



Detection of Plasmid-Mediated Mobile Colistin Resistance Gene (*mcr-1*) in *Enterobacteriales* Isolates from a University Hospital

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Purpose: Colistin represents one of the last treatment options for infections caused by multi-drug resistant (MDR) *Enterobacteriales*. The emergence of a plasmid-mediated mobile colistin resistance-1 (*mcr-1*) gene has raised serious concerns about its potential dissemination among bacteria.

Methods: In this study, we evaluated the chromogenic medium, CHROMID® Colistin Resistance (COLR) agar, for the rapid detection of colistin-resistant *Enterobacteriales* using broth microdilution (BMD) as a reference method. We also attempted to detect *mcr-1*, *-2*, *-3*, *-4*, and *-5* genes, as well as the insertion sequence *IS_{AplI}* via polymerase chain reaction (PCR), followed by sequencing of *mcr* gene(s).

Results: Among the 100 studied *Enterobacteriales* isolates, 53% of them were colistin-resistant, with higher rate among *Klebsiella pneumoniae* (75%) as compared to *Escherichia coli* (44.4%). The COLR agar showed 83.2% sensitivity and 97.9% specificity for the detection of colistin resistance. Among colistin-resistant isolates, *mcr-1* gene was only detected in four (7.5%) *E. coli* isolates. The *IS_{AplI}* was not found among *mcr-1* positive isolates. Sequencing of *mcr-1* gene revealed nucleotide sequence homogeneity with the wild-type *mcr-1* gene in BLAST.

Conclusion: The COLR agar is a promising phenotypic method for the detection of colistin-resistant *Enterobacteriales*. Multiplex PCR followed by sequencing can be used for *mcr* genes' detection and characterization.

Keywords: colistin resistance, *Enterobacteriales*, *mcr* genes, CHROMID® COLR agar, multiplex PCR, sequencing

Introduction

The ability of *Enterobacteriales* to acquire mobile genetic elements carrying antibiotic resistance has facilitated the development of resistance to multiple antibiotics with subsequent spread of multi-drug resistant (MDR) members of *Enterobacteriales*.¹ Colistin resistance has frequently been reported in areas with high rates of carbapenem-resistant Gram-negative bacteria with subsequent widespread use of colistin in clinical practice.² Polymyxins have gained attention as a last resort antibiotic for the treatment of infections caused by MDR Gram-negative bacteria.³ They have been introduced to the antibiotic armamentarium since 1950s.⁴ Polymyxin B and polymyxin E (colistin) are used in clinical practice, with almost similar biological activity and differentiated by only a single amino acid.⁵ These cationic polypeptides target the outer membrane of Gram-negative

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bacteria,⁶ therefore destabilizing the lipopolysaccharide (LPS) and consequently increasing the membrane permeability, leading to leakage of the cytoplasmic contents and ultimately causing cell death.⁷

Various bacterial species are intrinsically resistant to colistin, whereas acquired resistance has been developed due to chromosomal mutations or acquisition of genes carried on plasmids.⁸ The plasmid-mediated mobile colistin resistance-1 (*mcr-1*) gene was first recognized in China.⁵ It encodes the enzyme phosphoethanolamine transferase that modifies the lipid A in the LPS of the bacterial outer membrane, thus decreasing the attachment of colistin, and hence inhibits cell lysis.⁹

Enterobacterales are the main host of *mcr-1* gene which was first located in *Escherichia coli* then spread to other nosocomial bacterial pathogens; such as *Klebsiella pneumoniae* to become a source of multiple outbreaks.¹⁰ Other Plasmid-mediated colistin resistance genes, including *mcr-2*, *-3*, *-4*, and *-5*, have been discovered in *Enterobacterales* strains recovered from human hosts.¹¹ The prevalence of *mcr-6*, *-7*, *-8*, *-9*, and *-10* genes in *Enterobacterales* is very rare.^{12,13} It has been shown that *mcr-1* sequences are sometimes associated with an upstream and/or downstream *ISApII* copy, an insertion sequence that may play a pivotal role in the mobilization of *mcr-1* gene.^{14,15} However, some *mcr-1* sequences lack the insertion sequence *ISApII*.¹⁶

Detection of colistin-resistant *Enterobacterales* is usually done by reliable phenotypic techniques, such as the broth microdilution (BMD) method. However, it is quite laborious, time-consuming, and difficult to interpret, and therefore not suitable for most clinical microbiology laboratories.¹⁷ Agar dilution is another reference method that can test several strains on the same plate. However, it is time-consuming, and the plates must be used within a week of preparation.⁶ Disk diffusion is a simple and inexpensive method. However, polymyxins show slow diffusion through the agar which results in small inhibition zones, limiting the predictive accuracy of this method. Therefore, it is not recommended for susceptibility testing of polymyxins.⁶ Currently, broth disk elution and agar dilution MIC methods are acceptable for testing colistin susceptibility among *Enterobacterales*, however these methods yield inaccurate results with *Acinetobacter* spp.¹⁸ Alternatively, chromogenic media can be used for screening of colistin resistance among *Enterobacterales* as they are rapid, accurate, and affordable.¹⁹ Meanwhile,

genotypic detection of *mcr* genes can be achieved by the gold standard molecular techniques.²⁰

The aim of the current study was to evaluate the usefulness of CHROMID[®] Colistin Resistance (COLR) agar as a screening method for the detection of colistin resistance among *Enterobacterales* as compared to the gold standard BMD method. We also attempted to detect the presence of *mcr-1*, *-2*, *-3*, *-4*, and *-5* genes via multiplex polymerase chain reaction (PCR), to be followed by sequencing of the *mcr-1* gene. Finally, the study aimed to evaluate the association of the insertion sequence *ISApII* with *mcr-1* gene, if present.

Materials and Methods

This cross-sectional study was conducted during the period from September 2018 through March 2020 and included a total of 100 *Enterobacterales* isolates (72 *E. coli* and 28 *K. pneumoniae*), obtained from the Strain Bank of the Medical Microbiology and Immunology Department, Faculty of Medicine, Cairo University, Egypt. The isolates were cultured on MacConkey's agar plates (Oxoid, UK) and incubated aerobically at 37°C for 24 hours. Although the isolates obtained from the Strain Bank were previously identified, identification was confirmed by conventional microbiological methods including colony morphology, Gram staining, sugar fermentation, and oxidase test. Further identification up to the species level was done by conventional methods including culture on triple sugar iron agar (TSI), motility indole ornithine (MIO), lysine iron agar (LIA), urease test, and citrate test.^{21,22} These isolates were selected on the basis of being MDR; they constitute 93% ESBL producers, 5% carbapenem-resistant isolates, and 2% AmpC producers. The isolates in the Strain Bank were previously identified as ESBL producers by double disc diffusion²³ and by conventional PCR.²⁴ Carbapenem-resistance and AmpC production were detected using the automated Vitek 2 system (bioMérieux, France). The isolates were preserved at -20°C from urine samples collected from hospitalized patients at Kasr Al-Ainy Hospital.²⁵ Informed consent was obtained from each participant. The study protocol was approved by the Ethical Committee of the Medical Microbiology and Immunology Department, Cairo University.

Detection of Colistin Resistance by COLR Agar Medium

Enterobacterales isolates were tested for colistin resistance using COLR medium (bioMérieux, France, cat no. 421170) according to the manufacturer's instructions.

Detection of Colistin Resistance Using the Broth Microdilution (BMD) Method

The colistin minimum inhibitory concentrations (MICs) of all *Enterobacterales* isolates were determined by the BMD method according to the Clinical and Laboratory Standard Institute (CLSI) guidelines. Colistin MICs of $\leq 2 \mu\text{g}/\text{mL}$ were interpreted as susceptible, whereas MICs of $\geq 4 \mu\text{g}/\text{mL}$ were interpreted as resistant.²³ The colistin-susceptible *E. coli* ATCC 25922 strain, with a colistin MIC susceptibility ranging from 0.25–2 $\mu\text{g}/\text{mL}$, was used as a quality control.

Genotypic Detection of Plasmid-Mediated *mcr*-1, 2, 3, 4, and 5 Genes

Genotypic analysis methods were carried out in the molecular biology unit at the Medical Biochemistry Department, Faculty of Medicine, Cairo University. Genomic DNA was extracted from fresh bacterial isolates using QIAamp DNA Mini Kit (Qiagen, Germany, cat. no. 51306) according to the manufacturer's instructions. Multiplex PCR was performed for the amplification of *mcr*-1, -2, -3, -4, and -5 genes using the primers as previously described.¹¹ A total of 35 PCR cycles were done as follows: 94°C for 30 seconds, 58°C for 90 seconds, and 72°C for 60 seconds. An initial denaturation step at 94°C for 2 minutes and a final extension step at 72°C for 10 minutes were performed. Detection of PCR products was done by 2% agarose gel electrophoresis stained with ethidium bromide and visualized under UV illumination using a Biometra T11 Gel documentation system (Biometra, Germany). Visual detection of the expected DNA bands at 320 bp, 715 bp, 929 bp, 1,116 bp, or 1,644 bp was indicative of a positive isolate harboring *mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4, or *mcr*-5 gene, respectively.¹¹

Detection of Insertion Sequence IS*ApI* in *mcr*-1 Positive Isolates

Amplification of IS*ApI* insertion sequence was done on the extracted DNA from *mcr*-1 positive isolates using the previously published primers: IS*ApI*-*mcr*-F TGGACATT GGAAGCCGATA and IS*ApI*-*mcr*-R GCCACAAGA ACAACGGACT.²⁶ Detection of the amplified PCR product IS*ApI* (707 bp length) was done using 2% agarose gel electrophoresis.

DNA Sequencing of *mcr*-1 Gene

The amplified PCR products from *mcr*-1 positive isolates were sent to GATC Biotech, Germany for DNA

sequencing. Initially, purification of the amplified PCR products was done using the GeneJET PCR Purification Kit (Thermo scientific, USA, cat. no. K0702) according to the manufacturer's instructions. Then after, genomic DNA sequencing was done by Sanger's method in the forward direction using the *mcr*-1 gene forward primer; as described before.¹¹ The obtained sequences were compared to known sequences deposited at the GenBank BLAST program, National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>).

Statistical Analysis

All statistical analyses were performed using the statistical package SPSS (Statistical Package for the Social Sciences) version 25. Mean, standard deviation, median, and inter-quartile range (IQR) were used for quantitative data, while frequency (count) and relative frequency (percentage) were used for categorical data. Comparisons between quantitative variables were done using the non-parametric Mann Whitney *U*-test. For evaluation of the chromogenic agar in comparison to the BMD method, standard diagnostic indices including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy were calculated. The chi-square (χ^2) test was used to compare categorical data, but the Exact test was used instead when the expected frequency was less than 5. A *p*-value of ≤ 0.05 was considered as statistically significant.

Results

The present study was carried out on 100 *Enterobacterales* isolates retrieved from 36 male and 64 female patients; their ages ranged from 2–91 years (mean=47±23 years). The isolates were identified as 72 (72%) *E. coli* and 28 (28%) *K. pneumoniae*.

Detection of Colistin Resistance Using COLR Agar

Cultivation of the *Enterobacterales* isolates on COLR agar after 4–5 hours incubation in brain heart infusion (BHI) broth containing 10 μg colistin disk revealed that 45/100 (45%) of the isolates showed a positive growth, indicating colistin resistance. Twenty-nine *E. coli* isolates (29/72, 40.3%) and 16 *K. pneumoniae* isolates (16/28, 57.1%) were colistin-resistant.

Detection of Colistin Resistance Among Enterobacteriales Isolates Using BMD

Method

The BMD method showed that out of the 100 studied *Enterobacteriales* isolates, 53 (53%) colistin-resistant isolates were found, among which, 32/72 (44.4%) of *E. coli* and 21/28 (75%) of *K. pneumoniae* isolates were colistin-resistant. Interestingly, all of the five carbapenem-resistant isolates included in the study were colistin-resistant. On the other hand, colistin resistance rate among both ESBL and AmpC producers was approximately 50%.

Correlation Between the COLR Agar and BMD Method

Considering the BMD method as the gold standard test for the detection of colistin resistance, growth of *Enterobacteriales* isolates on the COLR agar showed 83.2% sensitivity, 97.9% specificity, 97.8% positive predictive value (PPV), 83.6% negative predictive value (NPV), and 90% accuracy (Table 1). The value of Kappa (statistical measurement of agreement) indicates a significant agreement between the COLR agar and BMD method ($p < 0.001$).

The MIC values were significantly higher in colistin-resistant *Enterobacteriales* isolates grown on COLR agar (median=4; range=2–32) compared to those that failed to grow (median=2; range=1–4) ($p < 0.001$). Interestingly, the COLR agar had high sensitivity (100%) in the detection of colistin-resistant *Enterobacteriales* isolates with MIC values ≥ 8 , its sensitivity decreased to 75.7% in isolates with colistin MIC=4. Moreover, 97.8% (46/47) of colistin-susceptible isolates (MIC<4) did not grow on the COLR agar. The distribution of MIC values of colistin is shown in Figure 1.

Table 1 Correlation Between CHROMID® COLR Agar and BMD Method

		Broth Microdilution Method	
		Resistant n (%)	Sensitive n (%)
CHROMID® COLR agar	Positive growth (Resistant)	44 (83%)	1 (2.1%)
	Negative growth (Sensitive)	9 (17%)	46 (97.9%)
	Total	53 (100%)	47 (100%)

Detection of Plasmid-Mediated mcr-I, 2, 3, 4, and 5 Genes

We found that out of the 53 colistin-resistant isolates detected by BMD method, only four (7.5%) isolates were positive for the presence of *mcr-I* gene (Figure S1). All the *mcr-I* positive isolates were *E. coli*, representing 5.6% (4/72) of the total *E. coli* isolates. Meanwhile, all colistin-resistant isolates were tested negative for the presence of other *mcr* genes (*mcr-2*, 3, 4, and 5). Moreover, in the 47 colistin-susceptible isolates, none of the five *mcr* genes could be detected.

Colistin MICs of the four *mcr-I* positive isolates ranged between 4–16 $\mu\text{g/mL}$. We also demonstrated significantly higher colistin MICs in *mcr-I* positive isolates (median=8, IQR=6–12) compared to *mcr* negative isolates (median=4, IQR=2–4) ($p = 0.022$).

Detection of Insertion Sequence ISApII Among mcr-I Positive Isolates

Conventional PCR was done to detect the presence of ISApII among the four *mcr-I* positive *E. coli* isolates. However, ISApII could not be detected in any of them.

Sequencing of mcr-I Gene

The four *mcr-I* positive *E. coli* isolates were further sequenced by an ABI 3730 XL automated DNA sequencer. Three isolates matched the sequence of *mcr-I* gene when compared to the prototype sequence of the wild *mcr-I* gene in the BLAST system (Figure S2), while only one isolate failed to be read by the sequencer, probably due to product degradation during transportation. Comparing the three chromatograms with the *mcr-I* wild gene sequence in BLAST using multiple alignment confirmed the homogeneity of the nucleotide sequence for the *mcr-I* gene (Table S1).

Discussion

Massive and inappropriate use of antibiotics generates a selective pressure that is followed by rapid emergence and spread of MDR *Enterobacteriales*.²⁷ Colistin is considered one of the last lines of therapy that was used separately or in combination with other antibiotics to effectively treat serious infections caused by MDR pathogens.²⁸ In the present study, we found that out of 100 *Enterobacteriales* isolates, 53% were colistin-resistant, with a 44.4% (32/72) resistance rate among *E. coli* isolates and a 75% (21/28) resistance rate among *K. pneumoniae* isolates. Approximately similar rates of colistin resistance

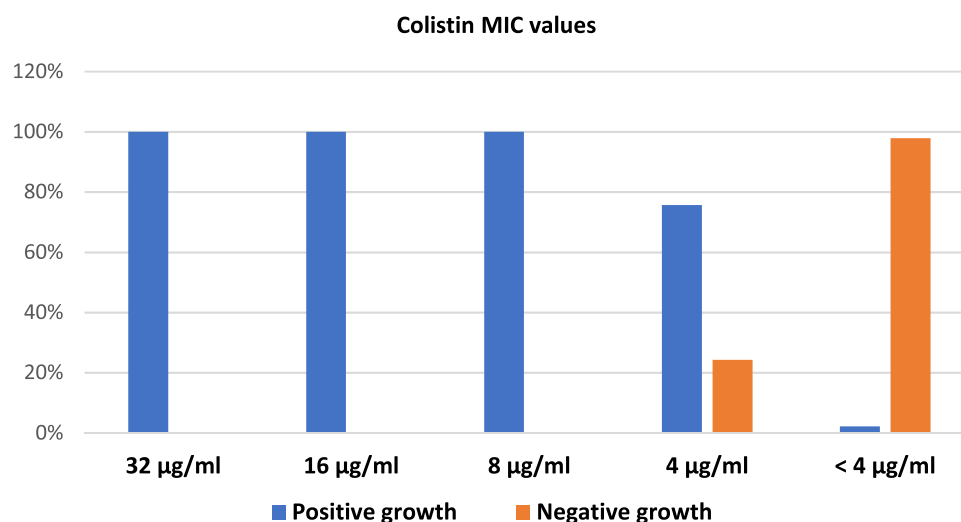


Figure 1 Colistin MIC values among *Enterobacteriales* in relation to the results of CHROMID® COLR agar.

Abbreviations: MIC, minimum inhibitory concentration; COLR, colistin resistance.

were reported by an earlier study conducted in France that detected a 53.1% resistance rate among *E. coli* and a 76% resistance rate among *K. pneumoniae* isolates, using the BMD method.²⁹ On the other hand, another study carried out in India demonstrated that out of 64 *Enterobacteriales* isolates tested for colistin MICs by BMD method, none of them were colistin-resistant.³⁰

The discrepancy in colistin resistance among different studies is explained by the diversity in the type of specimens, number of cases, the general condition of patients, geographical regions, various antibiotic policies, and compliance to the infection control measures. Meanwhile, the high rate of colistin resistance in the current study can be elucidated by the fact that the isolates were previously collected from hospitalized patients with urinary tract infection; where the resistance pattern is more common among hospitalized patients due to extensive antibiotic use, the presence of co-morbidities, and the existence of indwelling catheters.

In the present study, the COLR agar showed that 45 (45%) isolates were colistin-resistant, with MICs ranging from 2–32 µg/mL. Considering the BMD method as the gold standard for the detection of colistin resistance in the current study, the sensitivity, specificity, PPV, NPV, and accuracy of the COLR agar were 83.2%, 97.9%, 97.8%, 83.6%, and 90%, respectively, with 100% sensitivity in the detection of colistin-resistant *Enterobacteriales* isolates with MIC values ≥ 8 µg/mL. Failure of detection of low-level resistant isolates in the present study may be attributed to the high concentration (10 µg) of colistin used or other

constituents of the COLR agar that may have an antimicrobial effect. The efficacy of the COLR agar in the detection of colistin resistance among *Enterobacteriales* was in agreement with García-Fernández et al,¹⁹ who revealed that COLR agar has a sensitivity of 88% and a specificity of 100% when compared with the standard BMD method.

In the current study, the *mcr-1* gene was detected in four colistin-resistant *E. coli* isolates; 4% (4/100) of the total isolates, 7.5% (4/53) of colistin-resistant isolates, and 5.6% (4/72) of *E. coli* isolates. The remaining four *mcr* genes (*mcr-2*, 3, 4, and 5) were not detected in any of the studied isolates. All the four *mcr-1* positive isolates demonstrated significantly higher colistin MICs compared to *mcr-1* negative isolates ($p=0.022$). The inability to detect *mcr* genes among the remaining colistin-resistant isolates could be explained by either the presence of other plasmid or chromosomally mediated resistance mechanisms, extrusion of the drug by efflux pump, decreased permeability of bacterial cell membrane, or enzyme-mediated inactivation.

A closely similar rate was observed in a study done in Hong Kong³¹ which reported that out of 62 colistin-resistant *Enterobacteriales* isolates of human origin, 6.5% (4/62) of *E. coli* isolates were *mcr-1* positive. Similarly, a study conducted in Oman reported that out of 22 colistin-resistant *Enterobacteriales* isolates, a single (4.5%) *E. coli* isolate carrying *mcr-1* gene was detected, whereas none of them was proved to carry the *mcr-2* gene.³² A lower incidence rate of the *mcr-1* gene was detected by an Egyptian study³³ which revealed that among 241 Gram-

negative isolates collected from different hospitals, *mcr-1* was detected in only one *E. coli* (0.4%) isolated from sputum of a patient with bacteremia with no history of traveling abroad. A higher incidence rate of the *mcr-1* gene was reported by Rebelo et al,¹¹ who found that out of 42 *E. coli* isolates of animal origin, 14 (33.3%) isolates carried *mcr* genes and were distributed as follows: nine (21.4%) *E. coli* isolates harbored *mcr-1* gene, one (2.3%) isolate harbored *mcr-3*, and one (2.3%) isolate harbored *mcr-4*. Co-occurrence of *mcr-1* and *mcr-3* was observed in two (4.7%, 2/42) *E. coli* isolates, whereas a single (2.3%, 1/42) *E. coli* isolate harbored both *mcr-1* and *mcr-4* genes. The high prevalence of *mcr-1* in animal isolates compared with human clinical isolates worldwide indicates that animals and their products are possible sources of *mcr-1* in humans. Moreover, the misuse of colistin in agriculture and the poultry industry may be the main cause of the high incidence of *mcr-1* in bacteria isolated from animals and animal products.³⁴

The mobile colistin resistance-1 (*mcr*) gene is more commonly isolated from *E. coli* isolates than from *K. pneumoniae*, a finding that is supported by our study as well as by previous studies.^{11,19,34,35} Additionally, a previous Egyptian study found that the eight colistin-resistant *K. pneumoniae* isolates were negative for *mcr-1* gene.¹⁷ Another study showed that two *E. coli* isolates and two *K. pneumoniae* isolates were colistin-resistant and none of them was positive for *mcr-1* gene.³⁶

In the current study, sequencing of the *mcr-1* gene of the four positive isolates followed by multiple alignments of the chromatograms revealed homogeneity of the nucleotide sequence for *mcr-1* among three of the isolates when compared to the NCBI database. Consistent with our results, Johura et al³⁷ confirmed the homogeneity of the nucleotide sequence of *mcr-1* in 13 *E. coli* strains. Moreover, they reported that their *E. coli* strains were heterogenous, as confirmed by pulsed-field gel electrophoresis, suggesting horizontal transmission of colistin resistance. It is worth mentioning that several variants of *mcr-1* have been identified from *E. coli* strains isolated from animal, sewage, or human urinary tract samples in various countries.^{38–40}

In the present study, we could not detect the *ISAp11* in any of the four *mcr-1* positive isolates. This finding was consistent with a study done in Oman by Mohsin et al,³² who demonstrated the lack of *ISAp11* in the genetic surrounding of the *mcr-1* gene. Additionally, a study from the Czech Republic confirmed the absence of *ISAp11* element

upstream of *mcr-1* gene located on IncI2 plasmids.⁴¹ On the contrary, Snesrud et al¹⁶ found *ISAp11* in 23 (30%) out of 77 *mcr-1* positive isolates. However, Wang et al³⁵ did not find the *ISAp11* in 56.9% (260/457) of the *mcr-1* positive isolates, indicating that the *mcr-1* transposon may have been completely stabilized in their genomic background. Failure of the detection of the *ISAp11* in our study could be attributed to the small number of *mcr-1* positive isolates. Furthermore, the current study did not include other members of *Enterobacterales* which may have affected the actual prevalence of *mcr* genes among *Enterobacterales*.

The emergence of plasmid-mediated colistin resistance in *Enterobacterales* is currently a crucial issue owing to the high potential of their dissemination in clinical settings. It is important to critically develop proper guidelines against the use of this last-line treatment option, so that the spread of resistance can be limited. For better understanding of the actual status of the global colistin-resistance, development and implementation of rapid procedures to detect colistin resistance in clinical microbiology laboratories should be enhanced.

This study has two limitations. First, our investigation was restricted to *E. coli* and *K. pneumoniae* isolates obtained from urine samples only. However, *E. coli* and *K. pneumoniae* are the most common members of *Enterobacterales* recovered from clinical specimens. Moreover, urinary tract infections are the most common type of infections caused by *Enterobacterales* that are encountered in hospitals.⁴² Future studies investigating other members of *Enterobacterales* and different types of samples are required. Second, there is a need for overseas transportation of the isolates for sequencing of PCR products which leads to degradation of one of the isolate sequences and subsequent failure of sequence reading.

Conclusion

In summary, the chromogenic COLR agar medium offers a simple, easy, and inexpensive method for rapid detection of colistin-resistant *Enterobacterales*. However, it is not recommended as a screening test due to its inadequate sensitivity and accuracy. The BMD method remains the more sensitive standard test for colistin resistance. Multiplex PCR is suggested for the determination of the presence of *mcr* genes in laboratories with limited resources. However, its value for the detection of *mcr* genes is debatable due to the low prevalence of *mcr* genes in our hospital. Therefore, further studies using

larger sample size are recommended to elucidate the role of *mcr* genes in colistin resistance as well as to confirm or exclude the association between *mcr-1* gene and the insertion sequence IS*AplI*. In addition, future investigations will be necessary to detect other mechanisms of colistin resistance among *Enterobacterales* isolates negative for *mcr* genes, such as *mgrB* genes inactivation or the presence of insertion sequence disrupting this chromosomal gene.

Ethics Approval and Informed Consent

There is no ethical concern in this study, and this experiment was approved by the Ethical Committee of the Medical Microbiology and Immunology Department, Cairo University. Written informed consent was obtained from patients in accordance with the Declaration of Helsinki.

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Disclosure

The authors report no conflicts of interest in this work.

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