A Novel Chromogenic Culture Media (CHROMagar COL-*APSE*) for the Isolation and Differentiation of Colistin Resistant Gram-negative Pathogens

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INTRODUCTION

- Polymyxin E (colistin) and B are increasingly used as antimicrobials in the treatment of multi-drug resistant bacterial infections.
- Polymyxin resistance (PR), although intrinsic in Gram-positive and some Gram-negative species (*Proteus, Morganella, Serattia* spp), is now a problem in a number of other pathogens (*Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli, Salmonella enterica, Klebsiella pneumoniae*).
- Resistance arises due to mutations / insertions in genes involved in LPS biosynthesis (***lpx, pmrA/B, mgrB, phoP/Q)*** and / or the acquisition of phosphoethanolomine transferases **(PEtN)**.
- Of great concern is the recently described plasmid-encoded PEtN, **MCR-1**, now found worldwide in a range of animal, human and environmental bacterial isolates

AIM

To develop a new chromogenic culture media and evaluate its sensitivity and specificity in the detection of polymyxin-resistant pathogens.

MATERIALS AND METHODS

Media Preparation

- CHROMagar COL-*APSE* plates were prepared in-house using dehydrated CHROMagar base media (CHROMagar™, Paris, France) supplemented with CHROMagar COL-*APSE* supplement (Figure 1).
- This contains antimicrobials specifically selected to avoid potentiation (colistin sulfate / oxazolidonone) at concentrations designed to enhance the growth of PR Gram-negative species, and suppress the growth of Gram-positive bacteria.
- CHROMagar COL-*APSE* media for use with samples containing *Proteus* spp were prepared with the addition of 50 mg/L p-nitrophenyl glycerol (PNPG).

Bacterial Isolates and Determination of Polymyxin Minimum Inhibitory Concentration (MICs)

- Eighty-four isolates were used in the evaluation of CHROMagar COL-*APSE* media.
- 8 isolates with intrinsic COL resistance (1 clinical human and 7 type strains), 13 COL susceptible isolates (6 clinical human, 3 clinical veterinary and 4 type strains) and 63 isolates with acquired COL resistance (5 clinical human and 58 clinical veterinary) contained within our collections.
- The MICs of colistin (COL) and polymyxin B (POL) (0.006 – 256 µg/ml) were first determined by agar dilution on Mueller-Hinton II agar using a multi-point inoculator.
- Susceptibility (≤ 2-4 µg/ml) and resistance (> 2-8 µg/ml) to either COL or POL was interpreted according to current CLSI and / or EUCAST species specific breakpoints.

MCR1/2 Detection

- All clinical isolates were screened for the presence of the *mcr-1/2* genes by a multiplex PCR assay.

Lower Limit of Detection (LLD)

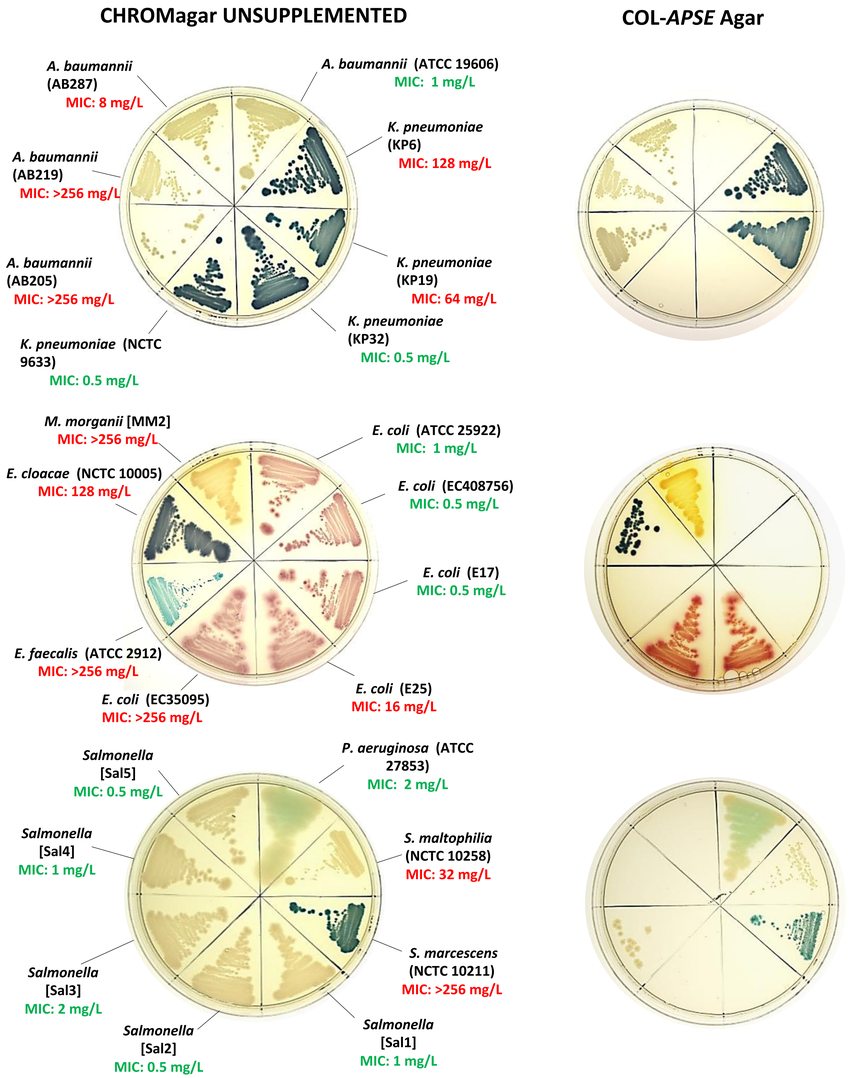
- The lowest limit of detection was assessed using serial dilutions (10^{-1} – 10^{-9}) of an overnight culture grown at 37 °C for 24 h in 3 ml of Luria-Bertani (LB) broth. Ten-fold serial dilutions (100 µl in 900 µl) were made in phosphate-buffered saline (PBS) and 20 µl of each dilution plated onto un-supplemented MH 2 (control) and CHROMagar COL-*APSE* plates using the Miles and Misra procedure.
- Colony counts obtained on CHROMagar COL-*APSE* were subtracted from the number recovered on MH 2 agar to quantify the total number of COL resistant organisms (CFU) within the total population plated, required for viable growth on each selective media (Table 1).

Isolation and Differentiation of Organisms in Mixed Culture

- Two pools containing mixtures of COL resistant (R) and COL susceptible (S) organisms were used to assess the performance of the media with complex polymicrobial samples (Figure 2).
- Pool 1 - *A. baumannii* AB205 (COL resistant), *E. coli* E7 (COL resistant), *E. cloacae* NCTC 10005 (COL resistant), *E. faecalis* ATCC 2912 (COL resistant), *K. pneumoniae* KP19 (COL resistant), *M. morgannii* MM2 (COL resistant), *P. aeruginosa* ATCC 27853 (COL heteroresistant), *S. marcescens* NCTC 10211 (COL resistant), *P. mirabilis* NCTC 13376 (COL resistant).
- Pool 2 - *A. baumannii* ATCC 19606 (COL susceptible), *E. coli* ATCC 25922 (COL susceptible), *E. faecalis* ATCC 2912 (COL resistant), *P. aeruginosa* ATCC 27853 (COL heteroresistant), *K. pneumoniae* ATCC 9633 (COL susceptible)
- Internal and external quality control of the stability of the media was assessed using *P. mirabilis* NCTC 13376, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 9633 and *E. faecalis* ATCC 2912. Media for use with pools containing *P. mirabilis* NCTC 13376 was supplemented with PNPG.

RESULTS

Figure 1. Comparative growth of CHROMagar base media with CHROMagar COL-*APSE*

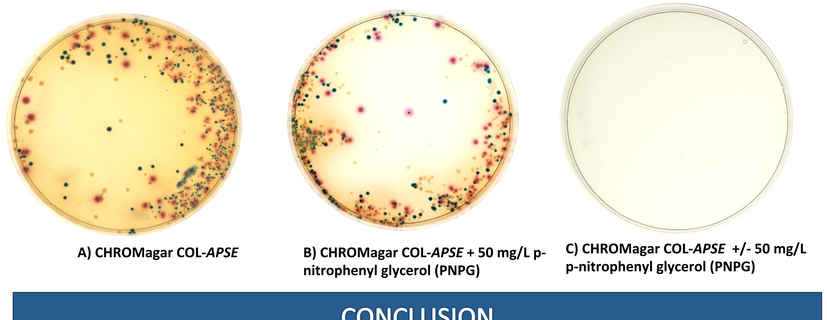


- The *mcr-1* gene was present in 61 of the *E. coli* strains, 58 of which were also phenotypically resistant to COL (MIC >2 µg/ml).
- The MICs of COL and POL asessed by agar dilution were comparable (+/- 1 dilution), including for three *E. coli* strains positive for *mcr-1* but with susceptibility to COL.
- CHROMagar COL-*APSE* was able to support the growth of all COL resistant strains with an inocula as low as 10^1 CFU, whilst suppressing the growth of all COL susceptible Gram-negative and COL resistant Gram-positive species. Notably, addition of PNPG was beneficial in suppressing swarming of colistin resistant *Proteus spp* without affecting the performance of the media.
- Proteus swarming (*Proteus mirabilis* NCTC 13376) was reduced significantly with the addition of PNPG (Figure 2B). No growth was seen in sensitive bacterial pools +/- *Proteus mirabilis* NCTC 13376 and +/- PNPG (Figure 2C).

Table 1. Lower Limit of Detection
Isolates were provided by *Denmark and #France

ISOLATE	MIC (mg/L)/ Agar dilution		Polymyxin Resistance Mechanism	Lowest Limit of Detection (CFU)
	Colistin	Polymxyin B		CHROMagar COL-*APSE*
Intrinsic Resistance to Polymyxins				
P. mirabilis NCTC 13376	>256	>256	Intrinsic	10^1
S. marcescens NCTC 10211	>256	>256	Intrinsic	10^1
M. morgannii MM2	>256	>256	Intrinsic	10^1
E. faecalis ATCC 2912	>256	256	Intrinsic	$>10^9$
E. gallinarum ATCC 49573	>256	>256	Intrinsic	$>10^8$
C. albicans ATCC 10231	256	256	Intrinsic	$>10^7$
E. cloacae NCTC 10005	128	256	Intrinsic	10^2
S. maltophilia NCTC 10258	8	64	Intrinsic	10^{1*}
Susceptible to Polymyxins				
P. aeruginosa ATCC 27853	2	2	NA	10^2
Salmonella Group D (non-Typhi) Sal3	2	2	N/A	10^4
E. coli E17	1	2	MCR-1	10^7
Salmonella enterica subsp. diarizonae Sal1	1	2	N/A	10^5
A. baumannii ATCC 19606	1	2	NA	10^6
E. coli E44	1	1	MCR-1	$>10^9$
Salmonella enterica subsp. diarizonae Sal4	1	1	N/A	10^5
K. pneumoniae KP32	0.5	1	NA	10^6
Salmonella enterica subsp. diarizonae Sal2	0.5	1	N/A	10^5
Salmonella Group D (non-Typhi) Sal5	0.5	1	N/A	10^3
E. coli ATCC 25922	0.5	0.5	NA	10^6
E. coli 40875	0.5	0.5	MCR-1	10^6
K. pneumoniae ATCC 9633	0.5	0.5	NA	$>10^9$
Acquired Resistance to Polymxins				
A. baumannii AB219	>256	>256	Unknown	10^1
A. baumannii AB205	>256	32	Unknown	10^1
A. baumannii AB287	8	4	Unknown	10^1
K. pneumoniae KP6	128	256	Unknown	10^1
K. pneumoniae KP19	64	64	Unknown	10^1
#*E. coli* 35095	>256	>256	MCR-1	10^1
#*E. coli* 35175	64	32	MCR-1	10^1
#*E. coli* (n=4)	32	32	MCR-1	10^1
**E. coli* (n=12)	32	16	MCR-1	10^{1*}
**E. coli* E27	16	32	MCR-1	10^1
*#*E. coli* (n=34)	16	16	MCR-1	10^1
*#*E. coli* (n=3)	16	8	MCR-1	10^1
**E. coli* E45	8	16	MCR-1	10^1
#*E. coli* 32218	8	8	MCR-1	10^1

Figure 2. Simulated bacterial pools inoculated using L-spreader method



A) CHROMagar COL-*APSE*

B) CHROMagar COL-*APSE* + 50 mg/L p-nitrophenyl glycerol (PNPG)

C) CHROMagar COL-*APSE* +/- 50 mg/L p-nitrophenyl glycerol (PNPG)

CONCLUSION

- **CHROMagar COL-*APSE*** is a **sensitive** and **specific media** for the growth of COL resistant bacterial pathogens with a lower limit of detection of 10^1 CFU
- **CHROMagar COL-*APSE*** may be useful as a **primary isolation media** in the **surveillance** and **recovery** of COL resistant bacteria from **complex human**, **veterinary** and **environmental samples** especially those with plasmid mediated MCR-1 or novel mechanisms of polymyxin resistance.

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