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Abstract

In this study, we assessed the selective effect of colistin orally administered to healthy weaned piglets harbouring an intestinal *mcr-1*-positive *Escherichia coli* strain. Maximum recommended dose and a higher dose often used in European pig farms were given by gavage. No selection of the *mcr-1*-positive strain was observed in our controlled conditions whatever the dose. Further investigations in real farming conditions seem necessary.

Keywords: colistin; *mcr-1; Escherichia col;* piglets; selection

1. Introduction

Colistin is an old polypeptidic antibiotic widely used in food-producing animals, especially in pig production as oral group treatment and metaphylaxis against Enterobacteriaceae digestive infections after weaning. Colistin is also used in human medicine as a last resort antibiotic against multi-drug bacteria. The first plasmid-mediated colistin resistance gene (mcr-1) discovered in China at the end of 2015 [1] has raised concern about the risk of spread of this resistance. Few months after, mcr-1 was detected in all continents, both in human and animals [2]. European Medicines Agency emphasized the need of reducing colistin use in animal and proposed to class colistin as critically important antimicrobials [3]. Scientific community lacks of in vivo data about *mcr-1*, especially in commensal Enterobacteriaceae to properly characterize the public health risk. We assessed here in piglets the selective effect of controlled colistin oral treatments on commensal intestinal *Escherichia coli* harbouring a *mcr-1*-positive strain with monitoring of faecal concentrations.

2. Materials and methods

2.1. Animals and housing

Fifteen Large White-Landrace-Piétrain piglets were used to carry out the experiment, with no history of antimicrobials treatments. They were weaned at 21 days old and then fed with a standard non-medicated ration and had free access to water. After 5 days of collective housing, they were put in individual boxes with no possible contact (8 days before treatment, D-8).

2.2. Bacterial strain and inoculation

The original strain was a colistin-resistant *E. coli* (MIC = 8 mg/L) isolated from the intestines of a healthy pig sampled in a French slaughterhouse [4]. This strain harbouring *mcr-1* (confirmed by PCR) was made rifampicin-resistant (MIC > 512 mg/L) by spontaneous mutation before inoculation and named ECmcr1+. The inoculation phase consisted of three gavages (at D-7, D-5 and D-2) of 5 mL of about 10^7 CFU/mL of ECmcr1+ in saline suspension.

2.3. Experimental treatment and sampling

Piglets were randomly divided into three groups of 5 animals and force-fed with colistin sulphate (Acti-coli, Biové, Arques, France) using a polyethylene tube, from D0 to D4 (5 days). RD group (for maximum Recommended Dosage) received 100 000 UI/kg/day [5] equivalent to 3 mg/kg/day of colistin base activity (CBA) [6], given twice a day as 1.5 mg/kg in 5 mL solution; HD group (for Higher Dosage often found in pig farms [7]) received 200 000 UI/kg/day equivalent to 6 mg/kg/day of CBA, given twice a day as 3 mg/kg in 5 mL solution; the placebo group received water. Fresh faecal samples were taken on mornings (after anal stimulation) from the day before ECmcr1+ inoculation (D-8) until 19 days after the

end of colistin treatment (D23). This experiment was approved by the ComEth Anses/ENVA/UPEC n°16 (French ethical committee) under the reference APAFIS#2905-2015112717486085.

2.4. Microbiological analysis and colistin assay

About 1 g of each fresh faecal sample was diluted in saline solution. Selected dilutions were plated on Mac Conkey agar (BD, Le Pont de Claix, France) alone or supplemented with 200 mg/L of rifampicin (Sigma, Saint-Quentin Fallavier, France) in order to count total *E. coli* and ECmcr1+, respectively.

Another specimen (1 g of faeces) from D0 to D9 was kept at -20°C until colistin was assayed by a LC-MS/MS method adapted from previous works [8, 9]. Briefly, faeces were mixed with blank plasma and diluted in 10 mL of acetonitrile with 6% of trichloroacetic acid. After vortexing, centrifugation and evaporation, dry matter was diluted in buffer (pH=7.2) with blank plasma and loaded on Oasis HLB Catridges (Waters, Milford, MA, USA). After washing and eluates evaporation, residues were analysed by HPLC-MS/MS with a limit of quantification (LOQ) of 1 µg/g of faeces. Quality controls were prepared at 2.5, 12.5 and 18.8 µg/g of faeces.

2.5. Statistical analysis

Mean values of colistin faecal concentrations of the two treated groups were compared using a Student-T test. Mean values of total faecal *E. coli* and of ECmcr1+ for treated groups were compared to those of the placebo group using a Student-T test. All statistical analyses were carried out using R 3.3.2 [10].

3. Results

High faecal colistin concentrations were measured with mean values greater than or equal to about 200 µg/g of faeces from D2 to D5 in the two treated-groups (Table 1). No significant differences were observed between these groups (T-test) due to the high inter-individual variability. Three days after the end of treatment (D7), faecal concentrations were reduced by about 100-fold and felt below the LOQ after 2 days more (D9).

No significant differences were found for total faecal *E. coli* (ECtot) population between groups before colistin administration (from D-8 to D0, Fig. 1A). We observed a slight but non-significant decrease of ECtot during the treatment phase for the placebo group. Conversely, a stronger reduction of ECtot was noticed for each colistin-treated group between D0 and D3 but only significant for HD group (compared to placebo, Fig. 1A). Then, a return to initial level of ECtot was observed 2 and 3 days after last colistin dose (D4) for RD and HD group, respectively.

After the inoculation phase (D-7 to D-2), ECmcr1+ reached up to 2 % of ECtot (Fig. 1B). However 7 of 15 piglets had ECmcr1+ count lower to the LOQ (1.2 log CFU/g of faeces) just before treatment ; for 5 of them the strain was undetectable during all the experiment (2 in HD group, 2 in placebo group and 1 in RD group). Mean ECmcr1+ levels were equivalent between the three groups before the first colistin administration (D0). Overall, ECmcr1+ level compared favourably between each group i.e. remained relatively constant during and after treatment.

4. Discussion

To our knowledge, this is the first in vivo study exploring the selective effect of colistin on *mcr-1*-positive *E. coli* in pigs. Previous studies already attested a rare emergence of colistin-resistant *E. coli* after oral colistin treatment of healthy [7] and sick piglets [11], therefore we neglected it. Exogenous resistant bacteria inoculation in treated pigs already supported the selective effect of other antimicrobials in these animals [12]. In order to control the colistin doses, we chose to force-feed the piglets for treatment. The absence of significant differences of faecal colistin concentrations between HD and RD groups was likely due to measurement uncertainty. However, these results seemed relevant: with a daily faecal excretion of piglets of around 250 g [13], we can estimate that about 60 and 95 % of the colistin dose was recovered in faeces for HD and RD group. This is consistent with the poor absorption of colistin sulphate after oral treatments in pigs [5]. In addition, we chose to induce a rifampicin resistance in ECmcr1+ in order to monitor precisely this strain and due to the poor accuracy of colistin-supplemented media for resistant Enterobacteriaceae isolation [7, 11].

The initial decrease of ECtot level before treatment and the slight continuous one of placebo group were probably due to the weaning process that disturbed the microbiota equilibrium [14]. The high colistin treatment induced a significant reduction of ECtot counts compared to the placebo group (Fig. 1A), but the recommended dose poorly affected it. Considering the high faecal concentrations during the treatment phase (> 200 µg/g), greater reduction of *E. coli* would be expected. Colistin high adsorption to faecal fibres could lead to a dramatic decrease of its effect [15]. In addition, for polymyxin B, up to 90 % of the initial dose could be reversibly bound to faeces [16]. Although this was shown with a bioassay method and not with colistin, the real active colistin concentrations in our experiment should be greater than 20 µg/g of faeces. This is normally still enough to reduce ECtot population as it is composed

146 of majority of colistin-sensitive strains (CMI ≤ 2 mg/L). Therefore, the absence of significant
147 difference of the ECtot evolution between the two treated groups is still unclear. The small
148 number of animal per group is perhaps a limiting factor. Moreover, inter and intra-individual
149 variabilities of faecal colistin concentrations were high, probably due to a heterogeneous
150 colistin distribution within faeces. Therefore, the actual effective concentrations of colistin, to
151 which bacteria were exposed within the digestive tract, are mostly unknown.
152
153 In the placebo group, the counts of ECmcr1+ were low but remained stable enough over the
154 experiment (Fig. 1B). No selective effect of colistin in treated piglets was observed as
155 ECmcr1+ stayed at a constant level over time, whatever the dose. This suggests that, in
156 standardized conditions, bacteria were exposed during a too short period to concentrations
157 within the selection window, i.e. between the MICs of indigenous *E. coli* and of ECmcr1+
158 (8 mg/L). In comparison, when colistin is administered on a large scale through water or feed
159 on pig farms, colistin concentration in faeces are lower [7] and much more variable [17];
160 therefore the probability to select strains harbouring *mcr-1* increases. Despite this, no real
161 outbreak of *mcr-1* positive strains exists in pig production in Europe [2] where a low
162 prevalence of *mcr-1* positive *E. coli* is found (e.g. 0.5 % in French pig farms) [4]. In contrast,
163 a high prevalence is observed in Asia (more than 20 % in China) [2], where colistin has been
164 used for decades as growth-promoter for piglets [18]. This sub-therapeutic dose (about 4-5
165 times lower than therapeutic use [19]) administered during a long period is likely to give
166 digestive concentrations reaching the selection window of *mcr-1* positive strains. Awareness
167 of this high risk led China to ban colistin as promoter very recently [20]. This hypothesis
168 should deserve further considerations.
169
170

5. Conclusions

In conclusion, under this experimental setting in piglets, oral dosing with colistin did not induce selection of *mcr-1* positive strains. Further investigations should be necessary to confirm this observation in farming conditions (with colistin given via food or water and piglets having contact between them). Meanwhile, a responsible and careful use of colistin is required to preserve this last-resort antimicrobial agent.

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Declarations

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Competing Interests: None

Ethical Approval: This experiment was approved by the ComEth Anses/ENVA/UPEC n°16 (French ethical committee) under the reference APAFIS#2905-2015112717486085

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254

255 **Figure Legend**

256 **Fig 1:** Mean (± SD) of counts of total faecal E.coli (A) and ECmcr1+ (B) before, during and
257 after oral colistin (or water) administrations for HD group (green), RD group (blue) and
258 placebo group (red). Vertical arrows indicate ECmcr1+ inoculations. The vertical dotted lines
259 indicate the treatment period (D0 to D4). Horizontal dashed line represents the limit of
260 quantification (log 1.2) and data below this value were arbitrary put to value log 0.6. Similar
261 profiles were obtained when data below LOQ were fixed at zero or at LOQ. Significant
262 differences from placebo group with Student test: * ($p<0.05$); ** ($p<0.01$)

263

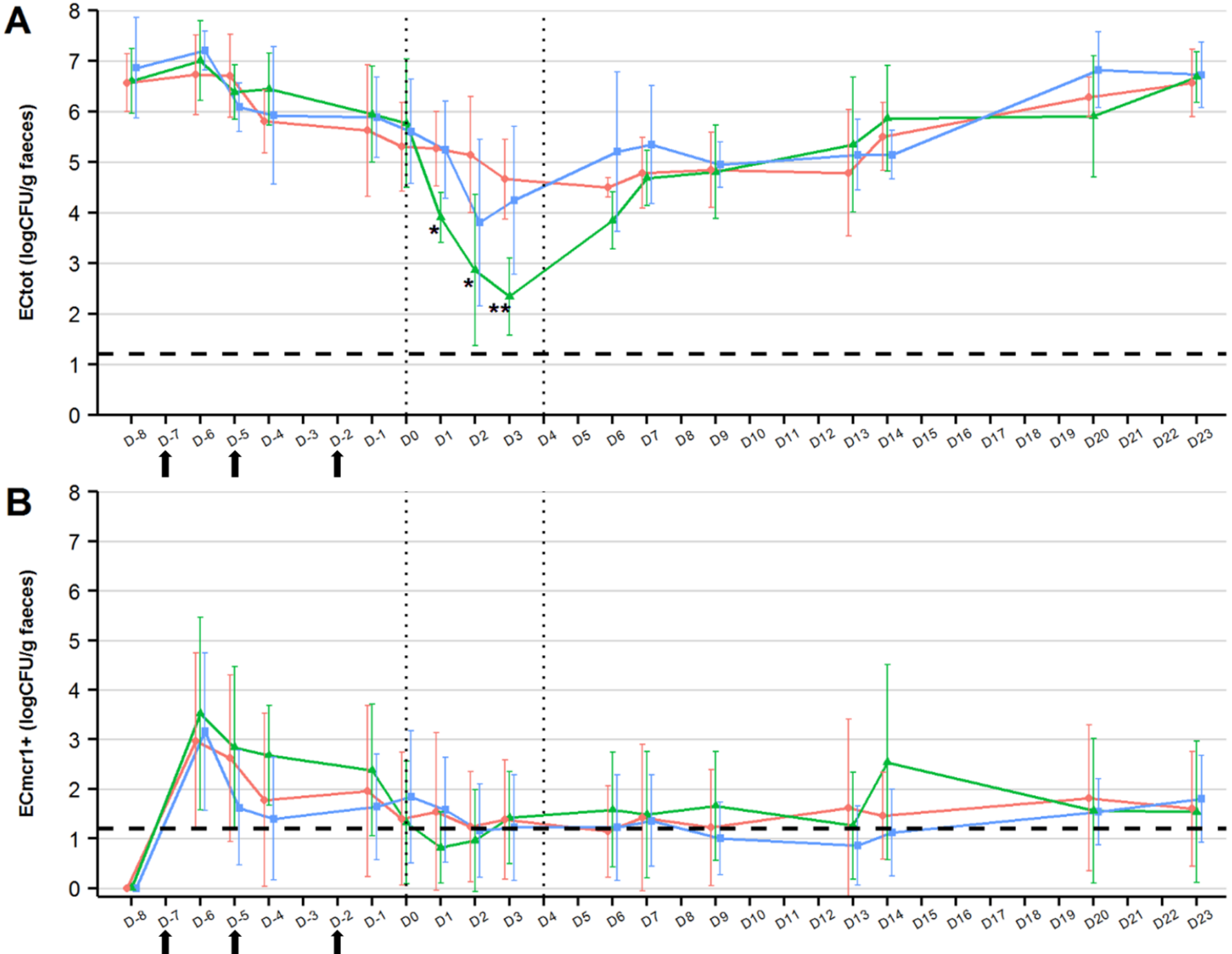
A
ECtot (logCFU/g faeces)
0
1
2
3
4
5
6
7
8
*
*
**
D-8 D-7 D-6 D-5 D-4 D-3 D-2 D-1 D0 D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 D13 D14 D15 D16 D17 D18 D19 D20 D21 D22 D23
B
ECmcr1+ (logCFU/g faeces)
0
1
2
3
4
5
6
7
8
D-8 D-7 D-6 D-5 D-4 D-3 D-2 D-1 D0 D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 D13 D14 D15 D16 D17 D18 D19 D20 D21 D22 D23

Table 1

TABLE 1: Faecal colistin concentrations over time in each treated-group[a].

	Mean ± SD (and range) in µg of colistin base/g of faeces per day.								
Treatment Group	D0	D1	D2	D3	D4	D5	D6	D7	D9
Recommended Dose (100,000 UI/kg/day)	0	93.2 ± 56.0 (28.1 - 149.9)	193.6 ± 144.5 (75.5 - 381.5)	217.4 ± 103.3 (74.2 - 327.0)	197.1 ± 54.7 (128.9 - 261.2)	262.5 ± 57.8 (212.4 - 342.1)	42.3 ± 22.8 (14.9 - 72.0)	1.8 ± 0.3 (1.6 - 2.2)	< LOQ[b]
High Dose (200,000 UI/kg/day)	0	119.6 ± 55.2 (61.2 - 188.9)	267.9 ± 78.3 (169.7 - 354.6)	268.9 ± 119.2 (155.0 - 470.9)	373.4 ± 190.2 (141.6 - 669.5)	249.7 ± 109.1 (106.3 - 372.4)	80.3 ± 103.8 (15.0 - 264.8)	3.4 ± 1.6 (1.7 - 5.7)	< LOQ[b]

[a] Placebo group is not mentioned as all concentrations were null

[b] LOQ: 1 µg/g of faeces