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A culture medium for screening 16S rRNA methylase-producing pan-aminoglycoside resistant Gram-negative bacteria

Patrice Nordmann [a,b,c,d,*], Alain Mazé [a,b], Esther Culebras [e], Jan Dobias [a,b], Aurélie Jayol [a,b,c], Laurent Poirel [a,b,c]

[a] *Medical and Molecular Microbiology Unit, Department of Medicine, Faculty of Science, University of Fribourg, Fribourg, Switzerland*
[b] *INSERM European Unit (LEA, IAME, France), University of Fribourg, Fribourg, Switzerland*
[c] *Swiss National Reference Center for Emerging Antibiotic Resistance (NARA), Fribourg, Switzerland*
[d] *Institute for Microbiology, University of Lausanne and University Hospital Centre, Lausanne, Switzerland*
[e] *Microbiology Department, Hospital Clínico San Carlos, Madrid, Spain*

The amikacin plus gentamicin-containing SuperAminoglycoside medium was developed for screening multiple-aminoglycoside resistance in Gram-negative bacteria (Enterobacteriaceae, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*). It was evaluated using aminoglycoside-susceptible (n=12) and aminoglycoside-resistant (n=59) Gram-negative isolates, including 16S rRNA methylase producers (n=20). Its sensitivity and specificity of detection were, respectively, of 95% and 96% for detecting multiple aminoglycoside-resistant methylase producers.

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16S rRNA methylases
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Susceptibility testing
Gentamicin
Amikacin
Tobramycin
Netilmicin
Kanamycin
Plazomicin

Multidrug resistance is now observed increasingly in nosocomial and community-acquired pathogens worldwide. These bacteria possess multiple resistance mechanisms, thus nullifying most of the major antibiotic classes such as the β-lactams and aminoglycosides. Considering the wide diffusion of extended-spectrum β-lactamase–producing enterobacterial isolates currently observed worldwide (Poirel et al., 2012), there is a significant need to rely on the use of aminoglycosides to treat infections caused by those multidrug-resistant isolates. Therefore, identifying and monitoring those isolates exhibiting resistance to aminoglycosides are of significant concern.

The most prevalent mechanisms of resistance to aminoglycosides (AG) in Enterobacteriaceae are nucleotidyltransferases (ANT), phosphotransferases (APH), and acetyltransferases (AAC) enzymes that modify the structures of AG (Ramirez and Tolmasky, 2010). Those enzymes are usually plasmid-encoded and may be co-associated in some isolates. However, they usually do not confer cross resistance to all AG molecules.

On the other hand, plasmid-mediated 16S rRNA methylases conferring a high level of resistance to multiple AG are also reported (Doi et al., 2016). Those enzymes methylate the 16S rRNA, which is the intracellular target of the AG. The 16S rRNA methylases described in Enterobacteriaceae are ArmA, RmtB to RmtH, and NpmA (only a single isolate reported so far for that latter enzyme). The ArmA methylase is the most frequently identified (Doi et al., 2016; Zhou et al., 2010). Those methylases confer resistance to almost all AG (amikacin, gentamicin, tobramycin, and kanamycin, except neomycin and apramycin), although NpmA confers additionally resistance to neomycin (given as a topical agent) and to apramycin (only used in veterinary medicine) (Wachino et al., 2007). They have been identified in Enterobacteriaceae, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*.

Taking into account the significance of a potential spread of 16S rRNA methylases, our aim was to develop a selective culture medium for screening multiple aminoglycoside-resistant bacteria in human and animal isolates.

1. Material and methods

The necessity for that medium to prevent contamination by Gram positives and fungi was taken in account. Based on our experience of development of several screening media, the optimal screening medium

* Corresponding author. Tel.: +41-26-300-9581.
E-mail address: patrice.nordmann@unifr.ch (P. Nordmann).

retained was based on the Eosine-Methylene-Blue (EMB) medium (product no. 70186; EMB Fluka, Saint-Louis, MO) that is selective for Gram negatives (Levine, 1943). This medium may also contribute to species identification by differentiating lactose fermenters (black colonies) from most of the nonfermenters (colorless or light lavender).

Since 16S rRNA methylase producers systematically exhibit high-level resistance to both amikacin and gentamicin, which is very rarely the case for isolates producing one or more aminoglycoside-modifying enzymes, those two antibiotics were chosen as selective markers in our medium. The optimal final concentration of amikacin and gentamicin (Sigma Aldrich, St. Louis, MO) was 30 μg/mL each taking into account the MIC cutoff values of amikacin and of gentamicin of susceptible and resistant strains (see below). Vancomycin (Sigma) was added as anti–Gram-positive molecule at a final concentration of 10 μg/mL, and amphotericin B (Bristol-Myers-Squibb, Rueil-Malmaison, France) was added as anti-fungi molecule at a final concentration of 5 μg/mL. Cultures were incubated at 37°C during 18 h. When no growth was observed after 18 h, incubation was extended up to 48 h to definitely assess that no growth actually occurred.

The instructions for the preparation of the stock solutions of amikacin, gentamicin, vancomycin, and amphotericin B are indicated in Table 1. According to our observations, the stock solutions may be kept at −20°C during 1 year. For preparing the SuperAminoglycoside medium, the diluted powder of EMB was autoclaved at 121°C for 15 min. After cooling the EMB medium for 1 h at 56°C, the antibiotic stock solutions were added (Table 1). Poured plates were stored at 4°C and protected from direct light exposure before use.

A total of 69 isolates of various species (Enterobacteriaceae, *P. aeruginosa*, and *A. baumannii)* were tested in this study to evaluate the performance of the SuperAminoglycoside medium (Table 2). Twelve isolates were susceptible to aminoglycosides, and 57 isolates were resistant to aminoglycosides (Table 2). The AG-resistant isolates were characterized at the molecular level for their resistance mechanism (Berçot et al., 2011). Twenty isolates produced 16S rRNA methylases, and 37 isolates produced aminoglycoside-modifying enzymes. A single isolate was a recombinant strain producing NpmA. The recombinant plasmid (pNpmA) was constructed by amplifying by polymerase chain reaction a DNA fragment containing the entire *npmA* gene with primers NpmA-Fw-HindIII (5′-ATATAAAGCTTGGCCAGTTATGGTAAAATAG-3′) and NpmA-Rv-BamHI (5′-ATATATGGATCCAAACAAAGAAATTGCTATAACC-4′) from recombinant plasmid pMCL-BE (Wachino et al., 2007) followed by its cloning into pACYC184 cloning vector giving rise to plasmid pFR2000 and its expression into *Escherichia coli* TOP10.

MICs of amikacin and gentamicin were determined using the broth microdilution method in cation-adjusted Mueller–Hinton broth, as recommended by the CLSI (CLSI, 2012). For each strain, an inoculum corresponding to 5×10^5 CFU/mL was distributed in the 96-well tray (Sarstedt, Nümbrecht, Germany). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as control strains (CLSI, 2014), and the evaluation of the selective medium was performed in triplicate.

Table 1
Composition of the SuperAminoglycoside medium.

Compound	Stock solution (mg/mL)	Quantity or volume to add[a]	Final concentration[b]
EMB agar powder	-	15 g	3.75%
Distilled water	-	400 mL	
Gentamicin	30 in distilled water	400 μL	30
Amikacin	30 in distilled water	400 μL	30
Vancomycin	20 in distilled water	200 μL	10
Amphotericin B	2 in D(+)-glucose 10%	100 μL	5

[a] The volume of 400 mL of medium was for 20 plates, considering plates of 100 mm diameter.
[b] Concentrations are in micrograms per milliliter unless noted otherwise.

According to the CLSI breakpoints (CLSI, 2014), Enterobacteriaceae, *P. aeruginosa,* and *A. baumannii* isolates with MICs of gentamicin ≤4 and of amikacin ≤16 μg/mL are categorized as susceptible, while those with MICs of gentamicin ≥16 and of amikacin ≥64 μg/mL are categorized as resistant.

2. Results

Using an inoculum with an optical density of 0.5 Mac Farland (inoculum of ~10^8 CFU/mL), serial 10-fold dilutions of the isolates were made in normal saline, and 100-μL portions were plated onto the SuperAminoglycoside medium.

To quantify the viable bacteria in each dilution, Trypticase soy agar was inoculated concomitantly with 100 μL of suspension and incubated overnight at 37°C. The lowest limit of detection for the tested strains was determined using the SuperAminoglycoside medium. The sensitivity and specificity cutoff values were set at 1×10^3 CFU/mL, i.e., a limit value of 1×10^3 CFU/mL and above was considered as "not efficiently detected" (Nordmann et al., 2012).

The lowest limit of detection was above the cutoff value of 10^3 CFU/mL, being ≥1×10^6 CFU/mL for all the aminoglycoside-susceptible isolates and for the isolates producing aminoglycoside-modifying enzymes (Table 2). On the contrary, the 16S rRNA methylase producers grew on the SuperAminoglycoside medium in 24 h, and the lowest limit of detection was below the cutoff value, except for the *E. coli* TOP10 isolate that produced NpmA (Table 2). Of note, two *E. coli* isolates that did not produce 16S rRNA methylases (HCSC-Ec22 and HCSC-Ec296) grew on the SuperAminoglycoside medium. This was somehow expected when considering that those isolates producing aminoglycoside-modifying enzymes exhibited high MIC values of both amikacin and gentamicin (Table 2). The high level of resistance to amikacin and gentamicin observed for those two isolates likely resulted from the production of aminoglyside-modifying enzymes (as indicated in Table 2) and possibly of permeability defects.

The sensitivity and specificity of the SuperAminoglycoside medium for selecting amikacin- and gentamicin resistant isolates producing 16S rRNA methylases were consequently 95% (19/20) and 96% (2/49), respectively, although a limited number of strains have been tested (Lopez-Diaz et al., 2017). When considering detection of amikacin- and gentamicin-resistant isolates only (regardless of production of 16S rRNA methylases), the specificity of the medium was 100%.

Similar results were obtained by using a Drigalski-containing medium or with other AG combination or AG concentration (data not shown). Spiked stools were also tested using this selective culture medium, done in triplicate. Spiked fecal samples were made by adding 100 μL of each strain dilution to 900 μL of fecal suspension that was obtained by suspending 5 g of freshly pooled feces from five healthy volunteers in 50 mL of distilled water, as done previously (Nordmann et al., 2016). A nonspiked fecal suspension was used as negative control. The lowest detection limit was determined by plating 100 μL of each dilution on the screening medium. The sensitivity and specificity were determined using the same cutoff value set at ≥10^3 CFU/mL (Nordmann et al., 2016). This value may correspond to a low-level carriage of resistant bacteria in stools. Sensitivity and specificity were similar to those found by using pure cultures of the strains, i.e., 96% and 96%, respectively. The spiked 16S rRNA methylase producers (except the NpmA producer) in stools grew with a lowest detection limit ranging from 10^1 to 10^2 CFU/mL (Table 2).

To assess the storage stability of the SuperAminoglycoside medium, *C. albicans*, *S. aureus*, and AG-susceptible *E. coli* ATCC 25922, *K. pneumoniae* R437, *E. cloacae* R713, and *P. aeruginosa* R191 were subcultured on a daily basis onto the SuperAminoglycoside agar plates from a single batch of medium stored at 4°C. Growth of those isolates was consistently inhibited during at least a 7-day period.

Table 2
MICs of amikacin and gentamicin for the studied strains and limit of detection of the aminoglycoside-containing culture medium EMB 30/30.[a]

Strain	Species	Mechanism of aminoglycoside resistance	MIC (μg/mL) of antibiotics[b]		Lowest detection limit (CFU/mL)[c]	Lowest detection limit in stools (CFU/mL)[c]
			Amikacin	Gentamicin		
Susceptible strains						
R2298	*E. coli* ATCC 25922	-	2	1	$>1 \times 10^8$	$>1 \times 10^8$
R2278	*E. coli* ATCC 35218	-	4	1	$>1 \times 10^8$	$>1 \times 10^8$
R1436	*E. coli*	-	2	1	$>1 \times 10^8$	$>1 \times 10^8$
R1438	*E. coli*	-	2	1	$>1 \times 10^8$	$>1 \times 10^8$
R347	*K. pneumoniae*	-	4	0.5	$>1 \times 10^8$	$>1 \times 10^8$
S155	*K. pneumoniae*	-	2	0.5	$>1 \times 10^8$	$>1 \times 10^8$
R1433	*E. cloacae*	-	4	2	$>1 \times 10^8$	$>1 \times 10^8$
R713	*E. cloacae*	-	4	0.5	$>1 \times 10^8$	$>1 \times 10^8$
HM	*E. cloacae*	-	4	2	$>1 \times 10^8$	$>1 \times 10^8$
R2260	*P. mirabilis*	-	4	4	$>1 \times 10^8$	$>1 \times 10^8$
R1395	*A. baumannii*	-	2	1	$>1 \times 10^8$	$>1 \times 10^8$
R191	*P. aeruginosa*	-	8	2	$>1 \times 10^8$	$>1 \times 10^8$
Aminoglycoside-resistant and non–methylase-producing strains						
pWP701	*E. coli*	AAC(3)-IV	1	128	9×10^7	$>1 \times 10^8$
pFCT4392	*E. coli*	AAC(3)-Ia	1	32	3×10^7	$>1 \times 10^8$
390	*E. coli*	AAC(3)-IV	8	64	$>1 \times 10^8$	$>1 \times 10^8$
HCSC-Ec90	*E. coli*	AAC(3)-IIa	4	64	$>1 \times 10^8$	$>1 \times 10^8$
22089	*E. coli*	AAC(3)-I + AAC(3)-V	8	64	$>1 \times 10^8$	$>1 \times 10^8$
97	*E. coli*	AAC(3)-IV	2	32	$>1 \times 10^8$	$>1 \times 10^8$
92/31	*E. coli*	AAC(3)-IV	4	16	$>1 \times 10^8$	$>1 \times 10^8$
4000	*E. coli*	APH(3')-I + AAC(3)-V	4	64	$>1 \times 10^8$	$>1 \times 10^8$
pFCT1163	*E. coli*	AAC(6')-Ia	128	4	1×10^8	$>1 \times 10^8$
1054a	*E. coli*	AAC(6')-I	8	8	$>1 \times 10^8$	$>1 \times 10^8$
HCSC-Ec235	*E. coli*	AAC(6')-Ib	8	8	$>1 \times 10^8$	$>1 \times 10^8$
HCSC-Ec296	*E. coli*	AAC(6')-Ib	32	128	3×10^2	3×10^2
1488	*E. coli*	AAC(6')-II	4	16	$>1 \times 10^8$	$>1 \times 10^8$
1054b	*E. coli*	AAC(6')-I	16	8	$>1 \times 10^8$	$>1 \times 10^8$
RP4	*E. coli*	APH(3')-Ib	1	2	7×10^7	$>1 \times 10^8$
HCSC-Ec52	*E. coli*	AAC(6')-Ib + APH(3')-Ia	8	64	$>1 \times 10^8$	$>1 \times 10^8$
122971	*E. coli*	ANT(2")	4	128	$>1 \times 10^8$	$>1 \times 10^8$
HCSC-Ec22	*E. coli*	AAC(6')-Ib + AAC(3)-IIa	64	64	2×10^2	3×10^2
HCSC-Ec28	*E. coli*	AAC(6')-Ib + ANT(2")-Ia	8	32	$>1 \times 10^8$	$>1 \times 10^8$
HCSC-Ec30	*E. coli*	AAC(3)-IIa + ANT(2")-Ia	4	64	$>1 \times 10^8$	$>1 \times 10^8$
HCSC-Ec33	*E. coli*	AAC(3)-IIa + APH(3')-Ia	2	64	$>1 \times 10^8$	$>1 \times 10^8$
HCSC-Ec78	*E. coli*	ANT(2")-Ia + APH(3')-Ia	2	128	$>1 \times 10^8$	$>1 \times 10^8$
HCSC-Ec246	*E. coli*	AAC(6')-Ib + ANT(2")-Ia	8	32	$>1 \times 10^8$	$>1 \times 10^8$
1800	*K. pneumoniae*	AAC(6')-I + AAC(3)-I	32	16	1×10^8	$>1 \times 10^8$
13000	*K. pneumoniae*	APH(3')-I + AAC(3)-V	0.5	128	$>1 \times 10^8$	$>1 \times 10^8$
22233	*K. pneumoniae*	AAC(3)-V	16	32	$>1 \times 10^8$	$>1 \times 10^8$
1110	*E. cloacae*	ANT(2")	1	32	$>1 \times 10^8$	$>1 \times 10^8$
BM2667	*P. stuartii*	APH(3')-VI + AAC(2')	64	8	1×10^8	$>1 \times 10^8$
531	*P. rettgeri*	AAC(2')	16	4	6×10^7	$>1 \times 10^8$
4290	*S. marcescens*	AAC(3)-I	4	64	4×10^7	$>1 \times 10^8$
BM2580	*A. baumannii*	APH(3')-VI	64	2	$>1 \times 10^8$	$>1 \times 10^8$
8541	*A. baumannii*	APH(3')-VI	128	0.5	$>1 \times 10^8$	$>1 \times 10^8$
4996	*A. baumannii*	AAC(6')-I	64	4	1×10^8	$>1 \times 10^8$
3656	*P. aeruginosa*	APH(3')-I + ANT(4')-II	128	8	5×10^7	$>1 \times 10^8$
3658	*P. aeruginosa*	APH(3')-I + ANT(4')-II	>128	4	$>1 \times 10^8$	$>1 \times 10^8$
28233	*P. aeruginosa*	APH(3')-I + AAC(3)-I	4	1	$>1 \times 10^8$	$>1 \times 10^8$
3655	*P. aeruginosa*	APH(3')-I + ANT(2")	1	4	$>1 \times 10^8$	$>1 \times 10^8$
16S rRNA methylase-producing strains						
pFR2000	*E. coli* TOP10	NpmA	64	32	1×10^7	1×10^7
R989	*E. coli*	ArmA	>512	>256	2×10^1	1×10^2
R1012	*E. coli*	ArmA	>512	>256	1×10^1	3×10^1
62Carb	*E. coli*	RmtB	>512	>256	2×10^2	2×10^2
E28	*E. coli*	RmtB	>512	>256	4×10^1	4×10^1
R107	*E. coli*	RmtB	>512	>256	2×10^1	2×10^1
R990	*E. coli*	RmtB	>512	>256	2×10^1	1×10^1
R262	*E. coli*	RmtC	>512	>256	3×10^2	3×10^2
R451	*E. coli*	RmtC	>512	>256	3×10^1	2×10^1
R500	*K. pneumoniae*	ArmA	>512	>256	2×10^2	2×10^2
R108	*K. pneumoniae*	ArmA	>512	>256	4×10^1	2×10^1
R2257	*K. pneumoniae*	ArmA	>512	>256	1×10^1	2×10^1
N34	*K. pneumoniae*	RmtC	>512	>256	3×10^1	1×10^2
R502	*K. pneumoniae*	RmtF	>512	>256	2×10^2	3×10^2
R2160	*K. pneumoniae*	RmtG	>512	>256	3×10^2	3×10^2
R2258	*E. cloacae*	ArmA	>512	>256	2×10^1	2×10^1
R265	*P. stuartii*	RmtC	>512	>256	3×10^2	3×10^2
R38	*P. stuartii*	RmtC	>512	>256	2×10^1	1×10^1

Table 2 *(continued)*

Strain	Species	Mechanism of aminoglycoside resistance	MIC (μg/mL) of antibiotics[b]		Lowest detection limit (CFU/mL)[c]	Lowest detection limit in stools (CFU/mL)[c]
			Amikacin	Gentamicin		
R263	*P. mirabilis*	ArmA	>512	>256	4×10^2	3×10^2
R264	*A. baumannii*	ArmA	>512	>256	3×10^2	3×10^2

AAC = *N*-acetyltransferases; ANT = *O*-adenyltransferases; APH = *O*-phosphotransferases.

[a] EMB 30/30 refers to the EMB culture medium supplemented with amikacin and gentamicin, 30 mg/L each.

[b] MICs of amikacin and gentamicin were determined using the broth microdilution technique

[c] Underlined CFU counts are considered as results below cutoff values set at ≥1×10^3 CFU/mL.

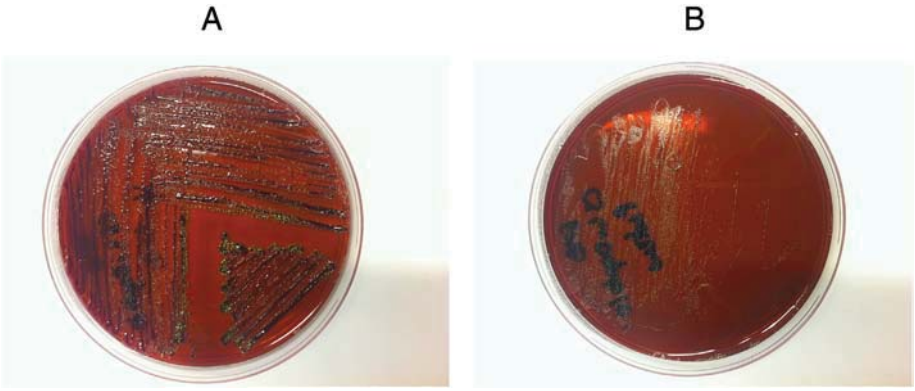

Fig. 1. 16S rRNA methylase-producing *K. pneumoniae* (A) and *P. mirabilis* (B) growing onto the SuperAminoglycoside medium.

3. Discussion

We developed here the very first selective medium allowing screening and detection of Gram-negative bacteria exhibiting high-level resistance to aminoglycosides, in particular 16S rRNA methylase producers. Those latter strains tend to emerge in different parts of the world, and it is necessary to use such a medium to perform prospective screening and epidemiological surveys.

The SuperAminoglycoside medium may detect not only 16S rRNA methylase producers but also the very rare isolates being resistant to both gentamicin and amikacin due to the combination of aminoglycosides-modifying enzymes or to specific aminoglycoside-modifying enzymes. For example, AAC(6')-Ib with Leu118 and Ser119 confers a high level of resistance to both gentamicin and amikacin (Casin et al., 2003). Failure to detect the NpmA producer tested was noticeable. However, i) the isolate tested was a recombinant strain, and MIC values of amikacin and gentamicin for this isolate were at the limit of the resistance cutoff value; ii) the resistance level to gentamicin and amikacin of the NpmA-producing clinical isolate is much higher (Wachino et al., 2007) and likely to be detected by the SuperAminoglycoside medium; and iii) NpmA producers seem to be exceptional (just a single NpmA producer reported so far) (Wachino et al., 2010). Overall, this medium offers the possibility to select for those multiple-aminoglycoside–resistant Enterobacteriaceae, *P. aeruginosa*, and *A. baumannii* from pure culture as well as from stools. It is foreseeable that similar results would be obtained by using rectal swabs as starting specimens. This medium contributes to species identifications according to the color of the colonies growing on the medium (Fig. 1A and B) and is able to differentiate several bacterial species growing on the medium.

The use of this selective medium may contribute to rapidly identify carriers of multidrug-resistant isolates producing plasmid-mediated 16S rRNA methylases and consequently to rapidly implement infection control measures in order to limit their spread. It may be useful in particular in the context of the spread of NDM-type carbapenemase producers since NDM and 16S rRNA methylases are often associated (Lopez-Diaz et al., 2017; Poirel et al., 2011; Rahman et al., 2015). Finally, this medium may be also interesting for screening the environmental and animal reservoirs for 16S rRNA methylase producers. Indeed, those resistance traits have been already identified in chicken for which aminoglycoside use may represent a selective driving force (Yang et al., 2015).

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Conflict of interest

An international patent form has been submitted on behalf of the University of Fribourg (Switzerland) corresponding to this selective culture medium.

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