Veterinary Microbiology 230 (2019) 164–170

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Impact of colistin administered before or after inoculation on the transmission of a *mcr*-1 colistin-resistant *Escherichia coli* strain between pigs

Gwenaëlle Mourand[a,b], Mathieu Andraud[a,b], Eric Jouy[a,b], Claire Chauvin[a,b], Laetitia Le Devendec[a,b], Frederic Paboeuf[a,b], Isabelle Kempf[a,b,*]

[a] ANSES, Laboratoire de Ploufragan-Plouzané-Niort, Ploufragan, France
[b] Université Bretagne Loire, France

ARTICLE INFO

Keywords:
Pig
Colistin
Resistance
mcr-1
Transmission
Treatment

ABSTRACT

Colistin resistance associated with plasmidic resistance genes is a serious public health issue. We aimed at studying the transmission of an *mcr*-1 colistin- and rifampicin-resistant *Escherichia coli* strain between inoculated pigs and sentinels in different controlled conditions. Three groups of four pigs were bred in separated animal rooms and inoculated on Day 0 (D0). In each inoculated group, six contact pigs were introduced on D2. The first inoculated-and-contact group was left untreated. The ten pigs in the second inoculated-and-contact group received colistin (100 000 IU/kg) before inoculation or contact (D-7 to D-5), simulating prophylactic administration. Pigs in the third inoculated-and-contact group were treated just after inoculation or before transfer (D0 to D2), simulating metaphylactic administration. Faecal samples were regularly collected and segments of intestinal tracts were obtained at necropsy, on D20-D22.

Samples were cultured on rifampicin-supplemented media, and PCR was used to detect the *mcr-1* gene. The kinetics of infection, based on culture results, were analysed using an SIR model.

The inoculated strain was detected in all inoculated and contact pigs. The SIR model showed that one infected pig could transmit the resistant bacteria to one susceptible individual in less than 3 h on average. Prophylactic administration significantly enhanced the transmission rate and resulted in more samples containing the *mcr-1* resistance gene at necropsy. No effect of metaphylactic administration could be detected on the transmission rate, nor on the carriage of the resistant strain.

Our study confirms that colistin should not be used in a prophylactic manner.

1. Introduction

Colistin resistance in *Enterobacteriales* is a veterinary and public health concern. Since the first report of the *mcr-1* plasmidic resistance gene in pigs, poultry and clinical isolates in China (Liu et al., 2016), many countries have described the presence of *mcr-1* and *mcr* variants in strains of human, animal, food and environmental origin (Wang et al., 2018). The *mcr* genes encode a phospho-ethanolamine–transferase, leading to the alteration of the lipid A subunit of the LPS, resulting in decreased attraction of polymyxins. This modification of the outer membrane is associated with a rather low level of resistance. Other mechanisms of resistance resulting from chromosomal mutations in the genes involved in modifications of lipid A, such as *pmrA, pmrB, phoP, phoQ,* and *mgrB*, have also been described. Recently, we showed that most colistin-resistant isolates from diseased pigs in France carry the *mcr-1* gene, sometimes with chromosomal mutations (Delannoy et al., 2017).

Like in other cases of resistance, the prevalence of colistin resistance in animals is probably associated with the use of polymyxins, or other antimicrobials in the case of co-selection of multi-drug-resistant strains (Haenni et al., 2016). The resistance gene, being carried by plasmids, can be transmitted both vertically through cell division or through conjugations between bacteria in animate and inanimate environments (Volkova et al., 2013). Between-host transmission of resistant bacteria therefore occurs by ingestion of contaminated faeces released into the environment by colonised animals. Selection pressure exerted by antimicrobial administration may also affect the transmissibility of resistant bacteria between animals. Therefore, a specific experimental trial was developed to quantify the transmission of an *mcr-1*-positive *E. coli* strain from inoculated to sentinel pigs. To evaluate the impact of colistin administration and to better cover diverse field conditions, different experimental designs were implemented, with colistin given or not given to the inoculated or exposed animals before or just after inoculation of the resistant strain.

* Corresponding author at: ANSES Laboratoire de Ploufragan-Plouzané-Niort, Zoopole Les Croix, 22440, Ploufragan, France.
E-mail address: isabelle.kempf@anses.fr (I. Kempf).

https://doi.org/10.1016/j.vetmic.2019.02.002
Received 31 October 2018; Received in revised form 17 January 2019; Accepted 3 February 2019

2. Material and methods

2.1. Animals and animal facilities

The trial was performed in accordance with French animal welfare regulations and the protocol approved by the ANSES/ENVA/UPEC Ethics Committee (ComEth authorisation 16-053 (APAFIS No. 2016060108594266). The trial was conducted at the ANSES Ploufragan animal facilities. Strict biosecurity measures were implemented in order to avoid contamination of the pigs, including the use of an air filtration system and airlocks for each unit, unit-specific clothes, and compulsory showering after visiting the pigs. The trials were conducted with specific-pathogen-free (SPF) Large White piglets.

The eight-week-old piglets were randomised before the experiment. The animals did not receive any antibiotic treatment prior to the trial and were given the same non-supplemented feed. Each room contained two pens.

The names of the groups are encoded according to their status (T for treated, NT for non-treated, I for inoculated, and E for exposed) and the time (in days) of the events (for example, T-7E2 means animals treated from Day -7 and exposed to inoculated animals on Day 2).

The experimental design (Figs. 1 and 2) was built to simulate transmission of a resistant strain from contaminated (inoculated) non-treated animals (NTI0), from animals treated and then inoculated (treated-inoculated (T-7I0)), or from animals inoculated and then treated (T0I0) to groups of contact pigs. The respective contact groups were NTE2 (non-treated pigs, put in contact with NTI0 on Day 2), T-7E2 (pigs treated from Day -7 and then introduced on Day 2 into the room of the T-7I0 group), and T0E2 (pigs treated from Day 0 and then introduced on Day 2 into the room of the T0I0 group).

The NTI0 / NTE2 setting simulates the transmission of a resistant strain in non-treated animals.

The T-7I0 / T-7E2 setting parallels prophylactic administration, when the drug is given to a group of animals before they are exposed to a pathogen. This situation was used to evaluate the colonisation by the resistant strain of inoculated pigs whose microbiota had previously been altered by colistin and might still contain low colistin concentrations, and the subsequent transmission of this strain to the microbiota of previously treated exposed pigs (T-7E2).

The T0I0 / T0E2 setting corresponds to metaphylactic administration, when the drug is given to animals, some of which are already contaminated (T0I0), while the others may be exposed in the following days (T0E2). In this case, the resistant strain is inoculated to pigs when the colistin concentration in the microbiota is high and alters the colistin-susceptible *Enterobacteriales* population (Fleury et al., 2016) and, depending on the colistin concentration in the gut, might also inhibit the colistin-non-susceptible *Enterobacteriales* population.

At the beginning of the trial (Days –27 to Day 2), the pigs were dispatched in six animal rooms, as indicated in Fig. 1. Pigs in groups T-7NI, T-7I0, and T-7E2 received colistin (Colivet solution, 2 000 000 IU/mL, CEVA, Libourne, France) by oral gavage at the recommended dosage (50 000 IU/kg of body weight twice a day) from Day -7 to Day -5. On Day 0, pigs in groups NTNI and T-7NI were orally inoculated with 10 mL of sterile broth. On the same day, pigs in groups NTI0, T-7I0 and T0I0 were orally inoculated with 10 mL of the *mcr-1* positive UB10-260Rif *E. coli* inoculum (Mourand et al., 2018). Pigs in groups T0E2 and T0I0 were given colistin on Days 0, 1 and 2, as previously described. On Day 0, the colistin was given at 10 A M and 5 PM, whereas the inoculation was performed at 11 A M. On Day 2, the exposed pigs were moved, as indicated in Figs. 1 and 2. Thus pigs NTE2, T-7E2 and T0E2 were introduced into rooms R4 to R6, respectively.

During the week, daily clinical examinations consisted of looking for general clinical signs and taking rectal temperatures. Individual faecal samples were collected from all pigs before the beginning of the trial, and on Days 4 to 8, 10, 13, 17 and 20. Inoculated pigs were also sampled on Days 1 and 2 at 12 A M, whereas exposed pigs were sampled on Day 2, four hours after their introduction into rooms R4 to R6.

The pigs were euthanised on Days 20 (pigs NTNI, T-7NI, T0I0 and T0E2), 21 (pigs T-7I0 and T-7E2) and 22 (pigs NTI0 and NTE2) and necropsied. Faecal samples obtained at different levels of the digestive tract (jejunum, ileum, caecum and colon) were collected.

2.2. Bacterial strain and media

As in a previous trial (Mourand et al., 2018), we used a rifampicin-resistant mutant (UB10-260Rif) of a previously characterized *E. coli* strain UB10-260, which was isolated from a diseased pig in 2010 (Delannoy et al., 2017). Briefly, the strain belongs to phylogenetic group A and is resistant to colistin (minimum inhibitory concentration (MIC): 4 mg/L), rifampicin, sulphamethoxazole, trimethoprim, tetracycline, chloramphenicol and ampicillin. It contains the *mcr-1* gene, and two mutations in the PmrB protein sequence (S138 N and V351I), whose impact on colistin resistance is unknown (Delannoy et al., 2017). No virulence factors could be detected. *In vitro*, it has been shown to be conjugative and able to transfer the *mcr-1* gene along with resistance to tetracycline, chloramphenicol and ampicillin (Delannoy et al., 2017). As previously described, the inoculum was prepared by culture in Mueller Hinton (MH) broth containing colistin (2 mg/L) and the bacterial pellet obtained after centrifugation was re-suspended in peptone buffered solution, so as to obtain for each pig, a 10 mL inoculum containing 9×10^8 CFU.

2.3. Samples analysis

Bacteriological analysis: faecal samples obtained during the trial or at post-mortem (PM) were diluted 1/10 in peptone buffered solution containing 20% glycerol. The diluted aliquots were stored at −70 °C. Before freezing, 100 µL of the decimal dilutions prepared from the faecal suspensions (10^{-1} to 10^{-3}) were deposited using EasySpiral® Dilute (Interscience, Saint-Nom-la-Bretèche, France) onto MH plates containing 250 mg/L rifampicin (MHR). The plates were incubated at 37 °C for 24 h, and colonies were enumerated using the Scan 1200® apparatus (Interscience). For negative samples, 200 µL of the 1/10 thawed dilutions was enriched overnight in 1.8 mL of colistin-supplemented broth (2 mg/L) before plating 10 µL onto MHR plates. The limit of detection was 10 CFU/g. To check that the colonies growing on MHR belonged to the inoculated strain, one random colony from each pig sampled on Days 1, 2, 4 and 17 was analysed: cellular lysates were prepared (Kellogg et al., 1990) and PCR tests were used to confirm that isolates belonged to the *E. coli* species (Furet et al., 2009), and to the same phylogenetic group as UB10-260Rif *E. coli* (Clermont et al., 2013), and the presence of the *mcr-1* gene was screened for by PCR (Liu et al., 2016).

The *mcr-1* gene was also screened for by real-time PCR in DNA prepared from all faecal samples, as previously described (Mourand et al., 2018). For all PM samples, enrichment was prepared and the *mcr-1* gene was tested for by PCR. Briefly, PM samples were diluted 1/10 in peptone buffer and 200 µL were added to 1.8 mL of LB broth containing 2 mg/L colistin. After incubation for 24 h at 37 °C, the suspensions were centrifuged for 10 min at 5 180 *g* and a cellular lysate was prepared (Kellogg et al., 1990) and submitted to *mcr-1* qPCR (Mourand et al., 2018).

2.4. Estimation of transmission parameters

The kinetics of infections observed in each group were analysed using an SIR model (Gallien et al., 2018 in press; Gallien et al., 2018). At each sampling time, the individuals were classified according to their bacteriological results (after enrichment) as susceptible (negative), infectious (shedding resistant bacteria), or recovered after the last positive sample. The actual infection times T_{Inf}, since they were not observed, were drawn from uniform distribution ranging between the times of the last negative and the first positive samples. The probability of infection π on a time interval Δt is expressed in terms of prevalence (proportion of shedding animals) as

A: Experimental design before transfer

Room	Pen 1	Pen 2
R1	6 non-treated, future-exposed pigs (NTE2)	
R2	6 treated, future-exposed pigs (T-7E2) Treatment on Days -7, -6 and -5	6 treated, future-exposed pigs (T0E2) Treatment on Days 0, 1 and 2
R3	2 control pigs (NTNI)	2 treated, non-inoculated pigs (T-7NI)
R4	2 non-treated, inoculated pigs (NTI0)	2 non-treated, inoculated pigs (NTI0)
R5	2 treated, then inoculated pigs (T-7I0)	2 treated, then inoculated pigs (T-7I0)
R6	2 inoculated, then treated pigs (T0I0) Treatment on Days 0, 1 and 2	2 inoculated, then treated pigs (T0I0) Treatment on Days 0, 1 and 2

B: Experimental design after transfer of exposed pigs on Day 2

Room	Pen 1	Pen 2
R3	2 control pigs (NTNI)	2 treated, non-inoculated pigs (T-7NI)
R4	2 non-treated, inoculated pigs (NTI0) 3 non-treated, exposed pigs (NTE_2)	2 non-treated, inoculated pigs (NTI0) 3 non-treated, exposed pigs (NTE2)
R5	2 treated, then inoculated pigs (T-7I0) 3 treated, exposed pigs (T-7E2)	2 treated, then inoculated pigs (T-7I0) 3 treated, exposed pigs (T-7E2)
R6	2 inoculated, then treated pigs (T0I0) 3 treated, exposed pigs (T0E2) Treatment on Days 0, 1 and 2	2 inoculated, then treated pigs (T0I0) 3 treated, exposed pigs (T0E2) Treatment on Days 0, 1 and 2

Fig. 1. Experimental design before and after transfer of exposed pigs on Day 2.

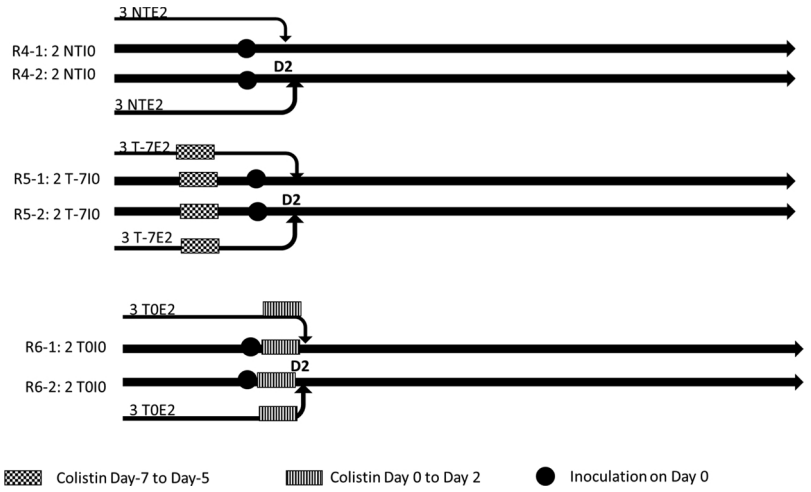

Fig. 2. Chronogram of the experiment.

$$\pi = 1 - exp\left(-\frac{\beta I}{N}\Delta t\right),$$

where β is the transmission rate (average number of new infections produced by one infectious individual per unit of time). With these notations, the probability of observing the infection kinetics given the parameters β and T_{Inf}, also called likelihood function, is expressed as:

$$L(\boldsymbol{T_{obs}}, I, N\beta, \boldsymbol{T_{Inf}}) = \prod_{i=1}^{Nc} \prod_{T_j = T_{obs_1}}^{T_j = T_{LastNeg_i}} \exp\left(-\frac{\beta I_j}{N}(T_j - T_{j-1})\right) * \left\{1 - \exp\left(-\frac{\beta I_{Inf}^{(i)}}{N}(T_{Inf}^{(i)} - T_{lastNeg_i})\right)\right\},$$

where I_j corresponds to the number of shedding animals over time interval $[T_{obs_{j-1}}, T_{obs_j}]$, and $I_{Inf}^{(i)}$ the number of shedders over the time interval during which individual i was infected. Parameter inference was performed using the Metropolis algorithm with uniform proposal distribution for the infection times and log-normal distribution for the transmission parameter. Non-informative priors were used with uniform and log-uniform distributions on large intervals ([-4,4] for $log(\beta)$ and $[T_{LastNeg}, T_{FirstPos}]$ for T_{Inf} . Three Markov chains were run with 10 000 iterations and a 1 000-iteration burn-in period, for which initial values were randomly drawn from prior distributions. Algorithm convergence was assessed using classical tests (Gelman-Rubin, Heidelberger, Geweke).

2.5. Statistical analysis

The Kruskal–Wallis non-parametric test was used to compare the weight gain of animals present in rooms R3 to R6 at Day 20. The distributions of pigs or isolates were analysed using the Chi2-test or the Fisher exact test when n $\leq$5 (https://biostatgv).

The culture titres were Log10 transformed, and the titres of samples positive only after enrichment were set at 1; titres of samples negative after enrichment were set at 0.

3. Results

3.1. Clinical signs and growth

No clinical signs were observed, except in one pig from the T0I0 group that suffered an accident and was euthanised on Day 10. The weight gains from Day -7 to Days 2, 7, 16 and 20 were compared between pigs in rooms R3 to R6. No statistically significant difference was detected ($p > 0.05$).

3.2. Culture results: fecal samples

Faecal samples collected from control pigs or collected before inoculation or transfer of contact pigs were negative. Titres obtained on MHR media for samples obtained from inoculated and contact pigs are given in Table 1. Analysis of 86 isolates obtained from live pigs directly or after enrichment showed that all were *E. coli* and that they shared the characteristics of UB10-260Rif *E. coli*: resistance to rifampicin, presence of the *mcr-1* gene, and the same phylogenetic group.

All inoculated pigs excreted the UB10-260Rif *E. coli* strain on all dates, except one pig in the T-7I0 group on Day 13, and one pig in the T0I0 group on Day 17 (Table 1). Thus, no significant differences between the numbers of positive samples were found between inoculated animals, either non-treated or treated before or after inoculation. Concerning exposed pigs, all were positive on at least one occasion. The numbers of positive samples for the NTE2, T-7E2 and T0E2 groups were 53/66 (80%), 38/66 (58%) and 49/66 (74%) (Chi2, $p = 0.01$), respectively. This shows that significantly fewer samples from the pigs treated before their transfer (T-7E2 group) contained the inoculated strain compared to the other exposed animals, being either non-treated or treated from Day 0.

The daily titres in the three inoculated groups (NTI0, T-7I0 and T0I0) did not differ significantly, except on Day 4, when pigs in group T-7I0 excreted significantly less resistant bacteria (Table 2). In each

Table 1
Culture on rifampicin-supplemented media of faecal and post-mortem samples, and *mcr-1* PCR results.

Room	Pen	Pig	Group	D1	H0	D2 H4	D2	D3	D4	D5	D6	D7	D8	D10	D13	D17	D20	Jejunum	Ileum	Caecum	Colon
R4	1	6390	NTI0	P*	P	X	P	P	P	P	P	P	P	P	P	P	P	N	P*	P	P*
		6450	NTI0	P*	P*	X	P*	P	P	P	P	P	P	P	P	P	P	N	P*	P*	P*
		6411	NTE2	X	X	P	P	P	P	P	P	P	P	P	P	P	P	N	P*	P*	P*
		6445	NTE2	X	X	P	P	P	P	P	P	P	P	P	P	P	P	N	P	P*	P*
		6462	NTE2	X	X	N	P	P	P	P	P	P	P	P	P	P	PE	N	N	P*	P*
	2	6418	NTI0	P*	P*	X	P	P	P	P	P	P	P	P	P	P	P	N	P*	P	P*
		6465	NTI0	P*	P	X	P	P	P	P	P	P	P	P	P	P	P	N	P	P	P*
		6437	NTE2	X	X	N	P	P	PE	N	P	P	P	N	PE	P	P	N	N	N	P*
		6449	NTE2	X	X	P	PE	P	PE	N	N	P	N	P	P	P	P	N	N	N	N
		6455	NTE2	X	X	N	N	N	PE	N	PE	P	PE	P	N	PE	P	N	N	N	P*
R5	1	6392	T-7I0	P*	P	X	P	P	P	P	P	P	P	PE	N	N*	P	N	*	*	P*
		6413	T-7I0	P	P*	X	P	P	P	P	P	P	P	P	P	P	P	N	P*	P*	P*
		6422	T-7E2	X	X	P	N	N	PE	N	P	N	N	N	P	PE	P	N	*	P*	P*
		6421	T-7E2	X	X	P	PE	PE	N	N	N	N	N	N	N	N	N	N	*	N	
		6460	T-7E2	X	X	N	P	N	N	P	P	N	N	N	P	N	N	*	*	*	
	2	6406	T-7I0	P*	P*	X	P	P	P	P	P	P	P	P	P	P	P	N	P*	P*	P*
		6456	T-7I0	P*	P	X	P	P	P	P	P	P	P	P	P	P	P	N	P*	P*	P*
		6435	T-7E2	X	X	P	P	P	PE	P	P	P	P	P	P	P	P	N	P*	*	P*
		6451	T-7E2	X	X	N	P	PE	N	N	N	N	P	P	P	N	N	*	*	*	
		6458	T-7E2	X	X	P	P	N	PE	PE	PE	P	P	P	P	P	N	P*	P*	P*	
R6	1	6398	T0I0	P*	P*	X	P*	P	P	P	P	P	P	X	X	X	N	P	P	N	
		6444	T0I0	P	P	X	P	P	P	P	P	P	P	P	N	P	N	P	P*	P*	
		6420	T0E2	X	X	P	P	P	P	P	P	P	P	P	N	P	N	P	P*	P*	
		6448	T0E2	X	X	P	P	P	P	P	N	P	P	P	P	P	P*	P*	P	P*	
		6459	T0E2	X	X	N	P	P	P	P	PE	N	P	P	N	P	N	N	N	P	
	2	6416	T0I0	P*	P	X	P*	P	P	P	P	P	P	P	P	P	N	P	P*	P*	
		6461	T0I0	P	P	X	P	P*	P	P	P	P	P	P	P	P	P*	P	P	P*	
		6438	T0E2	X	X	N	P	P	P	P	P	P	P	P	P	P	N	P	N	P	
		6452	T0E2	X	X	N	PE	P	P	P	P	P	N	P	P	P	P*	P*	N	P	
		6453	T0E2	X	X	N	N	N	N	P	N	P	N	N	N	N	N	N	N	N	

P: positive result, PE: positive only after enrichment, N: negative result, X: no sample.
*sample positive by PCR for *mcr-1*(for faecal samples, PCR was performed on DNA prepared from faecal samples; for PM samples, PCR was performed on enrichment broths). Absence of asterisk means negative *mcr-1* PCR result.
Grey cells: samples positive according to at least one method.

Table 2
Mean daily titres of faecal samples on rifampicin-supplemented media.

Room	Group (n)	D1**	D2 H0***	D2 H4***	D3	D4	D5	D6	D7	D8	D10	D13	D17	D20	Positive samples
R4	NTI0 (4)	6.4	5.3		4.7	4.5	4.3	3.9	4.1	3.9	3.8	3.7	3.8	3.9	48/48
	NTE2 (6)			1.75	2.4	2.4	1.7	1.7	2.6	2.8	1.9	2.6	2.2	2.1	53/66
R5	T-7I0 (4)	5.9	5.3		4.7	3.7	3.8	3.6	3.7	3.7	3.0	3.2	3.2	3.6	47/48
	T-7E2 (6)			1.99	2.2	0.7	0.5	0.8	1.2	0.7	1.2	1.3	2.0	1.0	38/66
R6	T0I0 (4)*	5.8	5.1		5.1	4.5	4.1	3.3	3.7	3.8	3.7	3.6	2.6	4.5	44/45
	T0E2 (6)			1.01	2.3	2.6	2.1	2.8	1.6	2.5	1.6	2.1	1.4	2.7	49/66

* One pig in the T0I0 group was euthanised on Day 10.
** Day 1.
*** Day 2, just before (H0) or 4 h (H4) after transfer into rooms containing inoculated pigs.

group, the maximum daily means (6.45, 5.93 and 5.82 Log10/g of faeces) were observed on the first day after inoculation, and decreased thereafter, but most often remained higher than 3 Log10/g up to the last sampling Day (Day 20). Conversely, for contact pigs, means of daily titres never reached 3 Log10/g, the maximum means being 2.8 on Day 8, 2.2 on Day 3, and 2.8 on Day 6 for groups NTE2, T-7E2 and T0E2, respectively. A comparison of these sentinel groups shows that the titres were significantly different on Days 4, 5 and 8, with lower titres in group T-7E2. On these days, the mean titres in this group were at least 1.2–1.8 Log10 lower than the mean titers in the other exposed groups.

3.3. Culture results: PM samples

Organs from all non-inoculated pigs were negative on MHR. For organs from inoculated and exposed pigs (Table 3), the ratio of positive jejunum samples was significantly lower than in other organs ($p < 0.001$), with only three samples from T0I0 or T0E2 animals being positive. No significant differences were seen between the other types of samples.

No significant difference in the ratio of total positive samples in the different groups of inoculated pigs could be detected ($p > 0.05$). This was also true for the groups of exposed pigs.

Overall, 34 out of 48 samples (71%) from inoculated animals were positive, thus significantly more than for the exposed pigs (30 out of 72 (42%), $p = 0.002$). Comparison of the titres obtained for each organ according to the different inoculated groups on the one hand, and the different exposed groups on the other, did not reveal any significant differences (data not shown).

3.4. mcr-1 PCR on DNA prepared from faecal samples

All the samples tested positive for the PCR internal control. All samples collected from non-inoculated pigs or before inoculation or exposure were negative for the *mcr-1* gene. A total of 19/339 samples were found to be positive (Table 1), all from inoculated pigs, and mostly during the first days after inoculation (9 on Day 1, 5 on Day 2, 3 on Day 3, 1 on Day 4, and 1 on Day 17). They were overall equally distributed between the groups, with 7 positive in the NTI0 group, and 6 in the T-7I0 and T0I0 groups. All *mcr-1*-positive samples were also positive according to culture on MHR, except for one faecal sample from pig 6392 (group T-7I0) on Day 17.

3.5. mcr-1 PCR on enrichment broths from PM samples

A total of 58/120 enrichment broths prepared from PM samples were found to be positive by PCR (Table 4). Jejuni were rarely positive, whereas 50% or more of the other organs were positive. No significant differences were found between the total numbers of positive samples in the inoculated groups. For the contact groups, the T-7E2 group yielded significantly more positive PCR results (16/24, 67%) than the other two exposed groups (NTE2: 9/24 (37.5%) and T0E2: 7/24 (29%)) ($p = 0.02$)). In all, 26 out of 48 samples (54%) from inoculated pigs were found to be positive compared to 32 out of 72 (44%) for exposed animals ($p > 0.05$). For the inoculated and exposed groups, the numbers of positive samples detected by culture and by PCR did not differ significantly.

3.6. Transmission

The transmission rate from the kinetics of infection observed in group T-7E2 was estimated to be 0.36 [0.12; 0.88] (animal/hour) (Fig. 3). In other words, one infected individual would be able to transmit resistant bacteria to one susceptible individual in less than 3 h on average. The estimates obtained in the other groups were significantly lower ($\beta = 0.08$ [0.03; 0.17] and $\beta = 0.06$ [0.02, 0.12] in groups NTE2 and T0E2, $p < 0.05$). All animals in group T-7E2 were infected within one day post-contact, leading to large credibility intervals for the parameter estimate, although convergence of the algorithm was achieved. The average time between infection and detection($T_{firstPos} - T_{Inf}$) ranged between 5 and 7 h, with 50% of the animals being infected within 3 h before detection, reflecting the proportion of positive animals at the first sampling time (4 h post-contact). However, the results

Table 3
Post-mortem samples: culture on rifampicin-supplemented media.

Room	Group	Jejunum	Ileum	Caecum	Colon	Total
R4	NTI0	0/4*	4/4	4/4	4/4	12/16
	NTE2	0/6	2/6	3/6	5/6	10/24
R5	T-7I0	0/4	3/4	3/4	4/4	10/16
	T-7E2	0/6	2/6	2/6	3/6	7/24
R6	T0I0**	1/4	4/4	4/4	3/4	12/16
	T0E2	2/6	4/6	2/6	5/6	13/24
	Total	3/30	19/30	18/30	24/30	64/120

The pigs were euthanised sacrificed on Day 20 (pigs NTNI, T-7NI, T0I0 and T0E2), Day 21 (pigs T-7I0 and T-7E2) and Day 22 (pigs NTI0 and NTE2). All samples from non-inoculated pigs were negative.
* Number of positive samples/number of tested samples.
** One pig in the T0I0 group was euthanised sacrificed on Day 10.

Table 4
mcr-1 PCR results on enrichment broths from post-mortem samples.

Room	Group	Jejunum	Ileum	Caecum	Colon	Total
R4	NTI0	0/4*	3/4	1/4	4/4	8/16
	NTE2	0/6	1/6	3/6	5/6	9/24
R5	T-7I0	0/4	4/4	4/4	4/4	12/16
	T-7E2	0/6	5/6	6/6	5/6	16/24
R6	T0I0**	1/4	0/4	2/4	3/4	6/16
	T0E2	2/6	2/6	1/6	2/6	7/24
Total		3/30	15/30	17/30	23/30	58/120

The pigs were euthanised on Day 20 (pigs NTNI, T-7NI, T0I0 and T0E2), Day 21 (pigs T-7I0 and T-7E2) and Day 22 (pigs NTI0 and NTE2). All samples from non-inoculated pigs were negative.
* Number of positive samples/number of tested samples.
** One pig in the T0I0 group was euthanised sacrificed on Day 10.

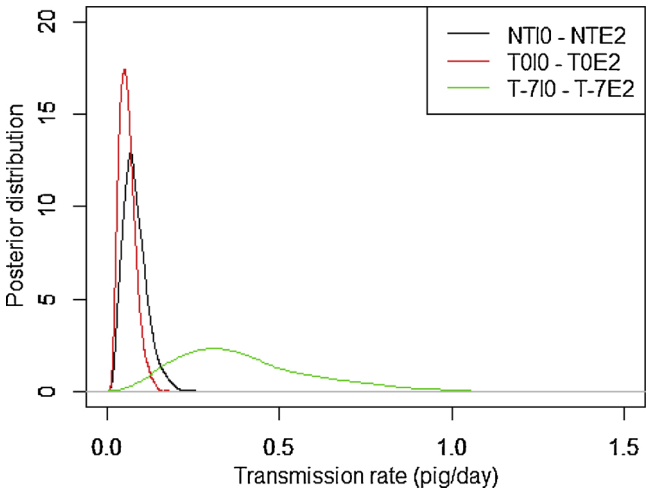

Fig. 3. Distribution of transmission rates according to treatment.

demonstrated significantly earlier infections in the pre-treated group (T-7E2; Cox proportional hazard model: HR = 0.73 [0.68, 0.78]), even though the exposure duration was similar in all groups.

4. Discussion

As previously observed (Mourand et al., 2018), inoculation with UB10-260Rif *E. coli* did not lead to clinical signs. After the inoculation of 9×10^8 CFU to the eight-week old pigs, the strain was regularly excreted by most inoculated pigs during the three-week trial, as observed in our previous assay using an inoculum of 2.5×10^8 CFU for seven-week-old pigs (Mourand et al., 2018).

No attempt to analyse faecal or PM samples with colistin-supplemented media was performed because of the poor sensitivity of such media based on our experience in previous trials (Mourand et al., 2018). Similarly, *mcr-1* qPCR on DNA extracted from faecal samples, which could be a measure of all *mcr-1*-containing bacteria, showed very poor sensitivity compared to culture on MHR media, with only 19 positive results out of 339 (5.6%) tested samples, compared to 279/339 (82.3%) for culture ($p = 5 \text{ x} 10^{-90}$). Conversely for PM samples, results from cultures on MHR media (53.3% positive samples) and PCR on enrichment broths (48.3% positive samples) were not significantly different, probably because the PCR was performed on cultures obtained after enrichment in colistin broths, resulting in a higher numbers of *mcr-1*-containing cells. Thus to determine transmission parameters from fecal samples, we favoured results obtained from culture on MHR media, although this medium did not enable us to detect possible transfers of the *mcr-1* gene from the inoculated strain to other commensal *Enterobacteriales,* as could be previously observed *in vitro* (Delannoy et al., 2017) or *in vivo* (Mourand et al., 2018). This means that the calculated transmission parameters are relevant for the inoculated strain and may underestimate transmission of colistin-resistant strains as we took into account only the inoculated strain, and not all the bacteria that may contain the *mcr-1* gene. Inversely, the loss of the *mcr-1* gene from UB10-260Rif *E. coli* is probably a rare event *in vivo* as it was not detected during the characterisation of all the 86 rifampicin-resistant *E. coli* re-isolated from this study, or the 261 samples obtained during the previous assay (Mourand et al., 2018). This means that the calculated transmission parameters probably do not overestimate the transmission of colistin-resistance.

Inoculated pigs had high titers compared to exposed pigs. This may be related to the titer of the inoculated culture (9×10^8 CFU per pig), probably much higher than the doses to which contact pigs were exposed, *via* contaminated feces from inoculated animals.

The comparison of faecal, PM cultural, and molecular results obtained for the different inoculated groups showed that the only significant difference was observed on Day 4, with T-7I0 pigs (treated on Days -7, -6 and -5) excreting less resistant bacteria compared to non-treated pigs or pigs treated on Days 0, 1 and 2. We could not determine the concentration of colistin in the gut of T-7I0 pigs at the time of inoculation, but Viel et al. (Viel et al., 2017) reported mean concentrations of 1.8 +/-0.3 µg/g of feces collected on Day 7 for pigs receiving the recommended dose (100 000 IU/kg/day) from Day 0 to Day 4. Thus the colistin concentrations in our pig guts five days after the last administration were probably very low. Thus we can hypothesise that the lower excretion of the inoculated strain could be due to an indirect effect of colistin, possibly associated with microbiota disruption and a more heterogeneous microbial population in treated animals as observed by Fleury et al. (Fleury et al., 2016). However no attempt to characterize the composition of the pig gut microbiota was done.

Similarly for sentinel pigs, group T-7E2 differed significantly from the other exposed groups, with a lower number of culture-positive faecal samples, and lower titres on Days 4, 5 and 8. However, the transmission parameter was significantly higher in this group of pigs treated seven days before inoculation and 9 days before exposure, suggesting higher receptivity of this group of exposed pigs, again probably in relation with microbiota disruptions induced by the preceding treatment. Moreover, at PM on Day 21, according to PCR on enrichment broths, this group yielded more positive samples than the other exposed groups. The discrepancy between the PM non-significantly-different culture results and the significantly-different PCR results, obtained on enrichment from organ samples, may lie in the sampling time, sampling method (faecal material or internal organs) and also in the target (only the inoculated strain for culture), but all *mcr-1*-positive bacteria for the PCR. Thus, as previously explained, we can hypothesise that at Day 2, *i.e.* 7 days after the last administration, the concentration of colistin was very low (Viel et al., 2017), and that the disruption of the intestinal microbiota of pigs treated on Days -7 to -5 resulted in lower colonisation during the very first few days after contact on Day 2; but ultimately, at PM, more *mcr-1*-resistant bacteria were present in the pigs that had received early administration of colistin. As a consequence of our findings, we can imagine that under field conditions, the higher contamination of internal organs by *mcr-1*-positive strains might result in more frequent contaminations of meat products by resistant isolates. Moreover, the higher transmission rate in case of early treatment may result in more pigs becoming contaminated, and thus in a higher impact on environmental contamination. Therefore, our observations reinforce the recommendations of the EMA/CVMP/ 231573/2016 not to use colistin in a prophylactic manner (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/07/WC500211080.pdf).

The absence of differences between NTI0 and T0I0 pigs means that the colistin treatment, given at the therapeutic dose during the first days of infection, had no detectable impact on the colonisation of the pigs by colistin-resistant UB10-260Rif *E. coli*. The colistin MIC for UB10-260Rif *E. coli* is 4 mg/L which is probably too high to inactivate the bacterium at the therapeutic dosage of 50 000 IU/kg BW twice a day. In *in vitro* pharmacodynamic studies, Guyonnet et al. (Guyonnet et al., 2010) showed that absence of re-growth of *E. coli* strains between 5 and 24 h required concentrations of 16 to 32 times the MIC of the strain, meaning that 64 to 128 mg/L are probably needed to completely inhibit the *in vitro* growth of UB10-260Rif *E. coli*. They also reported that after administration of 50 000 IU/kg, the gastro-intestinal tract concentration peaked at 43.6 mg/kg and a mean of 37.2 mg/kg at 0.5 h was achieved for a few hours. Viel et al. (Viel et al., 2017) found colistin mean concentration values of 93 to 262 mg/kg of faeces during the 5 days of administration of 100 000 IU/kg/day to pigs; however, no selective effect on the inoculated *mcr*-1-positive strain, presenting a MIC of 8 mg/L, was observed in their experiment, which they attributed to the fact that the bacteria were exposed for too short a period to concentrations within the selection window. Similar phenomena probably explain why, in our conditions, the therapeutic administration of colistin did not prevent colonisation by *mcr-1*-positive UB10-260Rif *E. coli*, but inversely did not induce its selection. As the colistin MICs of

most *E. coli* isolates harboring an *mcr-1* gene are slightly above the colistin cutoff (2 mg/L), it is probable that colistin given at the therapeutic dose does not select these resistant isolates but cannot have a protective effect against an intestinal infection caused by such isolates. Our observations, in line with Viel et al. (Viel et al., 2017), can be compared to the results of the *in vivo* experiment reported by Liu et al. (Liu et al., 2016) that used a mice thigh model. In this mice model, MCR-1 was shown to provide protection against colistin, but intravenous administration of colistin was still able to decrease the resistant population by more than one Log. However, this mice model differs in many ways (site of infection, route of administration, presence of microbiota, *etc.*) from our conditions.

5. Conclusion

The design of the trial allowed us to evaluate the transmission parameters and the persistence of *mcr-1*-positive commensal *E. coli* in pigs, either inoculated with or exposed to this strain and given colistin or not. The administration of colistin simulated either prophylactic or therapeutic administration. Our results showed that the prophylactic administration enhanced the transmission rate of the inoculated strain, and resulted at PM in more samples containing the *mcr-1* resistance gene. In our conditions, no effect of therapeutic administration could be detected on the transmission rate, nor on the persistence of resistance. However, other experiments using different strains and experimental models are needed to further evaluate the efficacy and selection impact of colistin administration in field conditions, in particular when administered in cases of post-weaning diarrhoea.

Conflict of interest statement

The authors indicate that there are no conflicts of interest

Funding

This research was supported by the Ecoantibio Plan—a public policy set up by the French Ministry of Agriculture, Agro-Food and Forestry to reduce the risks of antibiotic resistance in veterinary medicine— ANSES (the French Agency for Food, Environmental and Occupational Health & Safety) and the Côtes d'Armor General Council.

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Acknowledgment

The authors would like to sincerely thank Yann Bailly (ANSES Ploufragan) for technical assistance.

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