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Veterinary Microbiology 226 (2018) 81–88

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Development of a pig infection model with colistin-resistant *Escherichia coli*

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ARTICLE INFO

Keywords:
Escherichia coli
Pig
mcr-1
Experimental model
Post-weaning diarrhea

ABSTRACT

Colistin-resistant *Escherichia coli* are isolated from pigs suffering from post-weaning diarrhea (PWD). This study was designed to develop an experimental model of PWD using *mcr-1*-carrying shiga toxin-producing *E. coli* (STEC) or enterotoxigenic *E. coli* (ETEC), for the future evaluation of control measures. Three groups of eight piglets, kept in high biosecurity units, were orally inoculated with *mcr-1*-positive STEC or ETEC, and one unchallenged group was used as a control. Clinical signs were recorded. Regularly-collected fecal samples and samples obtained from the digestive tract of animals sacrificed one month after inoculation were cultured in selective media and isolates were characterized. Blood samples were used to genotype the polymorphisms of the pigs' intestinal receptors for F4 and F18 *E. coli* adhesins.

Diarrhea was more frequent and more fecal samples contained the inoculated strain in the group inoculated with the O149-F4 ETEC strain than with the O141-F18 or O139-F18 STEC strains. However, fewer positive samples were obtained from the two pigs with the F4 resistant genotype. The three inoculated strains could be re-isolated up to the end of the experiment. Excretion peaked on the first week after inoculation with the O149-F4 ETEC strain, and later for the other two. An *mcr-1* gene transfer to other commensal isolates was observed only for O139-F18 STEC, while the loss of *mcr-1* from the inoculated strain occurred in all groups. The O149-F4 ETEC challenge may be used to evaluate alternative solutions to combat PWD caused by colistin-resistant *E. coli* in pigs.

1. Introduction

Post-weaning diarrhea (PWD) and edema disease are common pathological conditions affecting piglets (Rhouma et al., 2017a). Various *Escherichia coli* strains cause these diseases. Indeed, different serogroups (O138, O139, O141, O149, O157…), adhesive factors (F4 (K88), F8, AIDA, Eae…), and other virulence factors (enterotoxins LT, STa, STb, EAST…) have been reported in these strains (Fairbrother and Gyles, 2012). The control of these infections must be based primarily on optimizing breeding management of the weaning piglets so as to avoid, insofar as possible, stressful conditions as they are separated from the sow, transported, mixed, placed in a new environment and provided with new feed. Prophylactic measures may also include vaccination of the sow (Rhouma et al., 2017a). However, these measures may be ineffective in preventing digestive troubles, and in the past, orally administered antimicrobials were almost systematically used to control colibacillosis. As a result, *E. coli* strains have developed antimicrobial resistance, particularly to the most frequently administered molecules. Of note, resistance to colistin (CST) had been detected at relatively low levels for years in commensal isolates, but prevalence was sometimes high for pathogenic strains (Kempf et al., 2016), and after a publication on the emerging *mcr-1* gene in China (Liu et al., 2016), it soon appeared that this gene was also present in many other countries, particularly in pig *E. coli* strains (Kempf et al., 2016). As these strains are frequently also resistant to many other antimicrobials (Delannoy et al., 2017), it became necessary to develop and evaluate novel control products or measures. To our knowledge, experimental models of colibacillosis in piglets have been developed with inoculation of CST-susceptible *E. coli* (Madec et al., 2000; Rhouma et al., 2016, 2017b) but not with CST-resistant strains. However we believe that such models are needed to evaluate the impact or the efficacy of antimicrobials, including colistin, and alternative strategies on infections caused by strains with resistance or reduced susceptibilities to a number of molecules. Furthermore, it has been shown that acquisition and expression of the *mcr-1* gene in a *E. coli* cell may result in gross morphological changes, including cell membrane impairment, and reduced fitness and attenuated virulence in

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https://doi.org/10.1016/j.vetmic.2018.10.011
Received 4 July 2018; Received in revised form 17 September 2018; Accepted 12 October 2018

a *Galleria mellonella* model. Moreover the *mcr-1* mediated LPS modification reduces the stimulation of macrophages, and a lower production of IL-6 and TNF was induced with LPS extracted from *mcr-1* strains compared to control LPS (Yang et al., 2017). Thus because of these potential differences of susceptibility and virulence of *mcr-1* strains, we decided to develop an experimental model of colibacillosis in weaned piglets using multi-drug-resistant *E. coli* strains isolated from clinical cases and carrying the *mcr-1* gene.

2. Material and methods

2.1. Preparation of strains, media and inocula and whole genome sequencing

Three *E. coli* strains (12–246, 12–269 and 13–220) from our previously characterized collection of *mcr*-1-positive *E. coli* isolates from diseased pigs were selected on the basis of their resistances, serogroups and virulence genes as detected by high-throughput real-time PCR microarray technology (Delannoy et al., 2017). The intent was to choose strains representative of CST-resistant isolates obtained from piglets with PWD. All three strains were hemolytic. Strains 12–246, 12–269, and 13–220 belonged to phylogenetic groups (PGG) A, A, and E, and had markers O141-F18, O149-F4, and O139-F18 respectively. All three strains were resistant to colistin, sulfamethoxazole, trimethoprim, tetracycline, and ampicillin. Strains 12–246 and 12–269 were also resistant to gentamicin and chloramphenicol.

A rifampicin-resistant mutant (12–246 M, 12–269 M, and 13–220 M) was prepared from each strain by culturing a large amount of inoculum on rifampicin-supplemented media (250 mg/L). DNA from the three mutants was prepared using QIAmp DNA mini kits (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Whole genome sequencing was performed with the ion proton system (Ion Torrent™, Thermofisher, Villebon sur Yvette, France).

We cleaned reads with Trimmomatic (Bolger et al., 2014), then used BWA-MEM (Li, 2013) to align them with the *E. coli* E455 strain (NCBI Reference Sequence NZ_JEND02000001.1) to obtain an estimated coverage depth of 80. We next performed SPAdes assemblies on cleaned reads (Bankevich et al., 2012) and MIRA assemblies (Chevreux et al., 1999) on related raw reads. Contigs were identified using Megablast on a local nt database (Chen et al., 2015), and those corresponding to "Uncultured soil fungus" were deleted.

Sequences were analyzed using a web tool (https://cge.cbs.dtu.dk/services) to detect antimicrobial resistance genes and verotoxin virulence genes, and to determine the ST of each strain (Thomsen et al., 2016). Other virulence genes, including *paa, sfpC, cnf2, AIDA-I, ecf1-1, stcE,* and *eibG* (Tseng et al., 2014), *sitA-D*, *iucA-D*, *iutA*, *hlyF*, *ompT*, *etsA-C*, *iss*, *iroB-E*, *iroN*, *cvaA-C*, *cvi*, *tsh*, and *eitA-D* (Touzain et al., 2018), *terE, ureD,* and *esc1763* (AE005174.2), *hlyA* (FM180012.1), *orfA* and *orfB* (GU810159), were sought using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

To prepare the inocula, each mutant was cultured in Mueller Hinton (MH) broth containing colistin (2 mg/L) for 18 h at 37 °C, under gentle agitation. Each bacterial pellet obtained after centrifugation (5180 *g*, 5 min) was re-suspended in peptone buffered solution in order to obtain a titer of 10^7 colony-forming units (CFU)/mL.

2.2. Experimental design

The experiment was performed in accordance with French animal welfare regulations and the protocol was approved by the ANSES/ENVA/UPEC ethical committee (ComEth authorization 17-059 (APAFiS no. 2017082408183121)). The experiment was conducted in the ANSES Ploufragan animal facilities. Strict biosecurity measures were implemented 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 29 specific-pathogen-free (SPF) Large White piglets. The SPF piglets are naturally born from hysterectomy-derived sows controlled for presence of classical and african swine fever, Aujesky's disease, foot-and-mouth disease, H1N1/H3N2 swine influenza, transmissible gastroenteritis/porcine respiratory coronavirus, porcine parvovirus, reproductive and respiratory syndrome virus, porcine circovirus type 2, Border disease, *Mycoplasma hyopneumoniae, Pasteurella multocida, Bordetella bronchiseptica, Actinobacillus pleuropneumoniae, Haemophilus parasuis, Streptococcus suis* Type 2*, Salmonella, Lawsonia intracellularis, Brachyspira hyodysenteriae, Yersinia, Campylobacter, Listeria monocytogenes, Balantidium coli* and *Trichomonas.* The piglets are left with the sow until weaning and bred in a pathogen-free environment.

Table 1
Arrangement of pigs inoculated with the different studied strains.

Room	Groups- inoculation	Pen D	Pen V
E1	G1- sterile buffer	3 control pigs (1 F4-R)[a]	2 control pigs (0 F4-R)
E2	G2- *E. coli* 12-246M	4 inoculated pigs (0 F4-R)	4 inoculated pigs (2 F4-R)
E3	G3- *E. coli* 12-269M	4 inoculated pigs (1 F4-R)	4 inoculated pigs (1 F4-R)
E4	G4- *E. coli* 13-220M	4 inoculated pigs (0 F4-R)	4 inoculated pigs (0 F4-R)

All pigs were susceptible to *E. coli* F18.
[a] Number of pigs resistant to *E. coli* F4.

The six-week-old piglets were randomized 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 (Table 1). On Day 0, five piglets in animal room E1 (group 1, five pigs) were orally inoculated with 10 mL of sterile broth. On the same day, eight piglets per room (four piglets per pen) in animal rooms E2, E3, and E4, (respectively named groups G2, G3, and G4, eight pigs each) were orally inoculated with 10 mL of the inocula prepared with strains 12–246 M, 12–269 M, and 13–220 M respectively.

The weight of each animal was recorded once a week. During the week, daily clinical examinations consisted in looking for general clinical signs and taking rectal temperatures. Individual fecal samples were collected from pigs on Days -4, 1, 2, 3, 4, 5, 7, 9, 11, 14, 17, 21, 24, and 28 (groups G2, G3, and G4), and on Days -4, 7, 14, 21 and 28 (group G1). The pigs were sacrificed on Day 35 (group G2), Day 36 (group G3) or Day 37 (group G4). Three pigs from the G1 group were also sacrificed on Day 35, the others being kept as controls for another assay and necropsied on Day 50 following euthanasia. The sacrificed animals were necropsied and samples of the duodenum, jejunum, ileum, cecum, colon, and rectum were collected. The samples collected post-mortem contained both fecal material and scraped mucosa.

2.3. Analysis of fecal and organ samples

For culture analysis, all the samples were diluted 1/10 in peptone buffered solution containing 20% glycerol. The 1/10 diluted aliquots were stored at -70 °C. Before freezing (fecal samples) or after thawing (organ samples), decimal dilutions were prepared. One hundred microliters of decimal dilutions were inoculated using EasySpiral® Dilute (Interscience, Saint-Nom-la-Bretèche, France) onto MacConkey media supplemented with rifampicin (250 mg/L) and incubated overnight at 37 °C. Moreover, 200 µL of the 1/10 thawed dilutions was enriched overnight in 1.8 mL of rifampicin-supplemented LB broth (250 mg/L) before plating on rifampicin-supplemented MacConkey plates. The limit of detection was 100 CFU without enrichment and 50 CFU/g after enrichment. Whenever possible, two colonies (from samples positive only after enrichment) or three colonies (from samples positive without enrichment) per sample were stored. PCR was used to confirm that they belonged to the *E. coli* species (Bej et al., 1991), to detect the presence

of the *mcr-1* gene (Liu et al., 2016), and to determine their phylogenetic group (Clermont et al., 2013). For isolates which did not exhibit the expected results (presence of *mcr-1* and phylogenetic group of the inoculated strain), ERIC-PCR (Rivera et al., 1995) and PFGE profiles (Ribot et al., 2006) after digestion with *Xba*I were studied. The susceptibility of some of these isolates was compared to the susceptibility of the inoculated strains by determining the minimum inhibitory concentrations (MIC) using a broth microdilution method on EUVSEC plates (Sensititre, ThermoFisher Scientific, Dardilly, France).

In addition, fecal samples collected on Days 2, 9 and 17 were inoculated, after thawing, onto the Chromagar Orientation™ agar medium (i2a, Montpellier, France) supplemented with colistin (8 mg/L) and vancomycin (8 mg/L) (CAO-CV) as previously described (Mourand et al., 2018) to detect colistin-resistant rifampicin-susceptible *E. coli* resulting from transfer of the *mcr-1* gene to commensal *E. coli*. When *E. coli* colonies were observed, two colonies per sample were stored and further analyzed. Their identity and the presence of the *mcr-1* gene were checked by PCR, and their resistance to rifampicin was tested by culture on rifampicin-supplemented MH media (250 mg/L). Moreover, 200 µL of the 1/10 dilutions was enriched overnight in 1.8 mL of colistin-supplemented LB broth (2 mg/L) before plating onto rifampicin-supplemented MacConkey plates (limit of detection: 50 CFU/g). The colonies were identified and analyzed for the presence of the *mcr-1* gene as previously described.

For molecular analysis, thawed fecal and organ samples were tested with a slightly modified version of the protocol described by Dona et al. (Dona et al., 2017). Briefly, 200 µL of the 1/10 diluted thawed fecal suspension was used to inoculate 1.8 mL of Luria Bertani (LB) broth supplemented with rifampicin (250 mg/L). After overnight culture at 37 °C under agitation, the broths were centrifuged at 5180 *g* for ten minutes and a cellular lysate was prepared from the pellet. *E. coli* DNA and the *mcr-1* gene were then detected by PCR in the lysate. PCR was performed on lysates obtained from enrichment in the colistin-supplemented broths (2 mg/L), like for samples collected on Days 2, 9, and 17.

2.4. Study of resistance polymorphism to adhesion factors in pigs

Blood was obtained from each pig just before euthanasia. These samples were used to genotype the pigs for the different polymorphisms linked to the intestinal receptors for F4 and F18 *E. coli* adhesins.

The porcine mucin 4 (*muc4*) gene has been mapped on the q1 region of the pig chromosome 13, significantly associated with *in vitro E. coli* F4ac adhesion to enterocytes, and MUC13 is the most likely responsible gene governing susceptibility towards enterotoxigenic *E. coli* (ETEC) F4ac diarrhea (Ren et al., 2012). The polymorphism of *muc4* was analyzed based on the test described by Rasschaert et al. (Rasschaert et al., 2007) to determine the F4ac/ab receptor status. Briefly, a fragment of the mucin 4 gene was amplified using Hemo Klen Taq polymerase (NEB, Evry, France) and digested with *Xba*I. The 367 bp amplified *muc4* PCR fragment of the resistant allele is indigestible, unlike the susceptible allele. MUC13 was genotyped by PCR to detect the presence of amplicons of 151 bp (MUC13 A allele) and 83 pb (MUC13B allele) as described by Ren et al (Ren et al., 2012). Susceptible animals usually carry at least one MUC13B allele.

The *E. coli* F18 receptor locus is closely linked to the fucosyltransferase gene, and we used the test developed by Meijerink et al. (Meijerink et al., 1997) in which amplification followed by restriction with *Cfo*I enables discrimination between the different alleles, based on the number of amplified fragments: two fragments for homozygous A/A, three for homozygous G/G and four for heterozygous. The resistance is associated with allele A (Meijerink et al., 1997).

2.5. Statistical analysis

The Kruskall-Wallis non-parametric test was used to compare weight gain. The distributions of pigs or isolates were analyzed using the Chi2-test or the Fisher exact test.

2.6. Accession numbers

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession QZVZ00000000, QZWA00000000 and QZVY00000000 (respectively 12-246M, 12-269M and 13-220M).

3. Results

3.1. Strain sequences

Sequencing enabled us to detect the resistance genes present in the isolates. Thus, beside the previously described *mcr-1* gene and the mutations in genes possibly involved in resistance to polymyxins (Delannoy et al., 2017), the strains harbored the genes coding for their other observed resistances: sulfonamides (*sul1, sul2* or *sul3*), trimethoprim (*dfrA1*), tetracycline (*tet(A)*), chloramphenicol (*catA1, cmlA1*), ampicillin (bla_{TEM1a} or bla_{TEM1b}) or aminoglycosides (*aac(3)-IIa, aac(3)-IVa, aadA1, aadA3, aadA24, aph(3′)-Ia, aph(3′)-1c, aph(4)-Ia, strA* or *strB)*.

The virulence genes are listed in Table 2. The genes previously detected by microarray (Delannoy et al., 2017) were confirmed to be present. The two F18 *stx2*-positive strains belonged to ST 1 and 10, whereas the O149 F4 strain containing genes coding for the LT, STa and STb toxins belonged to ST-100.

3.2. Inoculum titers

The inoculum titers were 1.06×10^7 CFU/mL, 7.68×10^6 CFU/mL, and 9.28×10^6 CFU/mL for 12–246 M, 12–269 M and 13–220 M respectively.

3.3. Analysis of pig genotypes

Only 27 pigs could be tested as no blood was obtained from the two control pigs sacrificed on Day 50. As expected, *muc4* and *muc13* gene results were in agreement, and only five animals were found to be of the genotype resistant to *E. coli* F4ac/ab: one in group G1, two in G2, and two in G3 (Table 1). For the F18 receptor, ten pigs were heterozygous AG and the other 17 were homozygous GG. Thus all pigs were susceptible to *E. coli* F18.

3.4. Clinical signs, growth and lesions

All pigs of group G1 had normal rectal temperature during the assay. Moderate pyrexia (40.1 °C–41.0 °C) was observed during the three days after inoculation in 4, 3 and 1 pigs in groups G2 to G4 respectively.

No clinical signs were observed in group G1. In groups G2 to G4, moist, cow-dung appearance feces were observed in all pigs during the third week after inoculation. Moreover, in group G3, two pigs had diarrhea during the four days following inoculation.

The mean weight gains of groups G1 to G4 from Day 0 to Day 4 were respectively 2.9 ± 0.3, 3.0 ± 0.4, 2.1 ± 0.9, and 2.0 ± 0.6 kg ($P = 0.005$). The Day 0-Day 4 weight gain of group G4, but not those of groups G2 and G3, was significantly different from the weight gain of the control group ($P = 0.01$). The weight gains from Day 0 to Day 32 of groups G1 to G4 were respectively 29.5 ± 3.1, 26.6 ± 3.4, 25.1 ± 3.6, and 27.2 ± 3.5 kg and did not differ significantly ($P > 0.05$).

No lesions could be observed post-mortem.

Table 2
Characteristics of the studied strains.

Strains	PGG	O and F antigens and ST	Resistances	Resistance determinants	Virulence or marker genes
12-246M	A	O141	CST	*mcr-1* (deletion in *pmrB* gene)[a]	*fedA, fedF, gad, hlyA, iha, iss, orfA, orfB, paa, stx2, terE, ureD*
		F18	SMX	*sul1, sul2*	
		ST-10	TMP	*dfrA1*	
			TET	*tetA*	
			CHL	*catA1*	
			AMP	bla_{TEM-1a}	
			GEN	*aph3'1c, aac3IIa, aadA1, strA, strB*	
12-269M	A	O149	CST	*mcr-1,* (mutations in PhoQ and PhoP)[a]	*astA, capU,* esc1763 (317/408 nt), *gad, hlyA, iha, K88ab, ltcA, sta1, stb, terE, ureD*
		F4	SMX	*sul1, sul2, sul3*	
		ST-100	TMP	*dfrA1*	
			TET	*tetA*	
			CHL	*cat, cmlA1*	
			AMP	bla_{TEM-1a}	
			GEN	*aac(3)-IIa, aac(3)-IVa, aadA1, aadA3, aph(3')-Ia, aph(4)-Ia, strA, strB*	
13-220M	E	O139	CST	*mcr-1,* (mutations in PmrB and PhoQ)[a]	AIDA (94.5% identity), *eilA, esc1763* (317/408 nt), *fedA, fedF, gad, hlyA, lpfA, orfA, orfB, stx2, terE*
		F18	SMX	*sul1, sul2, sul3*	
		ST-1	TMP	*dfrA1*	
			TET	*tetA*	
			AMP	bla_{TEM-1b}	
				aadA1, aadA24, strA, strB	

PGG: phylogenetic group (Clermont et al., 2013); CST: colistin; SMX: sulfamethoxazole; TMP: trimethoprim; TET: tetracycline; CHL: chloramphenicol; AMP: ampicillin; GEN: gentamicin; AA: amino acid.
[a] as determined previously, impact on colistin resistance unknown (Delannoy et al., 2017).

3.5. Analysis of fecal samples

3.5.1. Cultures on rifampicin-supplemented media

The results are presented in Tables 3 and 4. All cultures from non-inoculated pigs or from samples collected before inoculation were negative on rifampicin-supplemented media. Some of these samples yielded *E. coli* colonies after enrichment, but none of the isolates tested had the *mcr-1* gene. For cultures on rifampicin-supplemented media, with or without enrichment, and after *mcr-1*-PCR control of isolates, all inoculated pigs were observed to be positive at least once. In groups G2, G3, and G4, out of 12 sampling days after inoculation, the pigs were positive on 1 to 7 (mean 4.1), 3 to 11 (mean 6.9) and 1 to 8 (mean 4.5) different days respectively. The total positive samples were 33 out of 102, 55 out of 103, and 40 out of 104 tested samples in groups G2 to G4 respectively, the proportion in G3 being significantly higher than the other two groups (Chi-2 test, $P < 0.05$). In all groups, at least one pig was positive on the last sampling day, four weeks after inoculation. The maximum mean titers were observed on Day 11 (1.84) in group G2, on Days 4 and 5 (5.75 on each day) in group G3, and on Day 17 (1.73) for group G4.

3.5.2. Analysis of isolates obtained from fecal samples directly or after enrichment on rifampicin-supplemented media

Most isolates obtained directly on rifampicin-supplemented media (218/229, 95%) or after enrichment in rifampicin-supplemented media (70/83, 84%) harbored the *mcr-1* gene and showed the expected characteristics of the inoculated strains. Considering only isolates obtained without enrichment, for group G2, 52 out of 53 isolates shared the characteristics of inoculated strain 12–246 M, and one isolate obtained on Day 14, without enrichment, belonged to PGG A, had the ERIC and PFGE profiles and the antimicrobial susceptibility of 12–246 M, including resistance to colistin, although it was negative for the *mcr-1* gene.

In group G3, 98 out of 107 isolates obtained without enrichment shared the characteristics of inoculated strain 12–269 M, but nine isolates lacked the *mcr-1* gene. Six were fully analyzed and were found to belong to PGG A, and have the ERIC and PFGE profiles of the 12–269 M isolate. They were resistant to tetracycline and gentamicin but, contrary to 12–269 M, susceptible to sulfamethoxazole, trimethoprim, colistin and ampicillin. The *mcr-1*-negative isolates had been obtained on Day 3, 4, 5, 9 and 28 from three pigs bred in the two pens of the animal room.

In group G4, 62 out of 69 isolates obtained without enrichment had the expected characteristics (presence of the *mcr-1* gene and phylogenetic group E). Six had the *mcr-1* gene but belonged to PGG A, and their ERIC and PFGE profiles were identical to each other but different to that of 13–220 M. They were obtained on Day 2 and Day 5 from three pigs placed in the two pens of the animal room. One isolate obtained on Day 24 was *mcr-1*-negative. Its PFGE profile was identical to the inoculated

Table 3
Culture on rifampicin media (with or without enrichment in rifampicin-supplemented broth).

	Day (D)														
Group (strain)	D1	D2	D3	D4	D5	D7	D9	D11	D14	D17	D21	D24	D28	Total	*mcr*+ isolates
G2 (12-246 M)	3/8[a]	3/8	1/6	0/8	2/8	3/8	7/8	7/8	3/8	1/8	1/8	1/8	1/8	33/102[a]	52/53[c]
	0.96[b]	0.62	0.48	0.00	0.65	1.07	1.42	1.84	1.09	0.36	0.21	0.21	0.25		
G3 (12-269 M)	4/8	6/8	6/7	7/8	8/8	8/8	2/8	1/8	3/8	2/8	2/8	3/8	3/8	55/103	98/107
	1.55	4.89	4.41	5.75	5.75	4.16	0.84	0.37	0.89	0.46	0.54	0.61	0.97		
G4 (13-220 M)	1/8	1/8	1/8	0/8	4/8	3/8	4/8	3/8	3/8	5/8	5/8	6/8	4/8	40/104	68/69
	0.37	0.21	0.25	0.00	1.28	0.90	0.50	0.88	0.85	1.73	1.06	1.71	1.02		

[a]Number of samples containing rifampicin-resistant *mcr-1*-positive *E. coli*/number of tested pigs; [b]Mean titer obtained on rifampicin- supplemented MacConkey media; [c]Number of *mcr-1*-positive *E. coli*/number of tested *E. coli* (obtained without enrichment).
All pigs were negative before inoculation and all pigs from the G1 non-inoculated group were negative on all sampling days.

Table 4
Isolation from individual pigs.

Group	Pen	Pig	Days													Number of samples containing		
			1	2	3	4	5	7	9	11	14	17	21	24	28	RifR, *mcr+* *E. coli*	RifS, *mcr+* *E. coli*	RifR, *mcr⁻ E. coli*
G2	D	6553	A	A	A		A	B	B	B						7		
		6559		A					B	AB						3		
		6578		A				A	AF	A	A		B			6		
		6588	A		NS		A	A	AF	B						5		
	V	6551*	B						B					B	A	4		
		6558*								A						1		
		6577			NS				A	B	A	A				4		
		6584							B	A	AE					3		1
G3	D	6555	A	AF	A	A	A	A				B	A	A	AE	10		1
		6563*			A		AB	B								3		
		6581		AF	NS	A	A	A								4		
		6585	A		ABE	BE	ABE	A	AEF	A	A		AB	A	ABE	11		5
	V	6552		AF	A	A	A	A			A	AB		AB	AE	9		1
		6562*	A	A		AB	A	A								5		
		6580		AF	A	A	A	A								5		
		6590	A	AF	A	A	A	A	A		A					8		
G4	D	6554					C		B	A		AF	A	A	A	6	1	
		6557						A	B	A	A	AF	A	A		7	0	
		6583		D			C		AB					B		2	2	
		6586					B	A			A	AB	B	AE	A	7	0	1
	V	6561										AB	B	B	B	4	0	
		6564	A				D									1	1	
		6582						B	A	AB	A	AF	A	AB	A	8	0	
		6591			A											1	0	

A: sample containing rifampicin-resistant, *mcr-1*-positive *E. coli* of the expected phylogenetic group, obtained directly.
B: sample containing rifampicin-resistant, *mcr-1*-positive *E. coli* of the expected phylogenetic group, obtained after enrichment.
C: sample containing rifampicin-resistant, *mcr-1*-positive *E. coli* of an unexpected phylogenetic group and PFGE profile, obtained directly.
D: sample containing rifampicin-resistant, *mcr-1*-positive *E. coli* of an unexpected phylogenetic group and PFGE profile, obtained after enrichment (possibly transconjugant).
E: sample containing rifampicin-resistant, *mcr-1*-negative *E. coli* of the expected phylogenetic group and PFGE profile, obtained directly (possibly inoculated strain having lost the *mcr-1* gene).
F: sample containing rifampicin-resistant, *mcr-1*-positive *E. coli* detected on CAO-CV medium.
NS: no sample.
Grey cells: clinical signs (diarrhea or temperature > 40.0 °C).
*: pig resistant to *E. coli* F4.

strain and it shared the susceptibility of 13–220 M except for colistin resistance.

3.5.3. Culture on CAO-CV media

The results obtained for samples collected on Days 2, 9 and 17 and inoculated on CAO-CV agar plates showed that respectively 15, 13 and 19 samples out of 24 contained *E. coli*. However, after analysis of the isolates, only 16 out of 80 contained the *mcr-1* gene and all 16 were rifampicin-resistant. Thus, for group G2, only two pigs were positive on Day 9; for G3, five pigs were positive on Day 2, and another one on Day

9; and for G4, three pigs were positive on Day 17. Positive pigs were detected in the two pens for groups G3 and G4 (Table 4). There was no difference between the proportion of positive samples in the inoculated groups (Fisher's Exact Test, $P > 0.05$).

3.5.4. *mcr-1* PCR on enrichment broths in rifampicin-supplemented LB

All the samples collected before inoculation were negative. For group G2, nine out of 102 samples were positive; they were obtained on Days 1, 7, 11, and 24 from seven different pigs. For group G3, 14 samples out of 103 obtained on Days 4, 5, 7, 21, 24, and 28 were positive; seven pigs were positive. For the E4 group, 12 out of 104 samples were positive; they were obtained on Days 5, 11, 21, 24, and 28 from six different pigs. There was no difference between the proportion of positive samples in the inoculated groups (Chi 2 test, $P > 0.05$).

3.5.5. *mcr-1* PCR on enrichment broths in colistin-supplemented LB

Only samples collected on Days 2, 9, and 17 were analyzed. All the results were negative except three fecal samples collected on Day 17 from two pigs in group G3 and one in G4. For the same Days 2, 9 and 17, culture on rifampicin media detected 31 positive samples out of 72 samples from inoculated groups and was thus significantly more sensitive ($P < 0.001$).

3.6. Analysis of organs

The results obtained on rifampicin-supplemented MacConkey plates after enrichment in rifampicin-supplemented broth, are given in Table 5.

All the samples collected from the non-inoculated pigs and all the duodenum samples were negative. The proportion of positive samples in group G3 (3/48) was significantly lower than the proportion in G2 (13/48) and G4 (17/48) (Fisher exact test, $P < 0.01$). Besides the isolates with the characteristics of the inoculated strains, a few rifampicin-resistant *mcr-1*-negative isolates belonging to the phylogenetic group of the respective inoculated strains were obtained in the jejunum or cecum from three different pigs in group G2; in the ileum or cecum from three different pigs in group G3; and in the ileum, colon, rectum or cecum from three different pigs in group G4.

4. Discussion – conclusion

The three strains proposed as an experimental model are relevant because they have been reported in literature and seem to be frequently involved in pig diarrhea. Indeed sequencing confirmed that they were Shiga toxin-producing (STEC) (12–246 M and 13–220 M) or ETEC (12–269 M). O141:F18 12–246 M is of type ST-10, which has already been isolated from many different sources, such as humans, animals (including swine in Italy and China), food (including pork in the USA) and the environment (water in France) (http://enterobase.warwick.ac.uk/species/ecoli/search_strains). An ST-10 *mcr-1* and bla_{NDM-5} *E. coli* of human origin was also characterized by Zhang et al. (Zhang et al., 2017) and several *mcr-1* ST-10 *E. coli* of healthy swine origin have also been reported in China (Wang et al., 2016). The O149:F4 12–269 M *E. coli* belongs to ST-100, like several pig or bovine pathogenic isolates of serogroups O149:H10:F4ac reported in different European, American or Asian countries, or an O149 poultry APEC isolated in Germany. The O139:F18 13–220 M strain belongs to ST-1, like O139:F18 isolates obtained from pig diarrhea in Hungary in 1988 (http://enterobase.warwick.ac.uk/species/ecoli/search_strains?query=st_search). An analysis of the different contigs carrying *mcr-1* did not reveal other resistance or virulence genes or notifiable genes.

Symptoms, including hyperthermia and diarrhea, were more severe in group G3. This group also had the highest excretion titers in the first week, and more fecal samples were found positive. However, at necropsy—more than one month after inoculation—the O149:$F4^+$ inoculated strain was less frequently isolated than the other two inoculated strains, both of type $F18^+$ *fimbriae*. This may be related to the fact that susceptibility to $F18^+$ or $F4^+$ *E. coli* may vary with the pig's age. Verdonck et al. (Verdonck et al., 2002) previously observed a similar delay, with $F4^+$ ETEC peak excretion 2 days post-infection (PI), but no excretion 7 days PI, in contrast to the peak excretion of $F18^+$ VTEC infection observed between 3 and 5 days PI, and excretion persisting up to 9 days PI. Interestingly, in group G3, the only two pigs found to be genetically resistant to *E. coli* F4 had no diarrhea and gave only a total of eight positive fecal samples out of 26, whereas 47 out of 77 samples were positive for the six pigs which were genetically susceptible to *E. coli* F4 ($P = 0.007$); their mean log10 titer was 0.6 compared to a mean of 3.0 ($P = 0.0004$) for the other pigs in group G3. Although all the pigs were genetically susceptible to *E. coli* F18, the symptoms remained relatively moderate in groups G2 and G4. The experimental model could be improved by using higher inocula doses, intra-gastric inoculation, withdrawing feed before inoculation, cold stress, pretreatment with antibiotics, co-inoculation of viruses, or inoculating piglets when younger (Madec et al., 2000), or using pigs known to be genetically susceptible to the inoculated strains. It is acknowledged that post-weaning digestive disorders are generally encountered in inadequate breeding conditions, contrary to the high-standard environment offered to our weaning piglets.

Using rifampicin-supplemented media, we could evaluate the recovery of the inoculated strain, and search for the possible loss of the *mcr-1* gene from the inoculated strains. The use of colistin-supplemented media was used to try to detect the transfer of the *mcr*-1 gene from the inoculated strains to commensal ones. Detection of the *mcr-1* gene in colistin-enrichment broths was also performed but the sensitivity of this method was poor as previously observed in experiments with pigs inoculated with a commensal *mcr-1*-positive *E. coli* strain (Mourand et al., 2018), and needs further improvements. The three inoculated strains could be recovered from fecal and post-mortem samples up to four and five weeks respectively after inoculation. For group G4 only, a few isolates carrying the *mcr-1* gene were also obtained, but their other characteristics (phylogenetic group, PFGE or ERIC-PCR profiles, and antimicrobial susceptibility) were different from those of the inoculated strain. As no *mcr-1*-containing *E. coli* was present in the non-inoculated animals, the uncharacteristic isolates are very probably commensal isolates having acquired the *mcr-1* gene or plasmid from *E. coli* 13–220 M. Other analyses are necessary to

Table 5
Number of samples positive for rifampicin-resistant *mcr*-1-positive *E. coli* and number of *mcr-1*-positive lysates.

Group (strain)		Jejunum	Ileum	Colon	Cecum	Rectum	Total (organs)	Total (pigs)
G2 (12-246 M)	Culture	0/8[a]	5/8	3/8	4/8	1/8	13/48	6/8
	PCR	1/8	5/8	1/8	4/8	1/8	12/48	6/8
G3 (12-269 M)	Culture	0/8	1/8	1/8	0/8	1/8	3/48	3/8
	PCR	0/8	1/8	0/8	0/8	1/8	2/48	2/8
G4 (13-220 M)	Culture	0/8	4/8	5/8	4/8	4/8	17/48	6/8
	PCR	0/8	7/8	4/8	4/8	0/8	15/48	7/8

All samples from non-inoculated pigs were negative.
[a] Number of positive samples/number of tested samples.

determine the precise mechanisms of this transfer, which can occur through either conjugation or transposon mobilization. Interestingly, *in vitro*, the *mcr-1* gene was shown to be on a conjugative plasmid in *E. coli* 13–220, but not in *E. coli* 12–246 or *E. coli* 12–269. The transconjugant obtained *in vitro* from *E. coli* 13–220 acquired resistance to colistin, tetracycline, sulfamethoxazole and trimethoprim. Different studies have shown that the *in vitro* acquisition of an *mcr-1*-containing plasmid does not lead to a considerable fitness cost in *E. coli* as measured by growth kinetics, cytotoxicity, and virulence in a *Galleria mellonella* model (Tietgen et al., 2018; Zhang et al., 2017), but a high level of *mcr-1* expression compromises growth rate, fitness, and the membrane's structural integrity while increasing cellular death (Yang et al., 2017). In our experiment, most isolates from samples collected from the *E. coli* 13–220 M-inoculated pigs shared the characteristics of this strain, suggesting that the commensal strains which had acquired the *mcr-1* gene were perhaps not as competitive as *E. coli* 13–220 M.

In the three inoculated groups, few of the isolates obtained were resistant to rifampicin, shared the phylogenetic group and PFGE profile of the inoculated strain but lacked the *mcr-1* gene. Wu et al. (2018) studied the stability of various *mcr-1*-harboring plasmids and showed that the IncHI2, IncI2, IncX4 and IncY plasmids were maintained after 14 days of passage in a colistin-free medium, but the IncFII plasmid was lost from the *E. coli* DH5alpha transformant after day 5. The *in vivo* loss of the *mcr-1* gene has already been detected for other strains (Mourand et al., 2018). Interestingly, the loss of the *mcr-1* gene was not accompanied by a loss of resistance to colistin for 12–246 M, suggesting that the previously described deletion in the PmrB protein, or other unnoticed mutations, probably played a role in colistin resistance. For *E. coli* 12–269 M, the isolates lacking *mcr-1* were no longer resistant to colistin, trimethoprim, sulfamethoxazole, and ampicillin, suggesting that these resistance genes may be present on the same genetic element, but further analysis is needed to confirm this hypothesis, as the genes were present on contigs different from the *mcr-1* contig. The loss of the colistin resistance consecutive to the absence of the *mcr-1* gene also suggests that the previously detected PhoQ and PhoP mutations (Delannoy et al., 2017) have probably no impact on colistin resistance). Finally in group G4, the isolate lacking the *mcr-1* gene had only lost colistin resistance.

Globally, comparison of the clinical signs and culture results obtained in the inoculated and control groups, shows that the O149-F4 ETEC challenge, resulting in more clinical signs and excretion, particularly in pigs with a F4 susceptible genotype, may be used to evaluate alternative solutions to combat PWD caused by colistin-susceptible or colistin-resistant *E. coli* in pigs.

Conflict of interest statement

The authors indicate that there are no conflicts of interest.

Funding

This work was supportedby grant ANR-15-CE21-0015-02 from the French National Research Agency, COMPARE, in the framework of the European Union's Horizon 2020 program under grant agreement No. 643476, 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.

Acknowledgments

The authors would like to sincerely thank Yann Bailly (ANSES Ploufragan) for technical assistance. The authors thank Labocea 22 for providing the three *E. coli* strains through RESAPATH network.

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