Journal of Applied Biosciences 156: 16132 - 16138

ISSN 1997-5902

Phenotypic characterization of the resistance of *Salmonella – Shigella* isolates to colistin and detection of mcr1/2 genes

Rolande MABIKA MABIKA[1], Franck MOUNIOKO[2], Loïs Wenceslas MBOUMBA[1], Alain SOUZA[1], Jean Fabrice YALA[*1]

[1]Laboratoire de Biologie Moléculaire et Cellulaire, équipe de Microbiologie (LABMC), Unité de recherche Agrobiologie, Université des Sciences et Techniques de Masuku (USTM), BP 067 Franceville, Gabon.

[2]Laboratoire d'Écologie Vectorielle, LEV, Institut de Recherche en Ecologie Tropicale (IRET), BP 13354, Libreville, Gabon

***Corresponding Author :** Jean Fabrice YALA

Laboratoire de Biologie Moléculaire et Cellulaire ; Équipe de Microbiologie, Université des Sciences et Techniques de Masuku (USTM), BP 067 Franceville, Gabon. Correspondent E. mail: (yalalajeanfabrice@gmail.com) Tel: +241 66.18.84.88

Original submitted in on 15th October 2020. Published online at www.m.elewa.org/journals/ on 31st December 2020 **https://doi.org/10.35759/JABs.156.6**

ABSTRACT

*Objective***:** Colistin is one of the latest line of therapeutics used in the management of infections due to multi-resistant Gram-negative bacteria. The current emergence of colistin resistance, in particular through the mediation of plasmid resistance genes (mcr1 and mcr2) in intestinal bacteria is a worldwide concern. The objective of this study is to evaluate the sensitivity of *Salmonella* and *Shigella* strains to colistin and the detection of *mcr*1 and *mcr*2 genes within these strains.

Methodology and Results: The colistin sensitivity profile of 30 *Salmonella* strains and 5 *Shigella* strains was determined using the Minimum Inhibitory Concentrations in liquid medium of Mueller Hinton and the results were interpreted in accordance with the standards of the European Committee on Antimicrobial Susceptibility Testing Epidemiological cut-off 2020 version 10.0. Finally, the mcr1 and mcr2 genes were detected by a conventional PCR. Overall, a phenotypic resistance rate of 20% was recorded for *Salmonella-Shigella* pathogens, with a frequency of 17.1% for *Salmonella* and 2.9% for *Shigella*. Molecular screening of these isolates revealed a lack of detection of the *mcr*1 and *mcr*2 genes in their genetic heritage.

Conclusion and application of results: this study shows that Salmonella and Shigella strains are resistant to colistin, however the mcr 1 and 2 genes have not been amplified. To this end, the rational use of colistin must be applied in the human and animal field in order to curb the increase and spread of resistance to this molecule.

Keywords: Colistin, Gabon, mcr, resistance, *Salmonella-Shigella*

INTRODUCTION

Antimicrobial resistance is a topic of particular interest due to the widespread of serious infectious caused by enterobacteria such as, *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter*, *Serratia*, *Proteus*, *Morganella* and *Yersinia* (Dortetet al,, 2010). Among these *enterobacteriaceae* infections, *Escherichia*, *Salmonella* and *Shigella* cause severe forms of the digestive tract (e.g., gastroenteric) (Ayalu A *et al.,* 2011; Lamberti *et al.,* 2014). In addition, *Salmonella* is responsible for over 200 million cases of diarrhoea worldwide and over 3 million deaths. Whereas *Shigella* is responsible for over 650,000 deaths (Ayalu A *et al.,* 2011). In this context, both *Salmonella* and *Shigella* represent a real worldwide concern, particularly in developing countries (Mohammadmahdi *et al.,* 2020). β-lactams are the first-line molecules in the treatment of enterobacteriaceae infections due to their low toxicity and bactericidal potency (Robin, Gibold and Bonnet, 2012). However, the massive use of antibiotics has largely contributed to the emergence of enterobacteria resistance. In addition, several strains of enterobacteriaceae family are reported to have an extended spectrum of antibiotic resistance, in part due to the acquisition of genes of resistance and by their own resistance (Nordmann *et al.,* 2012). Moreover, the production of β-lactam inactivating enzymes (β-lactamases) contributed to the emergence and expansion of extended-spectrum β-lactamase producing enterobacteriaceae (EBLSE) or carbapenemase that are known as multi-strain resistant (Cattoir, 2013; Doit, 2015). This multi-resistance has been also reported recently from *Shigella* isolates (Mohammadmahdi *et al.,* 2020). The widespread of carbapenemase-producing *Enterobacteriaceae* limits the therapeutic approaches against serious infections caused by enterobacteria strains. This phenomenon is responsible of the significant reduction in the effectiveness of new molecules available (Cattoir, 2013). To face this problem, colistin antibiotic has been proposed as the last-resort therapy against Enterobacteriaceae Producing Carbapenemase (ECP) infections (L. Dortet *et al.,* 2016; Yi *et al.,* 2019). However, the great adaptability of these bacteria also favoured the selection of colistin-resistant mutants (Zhang *et al.,* 2018). Globally, the frequency of resistance to colistin remains relatively low although its impact is increasingly reported (Kim *et al.,* 2019). The most common enterobacteria strains are from *E. coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae* (Mezghani Maalej *et al.,* 2012). Additionally, *E. coli* resistance rates were found between 0.1% to 2% in North America, 0.3% in Europe (Gales, Jones and Sader, 2011; L Dortet *et al.,* 2016). Strains of *Klebsiella* spp showed rates of 1.8%, 1.5%, 2.1% and 0.8% respectively for North America, Europe, Latin America and Asia Pacific (Gales, Jones and Sader, 2011). In African areas, the few studies carried out in Nigeria, Egypt and Tunisia show extremely low prevalence of colistin resistance in enterobacteriaceae (L. Dortet *et al.,* 2016). In Tunisia, strains of *E. coli*, *K. pneumoniae* and *Enterobacter cloacae* showed prevalence of resistance to colistin of 0.09%, 1.2% and 1.5%, respectively (Mezghani Maalej *et al.,* 2012). However, studies in Nigeria and Egypt did not identify any resistant strains (Adelowo *et al., 2014*; Hasanin *et al.,* 2014). In Gabon, while little is known, however, a recent study from 20 isolates of *E. coli* from childhood diarrheal faeces showed 40% of resistance to colistin (Mabika Mabika *et al.,* 2019; Yala *et al.,* 2020). The main objective of this study is to evaluate the sensitivity of *Salmonella* and *Shigella* strains isolated from diarrheal faeces to colistin. This will be determined using their minimum inhibitory concentrations (MIC) and a conventional PCR for the detection of the *mcr*-1 and *mcr*-2 genes.

MATERIAL AND METHODS

Bacterial strains: *Salmonella* and *Shigella* strains isolated from diarrheal faeces in children aged 0 to 5 years in the city of Koula-Moutou were screened in this study at the phenotypic and genotypic levels. The strains of *Salmonella* genus consisted of 17 strains of *Salmonella* spp, 6 of *Salmonella enterica*, 4 of *Salmonella Typhi* and 3 of *Salmonella Paratyphi* A. The *Shigella* genus consisted of 3 strains of

Shigella spp and 2 of *Shigella sonnei*. These strains have been cryopreserved in the strain library of the Laboratory of Cell and Molecular Biology at Masuku University of Science and Technology.

Evaluation of strain sensitivity to colistin: The determination of minimum inhibitory concentrations (MICs) was carried out using the reference method to characterize the phenotypes of Salmonella and *Shigella* with colistin. Briefly, geometric dilution ranges of reason 2 between 8µg/ml-0.625µg/ml were carried out and standardised inoculi were inoculated at 37°C for 18-24h incubation. The MIC values of the European Committee on Antimicrobial Susceptibility Testing epidemiological cut-off (EUCAST ECOFF) were used for the interpretation of the MIC values obtained (EUCAST, 2020).

Detection of *mcr*-1 and *mcr*-2 genes in *Salmonella-Shigella* strains: The search for the *mcr*-1 and *mcr*-2 genes was carried out by conventional PCR on all 35 *Salmonella-Shigella* isolates. The specific primer sequences used were mcr-1 forward (5'-AGTCCGTTTGTTCTTGTGGC-3'), mcr-1 reverse (5'-GGGGCTTGATGCTCACT-3'); *mcr*-2 forward (5'-CAAGTGTTGCGCAGTT-3') and *mcr*-2 reverse (5'-CAAGTGTGTGTTGCGCAGTT-3') (Zhang *et al.,* 2018). The different amplification conditions were an initial denaturation at 95°C for 15 minutes followed by 35 denaturation cycles at 94°C for 15 seconds, a hybridization of 57°C of 30 seconds, an elongation of 68°C of 70 seconds and finally, the final elongation step at 72°C for 5 minutes. The amplicons obtained were separated on a 1.5% (m/v) agarose gel by electrophoresis and visualized under ultraviolet light.

RESULTS

Phenotypic prevalence of colistin resistance strains and of the *mcr*-1 and *mcr*-2 genes.

Table 1: Minimum inhibitory concentration values for strains of *Salmonella* and *Shigella*.

	Effective N	Break point CMI (µg.mL^{-1})		Percentage of resistance	Frequency of genes detected	
		Sensitivity	Resistant		*mcr-1*	*mcr-2*
		≤2 n (%)	>2 n (%)	n (%)	n (%)	n (%)
Salmonella enterica	**6**	5 (83.3)	1 (16.7)		-	-
Salmonella Paratyphi A	**3**	3 (100.0)	-	**6 (17.1)**	-	-
Salmonella spp	**17**	13 (76.5)	4 (23.5)		-	-
Salmonella Typhi	**4**	3 (75.0)	1 (25.0)		-	-
Total	**30**	**24 (80.0)**	**6 (20,0)**		**-**	**-**
Shigella sonnei	**2**	2 (100.0)	-	**1 (2.9)**	**-**	**-**
Shigella spp	**3**	2 (66.7)	1 (33.3)		-	-
Total	**5**	**4 (80.0)**	**1 (20.0)**		**-**	**-**
Global Total	**35**	**28 (80.0)**	**7 (20.0)**	**7 (20.0)**	**0 (0.0)**	**0 (0.0)**

The results in Table I show that 80.0% of *Salmonella* and *Shigella* strains are sensitive to colistin compared to a resistance rate of 20.0%. Specifically, 17.1% of *Salmonella* isolates are resistant to colistin, while only 2.9% of *Shigella* has this phenotype. Among the 4 species of *Salmonella*, *Salmonella* spp show the highest resistance rate (23.5%) followed by *Salmonella Typhi* (25.0%) and *Salmonella enterica* (16.7%). In contrast, only the *Shigella* spp strain is resistant to colistin (33.3%) and all *Shigella sonnei* isolates are susceptible. In addition, molecular screening of the 35 target isolates revealed the absence of the *mcr*-1 and *mcr*-2 genes, which are carried by the plasmids

DISCUSSION

The main objective of this study was to evaluate the sensitivity of *Salmonella* and *Shigella* strains isolated from diarrheal faeces to colistin, using their MIC and a conventional PCR for the detection of the *mcr*-1 and *mcr*-2 genes. Colistin is a cyclic cationic peptide that inhibits the growth of Gram-positive bacteria by disrupting the integrity of the outer membrane of these bacteria through the interaction of its positive electrostatic charges with the negative charges of the outer lipopolysaccharide membrane (Sun *et al.,* 2009). The evaluation of the sensitivity of *Salmonella* and *Shigella* to colistin revealed an average prevalence (20%) of resistance. These results are consistent with the work of (Morales *et al.,* 2012), in which the authors showed that colistin resistance prevalence ranged from 10.52% to 21% for the genus *Salmonella*. for the genus *Shigella*, the authors showed a colistin resistance rate of 20-27% (Morales *et al.,* 2012). However, these results are higher than those obtained in Europe and Asia where the authors found prevalence around 3%(Gales *et al.,* 2011; L Dortet *et al.,* 2016). A recent study in Gabon revealed a high prevalence (40%) of *E. coli* strains to colistin (Yala *et al.,* 2020). The average rate of resistance of colistin resistance recorded in this study would be justified by the fact that colistin, although considered as the last line of defense against severe infections, is not yet misused abused in Gabon. Indeed, previous studies showed a relationship between the emergence of the colistin resistance gene (*mcr*-1/2) and the overuse of colistin in the veterinary field (L. Dortet *et al.,* 2016)**.** This low use in rural areas could be an outcome of the limited access to colistin or polymixins for treatment of patients (L. Dortet *et al.,* 2016). In addition, this could also be correlated with the small sample sizes of this study. Clearly, it has been shown that the overall number of strains tested could potentially impact the prevalence of antimicrobial resistance (Frye and Jackson, 2013). As a major component of the outer membrane of Gram-negative bacteria, LPS is the primary target of polymixins. Colistin resistance could be justified by the addition of positive charges to lipid, which is a component of the LPS, since these covalent changes in the lipid A fraction by cationic substitution are the most common mechanism of colistin resistance by enterobacteriaceae (Kim *et al.,* 2019). These changes in the charges of lipid A lead to a reduction in the negative charge and consequently, a decrease in affinity or electrostatic interaction with colistin. On the other hand, these modifications of lipid A by phosphoethanolamine transferase (pEtN) can be encoded by an Epta chromosomal gene (pmrC) or a plasmid gene, *mcr* (Nang *et al.,* 2019). Furthermore, the modification of lipid A by 4-amino-4-deoxy-L-arabinose (L-Ara4n) is due to an exclusively chromosomal arnT (pmrk) gene (Nang *et al.,* 2019). Indeed, it has been shown in the literature that cationic sugars such as L-Ara4n at lipid A in *S. enterica* reduce the negative charge of the outer membrane (Zhang *et al.,* 2019). In addition, resistance in *Salmonella* and *Shigella* strains may be a consequence of specific mutations in the pmrAB and phoPQ genes (Kim *et al.,* 2019). In addition to the changes in lipid A by the addition of phosphate, phosphoethanolamine (pEtN) or 4-amino-4-deoxy-L-arabinose (L-Ara4n) groups, some studies highlight the deacylation or hydroxylation of lipid chains (L. Dortet *et al.,* 2016). Other studies highlighted also that specific mutations in two-component systems such as Pmrab and Phopq and their regulators Mgrb and Pmrd are associated with colistin resistance in *Enterobacteriaceae*, including *Klebsiella pneumoniae*, *K. aerogenes* and *Salmonella Enterica*, as well as *P. aeruginosa* and *A. baumanni* (Poirel*et al.,* 2017). The resistance observed in this study could be due to the acquisition of the plasmid *mcr* genes. Indeed, enterobacteriaceae have a great ability to easily acquire genetic material by horizontal inter- and intra-species transfer, the process of which most often involves mobile genetic elements and particularly concerns antibiotics resistance genes (Dortet*et al., 2013*). The results of the search for plasmid resistance genes in *Salmonella* and Shigella strains indicate the absence or non-detection of the *mcr*-1 and *mcr*-2 genes in this work. These results may have several explanations. The first explanation would the fact that colistin resistance in Gram-negative bacteria is mainly due to the acquisition of mutations in two-component systems (Poirel *et al., 2017*). Investigation of the origin of colistin resistance in 30 *E. coli mcr*-1 negative strains found that 22 strains carried amino acids in Pmrb, Phop, Phoq, Mgrb and/or Pmrd, while no mutation in any of these genes were found in the remaining eight isolates (Kim *et al.,* 2019). In addition, several studies showed that the acquisition of *mcr* plasmid genes is more prevalent in clinical *E. coli* strains (Sperandeo *et al.,* 2007; Zhang *et al.,* 2019). In addition, the prevalence of *mcr*-1 strains is low at 1.4% and 0.7% for clinical strains of *E. coli* and *K. pneumoniae* respectively (Yu *et al.,* 2015). The prevalence of *mcr-1* strains is low in humans compared to animals (Haenni *et al.,* 2016). These bacteria (Padilla *et al.,* 2010) would likely use other colistin resistance mechanisms such as synthesis or expression of efflux pumps. Ultimately, a

decrease in the net negative charge of the outer membrane, lipid A loss or efflux pumps cause colistin resistance.

CONCLUSION AND APPLICATION OF RESULTS

This study showed that *Salmonella* and *Shigella* isolated from diarrheal faeces were resistant to colistin, a molecule of the last therapeutic line. This resistance to colistin in the city of Koula-Moutou is thought to be mediated by chromosomal and non-plasmid alterations. For better discrimination of molecular resistance, it would be appropriate to screen all variants of the *mcr* gene as well as the determinants of chromosomal resistance. The results of this study provide a strong signal for monitoring the emergence of colistin resistance of enteric pathogens in Gabon.

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