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Performance of CHROMID® Colistin R agar, a new chromogenic medium for screening of colistin-resistant *Enterobacterales*

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ABSTRACT

Recent emergence of transferable plasmid-borne colistin resistance (*mcr* genes) raised fear for pan-resistance. We evaluated the performance of a new chromogenic medium [CHROMID® Colistin R agar (COLR)] for the screening of colistin-resistant *Enterobacterales*. Specificity was evaluated using 89 rectal swabs and 89 stools prospectively collected. COLR sensitivity was evaluated by seeding 59 negative clinical samples artificially contaminated (10^5 CFU/mL) with 59 colistin-resistant *Enterobacterales*, including 20 *mcr-1*–positive strains. Twelve samples with an *Enterobacterales* with nonintrinsic resistance to colistin were recovered during the specificity study, including one *mcr-1*–positive *Escherichia coli*, representing a 6.7% prevalence of colistin resistance in fecal carriage. Overall, specificity was 100.0% [95% CI: 97.8–100.0] and sensitivity yielded 88.1% [95% CI: 77.5–94.1]. False negatives corresponded to 3 *Enterobacter* spp. (MIC>64 mg/L), 2 *Salmonella* spp. (MIC = 16 mg/L), 1 *E. coli* (MIC = 4 mg/L), and 1 *K. pneumoniae* (MIC = 8 mg/L). COLR appears to be a sensitive and specific chromogenic agar for screening colistin-resistant *Enterobacterales*, including those carrying *mcr-1* gene.

1. Introduction

Increasing incidence of infections caused by multidrug-resistant Gram-negative bacteria is a worldwide problem, leaving only few antimicrobial agents to treat them (Glasner et al., 2013). For this reason, there has been a recent increase in the use of polymyxins as a last-resort treatment (Olaitan et al., 2014). However, the emergence of polymyxins resistance is increasing in Gram-negative bacteria, particularly in *Enterobacterales* (Bialvaei and Samadi Kafil, 2015). In addition to the intrinsic and chromosomal mutations-derived mechanisms of resistance to colistin, a horizontally transferable plasmid-borne colistin resistance (*mcr-1*) gene has been recently described in humans as well as in food animals in China (Liu et al., 2016). Since then, the *mcr-1* gene has been identified worldwide in isolates from animals, hospitalized patients and outpatients, and the environment (Nordmann et al., 2016). Furthermore, other plasmid-mediated genes have been described in the last years as *mcr-2, -3, -4*, and *-5* (Borowiak et al., 2017; Carattoli et al., 2017; Xavier et al., 2016; Yin et al., 2017). The improvement of laboratory methods to detect colistin resistance is an aspect on which we must focus our efforts.

The CHROMID® Colistin R agar (COLR) (bioMérieux, France) is a selective chromogenic medium recently marketed for the screening of colistin-resistant *Enterobacterales* from clinical stools and rectal swabs samples. The aim of this study was to evaluate its performance to screen colistin-resistant *Enterobacterales* in the Clinical Microbiology laboratory of a university hospital in Madrid, Spain. The study was performed i) to assess specificity using stools and rectal swabs samples collected from both hospitalized and outpatients and ii) to assess sensitivity using contrived stools samples and contrived rectal swabs with a collection of colistin-resistant *Enterobacterales* from human or animal species.

2. Material and methods

2.1. Study design

This was a prospective study conducted to evaluate the performance of the COLR in a tertiary hospital in Madrid, Spain, during the period from August 2017 to October 2017. Samples were fresh leftovers from routine practice, anonymized and with only 1 sample per subject. The results obtained during this trial did not interfere in the patient's management. The ethical committee of Ramón y Cajal University Hospital approved the study in June 2017 (no. 131-17).

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2.2. Samples and methodology

The study workflow is summarized in Fig. 1. A total of 178 clinical samples with a balanced distribution between stools ($n = 89$) and rectal samples ($n = 89$) were used for the evaluation of COLR specificity. Rectal swabs (within the 24 h since sampling time) were released directly in enrichment broth [9 mL BHI broth (bioMérieux, France) + a 10-μg colistin disk (Oxoid, UK) (BHI-Col)]. In the case of stool samples (within the 48 h since sampling time), 50 μL were directly inoculated in BHI-Col (from a previous suspension of 1 g of feces in 1 mL of sterile saline).

At the same time, stool samples collected for the specificity study were also used to produce contrive samples, excluding later those in which a positive result was obtain in the specificity study. Finally, a total of 59 negative stool samples were used to produce contrived samples (53% rectal swabs and 47% stool samples) to evaluate COLR sensitivity. For this purpose, a set of 59 colistin-resistant *Enterobacterales* (human and animal origin) was used to generate such simulated samples (Table 1). A suspension of 1.5×10^6 CFU/mL of each strain was prepared, from which 0.1 mL was mixed with 1 mL of stool sample (limit of detection ~10^5 CFU/mL). For 50% of these preparations, 50 μL was directly inoculated in BHI-Col, corresponding with contrived stool samples. For the remaining 50%, a blank sterile swab was dipped and released in BHI-Col, corresponding to contrived swab samples.

In both branches of the study, after 4–5 h of incubation at 35 ± 2 °C, 50 μL from the BHI-Col broth was streaked onto COLR. Reading of COLR plates was performed after 18–24 h of incubation at 35 ± 2 °C according to manufacturer's instructions. Briefly, *Escherichia coli* colonies produce a pink to burgundy color; *Klebsiella pneumoniae* and *Enterobacter* spp., blue–green colonies; and *Salmonella* spp., white to colorless colonies. *Proteeae* tribe colonies produced a beige–brown color. COLR inhibits the growth of most of the Gram-positive bacteria, as well as yeast and molds (additional information is shown in the product package insert).

Table 1
Details of colistin-resistant *Enterobacterales* used for the contrived samples in the sensitivity study.

Microorganisms	*n* (%)	Origin human/animal	MIC range (mg/L)	*mcr-1* gene positive *n* (%)
E. coli	19 (32.2%)	0 / 19	[4–64]	19 (100%)
Enterobacter spp.	7 (11.9%)	7 / 0	[8–≥64]	0 (%)
K. pneumoniae	22 (37.3%)	22 / 0	[16–≥64]	0 (%)
Salmonella Paratyphi B var. Java	11 (18.6%)	0 / 11	[4–64]	11 (100%)
Total	59 (100%)	29 / 30	[4–≥64]	30 (50.8%)

Colonies that grew on COLR were identified using MALDI-TOF MS (Bruker-Daltonics, Bremen, Germany). Antimicrobial susceptibility to colistin was performed by standard broth microdilution (BMD) in frozen 96-well plates prepared in-house by bioMérieux in compliance with ISO 20776-1 standards (International Organization for Standardization, 2006). Colistin range tested was 0.125 to ≥64 mg/L. Broth microdilution was performed i) in *Enterobacterales* species not naturally resistant to colistin recovered from the specificity study and ii) in the colistin-resistant *Enterobacterales* used for the sensitivity study. A positive result was considered when the growth of an *Enterobacterales* not naturally resistant to colistin [i.e., *E. coli*, *Klebsiella* spp., and *Enterobacter* spp. (named as targeted species)] was confirmed by MALDI-TOF MS and BMD; otherwise, a negative result was considered.

In those isolates resistant to colistin (MIC >2 mg/L, according to EUCAST breakpoints) recovered in the specificity study, *mcr-1* and *mcr-2* genes were investigated by polymerase chain reaction as previously described (Liu et al., 2016; Xavier et al., 2016).

E. coli ATCC 25922, *E. coli* NCTC 13846 (*mcr-1* positive), and *K. pneumoniae* CCUG 59348 (colistin resistant, *mcr* negative) were used as quality control of COLR plates, and results were interpreted

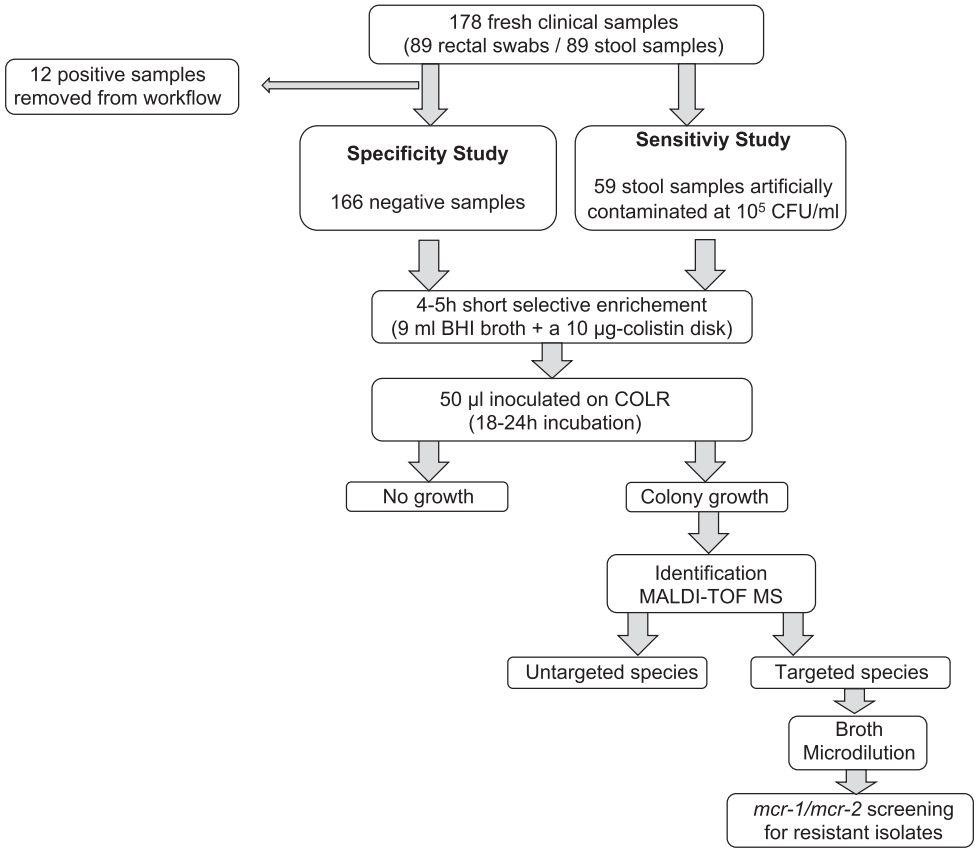

Fig. 1. Study workflow summary.

Table 2
Data from the colistin-resistant *Enterobacterales* isolated from clinical samples in the specificity study ($n = 12$).

Type of sample	Microorganism	MIC (μg/mL)	*mcr-1*/*mcr-2* genes
Rectal swab	*Klebsiella variicola*	16	Negative
Rectal swab	*Klebsiella pneumoniae*	≥ 64	Negative
Rectal swab	*Klebsiella pneumoniae*	32	Negative
Rectal swab	*Klebsiella pneumoniae*	16	Negative
Rectal swab	*Klebsiella pneumoniae*	≥ 64	Negative
Rectal swab	*Klebsiella variicola*	32	Negative
Rectal swab	*Escherichia coli*	8	Negative
Rectal swab	*Escherichia coli*	8	Negative
Rectal swab	*Klebsiella pneumoniae*	16	Negative
Rectal swab	*Klebsiella pneumoniae*	16	Negative
Stools	*Escherichia coli*	8	**Positive: *mcr-1***
Stools	*Enterobacter cloacae*	≥64	Negative

according to the product package insert. *E. coli* ATCC 25922, *E. coli* NCTC 13846, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control for each testing day of antimicrobial susceptibility testing (BMD), and results were interpreted according to EUCAST published ranges (v.7.1) (EUCAST, 2017).

2.3. Statistical analysis

The clinical specificity and sensitivity were calculated and presented along with its 95% confidence interval (CI) for COLR. CI was calculated as follows: if the percentage was in the range of 5%–95%, the Wilson score CI was performed; otherwise, the exact binomial CI was used.

In the eventuality that targeted species resistant to colistin were collected in the specificity study, they were i) not included in the calculation of the sensitivity but documented separately and ii) not included in spiking experiments aiming to assess the sensitivity of the agar.

3. Results

A total of 178 clinical samples (stool samples, $n = 89$; rectal swabs, $n = 89$) were processed during the specificity study. Twelve samples (12/178; 6.7%) produced growth of a targeted species confirmed to be resistant to colistin according MIC results (range from 8 to ≥64 mg/L), with 1 being *E. coli mcr-1* positive (Table 2). Consequently, the specificity was calculated over 166 negative samples composed of 87 stool samples and 79 rectal swabs. The specificity according to sample types reached 100.0% [95% CI: 95.8–100.0] and 100.0% [95% CI: 95.4–100.0] for stool samples and rectal swabs, respectively. When sample types were pooled, the specificity was 100% [95% CI: 97.8–100] (Table 3).

A total of 59 contrived samples (stool samples, $n = 28$; rectal swabs, $n = 31$) were included in the sensitivity study. Information of colistin-resistant *Enterobactericeae* used to contrived stools samples is shown in Table 1. Fifty-two strains produced a positive result in COLR, while 7 strains were not recovered, leading to false-negative results. False-negative results are detailed in Table 4. The sensitivity according to sample types reached 89.3% [95% CI: 72.8–96.3] and 87.1% [95% CI: 71.1–94.9] for stool samples and rectal swabs, respectively. When sample types were pooled, the sensitivity was 88.1% [95% CI: 77.5–94.1]. Sensitivity calculated as a function of the *mcr-1* gene pattern was as follows: for *mcr-1* negative, 86.2% [95% CI: 69.4–94.5] and for *mcr-1* positive, 90.0% [95% CI: 74.4–96.5]. Sensitivity by species tested was as follows: *K. pneumoniae*, 95.5% [95% CI: 77.2–99.9]; *E. coli*, 94.7% [95% CI: 75.4–99.1]; *Salmonella* Paratyphi, 81.8% [95% CI: 52.3–94.9]; and *Enterobacter* spp., 57.1% [95% CI: 25.1–84.2].

Table 4
Details of false-negative results obtained in the sensitivity study.

Microorganisms	Contrived sample type	MIC (μg/mL)	*mcr-1* gene
Enterobacter cloacae	Rectal swab	≥ 64	Negative
Enterobacter asburiae	Rectal swab	≥ 64	Negative
Enterobacter cloacae	Rectal swab	≥ 64	Negative
K. pneumoniae	Stool	8	Negative
Salmonella Paratyphi B var. Java	Stool	16	Positive
Salmonella Paratyphi B var. Java	Stool	16	Positive
E. coli	Rectal swab	4	Positive

4. Discussion

Since the description of the horizontally transferable plasmid-borne colistin resistance gene *mcr-1* by Liu et al. (2016) and later *mcr-2* to *-5*, *mcr* genes have been identified in all continents and from different sources (Nordmann et al., 2016). In a recent surveillance study performed in Europe looking for colistin-resistant and *mcr* genes in isolates recovered from healthy food-producing animals at slaughter, the presence of *mcr-1* in the 0.7% of *E. coli* and 0.1% of *Salmonella* isolates was observed, showing the presence of this resistant mechanism in the food chain (El Garch et al., 2018). The *mcr-1 gene* has been also identified in sewage water (Ovejero et al., 2017). The presence of *mcr-1* in clinical *E. coli* isolates from Spain has been identified in retrospective studies since 2012 (Prim et al., 2016). Moreover, the co-occurrence of multiple *mcr* genes has been found in a single isolate from cattle (Hernández et al., 2017). These findings highlight the necessity of performing screening studies with accurate tools to gain a better understanding of this health care problem.

Different methods to detect colistin resistance have been developed recently, some from both direct stool samples and colonies, such as culture-based methods like SuperPolymyxin medium (Nordmann and Poirel, 2016) and CHROMagar™ COL-APSE (Abdul Momin et al., 2017), as well as molecular tests like loop-mediated isothermal amplification (Zou et al., 2017). Others confirm resistance from colonies such as Rapid Polmyxin™ NP test (Poirel et al., 2018) or based on a microarray system such as CT103XL array (Bernasconi et al., 2017). The CHROMID® Colistin R agar evaluated in this study is a new chromogenic medium to detect colistin-resistant *Enterobacterales* from clinical samples (stools and swabs). COLR enables an easily identification of target species

Table 3
Summary of characteristic performances obtained during the evaluation of CHROMID® Colistin R agar after identification using MALDI-TOF.

	Specificity study				Sensitivity study			
	n	[a] True negative	[a] False positive	Specificity	*n*	[a] True positive	[a, b] False negative	Sensitivity
Pooled samples	166	166	0	100% [97.8–100.0]	59	52	7	88.1% [77.5–94.1]
Rectal swabs	79	79	0	100.0% [95.4–100.0]	31	27	4	87.1% [71.1–94.9]
Stools	87	87	0	100.0% [95.8–100.0]	28	25	3	89.3% [72.8–96.3]

[a] Confirmation of colistin resistance was performed by broth microdilution.
[b] The species that yielded a negative result were *Enterobacter* spp. ($n = 3$), *Salmonella* Paratyphi ($n = 2$), *E. coli* ($n = 1$), and *K. pneumoniae* ($n = 1$).

based on the different color of colonies. We are aware about the possible growth of species naturally resistant to colistin (i.e., *Aeromonas* spp., *Hafnia* spp., *Serratia* spp. *Proteus* spp., and *Morganella morganii*) that are frequently present in stools, but at least, the *Proteeae* tribe (*Proteus* spp., *Providencia* spp. and *M. morganii*) grows with a typical and recognizable beige–brown color.

The finding of 12 isolates resistant to colistin reflects an overall prevalence of 6.7% for fecal carriage of *Enterobacterales* with nonintrinsic resistance to colistin. In fact, 1 *E. coli* isolate (0.6%) was resistant due to acquired *mcr-1* gene, confirming the emergence of this plasmid-borne colistin resistance mechanism in our environment. Further studies on the resistance mechanisms on those isolates (e.g., *mcr*-3/4/5 genes or mutations in *pmrA*, *pmrB*, and *mgrB*) would have been helpful, but they were not under the scope of this clinical validation.

A 100% [95% CI: 97.8–100] specificity was obtained during the validation study, with identical results between rectal swabs and stool samples, suggesting that both types of specimens could be used in routine clinical microbiology laboratories to perform screening studies. There were no any *Enterobacterales* not naturally resistant to colistin that grow in COLR plates, yielded a MIC categorized as susceptible after the BMD analysis, so no false-positive results were obtained during the specificity study. Unfortunately, the use of a comparator resistance detection method was not included into the study design, which would have allowed a better assessment of specificity.

A final sensitivity of 88.1% [95% CI: 77.5–94.1] was observed when samples types were pooled, with the sensitivity being similar between rectal swabs and stool samples. A sensitivity of 90.0% [95% CI: 74.4–96.5] in species harboring a *mcr-1* gene shows a good sensitivity for this emerging resistance mechanism that is raising the risk of compromising the clinical utility of the last-resort polymyxins. Nevertheless, the inclusion of isolates with known *mcr-2*, *-3*, *-4*, and *-5* genes would help to demonstrate the ability of the screening agar to recognize these plasmid-borne variants.

Interestingly, the sensitivity varies if it is calculated by microorganism. The highest rates were obtained in *E. coli* and *K. pneumoniae*, with 95.5% and 94.7%, respectively. On the other hand, a final sensitivity of 57.1% in *Enterobacter* spp. suggests that this medium lacks the ability of recovering this specie despite they displayed elevated colistin MIC values.

In summary, the CHROMID® Colistin R agar represents a selective and specific method to perform surveillance studies to detect colistin-resistant *Enterobacterales* using both stool and swabs samples (with the exception of *Enterobacter* spp). The COLR is also an accurate and low-cost assay, affordable by clinical microbiology laboratories to carry out screening of this resistant mechanism in high-risk patients that, as other chromogenic media, can be easily integrated in the routine workflow.

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Conflict of interests

RC has participated in educational programs organized by bioMérieux. The other authors have no conflict of interests.

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