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Polymyxin resistance in Gram negatives: identification and screening

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Multidrug resistant (MDR) bacteria are of global concern, notably with the spread of carbapenemase producers. This is one of the reason why polymyxins such as colistin, that are old antibiotics, regain popularity as a last resort treatment to face the worddwide emergence of MDR Gram-negative bacteria. Colistin is a polycationic and bactericidal drug that targets the lipid A moiety of the lipopolysaccharide (LPS) moving its cationic charges and subsequently leading to cell wall lysis and bacterial death. The increasing use of colistin in animals since years but also nowadays in humans has led to emerging resistance to that antibiotic. Until recently, all mechanisms described involved chromosomal genes encoding proteins mainly related to regulatory systems of the LPS biosynthesis, such as the PmrAB and PhoPQ two-components systems in Enterobacteriales. In November 2015, the first plasmid-mediated colistin resistance gene, namely *mcr-1* that encodes a phosphoethanolamine transferase, was identified in enteobacterial isolates. Then *mcr*-like genes, namely *mcr-2* to *mcr-8*, have been identified. The *mcr* genes have been reported worldwide mostly in animal where the selection pressure by using polymyxin is high, and among environmental isolates. Although they are reported in clinical strains from humans in Switzerland, their spread among human pathogens in this country remains rare according to the results of recent surveys.
Resistance to colistin in human *E. coli* is mostly related to non-MCR and chromosome-encoded mechanisms. We are currently observing a worldwide spread of colistin-resistant strains (with a chromosome-encoded mechanism of resistance) among carbapenemase producers (KPC, OXA-48, NDM). Such strains have been identified in Switzerland resulting from an importation process (mostly from Italy) but also from direct selection in Switzerland following the treatment of patients with colistin.

A particular concern is that the majority of the MCR producers may be difficult to detect since they confer low MIC values (around 4 mg/L) while chromosomally-encoded resistance mechanisms confer much higher levels of resistance (usually > 64 mg/L).
So far resistance to polymyxins in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, is only chromosome-encoded with the exception of few plasmid-encoded MCR-4 in that latter species. They are mostly related to changes in LPS structure.

A-Infection

Testing shall be performed from isolated colonies

1- Identification

- MALDI-TOF
- or at the species level: Vitek (bioMérieux) or Phoenix (PMIC/ID Panels) for example

2- Susceptibility testing

2.1 MIC testing remains the gold standard for determining resistance to polymyxins. It must always be determined in liquid medium in cation-adjusted with a final composition of 20-25 mg/L of calcium and 10-12.5 mg/L of magnesium. Colistin sulphate must be used in untreated polystyrene plates without addition of any surfactant. One shall pay attention to the purity of the colistin (a natural product) that varies from one furnisher to another. We are using colistin tablets (ref. TAB/CO 0,8, Mast Group) as primary source of antibiotic. The E-test technique is not recommended for detecting colistin resistance due to the poor diffusion of colistin in solid agar.

The MIC breakpoints of colistin for Enterobacteriales, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are indicated below (EUCAST 2019 guidelines v.9.0)/
:

	MIC breakpoints (mg/L)	
	S ≤	**R >**
Colistin	**2**	**2**

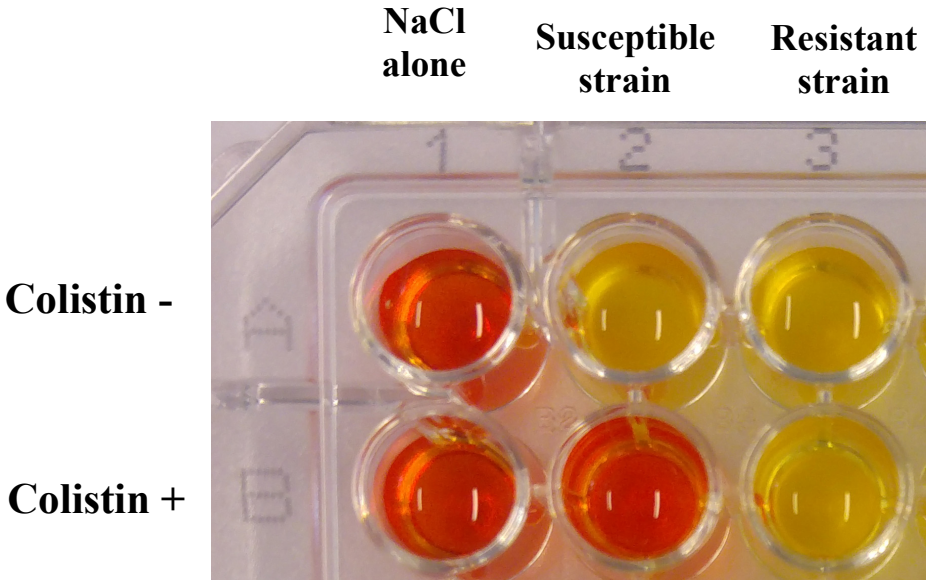
Results of susceptibility testing are obtained in 18 h. Since the MCR proteins are zinc-dependant enzymes, several studies reported the possibility to inhibit MCR activity and therefore to decrease the MICs of colistin by adding dipicolinic acid and EDTA. This inbibition may work for detecting MCR producers but only in *E. coli* and only with dipicolinic acid.

2.2. MIC determination is proposed by several companies. Those commercial devices are UMIC Colistine (Biocentric, France), MIC Strip Colistin (MERLIN, Germany), SensiTest Colistin (Liofilchem,Italy), Sensititre system (Thermo Fisher Scientific USA) and some automated systems. The UMIC Colistine consists of unitary tests containing dehydrated concentrations of colistin. It has been reported as reliable. The MIC strip colistin is based on the same principle. The Sensitest Colistin based also on the same principle consists of compact panel of 4 tests. The Sensitire system includes different antibiotics on 96-well trays with a customizable plate layout. The automated systems were developed to shorten results timeframes by increasing sensitivity, and also to avoid manipulation bias with incubation and real-time reading. However, they cannot give a real MIC value. The manufacturers are MicroScan Walkaway (Beckman Coulter, USA), Vitek (bioMérieux, France) and BD Phoenix (Becton Dickinson, France). Their performance are variable in particular for detecting resistance to polymyxins in non fermenters. With the exception of the Sensitest and the Sensititre methods, very major errors may occur with reports of false sensitivity for MCR producers, those latter often exhibiting low-level colistin resistance.

2.3. Rapid susceptibility testing; qualitative detection of colistin resistance

Rapid susceptibility tests for detection of colistin resistance have been developed for Enterobacteriales, *P. aeruginosa* and *A. baumannii* to provide faster results, obtained in 2-4 h. The Rapid Polymyxin NP test is based on a change of pH according to glucose metabolism related to bacterial growth. Detection of colistin resistance is made according to growth in presence of a given concentration of colistin. The test has been developed from bacterial cultures and can also be used directly using positive blood cultures. Compared to the broth microdilution susceptibility method, there is excellent agreement for detecting MCR-1 and MCR-2 producers. The Rapid Polymyxin NP test has also a good sensitivity to detect naturally-occurring polymyxin resistance mechanisms such as those observed in *Hafnia alvei*. However it fails to detect several colistin-resistant *Enterobacter* sp. isolates probably due to the frequent colistin heteroresistance pattern observed in this species. This test has been commercialized under the trade name of Rapid Polymyxin NP test (ELITECH Microbiology).

A rapid test was developed also for identification of polymyxin resistance in non fermenters (Reas Polymyxin *Acinetobacter*/*Pseudomonas* NP). This test detects viable cells after growth in a medium containing a defined concentration of colistin. The principle of this test is based on the visual detection of the reduction of the resazurin reagent, a viability colorant, as observed by its color change (blue to purple or pink). Its evaluation was performed by using colistin-resistant and colistin-susceptible *A. baumannii* and *P. aeruginosa* isolates. Sensitivity and specificity were found to be 100% and 95%, respectively, by comparison with the standard broth microdilution method. The Rapid Resa Polymyxin *Acinetobacter*/*Pseudomonas* NP test is inexpensive, easy to perform, highly sensitive and specific, and can be completed in 4 h. A derivative of the Rapid Polymyxin NP test has been developed (ELITECH Microbiology) that also shows good performances for detecting colistin resistance in those species.



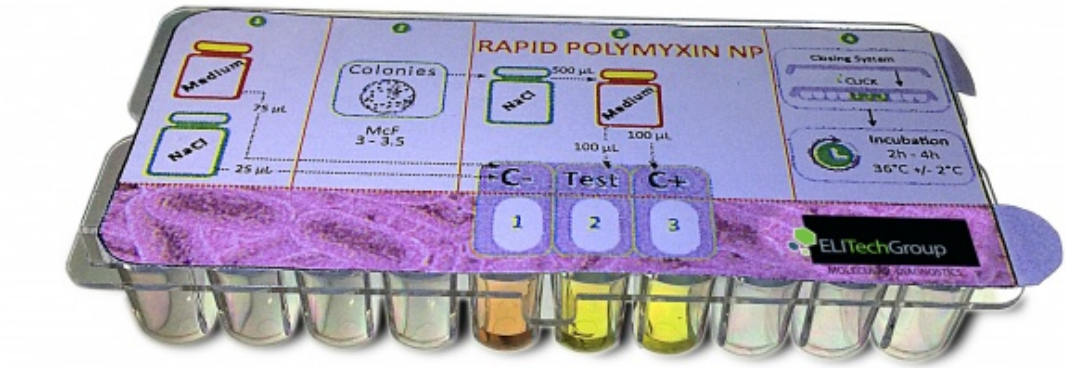


Figure 1. Rapid Polymyxin NP for detecting colistin resistance in Enterobacteriales. Note the lack of growth of colistin-susceptible strain in presence of colistin.

A MALDI-TOF technique (MALDIxin test) has been developed to detect chromosome and plasmid-mediated resistance in Enterobacteriaceae. It detects this resistance trait in 15 min. However possessing a MALDI-TOF machine is mandatory and the technique has to be settled in the lab.

3- Immunological detection of MCR producers. Lateral flow techniques have been commercialized for detecting MCR-1 producers such as that from NG Biotech (France). The test is rapid (15 min), sensitive and specific. It does not need any additional equipment for the reading.

Figure 2. Lateral-flow techniques for detecting MCR-1 producer

4- Molecular identification of polymyxin resistance. These methods are complementary to the phenotypic techniques. The main mutations for enterobacteriales are located on genes coding the two-component systems PmrA/PmrB and PhoP/PhoQ. Mutations in the negative feedback regulator of PhoPQ is the main mechanism of resistance to polymyxins in *K. pneumoniae*. Gene amplification and sequencing takes usually at least 3 days. Primers have been developed to identify the *mcr* genes and multiplex PCR genes for identification of those genes.
Real-time PCR techniques have been also developed for detection of *mcr-1*/*mcr-2* genes with excellent sensibility and specificity. Semi-automated PCR-based detection of *mcr* genes have been developed such as the CT103XL® microarray and the Eazyplex® (Amplex Biosystems, Germany) with good sensibility and specificity but remained not cost-effective.

Whole genome sequencing (WGS) may also be used, in particular for detecting unknown mechanisms of resistance to polymxyins. WGS so far can only be performed by expert laboratories and remains neither cost effective nor rapid.

We recommend therefore the following strategy for detection of polymyxin resistance;

1- Bacterial identification
2- Rapid Polymyxin NP test (and in the special case of detection of MCR- only, lateral flow technique)
3- Confirmation of resistance by performing susceptibility testing
4- PCR and sequencing of *mcr* genes or defined chromosomal genes to precisely determine the molecular bases of resistance (as determined by the NARA).

B - Screening (colonization)

1- Which patients ?

No recommendation consensus has been established for screening patients carrying colistin-resistant Gram-negative isolates. However, we recommend to screen contact patients of colonized or infected patient with a colistin- and carbapenem-resistant enterobacterial strain, or colistin-resistant and multidrug-resistant *P. aeruginosa* or *A. baumannii*.

2- Which samples ?

- Stools
- Rectal swabs of good quality
Repeated screening of samples may be recommended if patients are treated with antibiotics (false-negatives results) and for high-risk patients.

3- Which screening medium ?

- Several screening media have been developed for screening. All of them contain at least a selective concentration of colistin. The first screening medium that had been developed is the SuperPolymyxin medium subsequently commercialized by ELITECH Microbiology. It has the advantage of facilitating the visualization of *E. coli* strains (metallic green reflect) because its composition (EMB agar). It detects fermenters and

non-fermenters and strains displaying low-level resistance colistin. The CHROM agar COL-ASPE medium present the advantage to be chromogenic with the capacity to differentiate colistin-resistant non fermentative Gram-negative strains as well as Enterobacteriales. Recently, bioMérieux has also commercialized a screening medium. CHROMID R colistin R which detects colistin-resistant enterobacteriales.

- We showed very recently that recovery of colistin-resistant enterobacteriales may be enhanced by a pre-culture step (18 h) of stools in a broth containing 1 mg/L of colistin (unpublished data).

4- Recommendations

- Non outbreak situation (Fig 3); direct plating of rectal swabs or stools on chromogenic selective media.
- Outbreak situation (Fig. 4)
  - Direct plating on chromogenic selective media and/or use of molecular techniques if available
  - Concomitant cultures of the samples in an enrichment broth containing 1 mg/L of colistin.

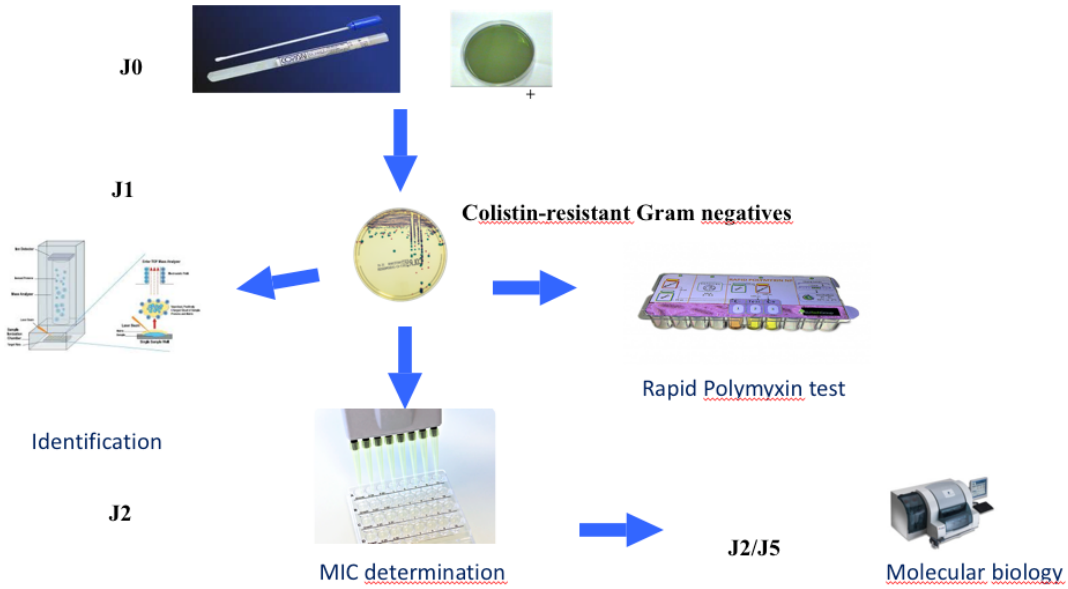


Figure 3. Detection of carriage of colistin-resistant Gram negatives (in the absence of an outbreak context)

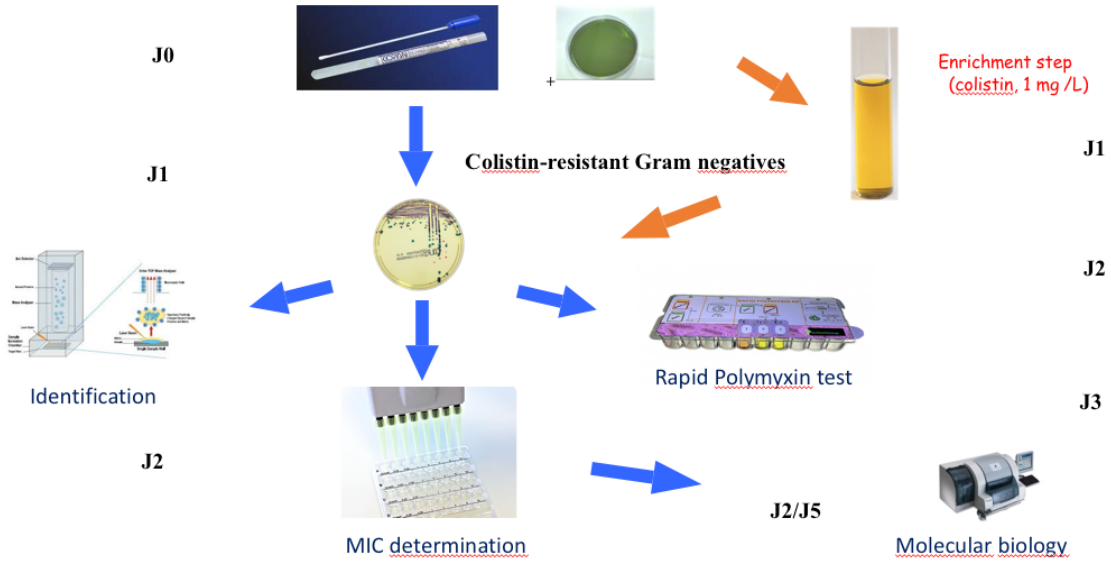


Figure 4. Detection of carriage of colistin-resistant Gram negatives (in the presence of an outbreak context)

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