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A Universal Culture Medium for Screening Polymyxin-Resistant Gram-Negative Isolates

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The colistin-containing SuperPolymyxin medium was developed for screening polymyxin-resistant Gram-negative bacteria. It was evaluated with 88 polymyxin-susceptible or polymyxin-resistant cultured Gram-negative isolates. Its sensitivity and specificity of detection were ca. 100%. The SuperPolymyxin medium is the first screening medium that is able to detect intrinsic and acquired polymyxin-resistant bacteria.

Emergence of polymyxin resistance (PR) is increasingly observed among Gram-negative bacteria (1). The identification of the plasmid-mediated colistin resistance determinant MCR-1 is an additional source of concern (2). Therefore, an early detection of carriers of PR isolates is becoming important.

Polymyxin-containing culture media are known for screening intrinsic PR bacterial species, such as *Serratia marcescens* and *Burkholderia cepacia* (3, 4). Those media containing high concentrations of colistin are not adapted for screening isolates with acquired PR that may be of a low level and may contain deoxycholic acids that may interfere with the growth of PR bacteria (18).

Therefore, our aim was to develop a selective culture medium for screening any type of PR Gram-negative bacteria. The design of this medium took into account the necessity to avoid swarming of *Proteus* spp. (naturally PR) and to prevent contamination by Gram-positive bacteria and fungi. The optimal screening medium retained was based on the eosin methylene blue (EMB) medium (product no. 70186; EMB Fluka, St. Louis, MO, USA) (5), which is selective for Gram-negative bacteria. This medium may also contribute to species identification by differentiating lactose fermenters (dark blue-brown colonies) from nonfermenters (colorless or light lavender) (Fig. 1). Moreover, differentiation of lactose fermenters was possible with *Escherichia coli* colonies displaying a characteristic metallic green sheen (Fig. 1A) and *Enterobacter* spp. and *Klebsiella* spp. giving brown, dark-centered, and mucoid colonies (Fig. 1B). The optimal colistin (Sigma-Aldrich, St. Louis, MO, USA) concentration was retained at 3.5 μg/ml. Since some *Streptococcus* and *Staphylococcus* strains may still grow on EBM medium, daptomycin (Novartis, Horsham, United Kingdom) was added at a concentration of 10 μg/ml. It is noteworthy that daptomycin and not vancomycin was added since vancomycin potentiates the activity of colistin against several Gram-negative bacteria (6, 7). Amphotericin B (Bristol-Myers Squibb, Rueil-Malmaison, France) was also added as an anti-fungi molecule at a concentration of 5 μg/ml.

TABLE 1 Preparation of the SuperPolymyxin medium

Compound	Stock solution (mg/ml)	Quantity or vol to add[a]	Final concn[b]
EMB agar powder		15 g	3.75%
Distilled water		400 ml	
Colistin sulfate	20 In water in glass tubes	70 μl	3.5
Daptomycin	20 In water	200 μl	10
Amphotericin B	20 In D-(+)-glucose 10%	100 μl	5

[a] The volume of 400 ml of SuperPolymyxin medium was for, i.e., 20 plates.
[b] Concentrations are in micrograms per milliliter unless noted otherwise.

The stock solutions of colistin, daptomycin, and amphotericin B were prepared as indicated in Table 1 and may be kept at −20°C for 1 year. Notably, glass tubes were used to prepare colistin stock solutions to avoid its binding to polystyrene. Colistimethate sulfate, a therapeutic prodrug of colistin, cannot be used. The diluted powder of EMB was autoclaved at 121°C for 15 min. After cooling

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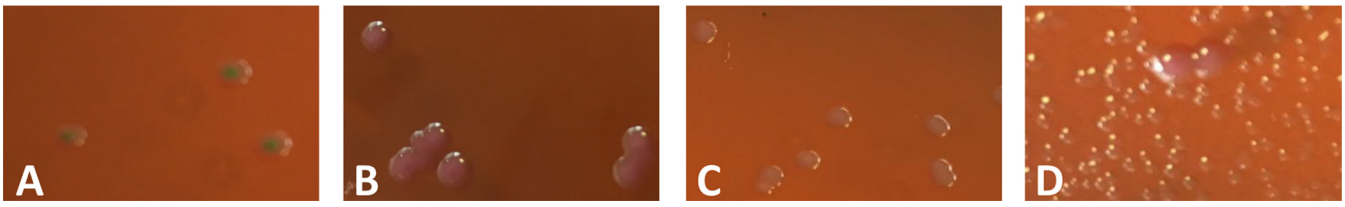

FIG 1 Polymyxin-resistant lactose-positive *E. coli* (A), polymyxin-resistant *K. pneumoniae* (B), polymyxin-resistant lactose-negative *E. coli* (C), and a mix of a heavy inoculum of *P. mirabilis* and a low inoculum of polymyxin-resistant *K. pneumoniae* (C) growing on the SuperPolymyxin medium.

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TABLE 2 MICs of polymyxins for the studied strains and limits of detection of the SuperPolymyxin culture medium

Strain[a]	Species	Origin	MIC[b]		Polymyxin resistance[c]	Mechanism of polymyxin resistance	Lowest detection limit (CFU/ml)[d]	Lowest detection limit in stools (CFU/ml)[d]
			Colistin	Polymyxin B				
Yeast isolates								
FR-A	*C. albicans*	France	NA	NA	NA	NA	$\geq 10^7$	ND
Gram-positive cocci isolates								
FR-B	*S. aureus*	France	NA	NA	NA	NA	$\geq 10^7$	ND
FR-C	*Staphylococcus epidermidis*	France	NA	NA	NA	NA	$\geq 10^7$	ND
FR-D	*Enterococcus faecium*	France	NA	NA	NA	NA	$\geq 10^7$	ND
FR-E	*Enterococcus faecalis*	France	NA	NA	NA	NA	$\geq 10^7$	ND
Gram-negative rod isolates naturally resistant to polymyxins								
FR-01	*M. morganii*	France	>128	>128	R	Intrinsic	10^1	10^1
FR-02	*P. mirabilis*	France	>128	>128	R	Intrinsic	10^1	ND
FR-03	*Proteus vulgaris*	France	>128	>128	R	Intrinsic	10^1	ND
FR-04	*Providencia stuartii*	France	>128	>128	R	Intrinsic	10^1	ND
FR-05	*S. marcescens*	France	>128	>128	R	Intrinsic	10^1	ND
FR-201	*B. cepacia*	France	>128	>128	R	Intrinsic	10^{1e}	ND
FR-202	*Burkholderia gladioli*	France	>128	>128	R	Intrinsic	10^1	ND
Gram-negative rod isolates with an acquired mechanism of resistance to polymyxins								
FR-06	*K. pneumoniae*	France	32	64	R	PmrA G53C	10^1	10^1
FR-07	*K. pneumoniae*	France	32	32	R	PmrA G53S	10^1	10^1
FR-09	*K. pneumoniae*	Turkey	32	64	R	PmrB L17Q	10^1	10^1
FR-10	*K. pneumoniae*	South Africa	16	8	R	PmrB T157P	10^1	10^1
FR-17	*K. pneumoniae*	Turkey	<128	128	R	PhoQ R16C	10^1	10^1
FR-21	*K. pneumoniae*	France	32	64	R	MgrB N42Y/K43I	10^1	10^1
FR-30	*K. pneumoniae*	France	>128	64	R	MgrB truncated	10^1	10^1
FR-31	*K. pneumoniae*	France	64	32	R	MgrB truncated	10^1	ND
FR-36	*K. pneumoniae*	Colombia	128	128	R	MgrB truncated	10^1	ND
FR-40	*K. pneumoniae*	France	64	64	R	MgrB IS*Ecp1*-$bla_{\text{CTX-M-15}}$	10^1	10^1
FR-41	*K. pneumoniae*	France	>128	128	R	MgrB IS*102*-like	10^1	ND
FR-47	*K. pneumoniae*	Turkey	64	32	R	MgrB I*3903b*-like	10^1	ND
FR-48	*K. pneumoniae*	Spain	128	128	R	MgrB IS*903*-like	10^1	ND
FR-49	*K. pneumoniae*	France	64	32	R	MgrB IS*5*-like	10^1	ND
FR-54	*K. pneumoniae*	Colombia	128	128	R	MgrB IS*Kpn13*	10^1	10^1
FR-56	*K. pneumoniae*	Spain	64	64	R	MgrB IS*Kpn26*	10^1	ND
FR-68	*K. pneumoniae*	Colombia	64	64	R	MgrB IS*Kpn14*	10^1	10^1
FR-70	*K. pneumoniae*	Colombia	128	128	R	*mgrB* promoter IS*10R*	10^1	ND
FR-71	*K. pneumoniae*	Turkey	32	32	R	*mgrB* promoter IS*Kpn14*	10^1	ND
FR-86	*K. pneumoniae*	Spain	64	64	R	*mgrB* deletion nt 100	10^2	ND
FR-89	*K. pneumoniae*	Colombia	>128	>128	R	*mgrB* deletion nt 23 to 33	10^1	ND
FR-92	*K. oxytoca*	Colombia	64	64	R	*mgrB* promoter IS*Kpn26*-like	10^1	10^1
FR-93	*E. coli*	France	4	4	R	Plasmid-mediated *mcr-1* gene	10^2	10^1
FR-94	*E. coli*	South Africa	16	16	R	Plasmid-mediated *mcr-1* gene	10^1	10^1
FR-98	*E. coli*	South Africa	8	8	R	Plasmid-mediated *mcr-1* gene	10^1	ND
FR-203	*A. baumannii*	Switzerland	128	128	R	PmrB G260D	10^2	10^1
FR-100	*K. pneumoniae*	France	64	64	R	Unknown	10^1	10^1
FR-102	*K. pneumoniae*	France	32	32	R	Unknown	10^1	ND
FR-113	*K. pneumoniae*	France	>128	>128	R	Unknown	10^1	ND
FR-114	*K. pneumoniae*	Colombia	64	128	R	Unknown	10^1	ND
FR-116	*K. pneumoniae*	France	64	32	R	Unknown	10^1	ND
FR-119	*E. coli*	France	8	4	R	Unknown	10^1	ND
FR-120	*E. coli*	France	8	4	R	Unknown	10^1	10^1
FR-121	*E. coli*	France	4	4	R	Unknown	10^1	ND

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TABLE 2 (Continued)

Strain[a]	Species	Origin	MIC[b]		Polymyxin resistance[c]	Mechanism of polymyxin resistance	Lowest detection limit (CFU/ml)[d]	Lowest detection limit in stools (CFU/ml)[d]
			Colistin	Polymyxin B				
FR-122	*E. cloacae*	Colombia	32	16	R	Unknown	10^1	10^1
FR-126	*E. cloacae*	France	>128	>128	R	Unknown	10^1	10^2
FR-135	*H. alvei*	France	16	8	R	Unknown	10^1	10^1
FR-204	*A. baumannii*	USA	>128	>128	R	Unknown	10^2	10^1
FR-205	*A. baumannii*	USA	8	8	R	Unknown	10^2	ND
FR-206	*A. baumannii*	USA	>128	>128	R	Unknown	10^2	ND
FR-207	*P. aeruginosa*	Colombia	64	64	R	Unknown	10^{2f}	10^1
FR-208	*P. aeruginosa*	France	>128	>128	R	Unknown	10^{1e}	ND
FR-209	*P. aeruginosa*	France	>128	>128	R	Unknown	10^{2f}	ND
FR-210	*S. maltophilia*	France	>128	>128	R	Unknown	10^{1e}	ND
FR-211	*S. maltophilia*	France	32	32	R	Unknown	5.10^{2e}	ND
Gram-negative rod isolates susceptible to polymyxins								
FR-136	*E. coli*	ATCC 25922	0.25	0.25	S	NA	$\underline{>10^7}$	ND
FR-212	*E. coli*	USA	0.12	0.12	S	NA	$\underline{>10^7}$	ND
FR-213	*E. coli*	Colombia	0.12	0.12	S	NA	$\underline{>10^7}$	ND
FR-214	*E. coli*	France	0.12	0.12	S	NA	$\underline{>10^7}$	ND
FR-215	*E. coli*	France	0.25	0.12	S	NA	$\underline{>10^7}$	ND
FR-216	*E. coli*	France	0.25	0.12	S	NA	$\underline{>10^7}$	ND
FR-217	*K. pneumoniae*	USA	0.12	0.12	S	NA	$\underline{5 \times 10^6}$	ND
FR-218	*K. pneumonia*	Colombia	0.12	0.5	S	NA	$\underline{>10^7}$	ND
FR-219	*K. pneumoniae*	Colombia	0.12	0.12	S	NA	$\underline{>10^7}$	ND
FR-220	*K. pneumoniae*	Colombia	0.5	0.25	S	NA	$\underline{5 \times 10^6}$	ND
FR-221	*K. pneumoniae*	Colombia	0.5	0.5	S	NA	$\underline{>10^7}$	ND
FR-222	*K. pneumoniae*	France	0.12	0.25	S	NA	$\underline{>10^7}$	ND
FR-223	*K. pneumoniae*	France	0.12	0.12	S	NA	$\underline{>10^6}$	ND
FR-224	*K. pneumoniae*	France	0.25	0.5	S	NA	$\underline{>10^6}$	ND
FR-225	*K. pneumoniae*	France	0.5	0.5	S	NA	$\underline{>10^7}$	ND
FR-226	*K. pneumoniae*	Spain	0.5	0.5	S	NA	$\underline{10^6}$	ND
FR-227	*K. pneumoniae*	Spain	0.5	0.5	S	NA	$\underline{10^7}$	ND
FR-228	*E. cloacae*	Colombia	0.12	0.25	S	NA	$\underline{>10^7}$	ND
FR-229	*E. cloacae*	Colombia	0.12	0.12	S	NA	$\underline{>10^7}$	ND
FR-230	*E. cloacae*	France	0.12	0.25	S	NA	$\underline{5 \times 10^6}$	ND
FR-231	*Enterobacter aerogenes*	France	0.12	0.12	S	NA	$\underline{>10^7}$	ND
FR-232	*Citrobacter freundii*	Colombia	0.25	0.25	S	NA	$\underline{>10^7}$	ND
FR-233	*C. freundii*	France	0.12	0.5	S	NA	$\underline{5 \times 10^6}$	ND
FR-234	*P. aeruginosa*	ATCC 27853	0.5	0.5	S	NA	$\underline{5 \times 10^6}$	ND
FR-235	*P. aeruginosa*	Colombia	1	0.25	S	NA	$\underline{>10^7}$	ND
FR-236	*P. aeruginosa*	Colombia	2	0.5	S	NA	$\underline{10^6}$	ND
FR-237	*P. aeruginosa*	Colombia	1	0.25	S	NA	$\underline{>10^7}$	ND
FR-238	*P. aeruginosa*	France	2	0.25	S	NA	$\underline{10^6}$	ND
FR-239	*A. baumannii*	Colombia	0.5	0.12	S	NA	$\underline{>10^7}$	ND
FR-240	*A. baumannii*	France	0.5	0.25	S	NA	$\underline{10^6}$	ND
FR-241	*A. baumannii*	USA	0.25	0.25	S	NA	$\underline{10^6}$	ND

[a] The strains FR-01 and FR-136 were used as positive and negative controls, respectively.
[b] MICs of colistin and polymyxin B were determined using the broth microdilution method. NA, not available.
[c] R, resistant; S, susceptible.
[d] Underlined CFU counts are considered to be results below cutoff values set at $\geq 1 \times 10^3$ CFU/ml. ND, not determinate.
[e] Positive culture after 36 h.
[f] Positive culture after 48 h.

the EMB medium for 1 h at 56°C, the antibiotic stock solutions were added. Poured plates were stored at 4°C, protected from direct light exposure for up to 1 week.

A total of 88 isolates were included in the study to evaluate the performance of the SuperPolymyxin medium that included 52 PR Gram-negative bacteria of worldwide origin (Table 2). Seven strains were intrinsic PR genders (*Morganella*, *Proteus*, *Providencia*, *Serratia*, and *Burkholderia*). Forty-five isolates with acquired PR were also included and consisted of 36 enterobacterial strains (*E. coli*, *Klebsiella* spp., *Enterobacter cloacae*, and *Hafnia alvei*) and 9 nonfermenters (*Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*). Thirty-one polymyxin-susceptible strains were included with 23 enterobacterial strains and 8 nonfermenters (Table 2).

MICs of polymyxins (colistin and polymyxin B) were determined using the broth microdilution method in cation-adjusted

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Mueller-Hinton broth as recommended by the CLSI (8). A final inoculum of 5×10^5 CFU/ml of each strain was distributed in the 96-polystyrene-well tray (Sarstedt, Nümbrecht, Germany). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as polymyxin-susceptible control strains (9), and all experiments were repeated in triplicate. The results were interpreted according to the CLSI breakpoints (9) for *A. baumannii* (susceptible [S], ≤2 μg/ml; resistant [R], ≥4 μg/ml) and for *Pseudomonas* spp. (S, ≤2 μg/ml; R, ≥8 μg/ml) and according to the EUCAST breakpoints for *Enterobacteriaceae* (i.e., S, ≤2 μg/ml; R, >2 μg/ml) (10). MIC values for colistin and polymyxin B were superimposable, permitting an easy classification between PR and polymyxin-susceptible strains (Table 2).

The known genes possibly involved in chromosomally encoded PR in *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *P. aeruginosa*, and *A. baumannii* were sequenced as described previously (11–15). The PCR detection of the *mcr-1* gene was carried out as described previously (2) (Table 2). MIC values of polymyxins for strains with acquired resistance were variable and were lower than those for intrinsically PR strains (Table 2).

Using an inoculum with an optical density of 0.5 McFarland standard (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 SuperPolymyxin medium. To quantify the viable bacteria in each dilution, Trypticase soy agar was inoculated concomitantly with 100 μl of suspension and was incubated overnight at 37°C. The number of viable colonies was counted after 24 h of culture at 37°C (and after 36 and 48 h for *B. cepacia*, *P. aeruginosa*, and *S. maltophilia*). PR and polymyxin-susceptible control strains were FR-01 (*Morganella morganii*) and FR-136 (*E. coli* ATCC 25922), respectively (Table 2). The lowest limit of detection for the tested strains was determined using the SuperPolymyxin 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 not efficiently detected (16). All of the PR strains grew on the SuperPolymyxin medium in 24 h except *P. aeruginosa*, *S. maltophilia*, and the intrinsically PR *Burkholderia* genus that grew in 24 to 48 h (Table 2). The lowest limit of detection was below the cutoff value for all PR strains, whereas the limit of detection of the polymyxin-susceptible strains was above the cutoff value at $\geq 1 \times 10^6$ CFU/ml (Table 1). The sensitivity and specificity of the SuperPolymyxin medium for selecting PR isolates were 100% in both cases. Moreover, this medium was tested with a light growth of a PR *K. pneumoniae* isolate (FR-10) among a heavier growth of *Proteus mirabilis* (FR-02) and revealed that it nicely discriminated between the two species (Fig. 1D).

Spiked stools were also tested with a representative collection of 22 PR isolates of various species with various levels and mechanisms of PR (Table 2). 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 described previously (17). A nonspiked fecal suspension was used as a negative control. The lowest detection limit of the PR isolate was determined by plating 100 μl of each dilution on the medium. The sensitivity and specificity were determined using the same cutoff value set at $\geq 10^3$ CFU/ml (17). This value may correspond to a low-level carriage of PR bacteria in stools. All of the PR isolates spiked in stools grew with a lowest detection limit ranging from 10^1 to 10^2 CFU/ml (Table 2).

Similar results were obtained with 20 colistin-susceptible and polymyxin B-susceptible strains and 20 colistin-resistant and polymyxin B-resistant strains by using polymyxin B at the same concentration instead of colistin sulfate.

Finally, to assess the storage stability of the SuperPolymyxin medium, *Candida albicans*, *Staphylococcus aureus*, and colistin-susceptible *E. coli* ATCC 25955 were subcultured daily onto SuperPolymyxin agar plates from a single batch of medium stored at 4°C. Growth of those isolates were consistently inhibited during at least a 7-day period.

The SuperPolymyxin medium constitutes a screening medium aimed to detect any PR bacteria regardless of its resistance mechanism and of its level. This medium may be used in human medicine for detecting carriers and in veterinary medicine for surveillance surveys.

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