Report on stability testing of isolates harbouring *mcr-1, mcr-2, mcr-3, mcr-4, mcr-5* and variants

Background

Antimicrobial susceptibility testing (AST) for colistin presents difficulties, which lead to lack of uniform results between laboratories, or in the same laboratory but at different times. The main justifications for the variability in AST results are the uneven diffusion of the antimicrobial molecule in culture media, the interaction with ions present in the media and the adsorption of the chemical in the surface of certain laboratory materials[1]. Currently, international standards (EUCAST and CLSI) recommend Minimum Inhibitory Concentration (MIC) by broth microdilution method as the only method for colistin susceptibility testing[2-4]. However, even when performing the tests according to the guidelines, laboratories still find a lack of reproducibility in the results. One possible explanation for MIC changes along time and space would be the variation of gene expression in bacterial samples, resulting from gene silencing[5] or plasmid loss[6] due to the lack of selective pressure, leading to a decrease in recorded MIC values. The aim of this study was to assess the MIC variation in samples with different genetic determinants for colistin resistance subjected to different incubation and storage conditions and to evaluate whether such variation was dependent on the genetic profile of the isolates.

Materials and methods

Ten bacterial isolates with different genetic determinants for colistin resistance were selected (Table 1). Pure fresh bacterial colonies of each of the ten isolates were selected from Blood Agar plates and used to perform MIC by broth microdilution method according to CLSI standards. The pure colonies were also used to inoculate three agar plates by multiple inoculation: one Blood Agar plate, one Biomérieux Chromid Colistin R Agar plate and one Cation-Adjusted Mueller Hinton agar plate with 2 mg/L of colistin (prepared in-house). Plates were incubated overnight and stored at 4° C for one week. After one week each of the ten inoculated isolates, from each of the three plates, were used to prepare new plates of the same type by multiple inoculation or manual re-streaking. The plates were incubated overnight and kept at 4° C for one week. The procedure was repeated three more times, corresponding to a total of four passages in antibiotic-containing or antibiotic-free media (Figure 1). After the last passage and overnight incubation MIC testing by broth microdilution was repeated.

Table 1. Bacterial isolates selected for testing

Isolate	Country	Host	Source	Resistance gene	Gene location
E. coli 2012-60-1176-27	Denmark	Chicken	Meat	*mcr-1*	ND
E. coli KP37	Belgium	Pig	Faeces	*mcr-2*	IncX4 plasmid
E. coli 2013-SQ352	Denmark	Unknown	Sewage	*mcr-3*	ND
E. coli 15-AB01299_0	Germany	Pig	Caecum	*mcr-4.2* (Q331R)	ND
Salmonella Paratyphi B dTa+ 13-SA01718	Germany	Chicken	Meat	*mcr-5*	ColE plasmid
Salmonella 4,12:i:-15Q003631	France	Pig	Carcass	*mcr-1*	ND
E. coli 15056414J9PUD1	Italy	Pig	Animal	*mcr-1.13*	ND
E. coli 15F001211	France	Calf	Animal	*mcr-3.2* (T488I)	ND
Salmonella Infantis 15Q004074	France	Calf	Carcass	*mcr-4.2* (Q331R)	ND
Salmonella Kedougou 151570	Spain	Pig	Carcass	*mcr-4.3* (V236F)	ND

ND – Not determined

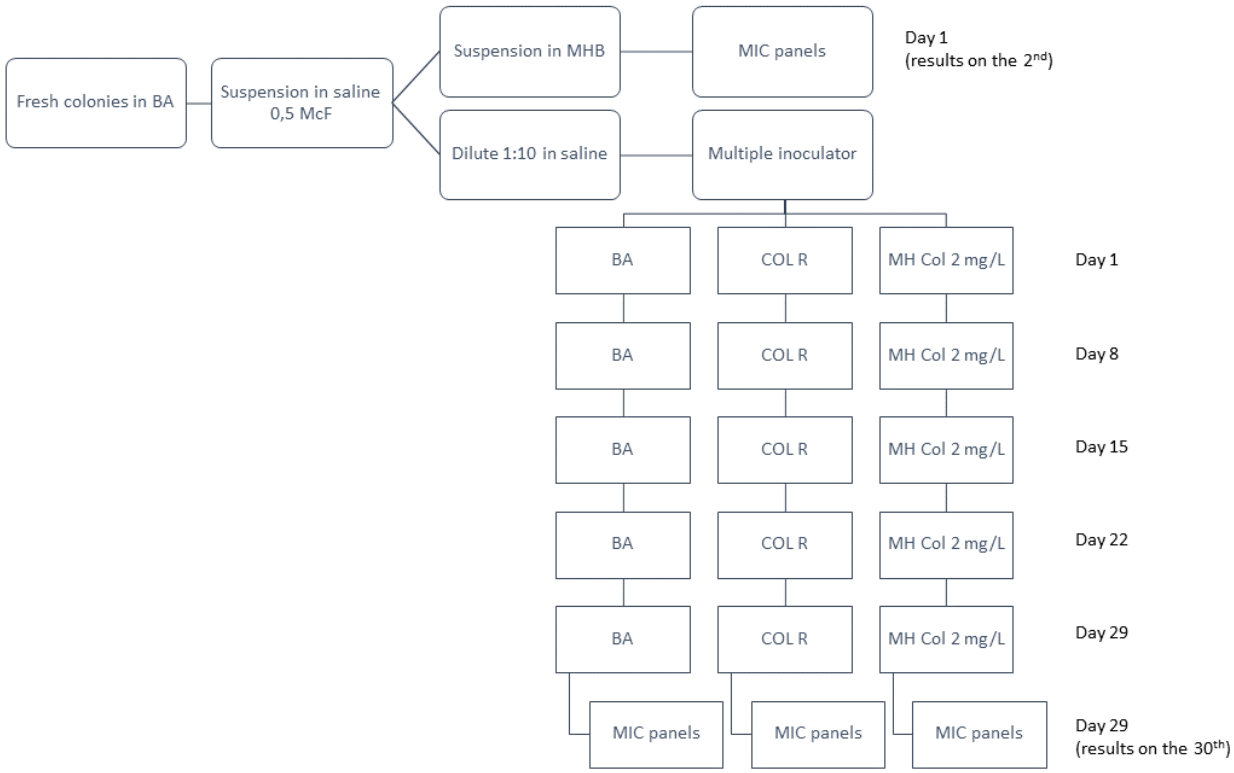

Figure 1. Schematic representation of laboratory procedure performed in this experience.

Results and discussion

No significant changes were observed in MIC values as no variation over one dilution was detected, and as such the results can be considered reproducible and constant according to ISO Standard 207762-1[7] (Table 2). Six isolates maintained a MIC of 4 mg/L regardless of antibiotic exposure. One isolate (*E. coli* 2013-SQ352) presented an apparent increase in MIC from 2 mg/L to 4 mg/L after one month in antibiotic-free media, while presenting MIC values of 2 mg/L or 4 mg/L in colistin-containing media. As such, it is believed the difference in MIC values is due to slight variations in media composition or laboratory procedure and not a result of altered mechanisms of gene expression or plasmid replication. The fact that this isolate has a colistin resistance gene but yielded a MIC of 2 mg/L on two occasions further corroborates the hypothesis that MIC results for colistin are slightly variable across time, and alteration is not due to biological changes. One isolate (*E. coli* 15-AB01299_0) appeared to suffer an increase in MIC values when exposed to the antibiotic, however the increase was only observed for one of the two agar plates with colistin. It is then believed that the increase is not significant but a result of differences in laboratory conditions, supported by again observing an initial MIC of 2 mg/L. One isolate (*E. coli* 15056414J9PUD1) presents a MIC of 2 mg/L while not exposed to colistin and shows an increase to 4 mg/L after one month in each of the colistin-containing agar plates. The result is not considered significant because two isolates with the same resistance gene (*mcr-1*, in isolates *E. coli* 2012-60-1176-27 and *Salmonella* 4,12:i:-15Q003631) do not show the same variation. One isolate (*Salmonella* Kedougou 151570) appears to show a decrease in MIC in all media from 4 mg/L to 2 mg/L, which is not concordant to the results observed in other isolates containing the same resistance gene (*mcr-4* in *E. coli* 15-AB01299_0 and *Salmonella* Infantis 15Q004074).

Table 2. MIC results for tested isolates

Isolate ID	**Colistin MIC (mg/L)**			
	Day 1 - BA[a]	**Day 29 - BA[b]**	**Day 29 - MH[c]**	**Day 29 - COL R[d]**
E. coli 2012-60-1176-27	4	4	4	4
E. coli KP37	4	4	4	4

E. coli 2013-SQ352	2	4	2	4
E. coli 15-AB01299_0	2	2	4	2
Salmonella Paratyphi B dTa+ 13-SA01718	4	4	4	4
Salmonella 4,12:i:-15Q003631	4	4	4	4
E. coli 15056414J9PUD1	2	2	4	4
E. coli 15F001211	4	4	4	4
Salmonella Infantis 15Q004074	4	4	4	4
Salmonella Kedougou 151570	4	2	2	2

[a] MIC results registered at the beginning of the experience, from bacteria grown in Blood Agar plates
[b] MIC results registered at the end of the experience, from bacteria kept in Blood Agar plates for one month
[c] MIC results registered at the end of the experience, from bacteria kept in Cation-Adjusted Mueller Hinton agar plates with 2 mg/L of colistin (prepared in-house) for one month
[d] MIC results registered at the end of the experience, from bacteria kept in Biomérieux Chromid Colistin R Agar plates for one month

Conclusion

Colistin MIC results do not seem to be influenced by storage of samples over time, nor by presence or absence of selective pressure by use of colistin or colistin-free agar media, respectively. Results do not vary according to *mcr*-gene and respective plasmid scaffold. It can be concluded that *mcr-1, mcr-2, mcr-3, mcr-4* and *mcr-5* genes are stable over time and that modifications of gene expression or plasmid loss are not responsible for variation in MIC results. It is also of note that colistin-resistant isolates can be misidentified as susceptible if only one MIC result is considered for classification, and it is recommended that antimicrobial susceptibility testing is repeated at least two times and preferably complemented by molecular biology or sequencing methods.
Limitations of this study are a lack of negative and positive controls, in particular colistin-susceptible strains to guarantee the selectivity of agar media and colistin-resistant strains with different mechanisms of resistance, such as chromosomal point mutations in the *pmrA/pmrB* system. As a complement to MIC testing, PCR could also be performed at the beginning and end of the experience to confirm the presence of the resistance genes. A larger collection of isolates could be tested to allow for statistical analysis of results. Plasmid characterization could be performed to evaluate if different plasmid types harboring the same *mcr* gene yield different results.

References

1. Poirel L, Jayol A, Nordmann P. Polymyxins: Antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. Clin Microbiol Rev. 2017;30(2):557-96. https://doi.org/10.1128/CMR.00064-16 PMID: 28275006

2. European Committee on Antimicrobial Susceptibility Testing (EUCAST). EUCAST warnings concerning antimicrobial susceptibility testing products or procedures. Växjö: EUCAST; Jun 2017. Available from: http://www.eucast.org/ ast_of_bacteria/warnings/

3. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Recommendations for MIC determination of colistin (polymyxin E). As recommended by the joint CLSIEUCAST Polymyxin Breakpoints Working Group. Växjö: EUCAST; Jun 2016. Available from: http://www.eucast.org/ fileadmin/src/media/PDFs/EUCAST_files/General_documents/ Recommendations_for_MIC_determination_of_colistin_ March_2016.pdf

4. Clinical and Laboratory Standards Institute (CLSI) Subcommittee on Antimicrobial Susceptibility Testing. CLSI AST News Update Volume 1, Issue 2. Philadelphia: CLSI; Dec 2016. Available from: https://clsi.org/media/1700/clsi-newswinter-2016.pdf

5. Enne VI, Delsol AA, Roe JM, Bennett PM. Evidence of Antibiotic Resistance Gene Silencing in Escherichia coli. Antimicrob Agents Chemother. 2006;50(9):3003-3010. doi:10.1128/AAC.00137-06.

6. Subbiah M, Top EM, Shah DH, Call DR. Selection Pressure Required for Long-Term Persistence of blaCMY-2-Positive IncA/C Plasmids. Appl Environ Microbiol. 2011;77(13):4486-4493. doi:10.1128/AEM.02788-10.

7. International Organization for Standardization (ISO). ISO International Standard ISO 20776-1:2006(E) – Clinical laboratory testing an *in* vitro diagnostic test systems – Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices – Part 1: Reference method for testing the *in vitro* activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases. Geneva: ISO; November 2006.