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Rapid Aminoglycoside NP test for rapid detection of multiple aminoglycoside resistance in *Enterobacteriaceae*

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The Rapid Aminoglycoside NP (Nordmann/Poirel) test was developed to rapidly identify multiple aminoglycoside (AG) resistance in *Enterobacteriaceae*. It is based on the detection of the glucose metabolism related to enterobacterial growth in presence of a defined concentration of amikacin plus gentamicin. Formation of acid metabolites was evidenced by a color change (orange to yellow) of the red phenol pH indicator. The Rapid Aminoglycoside NP test was evaluated by using bacterial colonies of 18 AG-resistant isolates producing 16S rRNA methylases, 20 AG-resistant isolates expressing AG-modifying enzymes (acetyl, adenyl and phospho transferases), and 10 isolates susceptible to AG. Its sensitivity and specificity were 100% and 97% respectively, as compared to the broth dilution method taken as the gold standard for determining aminoglycoside resistance. The test is inexpensive, rapid (<2 hours), and implementable worldwide.

INTRODUCTION

Extended-spectrum β -lactamases that hydrolyze extended-spectrum cephalosporins, and carbapenemases that hydrolyze in addition carbapenems are disseminating worldwide in *Enterobacteriaceae*, and therapeutic options are becoming limited (1). In those multidrug resistant isolates, aminoglycosides (AG) may still be considered as valuable treatment options (2), but their efficacy has been reduced by the surge and the dissemination of resistance.

The most prevalent mechanism of resistance to AG in *Enterobacteriaceae* are enzymes, that modify the chemical structure of aminoglycosides and can be nucleotidyltransferases, phosphotransferases, or acetyltransferases (3). The genetic determinants coding for those aminoglycoside-modifying enzymes are often present in various combinations in a given strain and are typically found on plasmids. They do not confer cross-resistance to all AG molecules.

However, plasmid-mediated 16S rRNA methyltransferases conferring high level of resistance to multiple AG is also reported (4), and they are identified at a high frequency, especially among producers of NDM-like carbapenemases (5). Enzymes encoded by these genes methylate the aminoglycoside tRNA recognition site (A-site) of the 16S rRNA, that is the intracellular target of the AG. The 16S rRNA methylases identified in *Enterobacteriaceae* are ArmA, RmtB to RmtF, and NpmA, with ArmA being the most frequently identified methylases (4). Those methylases exhibit a significant amino-acid heterogeneity, although sharing similar amino-acid motifs in their active sites. They confer resistance to almost all AG (amikacin, gentamicin, tobramycin, kanamycin, except neomycin), whereas NpmA confers additional resistance trait to neomycin (given as a topical agent) and to apramycin (not used for humans) (6).

In an area of paucity of novel molecules, rapidly identifying multiple resistance to AG by a simple test may be useful for implementing antibiotic stewardship and containment of those multidrug-resistant bacteria.

Thus, we developed a rapid colorimetric test based on the detection of the bacterial growth in presence of a defined concentration of a mix of two AG molecules (amikacin and gentamicin). Bacterial growth detection (or absence) was based on glucose metabolism (7). Acid formation related to glucose metabolism in *Enterobacteriaceae* may be observed through the color change of a pH indicator. This test is rapid (less than 4 h) and easy to handle.

MATERIAL AND METHODS

Isolates collection

A total of 48 isolates from clinical samples of worldwide origin were used to evaluate the performance of the Rapid Aminoglycoside NP (Nordmann/Poirel) test, including 28 isolates resistant to AG and 10 isolates susceptible to AG (Table). The AG resistant isolates were characterized at the molecular level for their resistance mechanisms with 18 isolates harboring 16S rRNA methylases and 20 others producing different AG-modifying enzymes (acetyl, adenylyl, and phosphoryl enzymes) (Table).

Minimum Inhibitory Concentrations (MICs) determination

MICs of amikacin and gentamicin were determined using the broth microdilution method in cation-adjusted Mueller-Hinton broth (MHB-CA), as recommended by the Clinical Laboratory Standard Institute (CLSI) guidelines (8). Amikacin and gentamicin sulfate (Fluorochem, Dertbyshire, UK) were tested over a range of dilutions from 0.25 to 256 µg/ml. A final inoculum of 5×10^5 CFU/ml of each strain was distributed in the 96 wells tray (Sarstedt, Nümbrecht, Germany). All experiments were repeated in triplicate and *E. coli* ATCC

25922 was used as quality control. According to the CLSI breakpoints (9), enterobacterial isolates with gentamicin MICs ≤ 4 and amikacin MICs ≤ 16 $\mu\text{g/ml}$ were categorized as susceptible, while those with gentamicin MICs ≥ 16 and amikacin MICs ≥ 64 $\mu\text{g/ml}$ were categorized as resistant.

Rapid Aminoglycoside NP test

(i) Reagents and solutions

Stock solutions of AG

Amikacin and gentamicin were diluted into MHB-CA medium in glass tubes to obtain stock solution at a concentration of 1.6 mg/ml each. The antibiotic powders and the stock solutions can be stored at 4°C and -20°C, respectively, before their use.

Rapid Aminoglycoside NP solution

To prepare 250 ml of the Rapid Aminoglycoside NP solution, the culture medium and the pH indicator were mixed in a glass bottle as follows: 6.25 g of MHB-CA powder, 0.0125 g of phenol red (Sigma Aldrich), and 225 ml of distilled water. The pH of the solution was adjusted to 6.7 by adding drops of 1 M HCl. This solution was then autoclaved at 121°C for 15 min. After cooling the solution to room temperature, 25 ml of D (+)-glucose anhydrous 10% (Roth, Karlsruhe, Germany) sterilized by filtration, was added. The final concentrations in the solution were consequently 2.5% of MHB-CA powder, 0.005% of phenol red indicator, and 1% of D (+)-glucose. This solution can be kept in 10 ml aliquots at 4°C for one week or at -20°C for one year. The thawed 10 ml aliquots shall be pre-warmed 10 min at 37°C before use to prevent bacterial growth delay resulting in a delay for obtaining the color change of the test.

AG-containing Rapid Aminoglycoside NP solution

Amikacin and gentamicin stock solution was added to the Rapid Aminoglycoside NP solution, before use, to a final concentration of 40 $\mu\text{g/ml}$ each.

(ii) Bacterial inoculum

A standardized enterobacterial inoculum was prepared using freshly-obtained (overnight) bacterial colonies grown on Luria-Bertani or Mueller-Hinton plates. The bacterial colonies were resuspended into sterile NaCl 0.85% to obtain a 3 to 3.5 McFarland optical density (ca. 10^9 CFU/ml) using a McFarland densitometer DEN-1B from Grant-bio (Grant instruments Ltd, England). A bacterial suspension was prepared for each isolate to be tested, and for the susceptible and resistant isolates used as controls (Table). *Escherichia coli* ATCC 25922 (FR136) was used as the negative control and an ArmA-producing *E. coli* strain from our collection (R989) was used as the positive control. The bacterial suspensions should be used to a maximum of 60 minutes after their preparation.

(iii) Tray inoculation.

The test was performed in a 96-well polystyrene microtest plate (round base, with lid, sterile, ref. 82.1582.001, Sarstedt, Nümbrecht, Germany). For each isolate, two wells are inoculated in parallel with the bacterial suspension, respectively with and without the AG mix. The Rapid Aminoglycoside NP test was performed as follows (Figure):

Step 1: 150 μ l of AG-free Rapid Aminoglycoside NP solution was transferred in the four wells of the first row.

Step 2: 150 μ l of Rapid Aminoglycoside NP solution supplemented with AG mix was transferred in the four wells of the second row.

Step 3: 50 μ l of sterile NaCl 0.85 % was added in the two wells of the first column.

Step 4: 50 μ l of the AG-susceptible isolate suspension used as negative control (*E. coli* ATCC 25922) was added in the two wells of the second column.

Step 5: 50 μ l of the AG-resistant isolate suspension used as positive control (16S rRNA methylase producer) was added in the two wells of the third column.

Step 6: 50 μ l of the tested isolate suspension was added in the two wells of the last column.

After mixing the bacterial suspension to the reactive medium by pipetting up and down, the final concentration of bacteria was ca. 10^8 CFU/ml in each well and the final concentrations of amikacin and gentamicin were 30 μ g/ml each.

(iv) Tray incubation

The inoculated tray was incubated up to 4 h at $35\pm 2^\circ\text{C}$ in ambient air, not sealed and without agitation. Non sealing of the tray was done due to the oxygen requirement for glucose metabolism of *Enterobacteriaceae*.

(v) Tray reading

Visual inspection of the tray was made after 10 min (to check for any spontaneous color change of the medium), and every hour up to 4 hours. The test was considered positive (AG resistant) if the isolate grew in presence of the AG mix while it was considered negative (AG susceptibility) if the susceptible isolate did not grow in presence of the AG mix. Cell growth was considered positive when the color of the assay reagent turned from orange to yellow. The color change indicates an acidification of the medium due to glucose metabolism that is used as a proxy for cell growth. Final reading was done when an assay of the second row turned yellow (positive) or at 4 hours in case of no color change (negative). The test was considered interpretable when (i) NaCl wells remained orange meaning the absence of medium contamination, (ii) S and R wells in first row turned from orange to yellow confirming the metabolism of glucose by the isolates, (iii) the S well in row 2 remained orange (no growth), and the R well in row 2 turned yellow (cell growth and glucose metabolism positive).

Other tested conditions

The Rapid Aminoglycoside NP test was also performed by testing other concentrations of the same mix of AG molecules or other AG mix such as tobramycin and kanamycin or amikacin and kanamycin. The test was performed also from overnight culture on different agar plates to

determine the potential impact of the culture medium prior to the realization of the test. The following culture media were tested; non-selective culture medium such Columbia agar + 5% sheep blood (bioMérieux), the non-selective chromogenic medium UriSelect 4 (Bio-Rad), chocolate agar + PolyVitex (bioMérieux), Eosine methylene blue agar (Sigma Aldrich), Drigalski agar (Bio-Rad), MacConkey agar (VWR BDH Prolabo, Leuven, Belgium) and bromocresol purple (bioMérieux).

RESULTS AND DISCUSSION

A total of 48 enterobacterial isolates were tested to evaluate the performance of the Rapid Aminoglycoside NP test, including 39 AG-resistant isolates and 10 AG-susceptible isolates (Table). Among the 38 AG resistant isolates, 18 isolates produced 16S rRNA methylases and exhibited high MIC values for both amikacin and gentamicin while 20 produced AG-modifying enzymes and exhibited various level of resistance for amikacin and gentamicin (Table).

A good correlation was obtained between multiple AG resistance and positivity of the test and conversely, non-multiple AG resistance and negativity of the test (Table). The sensitivity and the specificity of the test were 100% and 97% respectively compared with broth dilution taken as the gold standard for determining susceptibility and resistance to AG.

The reading of the color change in the wells every hour during four hours revealed that final results were obtained within 2 hours after inoculation for positive and negative strains when the tray was incubated at $35\pm 2^{\circ}\text{C}$ under an ambient atmosphere. The readings of the test should not be extended after 4 hours of incubation. After 4 hours, the dye reagent is not stable enough and inconsistent color shifts were observed under our experimental incubation conditions. The required test incubation time was therefore set to 2 hours. AG susceptibility

results obtained with the Rapid Aminoglycoside NP test were consequently obtained at least 16 h sooner than with disk diffusion method, E-test system and broth dilution method.

Solutions containing other combinations of AG or other AG molecules (kanamycin, tobramycin, streptomycin and apramycin) were compatible with the test in its current formulation. The test may be performed from cultured bacteria grown on media such as Luria Bertani, Mueller-Hinton, Uriselect-4, eosine-methylene blue medium, blood agar, and chocolate agar. Interference may be observed with colonies grown on acidifying culture media such as Drigalski, Mac Conkey and bromocresol purple. The test, as proposed here, has been optimized and other conditions, reagents and culture media might lead to a decrease of efficiency or stability of the test.

The study has two main limitations. First, the positivity of the test does not always superimpose the presence of 16S rRNA methylases. Indeed, combined production of nucleotidyl, phosphoryl and acetyltransferases in a given enterobacterial strain might confer resistance to both gentamicin and amikacin, although such combinations with MIC >30 µg/ml are rare. Second, this test is not compatible with non fermenters such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* species, which may also produce 16S rRNA methylases (10,11).

The Rapid Aminoglycoside NP test can be implemented for determination of multiple resistance to AG in particular in countries facing an endemic spread of carbapenemase producers (1). The use of the Rapid Aminoglycoside NP test may also contribute to rapidly identify carriers of MDR isolates producing plasmid-mediated 16S rRNA methylases, to rapidly implement infection control measures, and consequently to control their spread. In a world that is rapidly witnessing an evolution toward pandrug resistance, the Rapid Aminoglycoside test may guide prescription of novel broad-spectrum AG such as plazomicin by differentiating aminoglycoside modifying enzymes and methylase producers. Plazomicin

is indeed active against aminoglycoside-modifying enzymes such as the nucleotidyl, acetyl and phosphoryl enzymes (12), although plazomicin is inactive against those 16S rRNA methylase producers (13). This test may also support the development of such novel AG molecule facilitating patient enrollment in pivotal clinical trials.

CONCLUSION

The Rapid Aminoglycoside NP test is easy-to-perform, rapid (<2 h), sensitive, and specific. It detects multiple AG resistance among *Enterobacteriaceae* from selective and non-selective media prior to obtaining any antibiotic susceptibility testing results.

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FIGURE LEGEND

Figure. Representative results of the Rapid Aminoglycoside NP test.

The Rapid Aminoglycoside NP test was performed with a reference susceptible isolate (S) in the second column and with a reference 16S rRNA methylase isolate (ArmA) (R) in the third column, in a reaction medium without (upper line) and with (lower line) the AG mix (amikacin + gentamicin). Non-inoculated wells are shown in the first column (NaCl-containing wells). The tested isolate (T) in the fourth column grew in the presence of the mix of aminoglycosides and presented therefore a multiple resistance to aminoglycosides. The picture was taken after 2 hours of incubation.

Table. Rapid Aminoglycoside NP test results for AG-resistant enterobacterial isolates producers of 16S rRNA methylases and producers of AG-modifying enzymes, and for AG-susceptible enterobacterial isolates.

Isolate	Species	Mechanism of AG resistance	Amikacin		Gentamicin		Rapid Polymyxin NP ^b
			MIC ^a	Phenotype	MIC ^a	Phenotype	
AG susceptible isolates							
FR136	<i>Escherichia coli</i> ATCC 25922	-	2	S	0.5	S	-
R2278	<i>Escherichia coli</i> ATCC 35218	-	4	S	1	S	-
R1436	<i>Escherichia coli</i>	-	2	S	1	S	-
R1438	<i>Escherichia coli</i>	-	2	S	1	S	-
R347	<i>Klebsiella pneumoniae</i>	-	4	S	0.5	S	-
S155	<i>Klebsiella pneumoniae</i>	-	2	S	0.5	S	-
R1433	<i>Enterobacter cloacae</i>	-	4	S	2	S	-
R713	<i>Enterobacter cloacae</i>	-	4	S	0.5	S	-
HM	<i>Enterobacter cloacae</i>	-	4	S	2	S	-
R2260	<i>Proteus mirabilis</i>	-	4	S	4	S	-
AG resistant isolates producers of methylases							
R989	<i>Escherichia coli</i>	<i>armA</i>	>256	R	>256	R	+
R1012	<i>Escherichia coli</i>	<i>armA</i>	>256	R	>256	R	+
R500	<i>Klebsiella pneumoniae</i>	<i>armA</i>	>256	R	>256	R	+
R108	<i>Klebsiella pneumoniae</i>	<i>armA</i>	>256	R	>256	R	+
R2257	<i>Klebsiella pneumoniae</i>	<i>armA</i>	>256	R	>256	R	+
R2258	<i>Enterobacter cloacae</i>	<i>armA</i>	>256	R	>256	R	+
62Carb	<i>Escherichia coli</i>	<i>rmtB</i>	>256	R	>256	R	+
E28	<i>Escherichia coli</i>	<i>rmtB</i>	>256	R	>256	R	+
R107	<i>Escherichia coli</i>	<i>rmtB</i>	>256	R	>256	R	+
R990	<i>Escherichia coli</i>	<i>rmtB</i>	>256	R	>256	R	+
R262	<i>Escherichia coli</i>	<i>rmtC</i>	>256	R	>256	R	+
R451	<i>Escherichia coli</i>	<i>rmtC</i>	>256	R	>256	R	+
R265	<i>Providencia stuartii</i>	<i>rmtC</i>	>256	R	>256	R	+
R38	<i>Providencia stuartii</i>	<i>rmtC</i>	>256	R	>256	R	+
N34	<i>Klebsiella pneumoniae</i>	<i>rmtC</i>	>256	R	>256	R	+
R502	<i>Klebsiella pneumoniae</i>	<i>rmtF</i>	>256	R	>256	R	+
R2160	<i>Klebsiella pneumoniae</i>	<i>rmtG</i>	>256	R	>256	R	+

R2161	<i>Escherichia coli</i> TOP10	<i>npmA</i>	128	R	32	R	+
AG resistant isolates producers of AG-modifying enzymes							
pWP701	<i>Escherichia coli</i>	AAC(3)-IV	1	S	128	R	-
pFCT4392	<i>Escherichia coli</i>	AAC(3)-Ia	1	S	32	R	-
390	<i>Escherichia coli</i>	AAC(3)-IV	16	S	>128	R	-
22089	<i>Escherichia coli</i>	AAC(3)-I + AAC(3)-V	16	S	128	R	-
97	<i>Escherichia coli</i>	AAC(3)-IV	2	S	32	R	-
92/31	<i>Escherichia coli</i>	AAC(3)-IV	4	S	16	R	-
4000	<i>Escherichia coli</i>	APH(3')-I + AAC(3)-V	4	S	64	R	-
pFCT1163	<i>Escherichia coli</i>	AAC(6')-Ia	>128	R	4	S	-
1054	<i>Escherichia coli</i>	AAC(6')-I	8	S	8	I	-
1488	<i>Escherichia coli</i>	AAC(6')-II	4	S	16	R	-
1054	<i>Escherichia coli</i>	AAC(6')-I	16	S	8	I	-
RP4	<i>Escherichia coli</i>	APH(3')-Ib	1	S	1	S	-
122971	<i>Escherichia coli</i>	ANT(2'')	4	S	>128	R	-
1800	<i>Klebsiella pneumoniae</i>	AAC(6')-I + AAC(3)-I	32	I	16	R	-
13000	<i>Klebsiella pneumoniae</i>	APH(3')-I + AAC(3)-V	0.5	S	>128	R	-
22233	<i>Klebsiella pneumoniae</i>	AAC(3)-V	16	S	>128	R	-
1110	<i>Enterobacter cloacae</i>	ANT(2'')	1	S	32	R	-
BM2667	<i>Providencia stuartii</i>	APH(3')-VI + AAC(2')	64	R	16	R	-
531	<i>Providencia rettgeri</i>	AAC(2')	16	S	4	S	-
4290	<i>Serratia marcescens</i>	AAC(3)-I	4	S	64	R	-

AG, aminoglycosides; AAC, N-Acetyltransferases; ANT, O-Adenyltransferases; APH, O-Phosphotransferases; MIC, Minimum inhibitory concentrations; RANP, Rapid Aminoglycoside NP test.

^aMICs were determined by broth microdilution method.

^bResults of the RANP test after 2 hours of incubation at 35±2°C under ambient air.

