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RESEARCH ARTICLE

Detection of Colistin Resistance in *Mannheimia haemolytica* & *Pasteurella multocida* Isolates from Ruminants in Morocco

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ABSTRACT

Colistin is an important drug of last resort against lethal infections with multidrug-resistant gram-negative bacteria, this antibiotic interacts on phosphate groups of lipopolysaccharides present on the outer membrane of gram-negative bacteria. This first mechanism of transferable colistin resistance involves a gene called mcr-1. Here, we described different tools such as Colistin susceptibility test, Minimal Inhibitory Concentration to find the colistin profiles of *Mannheimia haemolytica* and *Pasteurella multocida* serogroup A Moroccan strain isolated from nasal swabs and lung taken from sheep, goat and cattle with respiratory diseases during January 2015 to December 2017 in six different regions, in addition, we investigated either real time PCR to detect mcr-1 gene. Antimicrobial sensitivity test, was achieved for Forty-one isolates, the resistance rates of isolates from *Pasteurellaceae* species were between 59 and 71%, which respectively correspond to *Mannheimia haemolytica* and *Pasteurella multocida* segroup A. The sensitive ratios were between 29 and 41% which respectively correspond to *Pasteurella multocida* segroup A and *Mannheimia haemolytica.* Also, the MIC test was done against colistin, the results showed a resistant profile with a MIC >=64 µg/ml except for 3 strains from ruminants which have a value <2 µg/ml. The real time PCR screening test was detected with ct values ranging from 23 to 31 and confirmed the results obtained by MIC test. To our knowledge the present study is the first study which reports the resistance to colistin in *Pasteurellaceae* species strains isolated from ruminants in Morocco, our results suggest the necessity and the urgency for establishing a national program for monitoring antibacterial resistance against colistin.

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INTRODUCTION

Colistin is a polypeptide antibiotic of the *polymyxin* family, of the *polymyxin E* group. It's a polycationic and both hydrophilic and lipophilic. Since the discovery of first plasmid-mediated colistin resistance gene mcr-1 in 2015 (Liu *et al.,* 2016), it has caught universal consideration. These polycationic sites interact with the phosphate groups of the lipopolysaccharides of the bacterial membrane structure, which increases membrane permeability and causes the leakage of intracellular content, resulting in the death of the bacteria (Liu *et al.,* 2016). Resistance to colistin is rare but well described in response to the emergence of antibiotic resistance (Jeannot *et al.,* 2017), including in multi-resistant Enterobacteriaceae with New Delhi metallo-beta lactamase, and following the recent discovery of a new mechanism of colistin resistance in bacteria (Lu *et al.,* 2017). The European Medicines Agency (EMA), assisted by the European Food Safety Authority (EFSA), updated in 2016 a scientific opinion dated 2013 on the use of the antibiotic colistin in animals. It is recommended that all veterinary use of this drug be discontinued (Rhouma *et al.,* 2016), except for clinical conditions for which there is no other effective treatment. This new form of antibiotic resistance is linked to the mcr-1 gene and has the potential to spread rapidly. On the basis

of the work carried out by Sebbar *et al*. (2019), the presence of MDRs was noted, so the antibiogram panel was completed with colistin, an antibiotic of the polymyxin family, which is one of the last effective medical remedies for the treatment of multi-resistant Gram-negative infections and which has a narrow spectrum of antibacterial activity limited to Gram-bacteria. In addition, the monitoring of using this type of products in Morocco demonstrated that the polypeptides family, especially colistin is the most commonly antibiotic used in ruminants after the tetracyclines family, especially oxytetracycline (Amine, 2018; El Majidi, 2018).

The aim of this study was to to determine the colistin profiles of forty-one strains of *Mannheimia haemolytica* and *Pasteurella multocida* serogroup A using two tools such as Colistin susceptibility test, Minimal Inhibitory Concentration and the detection of mcr-1 gene by real time PCR in Moroccan isolates outcomes from sick animals using a SYBR® Green technology.

MATERIALS AND METHODS

Isolates: Forty-one strains of *Pasteurella* species thirty-four *Mannheimia haemolytica* (twenty-eight nasal swabs and six lungs) and seven *Pasteurella multocida* (five nasal swabs and two lungs) were isolated from sheep, goat and cattle. The strains used in this study were identified among 162 samples collected from animals with respiratory diseases during January 2015 to December 2017 in six different regions of Morocco (Sebbar *et al.*, 2018, 2019)**.** Strain identification is summarized below in Table 1.

Colistin susceptibility test: Forty-one isolates have been the subject of a colistin susceptibility test (CT, 50 µg), this test was performed using Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Oxoid, UK) plate, described in Chapter 2.1.1 Laboratory methodologies for bacterial antimicrobial susceptibility testing 2009. *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were used as quality control strains. The rules for the technical implementation of the antibiogram in animal health are defined in the AFNOR NF U47-107 standard. The strains were classified as sensitive or resistant, using the zone diameter according to the Antibiogram Committee of the French Society of Microbiology (AC-FSM) veterinary recommendations (EUCAST, 2016).

Minimal Inhibitory Concentration (MIC): A standard microdilution technique was used to determine MICs. Antimicrobials were diluted either in sterile water, pH 8.0, microtiter plates were prepared using a dispenser. Each of the *Pasteurella* isolates was inoculated into 3.5 ml of Mueller-Hinton broth and incubated 4-6hr at 37°C to obtain a final concentration equal to a 0.5 McFarland nephelometer standard (10^8 colony-forming units [cfu]/ml). The adjusted culture was then diluted in Mueller-Hinton broth so that after inoculation each well contained approximately 5 x 10 cfu/ml. Plates were sealed and incubated at 37°C for 16-20hr. Then, plates were read with a reflective viewer, and the MIC was recorded as the lowest concentration of antimicrobial that completely inhibited all visible bacterial growth. Each lot of plates was subjected to internal quality control using a recommended reference strains such as *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) to ensure the validity of the results obtained (CLSI, 2019).

Interpretation of antimicrobial susceptibility was based on the guidelines of the Clinical Laboratory Standards Institute (*CLSI. Performance standards for antimicrobial susceptibility testing. 29th ed. M100. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute; .*, 2019). Susceptibility to colistin was interpreted according to the European Committee on Antimicrobial Susceptibility Testing breakpoint of >2 µg/ml.

Detection of the resistance Gene (mcr-1) by real time PCR: Extraction of bacterial DNA from the overnight cultures was performed using the Nucleospin Tissue Kit (Machery-Nagel, Germany) according to the manufacturer's instructions.

The Sybr®Green real time PCR (rt-PCR) assay for mcr-1 detection was performed with the primers mcr-1-FW (5′-AGTCCGTTTGTTCTTGTGGC-3′) and mcr-1-RV (5′-AGATCCTTGGTCTCGGCTTG -3′) described by (Rebelo Ana Rita, Bortolaia Valeria, Kjeldgaard Jette S, Pedersen Susanne K, Leekitcharoenphon Pimlapas, Hansen Inge M, Guerra Beatriz, Malorny Burkhard, Borowiak Maria, Hammerl Jens Andre, Battisti Antonio, Franco Alessia, Alba Patricia, Perrin-Guyomard A, 2018). Each 20 µl reaction mixture contained 0.4 µM of each primer, 1× SensiFAST SYBR® Lo-ROX Mix and 5 µl of DNA template. Experiments were performed with Agilent System (AriaMX) and data were analyzed with a first denaturation step (95°C for 2 min), followed by 40 cycles of denaturation (95°C for 5 s), annealing (60°C for 10 s) and extension (72°C for 20 s) and at the end the step for melting curve was added.

RESULTS

Colistin susceptibility test of *Mannheimia haemolytica* & *Pasteurella multocida* isolated from clinical nasal swabs and lung samples in Morocco: In our study according to antimicrobial results, for both *Pasteurellaceae* species, antimicrobial resistance ratios of isolates were between (59-71%) which respectively correspond to *Mannheimia haemolytica* and *Pasteurella multocida* segroup A.

The sensitive ratios were between (29-41%) which respectively correspond to *Pasteurella multocida* segroup A, and *Mannheimia haemolytica.* Results of antimicrobial susceptibility screening test are summarized in Table 2.

Minimal Inhibitory Concentration of colistin among *Mannheimia haemolytica* & *Pasteurella multocida* isolated from clinical nasal swabs and lung samples: The Forty-one strains tested by the MIC test against colistin showed a resistant profile with a MIC >=64 µg/ml except for three strains (7%) which have a value <2 µg/ml. Results of MIC test are summarized in Table 3.

Detection of resistance mcr-1 gene: The results obtained from the SYBR® Green PCR showed a cycle threshold (ct) ranging from 23 to 31 (Fig. 1), this effect confirms those obtained by the Minimal Inhibitory Concentration MIC test.

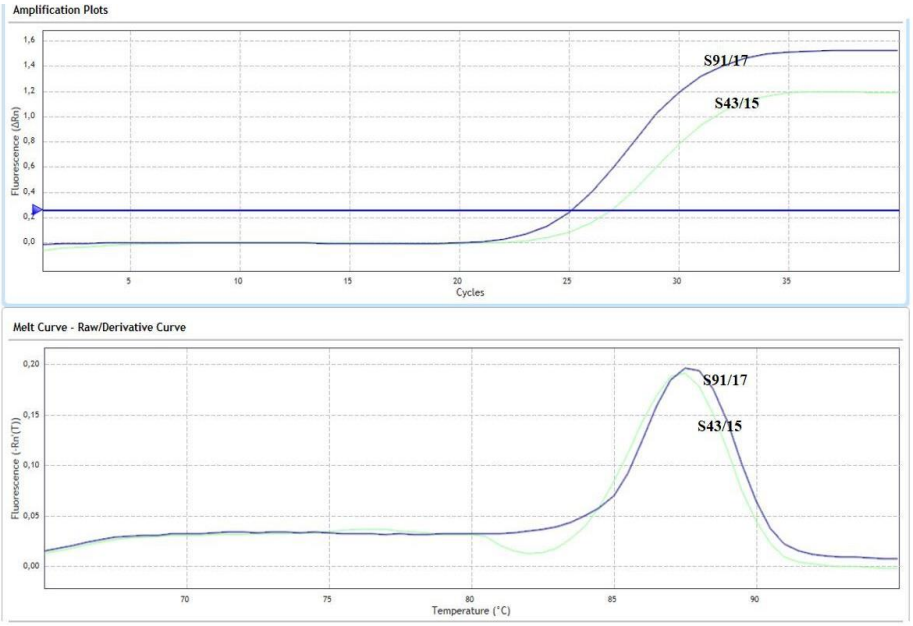

Fig. 1: SYBR green amplification curve and corresponding melting temperature curve. Amplification curves of two isolates (S43/15 (green) and S91/17(blue)); the curves were generated using SYBR® Green PCR.

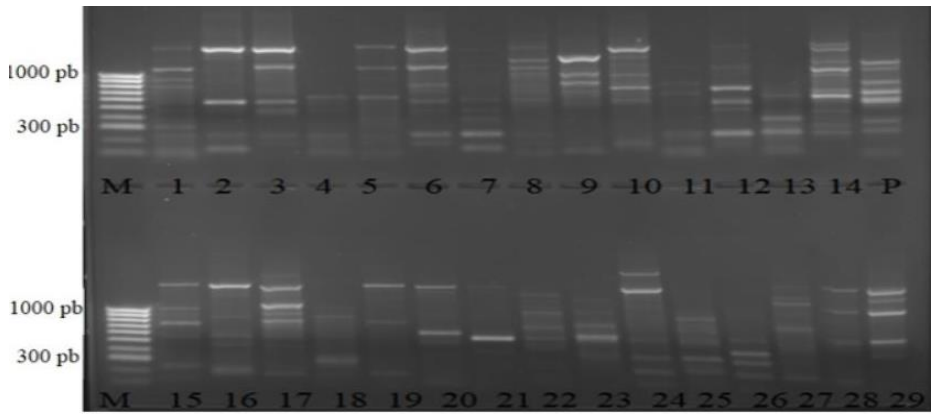

Fig. 2: Confirmation of the mcr-1 gene by gel based. M:Marqueur (100pb), 1:S7/15, 2:S27/15, 3:S30/15, 4:S34/15, 5:S41/15, 6:S42/15, 7:S43/15, 8:S1/16, 9:S2/16, 10:S4/16, 11:S8/16, 12:S10/16, 13:S12/16, 14:S13/16, 15:S14/16, 16:S15/16, 17:S4/17, 18:S6/17, 19:S11/17, 20:S15/17, 21:S16/17, 22:S20/17, 23:S38/17, 24:S42/17, 25:S46/17, 26:S73/17, 27:S91/17, 28:S93/17, 29:S96/17.

The measured fluorescence may be from non-specific amplification product. The specificity of the PCR products should therefore be verified. So, the PCR products are migrated to gel to ensure that the size of the amplified fragment is as expected. The product size of the amplicon was 320 bp with a melting temperature of 87°C. Migration on agarose gel based confirmed the presence of the mcr-1 gene in six strains which are (S43/15, S12/16, S13/16, S46/17, S73/17 and S91/17) (Fig. 2). The specificity of the primers has been confirmed by both traditional PCR and melting curve analysis.

DISCUSSION

Colistin is one of the few antibiotics still active in veterinary medicine used in the treatment of serious infections caused by multi-resistant bacteria, colistin resistance is complex and highly varied (Jeannot *et al.*, 2017), it has occurred via chromosomal mutations and the recent appearance of mcr-1 plasmid resistance. Concerning plasmid resistance, although it is more prevalent in the animal world, the plasmid has all the characteristics to spread to humans. Colistin resistance is a major public health problem and leads to a therapeutic impasse and an increased risk of mortality. This is why it is essential for laboratories to have reliable and reproducible techniques to properly detect this resistance in order to limit its spread and preserve colistin as an antibiotic of last resort.

The presence of MDRs resistant to at least three classes of antimicrobial agents (Sebbar *et al.*, 2019), this prompted us to complete the antibiogram panel with the polymyxin family of antibiotics, specifically colistin, which is one of the last effective medical remedies for the treatment of multidrug-resistant Gram-negative infections and which has a narrow spectrum of antibacterial activity limited to Gram-positive bacteria. The broth microdilution method according to ISO 20776-1 was chosen as the reference method.

Forty-one strains of gram-negative bacteria with variable colistin sensitivity profiles were tested. The results of antibiotic susceptibility testing indicated resistance rates of isolates from *Pasteurellaceae* species between (59-71%) which respectively correspond to *Mannheimia haemolytica* and *Pasteurella multocida* segroup A. The sensitive ratios were between (29-41%) which respectively correspond to *Pasteurella multocida* segroup A, and *Mannheimia haemolytica*, this has been reported in recent years (Humphries, 2014).

In addition all isolates were subjected to the Minimal Inhibitory Concentration against colistin and the results showed a resistant profile with a MIC >=64 µg/ml except for 3 strains which have a value <2 µg/ml, this has been reported in recent years (Li *et al.*, 2005; Osei Sekyere, 2019; Sebbar *et al.*, 2019) and demonstrated in this current study.

Table 1: Identification of isolates tested

Strain code	Organ type	Species	Origin
S6/15	Lung	Bovine	Kenitra
S7/15	Lung	Bovine	Larache
S27/15	Nasal swab	Sheep	Ouarzazate
S30/15	Nasal swab	Sheep	Tinghir
S32/15	Nasal swab	Sheep	Ouarzazate
S33/15	Nasal swab	Sheep	Ouarzazate
S34/15	Nasal swab	Sheep	Ouarzazate
S41/15	Nasal swab	Sheep	Casablanca
S42/15	Nasal swab	Sheep	Casablanca
S43/15	Nasal swab	Sheep	Casablanca
S1/16	Nasal swab	Bovine	Benslimane
S2/16	Nasal swab	Bovine	Berrchid
S4/16	Nasal swab	Bovine	Berrchid
S8/16	Nasal swab	Bovine	Berrchid
S10/16	Nasal swab	Bovine	Berrchid
S12/16	Nasal swab	Bovine	Tit Mellil
S13/16	Nasal swab	Bovine	Tit Mellil
S14/16	Nasal swab	Bovine	Berrchid
S15/16	Nasal swab	Bovine	Berrchid
S2/17	Nasal swab	Bovine	Rabat
S3/17	Nasal swab	Bovine	Rabat
S4/17	Lung	Bovine	Rabat
S6/17	Lung	Sheep	Rabat
S8/17	Nasal swab	Calves	Rabat
S11/17	Nasal swab	Calves	Khmiss zmamra
S13/17	Nasal swab	Calves	Rabat
S14/17	Nasal swab	Calves	Rabat
S15/17	Lung	Calves	LOUKKOUS
S16/17	Lung	Sheep	Oujda
S17/17	Lung	Sheep	Oujda
S20/17	Nasal swab	Bovine	Larache
S38/17	Nasal swab	Sheep	Tinghir
S42/17	Nasal swab	Sheep	Errachidia
S46/17	Nasal swab	Sheep	Errachidia
S49/17	Nasal swab	Sheep	Errachidia
S73/17	Nasal swab	Sheep	Tinghir
S86/17	Nasal swab	Goat	Khnefra
S91/17	Nasal swab	Ram	Khnefra
S93/17	Nasal swab	Goat	Khnefra
S96/17	Nasal swab	Lamb	Khnefra
S102/17	Lung	Goat	Khnefra

S: strain.

Colistin sensitivity tests are technically difficult because of several problems. First of all, colistin diffuses very poorly in agar. Second, the polymyxins bind to a range of plastics and glass including plastics most common (polystyrene and polypropylene) used in the trays of micro-dilution of laboratory sensitivity tests, which increases the problems reliability and reproducibility (*European Centre for Disease Prevention and Control (ECDC). RAPID RISK ASSESSMENT - Plasmid-mediated colistin resistance in Enterobacteriaceae.*, 2016; Humphries, 2015).

Currently, the MIC determination techniques (agar diffusion or concentration gradient) are no longer recommended because of the poor diffusion of colistin in agar (Gales *et al*., 2001). The correlation of MICs with disc diffusion zone diameters had already been considered to be the problem was identified more than 30 years ago. In addition, many studies have shown significant variability between Minimal Inhibitory Concentration results when using different methods and the presence of very large errors for some (Hindler and Humphries, 2013; Dafopoulou *et al.*, 2015).

These problems have been extensively studied by the European Committee on antimicrobial susceptibility (EUCAST) and the Clinical Laboratories Standards Institute (CLSI). Both recommend that, as the only method of testing for reliable and reproducible sensitivity, the determination of the MIC by the method of micro-dilution in broth according to ISO 20776-1 standard. They specify to use a cation-adjusted Mueller-Hinton liquid medium, perform the test with colistin sulphate and to use plates of polystyrene titration without pre-treatment. No other additives such as polysorbates should not be used (*European Committee on Antimicrobial Susceptibility Testing (EUCAST). Recommendations for MIC determination of colistin (polymyxin E) As recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group. . Disponible sur : http://www.eucast.org.*, 2016).

The High Council of Public Health, in 2016, has therefore taken decisions to detect these colistin-resistant strains associated with the mcr-1 gene. It is thus recommended to determine the MIC of colistin by the reference method of microdilution in liquid medium according to the recommendations of the CA-SFM/EUCAST 2017. Resistance is expressed most often at low levels with reported MIC values of 2 mg/L.

In parallel, the results obtained by MIC were confirmed by real-time PCR for the detection of the gene responsible for colistin resistance mcr-1. The results obtained from the SYBR® Green PCR showed a cycle threshold (ct) ranging from 23 to 31 as proved in previous studies (Poirel *et al.*, 2016; Enterobacteriaceae, 2019).

These results are in favour of those of the MIC test but do not forget that it is a PCR by an intercalating agent which is inserted between a double-stranded DNA whatever, so to conclude it is necessary to pass by a migration on gel and to see the size of the amplicons. The mcr-1 gene has been widely reported from all major continents, North America, North Africa, Southeast Asia, and Europe (Wang *et al.*, 2017).

In this study we might say that the first two techniques namely colistin susceptibility test and Minimal Inhibitory Concentration MIC are very heavy and disc susceptibility testing methods are unreliable at detecting colistin resistance (Tan and Ng, 2006). The best is to make a screening test by SYBR® Green PCR and his specificity was confirmed by migration on agarose gel based. According to EUCAST (2016) and CLSI (2019), our study confirmed that mcr-1-positive *pasteurella* strains tested are resistant to colistin as demonstrated in previous studies (Poirel *et al.*, 2016; Enterobacteriaceae, 2019).

Additional studies are underway to confirm the six strains by sequencing and comparing sequence homology with mcr-1 deposited in GenBank nucleotide database.

Table 2: Sensitivity rates of colistin among *Mannheimia haemolytica* & *Pasteurella multocida* isolated from clinical nasal swabs and lung samples from ruminants in Morocco

Family of Antibiotic	Antimicrobial agent	Disc potency (µg)	*Mannheimia haemolytica (n=34)*		*Pasteurella multocida (n=7)*	
			n (%) of S	n (%) of R	n (%) of S	n (%) of R
Polymyxines	Colistin (CT)	50	14 (41)	20 (59)	2 (29)	5 (71)

CT: Colistin, R: resistant, S: Sensitive, n: number, %; percentage.

Table 3: Results MIC test of colistin among *Mannheimia haemolytica* & *Pasteurella multocida* isolated from nasal swabs and lung samples

	Pasteurella spp (n= 41)	
	n (%)	[Concentration]
MIC (R)	38 (93)	>=64 µg/ml
MIC (S)	3 (7)	< 2 µg/ml.

MIC: Minimal Inhibitory Concentration, R: Resistant, S: Sensitive, n: number, %; percentage.

Conclusions: In conclusion we reported here the presence of resistance to colistin in Morocco and this study concludes that diffusion gradient tests underestimate MIC values and should therefore be avoided even when quality control results are within the limit values. It is immediately needed to reconsider the appropriate use of colistin in veterinary medicine. Such measures will reduce the zoonotic risk that may represent mcr-1 gene carrier bacteria especially in ruminants.

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Authors contribution: The work presented here was carried out in collaboration between all authors. G.S conceptualized and wrote the article; G.S and S.F did the analysis. All author's reviewed and approved the final manuscript.

Symbols and abbreviations: ATCC: American Type Culture Collection; °C: Celsius degree; CFU: Colony-forming units; DNA: Deoxyribonucleic acid; EMA: European Medicines Agency; CT: Colistin; hr: hour; µg: microgram; µM: micromolar; µl: Microliter; mcr-1: Mediated Colistin resistant-1; MDR: multidrug resistant; ml: milliliters; MIC: Minimal Inhibitory Concentration; min: minute; pb: pair base; PCR: Polymorphism chain reaction; pH: pression hydrolic; R: Resistant; S: Sensitive; s: second.

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