therapeutic options (2-4). Consequently, the Centers for Diseases Control and then the World Health Organization classified the carbapenem-resistant *A. baumannii* and *P. aeruginosa* among the most serious pathogens exhibiting multidrug resistance (5) (http://www.cdc.gov/ drugresistance/threat-report-2013/). Old antibiotics such as polymyxins (colistin and polymyxin B) are used as a last resort treatment for treating MDR *A. baumannii* and *P. aeruginosa* (5, 6). Unfortunately, resistance to polymyxins in those species is also on the rise, highlighting the importance of obtaining rapid results of polymyxin susceptibility to optimize the antibiotic stewarship.

The current standard method of detection for colistin susceptibility in Gram negatives is the determination of minimum inhibitory concentration (MIC) by the broth dilution method (BMD) (www.eucast.org). However, this procedure is time consuming, is impractical for most clinical laboratories and results are obtained in 24 h. Moreover, it is subjected to possible multiple technical problems, such as correct weighting of the colistin powder, variable activities of colistin-containing powder, or possible sticking of colistin onto some plastic plates. Other techniques such as disk diffusion and E-test are not recommended due to high rates of false-susceptibility results (up to 32%) (7, 8).

Recently, Nordmann *et al.* developed the Rapid Polymyxin NP, a liquid-based technique that identifies colistin-susceptible / -resistant enterobacterial isolates in less than 2 h (9). This test which is based on the visualization of glucose metabolization in presence of a pH indicator, can therefore not be applied to non-fermenting Gram-negative bacteria such as *A. baumannii* and *P. aeruginosa*.

Here we developed a new assay based on the utilization of resazurin (7-hydroxy-3H-phenoxazin-3 one 10-oxide), also referred as the alamarBlue® and the PrestoBlue®. Its principle is based on the fact that metabolically-active cells reduce the blue resazurin to the pink product resorufin. This reduction is proportional to the number of metabolically active cells (10). We developed this test for *A. baumannii* and *P. aeruginosa* isolates, based on a comparison of bacterial viabilities, after growth in medium with or without a defined concentration of colistin.

The objective of this study was to evaluate the performance of this assay by comparison with the BMD method using a collection of colistin-susceptible and -resistant *A. baumannii* and *P. aeruginosa* from human and environmental origin isolates.

MATERIALS AND METHODS

Bacterial strains. This study was carried out using 88 human, and 4 environmental isolates of *A. baumannii* (n=43) and *P. aeruginosa* (n=49) identified at the species level using the Microflex bench-top Matrix Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF) mass spectrometer (Brücker, Champs-sur-Marne, France). Of the 92 isolates tested, most were from clinical specimens (intestinal carriages and infections of hospitalized patients) and four from environmental samples (soils from Nigeria). Thirteen out of the 43 *A. baumannii* and 10 out of the 49 *P. aeruginosa* isolates were colistin-resistant according to BMD testing (Table 1). The colistin-susceptible strain *P. aeruginosa* ATCC 27853 and the colistin-resistant *Escherichia coli* Af23 were used as negative and positive controls for the determination of MIC of colistin, respectively. None of the colistin-resistant isolates carried a plasmid-borne *mcr*-like gene (*mcr-1* to *-5*) encoding colistin resistance determinant as assessed by negative PCR results using the multiplex PCR detecting *mcr-1* to *-5* (11) (data not shown).

In addition, a series of 32 enterobacterial isolates was tested, corresponding to *Escherichia coli* (n=16, among which 14 were resistant to colistin), *Klebsiella pneumoniae* (n=11, among which 10 were resistant to colistin), *Enterobacter cloacae* (n=1, susceptible to colistin), and *Salmonella* sp. (all resistant to colistin). Isolates producing MCR determinants were as follows; *E. coli* (12 MCR-1, 1 MCR-2, and 1 MCR-3), and *Salmonella* (1 MCR-1, 2 MCR-4, and 1 co-producing MCR-1 and MCR-5). No MCR-producing *K. pneumoniae* of *E. cloacae* was tested.

Susceptibility testing

Reference Antimicrobial Susceptibility Testing

The BMD method was performed in triplicate using homemade panels and interpreted according to the EUCAST/CLSI joint guidelines as described elsewhere. Isolates were considered as susceptible when MICs of colistin were ≤ 2 mg/L and resistant when MICs were > 2 mg/L (www.eucast.org). In case of discrepancies between the different replicate results, the median result was retained. Colistin sulfate (Sigma Aldrich) was diluted into MHB-CA medium in glass tubes to obtain a polymyxin stock solution at a concentration of 0.2 mg/ml. Those antibiotic powders can be stored at 4°C before their use while diluted polymyxin solutions realized may be kept at -20°C during one year.

Rapid ResaPolymyxin *Acinetobacter* /*Pseudomonas* NP test

We first compared different parameters using two colistin-susceptible isolates (*A. baumannii* N4 and *P. aeruginosa* ATCC 27853) and two colistin-resistant isolates (*A. baumannii* FR-259 and *P. aeruginosa* FR-274). These parameters included: resazurin dyes [(alamarBlue® and PrestoBlue®(ThermoFisher Scientifics, Waltham, Massachusetts, USA)], medium of growth [Luria Bertani (LB) (Sigma, Saint Louis, USA) agar plates and Mueller-

Hinton plates (Bio-Rad, Marnes la Coquette, France)], bacterial inoculum (0.5; 1.5 and 3.5 McFarland), and time of contact between colistin-containing medium before adding resazurin reagent (1, 2 and 3 hours). After comparison of the results with the different parameters, all experiments were performed in triplicate with the optimal conditions obtained, as described further. We did not observe any difference in the results obtained according to the medium growth used (LB or Mueller-Hinton).

Preparation of solution. For the test, we used Mueller-Hinton (MH) solution (Bio-Rad, Marnes la Coquette, France) with or without colistin sulfate (tablets, MAST DIAGNOSTICS, Merseyside, UK) at a defined final concentration of 3.75 mg/L.

Bacterial inoculum preparation. For each isolate to be tested and for the colistin-susceptible- and -resistant isolates used as controls, we prepared a standardized bacterial inoculum of 3.5 McFarland by using freshly obtained (overnight) bacterial colonies grown on UriSelect™. We used as positive controls (resistant isolates) *A. baumannii* FR-259 and *P. aeruginosa* FR-274, and as negative controls (susceptible isolates) *A. baumannii* N4 and *P. aeruginosa* ATCC 27853. We used the bacterial suspensions within 15 min of preparation and for no longer than 1 hour after preparation as recommended by the EUCAST guidelines for susceptibility testing (www.eucast.org).

Tray inoculation. We performed testing in a 96-well polystyrene microtest plate (round base, with lid, sterile; Sarstedt, Nümbrecht, Germany). For each isolate, bacterial suspension was inoculated in parallel into 2 wells, with and without colistin. For one isolate, the following steps of the Rapid ResaPolymyxin *Acinetobacter* / *Pseudomonas* NP test were performed as illustrated in the figure 1;

Step 1: 180 µl of colistin-free MH solution was transferred to wells A1, B1, C1 and D1.

Step 2: 180 µl of colistin-containing MH solution (4.16 mg/L to obtain a final concentration of 3.75 mg/L) was transferred to wells A2, B2, C2 and D2.

Step 3: 20 µl of NaCl 0.85% was added to wells A1 and A2.

Step 4: 20 µl of the colistin-susceptible isolate suspension used as negative control was added to wells B1 and B2.

Step 5: 20 µl of the colistin-resistant isolate suspension used as positive control was added to wells C1 and C2.

Step 6: 20 µl of the bacterial suspension to test was added to wells D1 and D2.

For each of the step 3 to 6, the bacterial suspension was mixed with the medium by pipetting up and down.

When several isolates were tested simultaneously, we paid attention not to exceed 15 min between the transfer of the colistin suspension in the microtest plate and the mix of the bacterial suspension.

Tray incubation. We incubated the inoculated tray at 35 ± 2°C, in ambient air, without being sealed and without agitation.

Adding of the resazurin. After 3 hours of incubation at 35 ± 2°C, the resazurin reagent PrestoBlue® was added at a concentration 10% V/V (i.e. 22 µl per well) and mixed each well by pipetting up and down (Figure 1).

Tray reading. After resazurin reagent adding, the tray was visually inspected every 15 min and then during 1 hour. During this time, the tray was maintained at 35 ± 2°C, in ambient air, without being sealed and without agitation. The test was considered positive (i.e. purple or pink) (polymyxin resistance) if the polymyxin-resistant isolate was viable in presence of colistin, and negative (i.e. blue) (polymyxin susceptibility) if the polymyxin-susceptible isolate was not viable in presence of colistin. We considered the test result interpretable if the following four conditions were met: 1) both wells with 0.85% NaCl without bacterial suspension (wells A1 and A2) remained blue (absence of medium contamination); 2) the wells with bacterial suspension and colistin-free MH solution (wells B1, C1 and D1) turned from blue to purple or pink, confirming the viability of the isolate cells; 3) the well with the colistin-susceptible reference bacterial suspension (negative control) and colistin-containing MH solution (well B2) remained blue, confirming the lack of growth of the isolate, and 4) the well with the colistin-resistant reference bacterial suspension (positive control) and colistin-containing MH solution (well C2) turned from blue to purple or pink, confirming the viability of the isolate in presence of colistin (Figure 1).

Result analysis. The results obtained with the Rapid ResaPolymyxin *Acinetobacter* / *Pseudomonas* NP test were compared to those obtained with the reference BMD method. Briefly, discrepancies were determined to assess the performances of the test to detect colistin resistance. Very major errors (VME) and major errors (ME) corresponding to false-susceptible and false-resistant results, respectively, were calculated as described elsewhere (12, 13).

RESULTS

A total of 43 *A. baumannii* and 49 *P. aeruginosa* isolates were included to evaluate the performance of the Rapid ResaPolymyxin *Acinetobacter* / *Pseudomonas* NP test (Table 1). Out of the 69 *A. baumannii* and *P. aeruginosa* isolates defined as colistin-susceptible according to the results of the BMD method (MICs of colistin ranging from less than <0.125 to 1 mg/L), all but three (MICs of 1, 0.25 and 0.25 µg/ml) were identified as susceptible by the Rapid ResaPolymyxin *Acinetobacter* / *Pseudomonas* NP test (Table 1). All the 23 colistin-resistant *A. baumannii* and *P. aeruginosa* isolates (MICs of colistin ranging from 4 to 128 mg/L) were identified as colistin-resistant by the Rapid ResaPolymyxin *Acinetobacter* / *Pseudomonas* NP test (Table 1). Consequently, out of the 92 *A. baumannii* and *P. aeruginosa* isolates, 3 ME (i.e. false resistance) but no VME (i.e. false susceptibility) were observed (sensitivity of 100% and specificity of 97%). All positive results were observed between 15 min and less than 1 hour after resazurin adding. Consequently, interpretation of the results was obtained in maximum 4 hours for all *A. baumannii* isolates and *P. aeruginosa* isolates.

In addition, by testing a collection of 32 enterobacterial isolates (among which 28 were resistant to colistin), all were perfectly detected as susceptible or resistant (100%). All the 18 MCR-producing and colistin-resistant isolates gave a positive result with the test, further assessing that plasmid-mediated resistance could also be perfectly detected using this test.

CONCLUSION

Out of the 92 *A. baumannii* and *P. aeruginosa* isolates, excellent sensitivity and specificity were respectively observed. This study showed that the Rapid ResaPolymyxin *Acinetobacter* / *Pseudomonas* NP test is a reliable tool for detecting resistance to colistin in *A. baumannii* and *P. aeruginosa* isolates in less than 4 hours. This test is inexpensive and easy to implement in numerous clinical laboratories. It complements the Rapid Polymyxin NP test which performs greatly with *Enterobacteriaceae* but was not appropriate for non fermenters. Of note, and as expected, the Rapid ResaPolymyxin *Acinetobacter* / *Pseudomonas* NP test was also nicely performing with the tested enterobacterial isolates, regardless of their resistance mechanisms.

Although MICs are not determined using this test, it gives results of susceptibility/resistance categorization very rapidly, which corresponds to the main relevant feature respect to the treatment strategy. Use of such rapid test may therefore contribute to optimize antibiotic stewardship. However, the relative small sample size of our collection may be considered as a limitation, and further studies with a broader set of resistant isolates will be needed to further validate the accuracy of that test.

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Figure legend

Figure 1. Representative results of the Rapid ResaPolymyxin *Acinetobacter* / *Pseudomonas* NP test. Non-inoculated wells are shown as controls of the medium and the color change (first row). The Rapid Resazurin *Acinetobacter* and *Pseudomonas* NP test was performed with a reference colistin-susceptible isolate (second row) and with a reference colistin-resistant isolate (third row) in a reaction without (first column) and with (second column) colistin. The tested isolate grew in the presence and absence of colistin (wells D1 and D2, respectively) and was therefore reported to be colistin-resistant.

Table 1. MICs of colistin (µg/ml) using the BMD method and results of the Rapid ResaPolymyxin *Acinetobacter* /*Pseudomonas* NP test

Isolate	Species	Geographic origin	Type of isolate	Phenotype	BMD MIC colistin (mg/L)	Rapid ResaPolymyxin *Acinetobacter*/*Pseudomonas* NP Test	
						Result	Discrepancies with BMD MIC colistin result
FR-242	*A. baumannii*	Switzerland	Clinical	S	<0.125	Negative	No
FR-243	*A. baumannii*	Turkey	Clinical	S	<0.125	Negative	No
FR-244	*A. baumannii*	Turkey	Clinical	S	<0.125	Negative	No
FR-245	*A. baumannii*	Turkey	Clinical	S	<0.125	Negative	No
FR-246	*A. baumannii*	Turkey	Clinical	S	<0.125	Negative	No
FR-247	*A. baumannii*	Turkey	Clinical	S	<0.125	Negative	No
FR-248	*A. baumannii*	Turkey	Clinical	S	<0.125	Negative	No
N4	*A. baumannii*	Switzerland	Clinical	S	0.25	Negative	No
N14	*A. baumannii*	Switzerland	Clinical	S	<0.125	Negative	No
N101	*A. baumannii*	Switzerland	Clinical	S	<0.125	Negative	No
Ab19	*A. baumannii*	France	Clinical	S	<0.125	Negative	No
AS1	*A. baumannii*	France	Clinical	S	<0.125	Negative	No
1279 Bahe	*A. baumannii*	France	Clinical	S	<0.125	Negative	No
FER	*A. baumannii*	France	Clinical	S	<0.125	Negative	No
CH17	*A. baumannii*	France	Clinical	S	<0.125	Negative	No
MAD	*A. baumannii*	France	Clinical	S	<0.125	Negative	No
CLA-1	*A. baumannii*	France	Clinical	S	<0.125	Negative	No
Ab21	*A. baumannii*	France	Clinical	S	<0.125	Negative	No
183 Italie	*A. baumannii*	France	Clinical	S	<0.125	Negative	No
NRZ	*A. baumannii*	France	Clinical	S	<0.125	Negative	No
ALL	*A. baumannii*	France	Clinical	S	<0.125	Negative	No
BCH	*A. baumannii*	France	Clinical	S	<0.125	Negative	No
GEN	*A. baumannii*	France	Clinical	S	<0.125	Negative	No

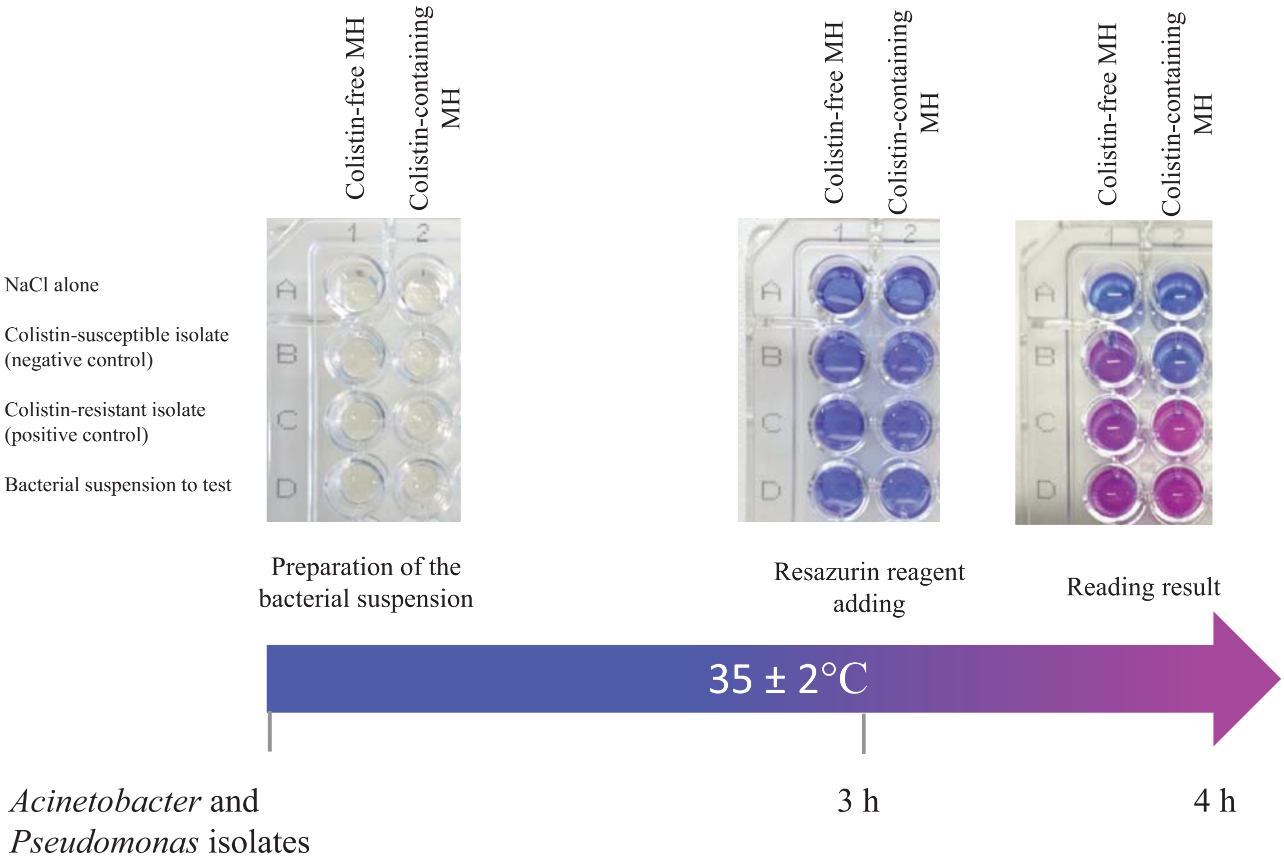
FR-282	*A. baumannii*	Nigeria	Environmental	S	0.5	Negative	No
FR-283	*A. baumannii*	Nigeria	Environmental	S	1	Positive	**Yes, ME**
FR-284	*A. baumannii*	Nigeria	Environmental	S	1	Negative	No
R2536	*A. baumannii*	France	Clinical	S	0.5	Negative	No
Ab10	*A. baumannii*	France	Clinical	S	2	Negative	No
Ab11	*A. baumannii*	France	Clinical	S	0.5	Negative	No
577	*A. baumannii*	Switzerland	Clinical	S	2	Negative	No
ATCC 27853	*P. aeruginosa*	USA	Reference	S	<0.125	Negative	No
FR-263	*P. aeruginosa*	France	Clinical	S	<0.125	Negative	No
FR-264	*P. aeruginosa*	France	Clinical	S	<0.125	Negative	No
FR-265	*P. aeruginosa*	France	Clinical	S	<0.125	Negative	No
FR-266	*P. aeruginosa*	France	Clinical	S	<0.125	Negative	No
FR-267	*P. aeruginosa*	France	Clinical	S	<0.125	Negative	No
FR-268	*P. aeruginosa*	France	Clinical	S	<0.125	Negative	No
FR-269	*P. aeruginosa*	France	Clinical	S	<0.125	Negative	No
FR-270	*P. aeruginosa*	France	Clinical	S	0.25	Negative	No
FR-271	*P. aeruginosa*	France	Clinical	S	0.25	Negative	No
FR-287	*P. aeruginosa*	Portugal	Asymptomatic carriage	S	0.25	Negative	No
41437	*P. aeruginosa*	France	Clinical	S	<0.125	Negative	No
CAS	*P. aeruginosa*	France	Clinical	S	<0.125	Negative	No
MES	*P. aeruginosa*	France	Clinical	S	0.25	Negative	No
Ka 209	*P. aeruginosa*	France	Clinical	S	0.5	Negative	No
Col-1	*P. aeruginosa*	France	Clinical	S	0.25	Negative	No
PO510	*P. aeruginosa*	France	Clinical	S	0.25	Positive	**Yes, ME**
12870	*P. aeruginosa*	France	Clinical	S	<0.125	Negative	No
PAM13	*P. aeruginosa*	France	Clinical	S	0.25	Negative	No
PAM 10	*P. aeruginosa*	France	Clinical	S	0,5	Negative	No
PA 13	*P. aeruginosa*	France	Clinical	S	0,5	Negative	No
PA 1	*P. aeruginosa*	France	Clinical	S	0,5	Negative	No
P16 Bre	*P. aeruginosa*	France	Clinical	S	0.5	Negative	No
PAO1-11B	*P. aeruginosa*	France	Clinical	S	0.25	Negative	No
4098 E	*P. aeruginosa*	France	Clinical	S	0.25	Negative	No

PAO1-T	*P. aeruginosa*	France	Clinical	S	<0.125	Negative	No
H729	*P. aeruginosa*	France	Clinical	S	1	Negative	No
PaeB-01	*P. aeruginosa*	France	Clinical	S	0.25	Positive	**Yes, ME**
PaeB-03	*P. aeruginosa*	France	Clinical	S	<0.125	Negative	No
PaeB-10	*P. aeruginosa*	France	Clinical	S	0.25	Negative	No
1782	*P. aeruginosa*	France	Clinical	S	0.125	Negative	No
COL-1	*P. aeruginosa*	France	Clinical	S	0.125	Negative	No
Bre	*P. aeruginosa*	Brazil	Clinical	S	0.125	Negative	No
5534	*P. aeruginosa*	Brazil	Clinical	S	0.125	Negative	No
PAO38	*P. aeruginosa*	France	Clinical	S	0.125	Negative	No
NEA	*P. aeruginosa*	Italy	Clinical	S	0.125	Negative	No
NTU	*P. aeruginosa*	France	Clinical	S	0.125	Negative	No
REZ	*P. aeruginosa*	France	Clinical	S	0.125	Negative	No
ECHE	*P. aeruginosa*	France	Clinical	S	0.125	Negative	No
FR-250	*A. baumannii*	Italy	Clinical	R	8	Positive	No
FR-252	*A. baumannii*	Italy	Clinical	R	64	Positive	No
FR-253	*A. baumannii*	Spain	Clinical	R	4	Positive	No
FR-254	*A. baumannii*	Spain	Clinical	R	16	Positive	No
FR-255	*A. baumannii*	Switzerland	Clinical	R	128	Positive	No
FR-256	*A. baumannii*	Turkey	Clinical	R	16	Positive	No
FR-257	*A. baumannii*	Turkey	Clinical	R	8	Positive	No
FR-258	*A. baumannii*	Turkey	Clinical	R	32	Positive	No
FR-259	*A. baumannii*	Turkey	Clinical	R	32	Positive	No
FR-260	*A. baumannii*	Turkey	Clinical	R	>128	Positive	No
FR-261	*A. baumannii*	Turkey	Clinical	R	4	Positive	No
FR-262	*A. baumannii*	Turkey	Clinical	R	>128	Positive	No
FR-286	*A. baumannii*	Nigeria	Environmental	R	32	Positive	No
FR-274	*P. aeruginosa*	France	Clinical	R	4	Positive	No
FR-275	*P. aeruginosa*	France	Clinical	R	32	Positive	No
FR-276	*P. aeruginosa*	France	Clinical	R	32	Positive	No
FR-277	*P. aeruginosa*	France	Clinical	R	16	Positive	No
FR-278	*P. aeruginosa*	France	Clinical	R	128	Positive	No

FR-279	*P. aeruginosa*	France	Clinical	R	8	Positive	No
FR-281	*P. aeruginosa*	France	Clinical	R	4	Positive	No
FR-288	*P. aeruginosa*	Portugal	Asymptomatic carriage	R	128	Positive	No
RNL-1	*P. aeruginosa*	Turkey	Clinical	R	8	Positive	No
FER	*P. aeruginosa*	France	Clinical	R	4	Positive	No

Colistin-resistant isolates are shaded in grey.

ME: major error; S, susceptible; R, resistant;


Colistin-free MH
Colistin-containing MH
NaCl alone
Colistin-susceptible isolate (negative control)
Colistin-resistant isolate (positive control)
Bacterial suspension to test
Preparation of the bacterial suspension
Resazurin reagent adding
Reading result
35 ± 2°C
Acinetobacter and *Pseudomonas* isolates
3 h
4 h