Vaccine 35 (2017) 353–360

Contents lists available at ScienceDirect

Vaccine

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

Immunogenicity and protective efficacy of a single-dose live non-pathogenic *Escherichia coli* oral vaccine against F4-positive enterotoxigenic *Escherichia coli* challenge in pigs

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ARTICLE INFO

Article history:
Received 27 May 2016
Received in revised form 8 November 2016
Accepted 10 November 2016
Available online 1 December 2016

Keywords:
Live vaccine
Enterotoxigenic *Escherichia coli*
Pig
Post-weaning diarrhea
Immune response
F4 (K88) fimbriae

ABSTRACT

Enterotoxigenic *Escherichia coli* strains expressing F4 (K88) fimbriae (F4-ETEC) are one of the most important causes of post-weaning diarrhea (PWD) in pigs. F4, a major antigen, plays an important role in the early steps of the infection. Herein, the efficacy of a live oral vaccine consisting of a non-pathogenic *E. coli* strain expressing F4 for protection of pigs against PWD was evaluated. Three blinded, placebo-controlled, block design, parallel-group confirmatory experiments were conducted, using an F4-ETEC PWD challenge model, each with a different vaccination-challenge interval (3, 7, and 21 days). The pigs were vaccinated via the drinking water with a single dose of the Coliprotec® F4 vaccine one day post-weaning. Efficacy was assessed by evaluating diarrhea, clinical observations, intestinal fluid accumulation, weight gain, intestinal colonization and fecal shedding of F4-ETEC. The immune response was evaluated by measuring serum and intestinal F4-specific antibodies. The administration of the vaccine resulted in a significant reduction of the incidence of moderate to severe diarrhea, ileal colonization by F4-ETEC, and fecal shedding of F4-ETEC after the heterologous challenge at 7 and 21 days post-vaccination. The 7-day onset of protection was associated with an increase of serum anti-F4 IgM whereas the 21-day duration of protection was associated with an increase of both serum anti-F4 IgM and IgA. Significant correlations between levels of serum and intestinal secretory anti-F4 antibodies were detected. Maternally derived F4-specific serum antibodies did not interfere with the vaccine efficacy. The evaluation of protection following a challenge three days after vaccination showed a reduction of the severity and the duration of diarrhea and of fecal shedding of F4-ETEC. The 7-day onset and the 21-day duration of protection induced by Coliprotec® F4 vaccine administered once in drinking water to pigs of at least 18 days of age were confirmed by protection against F4-ETEC and induction of F4-specific protective immunity.

1. Introduction

PWD[1] in pigs is a worldwide economically important disease due to mortality, weight loss, slow growth, treatment costs, and weight heterogeneity [1–6]. Current control strategies usually involve the use of antimicrobials; however, the emergence of antimicrobial resistance among *Escherichia coli* isolates from cases of PWD highlights the need for alternative control measures [7–10].

PWD typically occurs in the first weeks after weaning and is characterized by a marked decrease in feed consumption and diarrhea. Pigs become rapidly dehydrated and depressed; and they may die suddenly or after a short illness [11]. PWD is mainly caused by ETEC[2] that express F4 or F18 adhesive fimbriae, required for the colonization of the intestinal mucosa, and enterotoxins that induce secretory diarrhea [12–14]. Susceptibility of pigs to diarrhea due to F4-ETEC has been linked to a polymorphism on the MUC4 gene [15,16].

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[1] Post-Weaning Diarrhea.
[2] Enterotoxigenic *Escherichia coli*.

http://dx.doi.org/10.1016/j.vaccine.2016.11.045
0264-410X/

ETEC neonatal diarrhea can be effectively controlled by vaccination of pregnant sows [17,18]. However, passive lactogenic protection is rapidly lost after weaning and an active mucosal immunity has to be elicited to protect pigs against PWD [19]. To this end, oral vaccination of pigs represents a sustainable, practical, and effective approach.

Here, we report results demonstrating the efficacy of Coliprotec® F4, an oral vaccine for active immunization of pigs against PWD caused by F4-ETEC. These studies were designed to demonstrate the onset and duration of protective immunity using an F4-ETEC PWD porcine challenge model.

2. Materials and methods

2.1. Vaccine and challenge strains

Coliprotec® F4 (Prevtec Microbia GmbH, Germany) is a single-dose vaccine consisting of a live non-pathogenic *E. coli* O8:K87 (O and K antigens) lyophilisate that was reconstituted and diluted in water prior to its oral administration to pigs. The vaccine strain is positive for F4 fimbriae (formerly K88) and negative for toxins.

The F4-ETEC EcL8559 O149 challenge strain (Prevtec Microbia Inc., Canada) is nalidixic acid resistant, *Beta*-haemolytic, and positive for the enterotoxins STa, STb, LT and East-1 [20]. The challenge strain was prepared from one frozen seed vial that was grown in 500 ml of tryptic soy broth (TSB) for 4 h at 37 °C under 150 rpm agitation. Optical density was measured, the fresh culture was diluted with TSB and then administered.

2.2. Animals

Studies were performed at the Université de Montréal (UdeM, Canada) after being approved by the university animal care and ethics committee following the Canadian Council on Animal Care guidelines. A total of 80 castrated male and female pigs born to crossbred sows (Yorkshire-Landrace × Pen Ar Lan Naïma) were obtained from a conventional pig farm. All gilts of the herd were vaccinated against ETEC causing neonatal diarrhea prior to farrowing. Piglets were weaned at 17 or 18 days of age. Before weaning, susceptibility of piglets to F4-ETEC infection was investigated by analyzing the *MUC4* polymorphism [21]. Only susceptible pigs were selected and are referred to as F4R+ pigs. Animals had *ad libitum* access to water and to non-medicated feed.

2.3. Experimental design

Three blinded, placebo-controlled, block design, parallel-group confirmatory experiments were conducted using an F4-ETEC PWD challenge model, each with a different vaccination-challenge interval set at 3, 7, and 21 days, respectively (Table 1). Pigs were distributed between vaccine or control groups following a randomized complete block design within litters (1:1 ratio) with the generation of block size n = 4.

Experiments with challenge at 7 and 21 days post-vaccination (dpv) were done concurrently. Forty F4R+ pigs from 8 litters were transferred into the experimental units at weaning. The vaccinated pigs of both experiments (10 pigs each) were commingled in a single experimental unit and the unvaccinated pigs (controls) of both experiments (10 pigs each) were commingled in another experimental unit until their respective challenge phase. The vaccine was administrated via the drinking water using 150 ml per pig in a snap-in nursery feeder bowl. Control animals received 150 ml of filtered water per pig. The vaccine/control was consumed within 4 h. At 7 or 21 dpv, 10 vaccinates and 10 controls were transferred into a challenge room. The challenge strain was administered to each pig using an oesophageal tube after the administration of 10 ml of a 1.2% carbonate solution. Pigs were monitored for 3 days post-challenge (dpc) and then euthanized. Necropsy was performed on each animal.

Another similar experiment involved an early challenge performed at 3 dpv. Forty F4R+ piglets from 10 litters were transferred at weaning into the experimental units. Treatments were administered as previously described. Pigs were transferred at 3 dpv into a challenge room, challenged as previously described and monitored for 5 days before being euthanized. No necropsy was done.

2.4. Clinical and pathological examination

Animals were monitored once daily before and twice daily after the challenge for general health, behavior, appetite, body condition, and hair coat. Fecal consistency score and dehydration were evaluated once per day throughout the experiments. The fecal consistency was evaluated on a continuous scale with 5 levels: (0) normal, (1) pasty, (2) presence of liquid but more solid particles than liquid, (3) presence of more liquid than solid particles, and (4) totally liquid. Fecal consistency scores of 2, 3 and 4 were considered as mild, moderate and severe diarrhea, respectively. In the early challenge experiment, the severity and the duration of diarrhea were assessed as the post-challenge period was longer. The severity of diarrhea is the mean of the fecal consistency scores for each dpc. Duration of diarrhea is the number of days from the first to the last day showing diarrhea. For all challenge experiments, pigs were weighed on arrival at the facility, on the challenge day and at the study completion. For the 21-dpv challenge experiment, pigs were also weighed at 7 and 14 dpv.

Gross pathological findings were evaluated at necropsy for the 7-dpv and the 21-dpv challenge experiments. The consistency of the contents from the jejunum, ileum, caecum, colon and rectum was scored on an ordinal scale with 4 levels: (0) normal, (1) pasty, (2) presence of liquid but more solid particles than liquid, (3) presence of more liquid than solid particles or totally liquid. An intestinal segment with a consistency score >1 was considered having fluid accumulation and pigs presenting 2 or more intestinal

Table 1
Design of the Coliprotec® F4 vaccine efficacy experiments. Vaccine and challenge doses are reported as Colony Forming Unit (CFU) administered per pig. Challenges were performed using the F4-ETEC EcL8559 strain.

Experiment	Group	Number of animals	Treatment (lot number)	Vaccine dose (CFU)	Vaccination-challenge interval (days)	Challenge dose (CFU)	Challenge-euthanasia interval (days)
7-dpv challenge	Vaccine	10	Coliprotec® F4 (121811)	1.3×10^8	7	1.5×10^9	3
	Control	10	Water	Not applicable	7	1.5×10^9	3
21-dpv challenge	Vaccine	10	Coliprotec® F4 (121811)	1.3×10^8	21	9.5×10^8	3
	Control	10	Water	Not applicable	21	9.5×10^8	3
3-dpv challenge	Vaccine	20	Coliprotec® F4 (122964)	5.9×10^7	3	1.7×10^9	5
	Control	20	Water	Not applicable	3	1.7×10^9	5

segments with fluid accumulation were considered to have hypersecretion.

2.5. Bacteriological examination

Fecal shedding of the challenge strain was quantified using a standard live bacterial count method. Fresh fecal samples were collected daily from the day of the challenge to the study completion using pre-weighed sterile rectal swabs and transportation tubes. After weighing fecal samples, phosphate-buffered saline 0.1 M, pH 7.4 (PBS) was used to suspend feces and for ten-fold serial dilutions. Counting of typical colonies on tryptic soy agar with 5% sheep blood and 5 mg/L nalidixic acid was done after an overnight incubation at 37 °C. To evaluate ileum colonization by the challenge strain, approximately 2 cm of the ileum was sampled at about 10 cm from the ileocecal valve. A piece of ileum was weighed (between 0.1 g and 0.2 g) and was suspended in 1 ml of PBS, ground with a homogenizer and used for the bacterial count as described for fecal shedding. CFU/g of fecal or ileum sample result was calculated using the CFU/ml and weight of the sample.

2.6. Antibody analyses

Sera were prepared with blood collected from the cranial vena cava on the respective experiment days of vaccination, challenge and completion of the study for the 7-dpv and 21-dpv challenge experiments only. Serum samples were stored at −75 ± 5 °C until analysis. Levels of serum anti-F4 IgM, IgA and IgG were quantified using indirect ELISA as previously described [22] with the following modifications. Microlon® microplates (Greiner Bio-One, Germany) were coated with 2.5 μg/ml F4 fimbriae. The blocking solution was prepared with 1% bovine serum albumin and 0.2% Tween 80. Serum samples were diluted 1:10, 1:20 and 1:1200 and the HRP conjugated secondary antibodies 1:15,000, 1:3000 and 1:20,000 for anti-F4 IgM, IgA or IgG, respectively. The ABTS single reagent (EMD Millipore, USA) was incubated for one hour at room temperature before reading optical densities at 405 nm. Three replicates for each serum sample were analyzed and each microplate contained samples of a strong positive, a weak positive and a negative control (Prevtec Microbia Inc., Canada). Antibody levels were reported as percent positivity (PP) of the respective strong positive control [23].

To determine intestinal anti-F4 IgM and IgA, 10–20 ml of the jejunum and ileum contents were collected at necropsy, prepared [24], frozen at −75 ± 5 °C, concentrated 5-times by freeze drying and rehydrated with sterile water. Intestinal antibodies were quantified using the same ELISA method used for the respective serum antibody, except that the optical density of the strong positive serum control was divided by 5 before calculating PP results. Since antibodies in the contents are not confined to their secretion site due to intestinal transit, group comparative analyses were performed using the highest PP result among the intestinal segments of each animal (hereafter "pig's highest intestinal level") in addition to the PP of the jejunum and of the ileum.

2.7. Statistical analysis

Reduction of diarrhea and intestinal hypersecretion were evaluated with the Cochran-Mantel-Haenszel test [25] with litter and pen respectively as the stratification variable, using the mghr function from the Hmisc package [26] of R [27]. Intestinal segment fluid accumulation was evaluated using Friedman's test with litter as the stratification variable. Reduction of fecal shedding and ileum colonization by the challenge strain, and weight loss were evaluated by ANOVA for block design. Serum antibody levels, their evolution over time and intestinal antibody levels were also evaluated with an ANOVA for block design. Correlation between the highest intestinal antibody and serum antibody levels were performed using Pearson's correlation coefficient. All analyses were performed with R statistical software [27] using a statistical significance of 0.05.

3. Results and discussion

3.1. Diarrhea, intestinal fluid accumulation and hypersecretion

A total of 5 vaccinates and 3 controls of the 7-dpv challenge experiment and 4 vaccinates and 3 controls of the 21-dpv challenge experiment presented mild diarrhea (score 2) before the challenge. Therefore, only scores of 3 (moderate) and 4 (severe) were classified as post-challenge diarrhea for all experiments. Moderate to severe diarrhea is hereafter referred to as diarrhea for brevity. The incidence of pigs showing diarrhea at least once after the 7-dpv challenge was 11% (1/9) for vaccinates and 40% (4/10) for controls, with a statistically significant difference on the third dpc at the peak of diarrhea (Table 2). Similar results were observed after the 21-dpv challenge, with a significant reduction of vaccinated pigs showing diarrhea at least once after challenge (0% vaccinates vs 33% controls, $p = 0.05$). After the early 3-dpv challenge, 47% vaccinates and 74% controls had diarrhea at least once. Even though this difference is not statistically significant ($p = 0.104$), control pigs were affected more severely and Coliprotec® F4 partially protected the pigs, which was demonstrated by a significant reduction, at the peak of diarrhea (2 dpc), of the incidence of diarrheic pigs and the severity of diarrhea (Table 3). The

Table 2
Incidence of pigs (percentage) with diarrhea or with fluid accumulation for the 7-days post-vaccination (dpv) and 21-dpv challenge experiments. An asterisk denotes a significant difference ($p \leqslant 0.05$).

	7-dpv challenge experiment			21-dpv challenge experiment		
	Vaccine (n = 9)	Control (n = 10)	*p*-value	Vaccine (n = 10)	Control (n = 9)	*p*-value
Diarrhea for each day post-challenge						
0	0	0	–	0	0	–
1	11	10	0.808	0	33	0.050*
2	11	20	0.808	0	11	0.317
3	0	40	0.041*	0	0	–
Fluid accumulation in each intestinal segment						
Jejunum	11	30	0.098	20	11	0.399
Ileum	12	78	0.003*	22	38	0.281
Caecum	22	70	0.003*	20	33	0.251
Colon	11	70	0.001*	0	11	0.080
Rectum	0	40	0.002*	0	0	0.827

Table 3
Incidence of pigs (percentage) with diarrhea and severity of diarrhea (mean of scores) for the 3-day post-vaccination challenge experiment. An asterisk denotes a significant difference ($p \leqslant 0.05$).

Day post-challenge	Incidence			Severity		
	Vaccine (n = 20)	Control (n = 20)	*p*-value	Vaccine (n = 20)	Control (n = 20)	*p*-value
0	5	0	–	0.4	0.2	0.458
1	20	25	0.691	0.9	1.3	0.412
2	25	63	0.026*	1.2	2.4	0.023*
3	20	42	0.118	1.1	1.9	0.076
4	15	26	0.362	1.1	1.6	0.302
5	0	11	0.157	0.5	1.1	0.087

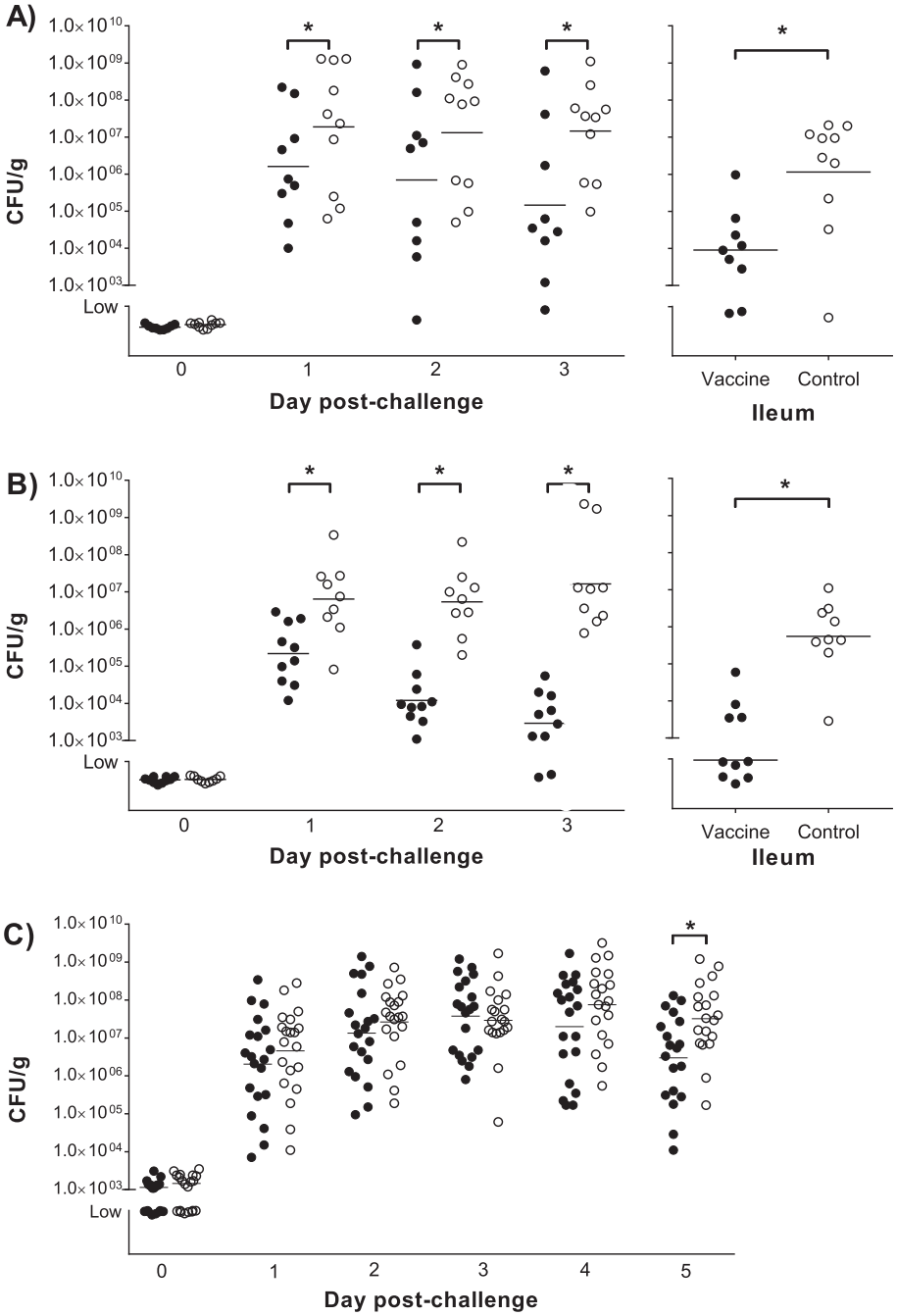

Fig. 1. Fecal shedding levels of the challenge strain for the 3 days post-challenge (dpc) and colonization levels of the ileum by the challenge strain at necropsy (day 3 post-challenge) for the 7-day post-vaccination (dpv) challenge experiment (A) and the 21-dpv challenge experiment (B). Fecal shedding levels of the challenge strain for the 5 dpc during the 3-dpv challenge experiment (C). Horizontal lines represent the geometric mean of CFU per gram of fecal material or ileum tissue. Open circles represent animals of the control group; solid circles represent animals of the vaccinated group. An asterisk denotes a significant difference ($p \leqslant 0.05$) between vaccinated and control pigs.

duration of diarrhea was also significantly lower ($p = 0.04$) for vaccinates (1.4 day) than controls (2.4 days) after the 3-dpv challenge.

The incidence of pigs showing intestinal hypersecretion after the 7-dpv challenge was significantly lower ($p = 0.009$) for vaccinates (11%) than for controls (70%), which was reflected by a significant reduction of pigs showing fluid accumulation in the ileum, caecum, colon and rectum lumen (Table 2). However, the incidence of pigs with intestinal hypersecretion was low at necropsy after the 21-dpv challenge for both treatment groups, without significant difference for fluid accumulation in each intestinal segment (Table 2).

Diarrhea caused by F4-ETEC is a result of multiple factors and is clinically observed when the amount of fluid in the small intestines exceeds the intestinal absorption capacity [28]. Though 70% of controls showed fluid accumulation in the ileum, the caecum and the colon after the 7-dpv challenge, only 40% did so in the rectum, in accordance with the incidence of diarrheic pigs on the necropsy day. In contrast, only one vaccinate showed hypersecretion and another one had diarrhea. Contrary to results after the 7-dpv challenge, control pigs developed diarrhea the first day after the 21-dpv challenge and recovered rapidly, resulting in 22% of controls with hypersecretion at necropsy.

It was previously suggested that susceptibility to F4-ETEC diarrhea decreases with age of pigs or with the increase of the weaning-challenge interval [29,30]. Results from the current three studies are consistent with this hypothesis as 74%, 40% and 33% of the ≈ 21-day-old (3 dpv), ≈ 25-day-old (7 dpv) and ≈ 39-day-old (21 dpv) control pigs had diarrhea after the challenge, respectively.

3.2. Fecal shedding and colonization of the ileum

In accordance with the clinical protection, Coliprotec® F4 significantly reduced the fecal shedding of F4-ETEC as early as the first day following the 7-dpv and 21-dpv challenges (Fig. 1A and B). The fecal shedding of F4-ETEC was significantly reduced by vaccination for the 3 days following the 7-dpv challenge ($p = 0.044$, 0.004 and 0.004, respectively) and the 21-dpv challenge ($p = 0.002$, $p < 0.001$, and $p < 0.001$, respectively) experiments (Fig. 1). These reductions reached $2.0 \log_{10}$ and $3.7 \log_{10}$ on the third day following the 7-dpv and 21-dpv challenges, respectively. However, following the 3-dpv challenge, the shedding was significantly reduced by vaccination only for the last day (5 dpc) of the study ($1.0 \log_{10}$; $p = 0.004$) (Fig. 1C).

The ileal colonization by the challenge strain was also significantly reduced for vaccinates compared to the controls following the 7-dpv challenge ($p = 0.001$) and the 21-dpv challenge ($p < 0.001$) (Fig. 1), reaching a difference of $2.1 \log_{10}$ and $2.8 \log_{10}$ respectively.

3.3. Weight gain

Reduction in weight gain is part of the economic losses caused by PWD, and delayed growth, which usually follows diarrheal episodes, makes the losses even worse [28]. Vaccinates challenged at 7 and 21 dpv showed a post-challenge DWG of 140 g and 175 g higher than for controls, respectively (Table 4). Even though the differences of DWG after the challenge are not statistically significant, they are relevant since 40% and 11% of the controls challenged at 7 and 21 dpv, respectively, lost weight whereas no vaccinate did. These differences in DWG resulted in vaccinates heavier than controls by 517 g and 1117 g at the end of the 7-dpv and 21-dpv challenge experiments, respectively (Table 4).

The DWG during the post-challenge period of the 3-dpv challenge experiment was significantly higher for vaccinates (29 g) than for controls (−53 g) (Table 4). The Coliprotec® F4 partial clinical protection observed after the 3-dpv challenge experiment resulted in an average weight gain for the entire study period of 311 g for vaccinates compared to an average weight loss of 197 g for the controls.

3.4. Antibody responses

Antibody responses to the F4 antigen were analyzed for the 7 and 21 dpv challenge experiments. Serum levels of anti-F4 IgM and IgA were low at vaccination. Statistically significant differences in evolution over time of serum anti-F4 IgM ($p < 0.001$ for both experiments) and IgA ($p = 0.025$ and $p = 0.001$ for 7 and 21 dpv challenge experiments, respectively) were observed between vaccinates and controls (Fig. 2). Significantly higher levels of serum antibodies were detected for vaccinates compared to controls during the 7-dpv challenge experiment at 7 dpv (IgM, $p = 0.05$) and 10 dpv (IgM, $p < 0.001$; IgA, $p = 0.04$). Vaccinates of the 21-dpv challenge experiment showed significantly higher serum anti-F4 IgM and IgA than controls from 10 dpv onward (IgM, p-values < 0.001, <0.001, 0.02 and 0.03; IgA, $p = 0.02$, 0.01, 0.007, 0.002, respectively at 10, 14, 21 and 24 dpv).

Marginally significant higher levels of anti-F4 IgM ($p = 0.061$) and IgA (p = 0.093) were detected in the jejunum of vaccinates necropsied at 10 dpv (Fig. 2A). Vaccinated pigs necropsied at 24 dpv showed significantly higher levels of anti-F4 IgM and IgA

Table 4
Bodyweight of pigs at the day before vaccination (day -1) and at the end of the respective experiment (8, 10 or 24 days post-vaccination [dpv]) and daily weight gain (DWG) for the entire study period and the post-challenge period for the 3-dpv, 7-dpv and 21-dpv challenge experiments, respectively. An asterisk denotes a significant difference ($p \leqslant 0.05$).

	Bodyweight (kg)		DWG (g)	
	day -1	end of the study	entire study period	post-challenge period
7-dpv challenge experiment				
Vaccine	6.330	8.545	188	293
Control	6.323	8.028	155	153
Difference	+0.007	+0.517	+33	+140
p-value	0.990	0.517	0.321	0.153
21-dpv challenge experiment				
Vaccine	5.845	15.551	388	541
Control	5.797	14.434	339	366
Difference	+0.048	+1.117	+49	+175
p-value	0.923	0.280	0.056	0.071
3-dpv challenge experiment				
Vaccine	5.458	5.769	30	29
Control	5.884	5.687	−16	−53
Difference	−0.426	+0.082	+46	+82
p-value	0.027*	0.739	0.019*	0.007*

in the jejunum compared to controls ($p = 0.046$ and 0.006, respectively) (Fig. 2B). When the pig's highest intestinal level was considered, anti-F4 IgA were significantly higher for vaccinates than controls at 10 dpv (7-dpv challenge experiment; $p = 0.025$) or marginally higher at 24 dpv (21-dpv challenge experiment; $p = 0.067$).

Oral immunization of pigs with F4 antigen, whether with F4 fimbriae [31], recombinant FaeG F4 fimbriae [32,33], or a live F4-positive *E. coli* strain [24], induces specific systemic and mucosal immune responses and fimbriae-specific mucosal antibodies prevent colonization of F4-ETEC [24]. Vaccination with

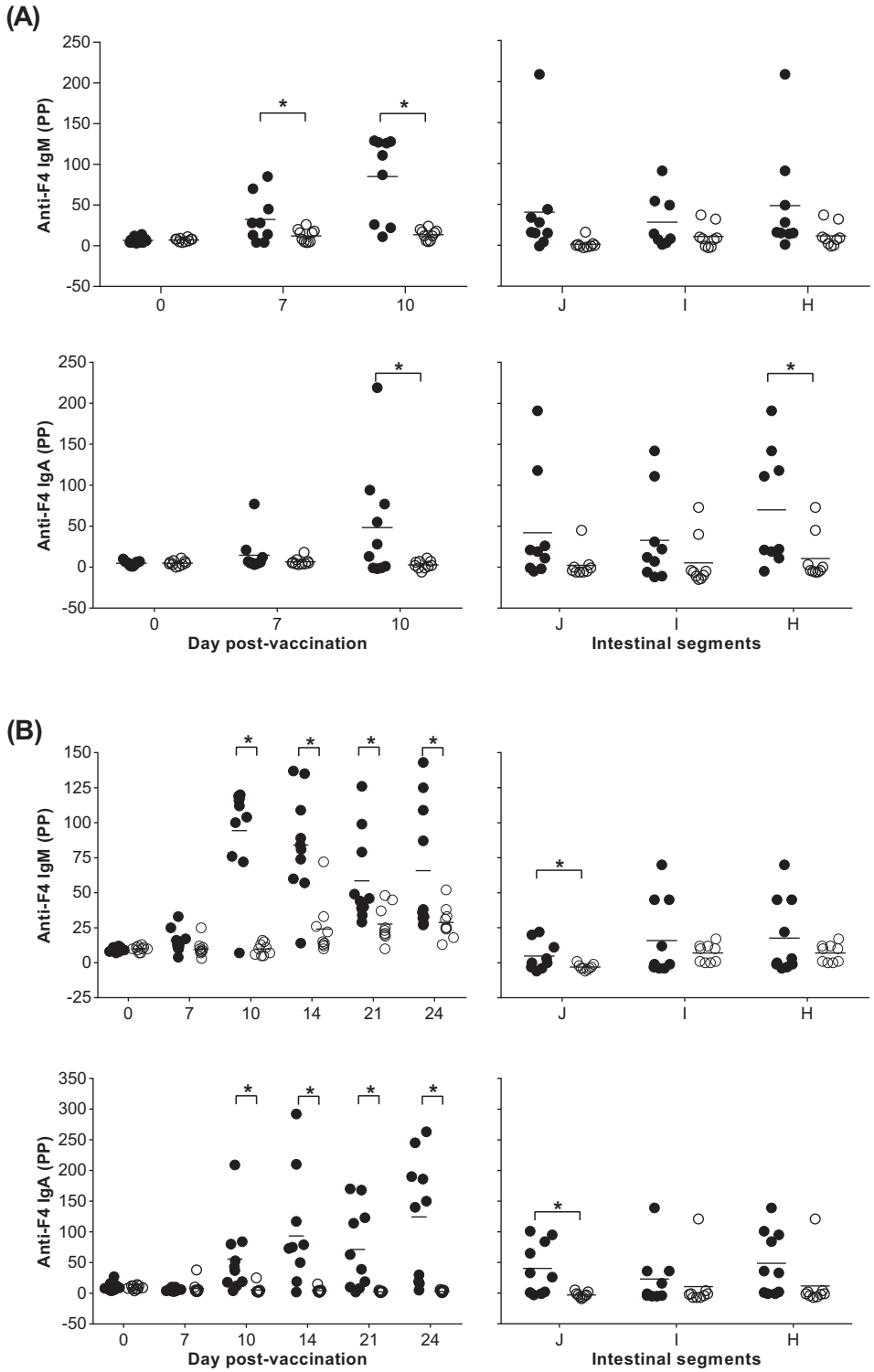

Fig. 2. Specific anti-F4 IgM and IgA antibodies in serum and in the intestinal contents at necropsy (jejunum; J, ileum; I and highest of the 2 segments; H) for the 7-day post-vaccination (dpv) challenge experiment (A) and the 21-dpv challenge experiment (B). The horizontal line represents the mean. Open circles represent results from animals of the control group; solid circles represent results from animals of the vaccinated group. PP refers to the Percentage of Positivity. Negative results refer to samples with data that were lower than the blank value. An asterisk denotes a significant difference ($p \leqslant 0.05$) between vaccinated and control pigs.

Coliprotec® F4 was thus characterized by the induction of serum anti-F4 IgM from 7 dpv that peaked around 14 dpv and anti-F4 IgA from 10 dpv that were maintained until 24 dpv (study completion of the 21-dpv challenge experiment). Results showed a significant correlation between the serum and the pig's highest intestinal antibody levels for anti-F4 IgM ($p = 0.016$) and IgA ($p = 0.002$) at 10 dpv, as well as for IgA ($p < 0.001$) at 24 dpv.

IgM is important as a secreted mucosal isotype in young pigs [34]. Though the shift from IgM to IgA as the predominant porcine intestinal isotype was reported to occur at about 12 weeks of age [35], a more rapid switch was observed with Coliprotec® F4. Verdonck et al. [24] demonstrated induction of mucosal and systemic F4-specific antibodies as early as 4 days after oral inoculation with a live F4-ETEC, which is 3 days earlier than the 7 dpv timepoint monitored in our experiments. No blood sampling of pigs was carried out in the 3-dpv challenge experiment and the contribution of an early production of anti-F4 antibodies in the observed partial protection is unknown. Competitive exclusion of F4-ETEC by the vaccine strain, reducing their intestinal colonization, is plausible shortly after vaccination, as the vaccine strain was detected at 3 dpv by PCR in fecal samples of 17 of the 20 vaccinated pigs (data not shown). However, fecal shedding of the F4-ETEC challenge strain was significantly reduced by vaccination only on the fifth day following the 3-dpv challenge, thus at 8 dpv. This timepoint is in accordance with the 7-day onset of immunity and protection demonstrated when pigs were challenged at 7 dpv. Competitive exclusion would not be an important mechanism of protection later on as only 3 out of 9 and 0 out of 10 vaccinated pigs were positive for the vaccine strain at 7 dpv and 21 dpv, respectively.

As expected, high levels of serum anti-F4 IgG were detected in both vaccinates and controls before the vaccination (data not shown) since pigs originated from a farm where gilts were immunized using a vaccine containing the F4 antigen. No interference of these maternally derived antibodies with the Coliprotec® F4 efficacy was observed. It was recently demonstrated that serum anti-F4 maternal antibodies enhance the secondary systemic immune response after oral immunization of pigs with F4 fimbriae [36].

3.5. Mortality and adverse event

One vaccinate of the 7-dpv challenge experiment was euthanized on the vaccination day due to severe lameness of the right hind limb that started the day before the vaccination after handling the animal. One control animal of the 21-dpv challenge experiment was euthanized at day 8 due to lateral decubitus and severe dyspnea. The pathology confirmed purulent rhinitis and severe bronchopneumonia caused by a complex of *Bordetella bronchiseptica*, *Pasteurella multocida* and *Streptococcus suis*. During the 3-dpv challenge experiment, one control pig was euthanized on the second dpc due to severe PWD caused by the challenge strain and one vaccinated animal was euthanized on the fourth dpc. The diagnostic testing showed a mixed infection of chronic interstitial pneumonia, suppurative bronchopneumonia due to *S. suis* and *B. bronchiseptica*, non-pandemic H1N1 porcine influenza and PWD caused by the challenge strain. All euthanasia were done for animal welfare reasons and all the events are considered not to be related to vaccination.

4. Conclusion

The present studies evaluated the efficacy of a live oral *E. coli* single-dose vaccine (Coliprotec® F4) to protect pigs against a F4-ETEC oral challenge at 3, 7 or 21 dpv and monitored specific immune responses. Results demonstrated that in pigs of at least 18 days of age, the administration of a single dose of Coliprotec® F4 via drinking water induces clinical protection against F4-ETEC within 7 days of vaccination and that protection is maintained until 21 days post-vaccination. The protection was demonstrated by a significant reduction of pigs having moderate to severe post-weaning diarrhea, ileum colonization by F4-ETEC and fecal shedding of F4-ETEC in infected pigs. A partial protection was observed after an early challenge at 3 dpv.

The 7-day onset of clinical protection was associated with an increase of anti-F4 IgM. Anti-F4 IgA induced from 10 dpv, along with anti-F4 IgM, were associated with the 21-day duration of protection. Significant correlations between serum and intestinal secretory anti-F4 antibodies were shown at 10 and 24 dpv.

Finally, it is worth noting that, though not statistically significant, vaccinated pigs were heavier than control pigs at the end of all studies.

Role of the funding source

This project was sponsored by Prevtec Microbia Inc. The sponsor made substantial contributions to the study design, conduct of the study, collection, analysis, and interpretation of the data, and the writing of the manuscript.

Author contribution

JMF, EN and KH served as the principal investigator, the sponsor responsible and the principal monitor of the study, respectively. JMF, EN, LB, DT, KH and RW conceived the studies. EN, LB, CLT, and DT contributed to study conduct and analysis. All authors contributed to data interpretation, writing, revision and approval of the manuscript.

Conflict of interest statement

The sponsor is the market authorization holder of the Coliprotec® F4 vaccine. EN, LB, CLT, DT and MB are current employees of the study sponsor. JMF and EN are co-inventors of Coliprotec® F4 and shareholders of the study sponsor, a university spin-off company. KH and JMF were compensated by the study sponsor for their participation as the study principal monitor and principal investigator, respectively. AH is a current employee of the European distributor of Coliprotec® F4.

Acknowledgement

We applied the "sequence-determines-credit" approach (SDC) for the sequence of authors. The authors wish to thank Denis Haine for statistical analyses, and Brigitte Lehoux, Ghyslaine Vanier, Steve Vo, Katherine Goyette, Frank van Lier, Éric Rasolofo, and Éric Allard for their assistance.

References

[1] Hoa NX, Kalhoro DH, Lu C. Distribution of serogroups and virulence genes of *E. coli* strains isolated from porcine post weaning diarrhea in Thua Thien Hue province Vietnam. Tạp chí Công ngh Sinh học 2013;11:665–72.
[2] Zhang W, Zhao M, Ruesch L, Omot A, Francis D. Prevalence of virulence genes in *Escherichia coli* strains recently isolated from young pigs with diarrhea in the US. Vet Microbiol 2007;123:145–52. http://dx.doi.org/10.1016/j.vetmic.2007.02.018.
[3] Lyutskanov M. Epidemiological characteristics of post-weaning diarrhoea associated with toxin-producing *Escherichia coli* in large intensive pig farms. Trakia J Sci 2011;9:68–73.
[4] Svensmark B, Jorsal SE, Nielsen K, Willeberg P. Epidemiological studies of piglet diarrhoea in intensively managed Danish sow herds. I. Pre-weaning diarrhoea. Acta Vet Scand 1989;30:43–53.
[5] Tubbs RC, Hurd HS, Dargatz D, Hill G. Preweaning morbidity and mortality in the United States swine herd. Swine Heal Prod 1993;1:21–8.

[6] USDA. Part II. Reference of Swine Health and Health Management in the United States, 2000. USDA:APHIS:VS, CEAH, National Animal Health Monitoring System, Fort Collins, CO. vol. #N355.0202; 2002.
[7] Jahanbakhsh S, Smith MG, Kohan-Ghadr HR, Letellier A, Abraham S, Trott DJ, et al. Dynamics of extended-spectrum cephalosporin resistance in pathogenic *Escherichia coli* isolated from diseased pigs in Quebec, Canada. Int J Antimicrob Agents 2016;48:194–202. http://dx.doi.org/10.1016/j.ijantimicag.2016.05.00.
[8] Luppi A, Bonilauri P, Dottori M, Gherpelli Y, Biasi G, Merialdi G, et al. Antimicrobial resistance of F4+ *Escherichia coli* isolated from swine in Italy. Transbound Emerg Dis 2015;62:67–71. http://dx.doi.org/10.1111/tbed.12081.
[9] Abraham S, Jordan D, Wong HS, Johnson JR, Toleman MA, Wakeham DL, et al. First detection of extended-spectrum cephalosporin- and fluoroquinolone-resistant *Escherichia coli* in Australian food-producing animals. J Glob Antimicrob Resist 2015;3:273–7. http://dx.doi.org/10.1016/j.jgar.2015.08.002.
[10] Abraham S, Trott DJ, Jordan D, Gordon DM, Groves MD, Fairbrother JM, et al. Phylogenetic and molecular insights into the evolution of multidrug-resistant porcine enterotoxigenic *Escherichia coli* in Australia. Int J Antimicrob Agents 2014;44:105–11. http://dx.doi.org/10.1016/j.ijantimicag.2014.04.011.
[11] Svendsen J, Larsen JL, Bille N. Outbreaks of post weaning *Escherichia coli* diarrhoea in pigs. Nord Vet Med 1974;26:314–22.
[12] De la Fé Rodríguez PY, Coddens A, Del Fava E, Abrahantes JC, Shkedy Z, Martin LOM, et al. High prevalence of F4+ and F18+ *Escherichia coli* in Cuban piggeries as determined by serological survey. Trop Anim Health Prod 2011;43:937–46. http://dx.doi.org/10.1007/s11250-011-9786-4.
[13] Frydendahl K. Prevalence of serogroups and virulence genes in *Escherichia coli* associated with postweaning diarrhoea and edema disease in pigs and a comparison of diagnostic approaches. Vet Microbiol 2002;85:169–82. http://dx.doi.org/10.1016/S0378-1135(01)00504-1.
[14] Fairbrother JM, Nadeau É, Gyles CL. *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Anim Heal Res Rev 2005;6:17–39. http://dx.doi.org/10.1079/AHR2005105.
[15] Goetstouwers T, Van Poucke M, Coppieters W, Nguyen VU, Melkebeek V, Coddens A, et al. Refined candidate region for F4ab/ac enterotoxigenic *Escherichia coli* susceptibility situated proximal to MUC13 in pigs. PLoS ONE 2014;9:e105013. http://dx.doi.org/10.1371/journal.pone.0105013.
[16] Li H, Li Y, Qiu X, Niu X, Liu Y, Zhang Q. Identification and screening of gene(s) related to susceptibility to enterotoxigenic *Escherichia coli* F4ab/ac in piglets. Asian Australas J Anim Sci 2008;21:489–93. http://dx.doi.org/10.5713/ajas.2008.70464.
[17] Rutter JM, Jones GW. Protection against enteric disease caused by *Escherichia coli* - a model for vaccination with a virulence determinant? Nature 1973;242:531–2.
[18] Deprez P, Den Van, Hende C, Muylle E, Oyaert W. The influence of the administration of sow's milk on the postweaning excretion of hemolytic *E. coli* in the pig. Vet Res Commun 1986;10:469–78. http://dx.doi.org/10.1007/BF02214010.
[19] McGhee JR, Mestecky J, Dertzbaugh MT, Eldridge JH, Hirasawa M, Kiyono H. The mucosal immune system: from fundamental concepts to vaccine development. Vaccine 1992;10:75–88.
[20] Daudelin J-F, Lessard M, Beaudoin F, Nadeau É, Bissonnette N, Boutin Y, et al. Administration of probiotics influences F4 (K88)-positive enterotoxigenic *Escherichia coli* attachment and intestinal cytokine expression in weaned pigs. Vet Res 2011;42:69. http://dx.doi.org/10.1186/1297-9716-42-69.
[21] Jensen GM, Frydendahl K, Svendsen O, Jørgensen CB, Cirera S, Fredholm M, et al. Experimental infection with *Escherichia coli* O149:F4ac in weaned piglets. Vet Microbiol 2006;115:243–9. http://dx.doi.org/10.1016/j.vetmic.2006.01.002.
[22] Delisle B, Calinescu C, Mateescu MA, Fairbrother JM, Nadeau É. Oral immunization with F4 fimbriae and CpG formulated with Carboxymethyl Starch enhances F4-specific mucosal immune response and modulates Th1 and Th2 cytokines in weaned pigs. J Pharm Pharm Sci 2012;15:642–56.
[23] Wright PF, Nilsson E, Van Rooij EM, Lelenta M, Jeggo MH. Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. Rev Sci Tech 1993;12:435–50.
[24] Verdonck F, Cox E, van Gog K, Van der Stede Y, Duchateau L, Deprez P, et al. Different kinetic of antibody responses following infection of newly weaned pigs with an F4 enterotoxigenic *Escherichia coli* strain or an F18 verotoxigenic *Escherichia coli* strain. Vaccine 2002;20:2995–3004.
[25] Mantel N, Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. J Natl Cancer Inst 1959;22:719–48. http://dx.doi.org/10.1016/0021-9681(79)90031-6.
[26] Greenland S, Robins JM. Estimation of a common effect parameter from sparse follow-up data. Biometrics 1985;41:55–68. http://dx.doi.org/10.2307/2530643.
[27] R Development Core Team. R: a language and environment for statistical computing. R Found. Stat. Comput. Vienna Austria, vol. 0, Vienna: 2015, p. ISBN 3-900051-07-0. doi: ISBN 3-900051-07-0.
[28] Nagy B, Fekete PZ. Enterotoxigenic *Escherichia coli* (ETEC) in farm animals. Vet Res 1999;30:259–84.
[29] Jin LZ, Zhao X. Intestinal receptors for adhesive fimbriae of enterotoxigenic *Escherichia coli* (ETEC) K88 in swine–a review. Appl Microbiol Biotechnol 2000;54:311–8. http://dx.doi.org/10.1007/s002530000404.
[30] Madec F, Bridoux N, Bounaix S, Cariolet R, Duval-Iflah Y, Hampson DJ, et al. Experimental models of porcine post-weaning colibacillosis and their relationship to post-weaning diarrhoea and digestive disorders as encountered in the field. Vet Microbiol 2000;72:295–310. http://dx.doi.org/10.1016/S0378-1135(99)00202-3.
[31] Van Den Broeck W, Cox E, Goddeeris BM. Receptor-dependent immune responses in pigs after oral immunization with F4 fimbriae. Infect Immun 1999;67:520–6.
[32] Verdonck F, Cox E, Van der Stede Y, Goddeeris BM. Oral immunization of piglets with recombinant F4 fimbrial adhesin FaeG monomers induces a mucosal and systemic F4-specific immune response. Vaccine 2004;22:4291–9. http://dx.doi.org/10.1016/j.vaccine.2004.04.016.
[33] Verdonck F, Snoeck V, Goddeeris BM, Cox E. Cholera toxin improves the F4 (K88)-specific immune response following oral immunization of pigs with recombinant FaeG. Vet Immunol Immunopathol 2005;103:21–9. http://dx.doi.org/10.1016/j.vetimm.2004.08.012.
[34] Snoeck V, Verfaillie T, Verdonck F, Goddeeris BM, Cox E. The jejunal Peyer's patches are the major inductive sites of the F4-specific immune response following intestinal immunization of pigs with F4 (K88) fimbriae. Vaccine 2006;24:3812–20. http://dx.doi.org/10.1016/j.vaccine.2005.07.025.
[35] Bianchi ATJ, Scholten JW, Moonen Leusen BHWM, Boersma WJA. Development of the natural response of immunoglobulin secreting cells in the pig as a function of organ, age and housing. Dev Comp Immunol 1999;23:511–20. http://dx.doi.org/10.1016/S0145-305X(99)00026-9.
[36] Nguyen UV, Melkebeek V, Devriendt B, Goetstouwers T, Van Poucke M, Peelman L, et al. Maternal immunity enhances systemic recall immune responses upon oral immunization of piglets with F4 fimbriae. Vet Res 2015;46:72. http://dx.doi.org/10.1186/s13567-015-0210-3.