

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

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ABSTRACT - Purpose. F4 *fimbriae* are a potential candidate for an oral subunit vaccine for prevention of post-weaning diarrhea in swine due to infection with F4-positive enterotoxigenic *Escherichia coli*. However, large quantities of F4 *fimbriae* are required to induce a specific antibody response. The aim of the present study was to evaluate the effect of supplementation of F4 *fimbriae* with Cytosine-phosphate-Guanosine-oligodeoxynucleotide (CpG-A D19) or with complete cholera toxin (CT) as adjuvants on the F4-specific antibody response and cytokine production in weaned pigs following oral administration of F4 fimbrial antigen formulated with Carboxymethyl Starch (CMS). Methods. Oral dosage forms of F4 *fimbriae* alone or supplemented with CpG-A D19 or with CT were formulated with CMS as monolithic tablets, obtained by direct compression, and administered to weaned pigs. Blood and faecal samples were collected to determine the systemic and mucosal immune status of animals at various times until necropsy. During necropsy, contents of the jejunum and ileum were collected for determination of mucosal F4 specific antibodies. Segments of jejunum and ileum were also used to measure mRNA cytokine production. Results. The presence of CpG in the formulation of the *fimbriae* significantly increased F4-specific immunoglobulin (Ig) IgM and IgG levels in intestinal secretions, and enhanced Th1 (Interferon-gamma / IFN- γ , Tumour Necrosis Factor-alpha / TNF- α , Interleukin-12p40 / IL-12p40, IL-1 β) and Th2 (IL-4, IL-6) cytokine production in intestinal tissues. Supplementation with CT did not result in induction of F4-specific antibodies in secretions, although a significant Th1 response (IFN- α , IFN- γ , IL-18) was detected in tissues. Neither F4-specific systemic antibodies, nor intestinally secreted IgA were detected throughout the immunization trial for all groups. Conclusions. CpG-A D19 appeared to be a promising adjuvant for an oral F4 subunit vaccine formulated with CMS excipient as monolithic tablets. This matrix afforded gastro-protection and delivered the F4 *fimbriae* at their intestinal sites.

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INTRODUCTION

F4-positive enterotoxigenic *Escherichia coli* (ETEC:F4) infections represent a major cause of post-weaning diarrhea (PWD) in pigs. PWD has a significant mortality rate and most often results in reduced gain of weight in pigs, leading to significant economic losses in swine production (1). F4 *fimbriae* are proteinaceous surface antigens composed of repeated subunits (2). They mediate bacterial binding to F4-specific receptors (F4R) located on the brush border of small intestinal enterocytes and subsequent colonization of the

small intestine. Several antimicrobial agents are often used to control PWD. However, increasing bacterial resistance to antibiotics underlines the need for new approaches. Oral vaccination is a promising alternative for the control of this disease. Advantages include a strong acquired mucosal immune response to administered antigen, easier administration procedures, reduced side effects and

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more convenient conditions of production (3).

F4 *fimbriae* have been proposed as a potential candidate for an oral subunit vaccine, due to their role in bacterial adherence and because they elicit a rapid and strong host immune response (4). Van den Broeck *et al.* (5-7) demonstrated that oral immunization of weaned pigs with F4 *fimbriae* induced systemic F4-specific antibody responses and F4-specific antibody secreting cells in intestinal tissues. However, the proposed immunization procedure required large quantities of F4, this antigen not being protected against degradation by the low pH of the stomach and by the presence of digestive enzymes. This disadvantage may be overcome by incorporating the F4 into a delivery system able to protect the antigen during gastric transit and to deliver it to the specific intestinal sites of F4-specific antibody response (8). The potential of carboxymethyl high amylose starch excipient (CMS) as delivery system for *fimbriae* has already been shown *in vitro* (9). The protection of F4 *fimbriae* provided by the biodegradable CMS matrix could offer a more effective piglet vaccination. Due to its functionality dependant of pH, CMS is an excipient able to afford gastro-protection of bioactive agents (9-10).

The amount of *fimbriae* required to stimulate an adequate oral immune response (5) may also be reduced by supplementation with an adjuvant to boost the immune response. Cholera toxin (CT), a potent immunostimulating adjuvant, has been used in several oral vaccines (11-13). It is a multimeric protein composed of A and B subunits. The B subunits bind the monosialogangliosides present on the surface of mammalian epithelial and hematopoietic cells, whereas the A subunits enter the cytosol and activate ADP-ribosylation of G protein, causing the elevation of cyclic AMP (14). Cytosine-phosphate-Guanosine-Oligodeoxynucleotides (CpG-ODNs) are another potent class of immunomodulators for mucosal adjuvants in vaccines (15). CpGs interact with pattern recognition receptors on immune cells and are recognized as danger signals that lead to the stimulation of the innate and adaptive host immune defence system. The CpG-A D19 stimulated the *in vitro* proliferation of peripheral blood mononuclear cells (PBMC) with a higher production of Interleukin-6 (IL-6), IL-12 and Tumour Necrosis Factor-alpha / TNF- α (16), and an increased level of Interferon-gamma / INF- γ (17). Although CpG

type-A is usually associated with the stimulation of Th1 cells by production of high levels of IFN- α (18), Van der Stede *et al.* (19) showed *in vitro* that CpG-A D19 can also stimulate B-cell proliferation and enhance expression of the major histocompatibility complex II (MHC II). They also found that intramuscular administration of F4 *fimbriae* and CpG-A D19 in pigs enhanced F4-specific proliferation of PBMC (20). Recently, Zhang *et al.* (21) demonstrated that subcutaneous administration of a CpG-A D19 equivalent, containing multiple CpG motifs, stimulated the innate immune system in weaned pigs. To date, the adaptive response and intestinal cytokine production in pigs following oral administration of CpG-A D19 with F4 *fimbriae*, has not been described. The aim of the present study was to orally administrate the F4 fimbrial antigen formulated with CMS excipient as monolithic tablets and to evaluate the effect of supplementation of F4 *fimbriae* with CpG-A D19 or CT on the F4-specific antibody response and cytokine production in weaned pigs. The novelty of our work is the finding that F4 *fimbriae* in the presence of CpG adjuvant, both formulated with CMS excipient (monolithic tablets), could be an effective oral subunit vaccine.

MATERIALS AND METHODS

Purification of F4 *fimbriae*

F4 *fimbriae* were purified as previously described (22), with some modifications. Briefly, the F4-positive bacterial suspension was centrifuged at 7000 rpm for 15 min, and the pellet was left to stand overnight at 4°C. The pellet was homogenized twice for 5 min on ice at maximum speed using an X-120 homogenizer (Polyscience, Staufen, Germany), with a 5 min cooling period on ice between each homogenization. The homogenate was then centrifuged at 11000 rpm for 20 min and the supernatant was precipitated with 20 % ammonium sulphate for 16 to 18 h at 4 °C under agitation. The suspension was then centrifuged at 10000 rpm for 1 h. The pellet containing the *fimbriae* was dissolved in a Phosphate Buffer Saline (PBS, 10 mM, pH 7.0) and dialyzed against water for 7 h, then against PBS, for 16 to 18 h. The suspension was adjusted to pH 4.0 with 2.5 % citric acid, agitated for 2 h at 4 °C, and centrifuged at 11000 rpm for 15 min. Finally, the pellet containing

the *fimbriae* was lyophilized. The protein concentration of the semi-purified fimbrial preparation was determined by the modified Lowry method (23) and the purity was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 12 % polyacrylamide gels.

Synthesis of CMS polymeric excipient

The CMS excipient was synthesized by etherification of high amylose starch (Hylon VII) with monochloroacetic acid in an alkaline reaction medium, as previously described by Calinescu *et al.* (9). 70 g Hylon VII were suspended in 170 mL of distilled water and warmed at 50°C under continuous stirring. Then, a volume of 235 mL of an aqueous 1.45 M NaOH solution was added to the reaction medium for starch gelatinisation (20 min, 50°C). To favour the carboxymethylation (nucleophilic substitution), 55 mL of 10 M NaOH solution were added to the reactional medium to transform the starch into a more reactive alkoxide form. 45.5 g of monochloroacetic acid, freshly dissolved in a minimal volume of water, were then added to the medium, and the reaction was carried on for 1 h at 50 °C under continuous stirring. After the substitution step, the gel-slurry was neutralized with an acetic acid solution, and finally, it was precipitated with acetone. At the end of this procedure, the polymeric derivative was washed with acetone:water (60:40, v/v) and filtrated for several times. The final drying was realized with pure acetone. CMS was then filtrated, and the obtained powder was air exposed at room temperature for drying, and sieved to obtain a powder with particles granulometrically smaller than 300 µm. This powder was used to prepare the monolithic tablets. The degree of substitution of CMS was determined by direct titration with 0.1 M NaOH solution, as previously described (10).

F4 *fimbriae* vaccine preparations

Lyophilised F4 *fimbriae* were exposed to 5 kGray (kGy) gamma-irradiation from Cobalt 60 to eliminate live F4⁺ bacteria surviving the purification process. Sterility of the irradiated F4 preparation was confirmed by absence of bacterial growth on blood agar and Luria-Bertani broth following inoculation of irradiated *fimbriae*. In addition, the integrity of the irradiated *fimbriae* was confirmed by SDS-PAGE and western blot, and with an

indirect immunofluorescence adhesion test using intestinal sections of previously identified F4R-positive pigs (24).

Fabrication of CMS monolithic tablets containing *fimbriae* and adjuvant

Irradiated lyophilized F4 *fimbriae*, alone or with complete CT (Sigma, St-Louis, USA) or with CpG-A D19 (5'-ggTGCATCGATGCAGggggg-3', BioCorp[®] AND inc., Montreal, Canada) were incorporated into 200 mg monolithic tablets formulated with CMS excipient. Thus, four different CMS formulations were obtained: (1) Formulation without F4 *fimbriae* and adjuvant (*unvaccinated control group*), (2) Formulation with 500 µg F4 *fimbriae*/dosage form (tablet) without adjuvant (*F4 group*), (3) Formulation with 500 µg F4 *fimbriae* associated with 25 µg complete CT adjuvant/tablet (*F4-CT group*) and (4) Formulation with 500 µg F4 *fimbriae* associated with 50 µg CpG-A D19 adjuvant/tablet (*F4-CpG group*). The tablets (200 mg) used for vaccination of piglets were obtained by direct compression at 2.5 T (Carver press, Wabash, IN, USA) of powders containing the F4 *fimbriae* bioactive agent, with or without CT or CpG adjuvant, and CMS excipient. For the unvaccinated control group, the monolithic tablets were obtained by direct compression (2.5 T) of 200 mg CMS excipient only.

Determination of F4R-positive piglets

To identify F4R-positive pigs to be included in the immunization assays, blood was collected from 2 weeks old nursing piglets in tubes containing EDTA (1.8 mg/mL, 7 mL) as anti-coagulant. Complete DNA genome was extracted from whole blood using a Qiamp DNA mini-kit (Qiagen Inc, Mississauga, Canada) and analyzed by a Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) assay as described by Jensen *et al.* (25).

Animal housing and necropsies

Pigs were handled throughout the study in accordance with the guidelines of the Canadian Council for Animal Care. Approval to carry out this *in vivo* study was obtained from the Institutional Animal Ethics Committee of the Faculty of Veterinary Medicine (Université de Montréal, Québec, Canada).

A total of 22 F4R-positive piglets (*Landrace X Duroc*) were selected and weaned at 28 days of age. Upon arrival in the animal care facilities, all pigs were treated orally with 150000 U/Kg body weight/day colistin (Pharmacie D. Bond and F. Beaulac, Actonvale, Canada) for 5 days to control the ETEC strains potentially present in the intestinal microflora. After 2 days of treatment, the pigs were transferred into isolation units. The presence of F4-positive ETEC in faecal swabs from the pigs was determined by PCR on arrival and subsequently at three day intervals. Animals were weighed and separated into 3 vaccinated groups of 5 pigs/group and one unvaccinated group of 7 animals. Food and water were administered to the pigs *ad libitum*. Blood and faecal samples were collected to determine the systemic and mucosal immune status of animals at the end of the colistin treatment, prior to the initial immunization (day 0), and at various times until necropsy (26 days post-initial immunization). Prior to necropsy, pigs were tranquillized with a mixture of 10 mg ketamine hydrochloride (Biomeda-MTC, Cambridge, Canada) and 20 mg xylazine (Bayer, Toronto, Canada) per kg of body weight and euthanized by intravenous injection of 108 mg per kg of body weight of sodium pentobarbital (Biomeda-MTC, Cambridge, Canada). During necropsy, contents of the jejunum and ileum were collected in 50 mL tubes, kept on ice, and then stored at -80°C until determination of mucosal F4-specific antibodies. Segments of jejunum and ileum (1x2 cm) with (PP+) or without (PP-) Peyer's patches (PP) were sampled to measure mRNA cytokine production. All tissue samples were placed in cryotubes, snap-frozen in liquid nitrogen, and stored at -80°C. In addition, 3 mm transversal sections of jejunum were embedded in an optimal cutting temperature Sakura Tissue-Tek® O.C.T. compound (Somagen Diagnostics, Edmonton, Canada), snap-frozen in liquid nitrogen, and stored at -80 °C until testing to confirm the F4-receptor (F4R) status of animals using an F4 indirect immunofluorescence assay (24).

Immunization procedure

All pigs were orally immunized twice. Each immunization consisted of administration of one pill (placebo or containing F4 *fimbriae* with and without adjuvant) per day for 3 successive days. The initial immunization (day 1) started in pigs of

35 days of age and the second immunization started on day 14 post-initial immunization.

Detection of F4-specific antibodies

Sample preparation

Serum

Blood was taken from the jugular vein on the day prior to the initial immunization and at 5, 8, 13, 16, 20, 23 and 26 days post-initial immunization, kept on ice, and then centrifuged to obtain serum that was then stored at -20 °C until analysis was carried out.

Intestinal contents

At 26 days post-initial immunization, the animals were euthanized and a necropsy was performed. The intestinal contents were collected from the jejunum and ileum and stored at -80°C until processing as described by Wenneras *et al.* (26), with slight modifications. Briefly, collected intestinal contents were treated by addition of 100 µg/mL (final concentration) soybean trypsin inhibitor (Sigma, St. Louis, USA) and 0.05 M (final concentration) EDTA (Sigma, St. Louis, USA) and kept on ice for 15 min. Then, the samples were centrifuged at 1000 x g for 15 min, and the supernatant of each sample was treated by adding 2 mM (final concentration) phenylmethylsulfonyl fluoride (Sigma, St. Louis, USA) and left to stand at room temperature for 15 min. Bovine serum albumin (Sigma) and sodium azide (Sigma) were then added to final concentrations of 1 mg/mL and 0.02% (w/v), respectively. The intestinal contents were freeze-dried and reconstituted with PBS (1:20) before analysis by ELISA.

Detection of F4-specific antibodies by ELISA

Systemic and secreted intestinal F4-specific antibodies were detected using an indirect ELISA test. The wells of the microtitre plates (Nalge Nunc, VWR International Co., Mississauga, Canada) were coated overnight at 4 °C with F4 *fimbriae* (5 µg/mL in 0.1 M carbonate buffer, pH 9.4) and non-specific binding sites were blocked by PBS supplemented with 0.2 % Tween-80 (v/v). Samples of serum and intestinal contents were first diluted with PBS (1:10 dilution for serum and 1:20 dilution for intestinal contents), then two fold-serial dilutions were performed in PBS containing 0.2 % Tween-20 (v/v) and 3 % bovine serum albumin (w/v). Microtiter plates were then incubated for 1 h at 37 °C. Optimal

dilutions (1:2500 for each conjugate) of goat anti-swine IgM, IgG or IgA horseradish peroxidase conjugates (Bethyl Laboratories, Cedarlane Laboratories Ltd., Burlington, Canada) were added to the wells and the microtiter plates were incubated for 1 h at 37 °C. Then, the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) substrate was added, and the optical density (OD) was measured at 405 nm after an incubation of 15 min at 37 °C. Between the incubation steps, the plates were washed three times with PBS containing 0.2 % Tween-20 (v/v). The cut-off values, being the mean OD at day 0 plus 2 times the standard deviation, were 0.32 for F4-specific IgM and IgG and 0.30 for IgA. The antibody titre was the inverse of the highest dilution which still had an OD higher than the cut-off value. When the titre was below the detection limit, it was arbitrarily set to the log₁₀ of one-half the detection limit for display purposes and mean calculation (27).

Porcine cytokine semi-quantitative reverse transcription polymerase chain reaction (Reverse Transcription-PCR)

Reverse Transcription-PCR was used to measure semi-quantitatively mRNA production for 10 cytokines (IFN- α , IFN- γ , TNF- α , IL-6, IL-10, IL-1 β , IL-4, IL-12p40, IL-18 and IL-8) in tissues. Approximately 50 mg of frozen tissue was immediately placed in liquid nitrogen to obtain a dry powder using a mortar and a pestle. Then, the tissue homogenate was transferred into a tube containing 1 mL of Trizol (Invitrogen, Burlington, Canada), and the extraction was carried out as described by the manufacturer. The RNA pellet was then resuspended in 25 μ L Promega nuclease-free water (Fisher Scientific Ltd., Ottawa, Canada) and heated at 65 °C for 10 min. Samples were treated with Turbo DNase (Ambion/Applied Biosystems Canada, Streetsville, Canada) according to the manufacturer's protocol, followed by precipitation using a solution of 10 % sodium acetate. RNA samples were resuspended in nuclease-free water to obtain approximately 300 ng/ μ L of RNA. Reverse transcription was carried out using approximately 1 μ g of total RNA and primed by random hexamer priming (Roche Diagnostics, Laval, Canada). Other reagents for reverse transcription, including RNase inhibitor, dNTP and SuperScript II RT (Invitrogen, Burlington, Canada), were used according to the

manufacturer's instructions. At the end of the reverse transcription, residual RNA was eliminated by adding 2 U of RNase H (Epicentre Biotechnologies, WI, USA), and the sample volume was adjusted to 100 μ L with nuclease-free water. PCR was carried out using Biotools Taq enzymes (Biotools B&M Labs., Madrid, Spain), PCR buffer (10 mM Tris-HCl, pH 9.0), 1.5 mM MgCl₂, 10 mM dNTP, 100 pmol of sense and anti-sense primers for each cytokine, 2.5 U Taq DNA polymerase, and 10 μ L of sample cDNA template in a 50 μ L reaction volume. RT-PCR and PCR were performed in a Biometra thermocycler (Göttingen, Germany) using the same primers and conditions similar to those described previously for IL-1 β , IL-4, IL-6, IL-8, IL-10, TNF- α , IFN- γ , cyclophilin (28), IL-12p40 (29), IL-18 (30) and IFN- α (31). An aliquot of each PCR sample was migrated on a 1.8 % Tris-Borate EDTA-agarose gel. The gels were stained with ethidium bromide and photographed using an alpha-imager (Alpha Innotech, San Leandro, USA). The relative amount of each product was determined by measuring the density of the band using BioNumerics software (Applied-maths Inc., Austin, USA). For each cytokine, the amount of the RT-PCR product was normalized to the value of cyclophilin, a constitutively expressed housekeeping gene used as standard for each sample.

STATISTICS

Differences between groups in antibody production and in cytokine mRNA expression ratio for combined results of jejunum and ileum were analyzed for statistical significance with the General Linear Model (Repeated Measures Analysis of Variance) test, and considered as duplicate sampling for statistical analysis.

RESULTS

CpG enhances the production of mucosal specific anti-F4 IgM in the small intestine

Upon necropsy of the pigs (26 days after initial immunization), we observed a significant increase ($P=0.01$) in the mucosal anti-F4 IgM titre in small intestinal contents of the F4-CpG group compared to that of the unvaccinated or the F4 group (Figure 1).

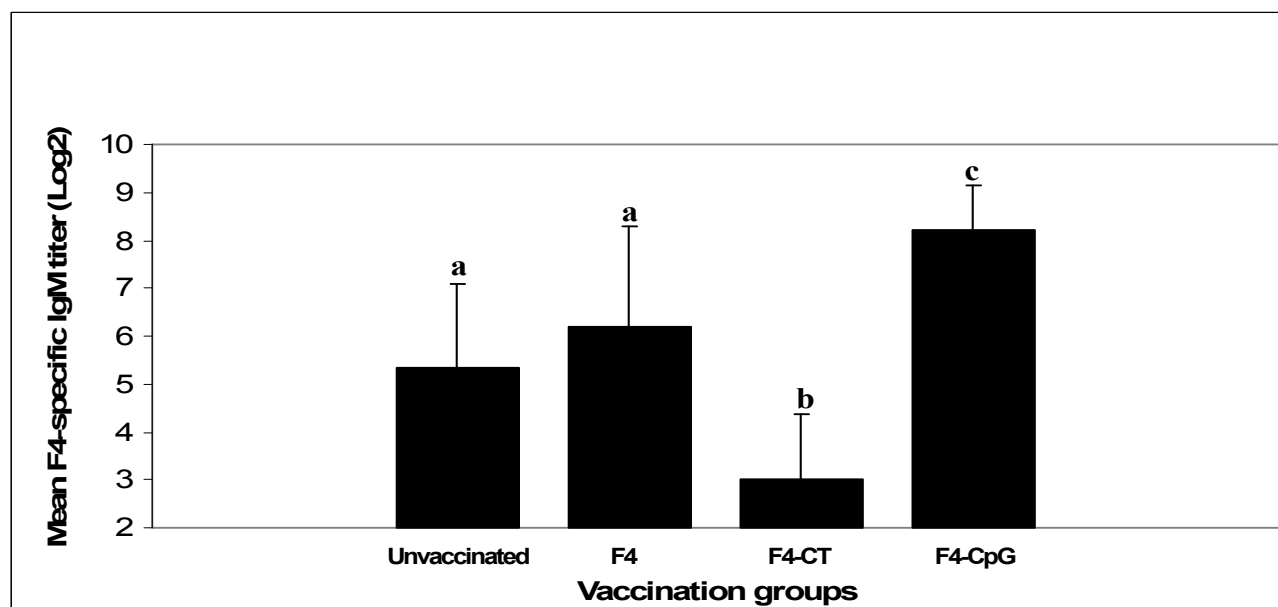


Figure 1. Modulation of specific anti-F4 IgM mucosal antibody by cholera toxin and CpG adjuvants after oral administration of F4 fimbriae formulated with carboxymethyl high amylose starch (CMS). Unvaccinated group: CMS monolithic tablets (200 mg). Vaccinated groups: CMS monolithic tablets (200 mg) containing 500 µg F4 fimbriae (F4 group), 500 µg F4 fimbriae and 25 µg complete cholera toxin (F4-CT group), 500 µg F4 fimbriae and 50 µg Cytosine-phosphate-Guanosine-Oligodeoxynucleotide (F4-CpG group). Letters a, b and c represent significantly different ($P \leq 0.05$) groups ($n=5$ pigs/group for vaccinated groups and $n=7$ pigs/group for unvaccinated group). Degree of substitution of CMS: 0.157.

In contrast, the mucosal anti-F4 IgM titre in the intestinal contents of the F4-CT group was significantly lower ($P=0.0004$) than that of the F4 group. No significant increase in the level of anti-F4 IgM was observed in the small intestinal contents of pigs of the F4 group compared to that of the unvaccinated group.

Production of mucosal specific anti-F4 IgG in the small intestine

In the intestinal contents, a significant increase ($P=0.04$) in the anti-F4 IgG titre was observed for the F4 group, compared to that of the unvaccinated group (Figure 2). Also, the anti-F4 IgG titre for the F4-CpG group was significantly higher ($P=0.0004$) than for the unvaccinated or the F4 groups, whereas the anti-F4 IgG titre for the F4-CT group was significantly lower ($P=0.002$) than for the F4 group. Mucosal anti-F4 IgA was not detected in the intestinal contents of any group.

Absence of serum F4-specific antibody after oral immunization of pigs

No significant difference in specific serum anti-F4 IgM response was observed between the vaccinated

(F4, F4-CpG, or F4-CT) and unvaccinated groups up to 26 days after initial oral immunization (Figure 3). Similarly, no significant difference in specific serum anti-F4 IgA response was observed between the vaccinated and unvaccinated groups (data not shown).

The serum anti-F4 IgG titres were not determined because pigs originated from commercial farms where sows are immunized with a commercial vaccine containing F4 *fimbriae* for the prevention diarrhea due to ETEC-F4 in newborn piglets. Commonly used commercially available vaccines are given parenterally and may be killed whole cell bacterins or purified fimbrial vaccines. This vaccination results in the production of high levels of specific IgG which is transferred to the piglets via the colostrum. This resulted in a high serum anti-F4 IgG antibody level in all animals before the vaccination procedure started, preventing the setting up of a realistic cut-off value for specific anti-F4 IgG.

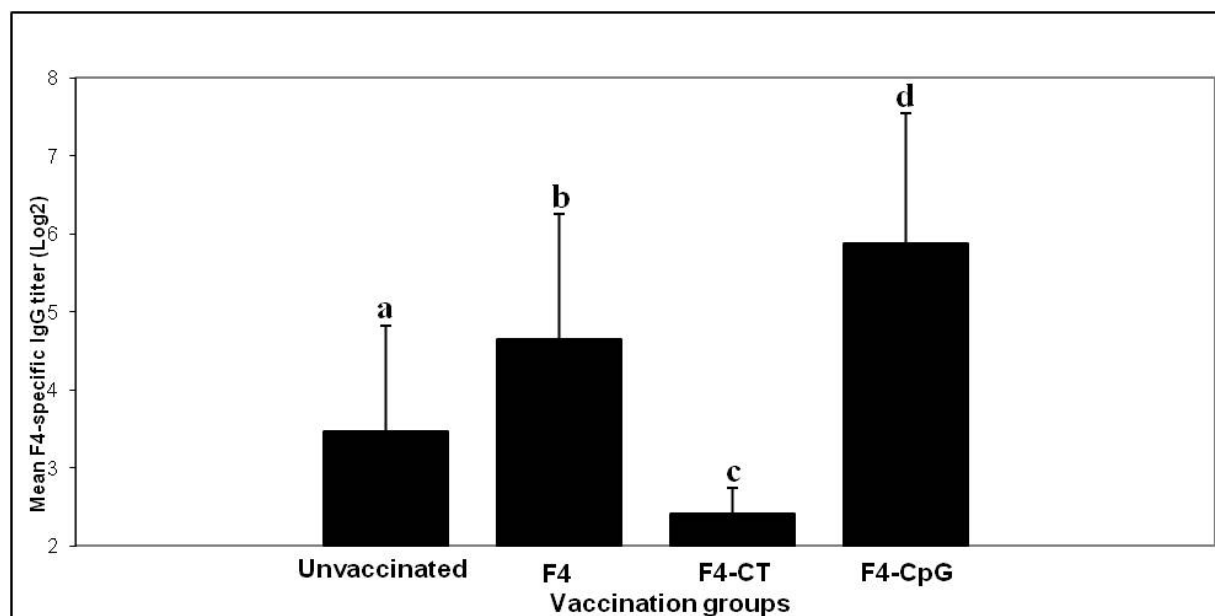


Figure 2. Modulation of specific anti-F4 IgG mucosal antibody by cholera toxin and CpG adjuvants after oral administration of F4 fimbriae formulated with carboxymethyl high amylose starch (CMS). Unvaccinated group: CMS monolithic tablets (200 mg). Vaccinated groups: CMS monolithic tablets (200 mg) containing 500 μ g F4 fimbriae (F4 group), 500 μ g F4 fimbriae and 25 μ g complete cholera toxin (F4-CT group), 500 μ g F4 fimbriae and 50 μ g Cytosine-phosphate-Guanosine-Oligodeoxynucleotide (F4-CpG group). Letters a, b, c, and d represent significantly different ($P \leq 0.05$) groups ($n=5$ pigs/group for vaccinated groups and $n=7$ pigs/group for unvaccinated group). Degree of substitution of CMS: 0.157.

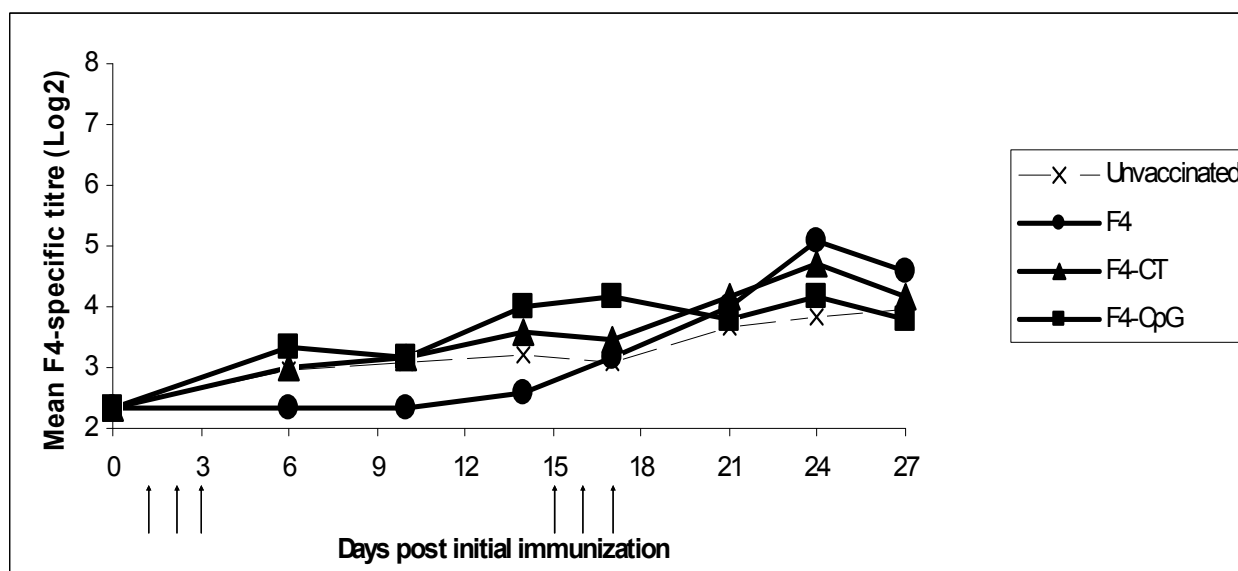


Figure 3. Levels of anti-F4 IgM serum antibody following oral administration of F4 fimbriae formulated with carboxymethyl high amylose starch (CMS). Unvaccinated group: CMS monolithic tablets (200 mg). Vaccinated groups: CMS monolithic tablets (200 mg) containing 500 μ g F4 fimbriae (F4 group), 500 μ g F4 fimbriae and 25 μ g complete cholera toxin (F4-CT group), 500 μ g F4 fimbriae and 50 μ g Cytosine-phosphate-Guanosine-Oligodeoxynucleotide (F4-CpG group). No significant difference in mean F4-specific IgM serum antibody titres was observed between the F4, F4-CT, F4-CpG and unvaccinated groups ($n=5$ pigs/group for vaccinated groups and $n=7$ pigs/group for unvaccinated group). Degree of substitution of CMS: 0.157. Arrows indicate consecutive days of the homologous oral immunization procedure (see materials and methods). Day 1: initial immunization, Day 26: necropsy.

CpG and CT up-regulate cytokine mRNA in different tissues

Cytokine mRNA expression in PP-negative (PP-) and PP-positive (PP+) intestinal tissues following necropsy at 26 days post-initial immunization was examined in order to explore the mechanisms for the observed modulation of specific mucosal antibody response to F4 *fimbriae*. No significant difference in expression of cytokines was observed between the F4 and unvaccinated groups, for either PP+ or PP- tissues. However, a significantly greater expression of IFN- γ and IL-4 was observed for the F4-CpG group than for the F4 group, in both the PP- and PP+ tissues (Table 1). Other Th1 and Th2 cytokines, such as IL12p40, TNF- α and IL-6 in the

PP- tissues and IL-1 β in the PP+ tissues, were also significantly up-regulated for the F4-CpG group. For the F4-CT group, significantly up-regulated pro-inflammatory cytokines were: IFN- γ in PP- and PP+ tissues and IFN- α and IL-18 in PP- tissues (Table 1). Overall, cytokine mRNA expression was higher in PP+ than in PP- tissues for IL-6, IL-10, IL-12 and TNF- α (Figure 4). The mRNA for IL-8 cytokine was highly expressed in a constitutive manner, with small differences between groups with or without adjuvant, in all tissues analyzed at 26 days after initial immunization (data not shown). In all groups and tissues, the cyclophilin housekeeping gene was expressed at a similar level.

Table 1. Up-regulation of cytokine mRNA expression in intestinal tissues of pigs immunized with F4 *fimbriae* in the presence of CpG or CT

Cytokine type		Up-regulation ^a of cytokine expression in various intestinal sites of pigs immunized with:			
		F4-CpG		F4-CT	
		PP-negative ^b	PP-positive ^c	PP-negative ^b	PP-positive ^c
Th1	IFN- γ	0.271 $\pm$ 0.0839 p=0.005	0.269 $\pm$ 0.0713 p=0.001	0.225 $\pm$ 0.0839 p=0.02	0.235 $\pm$ 0.0713 p=0.004
	IL12p40	0.0536 $\pm$ 0.0233 p=0.03	—	—	—
	TNF- α	0.0432 $\pm$ 0.0159 p=0.01	—	—	—
	IL-1 β	—	0.0525 $\pm$ 0.0221 p=0.03	—	—
	IFN- α	—	—	0.143 $\pm$ 0.0575 p=0.02	—
	IL-18	—	—	0.136 $\pm$ 0.0471 p=0.01	—
Th2	IL-4	0.0976 $\pm$ 0.0261 p=0.001	0.103 $\pm$ 0.0342 p=0.007	—	—
	IL-6	0.0939 $\pm$ 0.0430 p=0.04	—	—	—

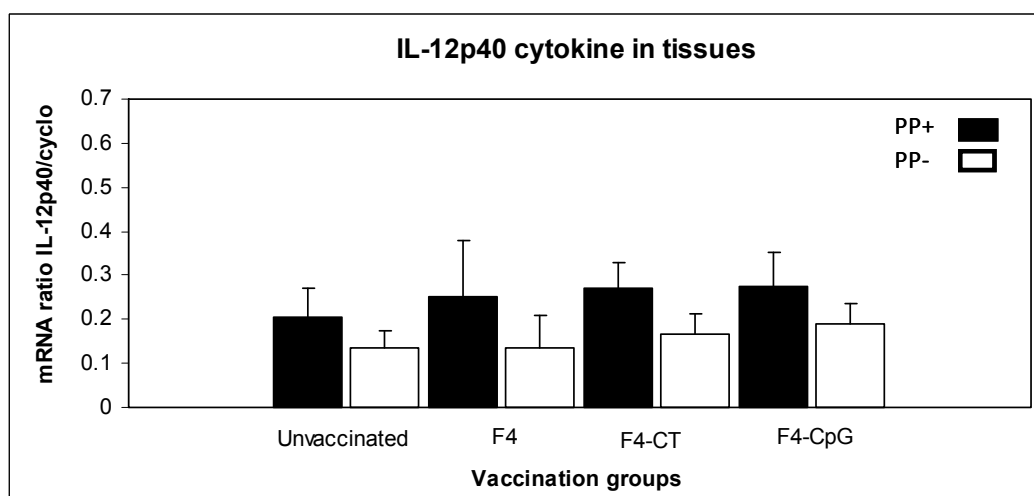
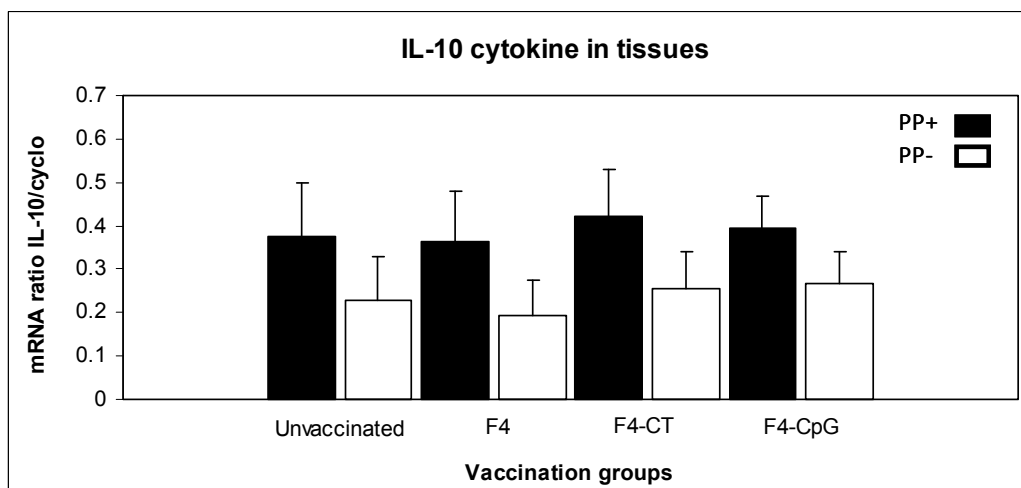
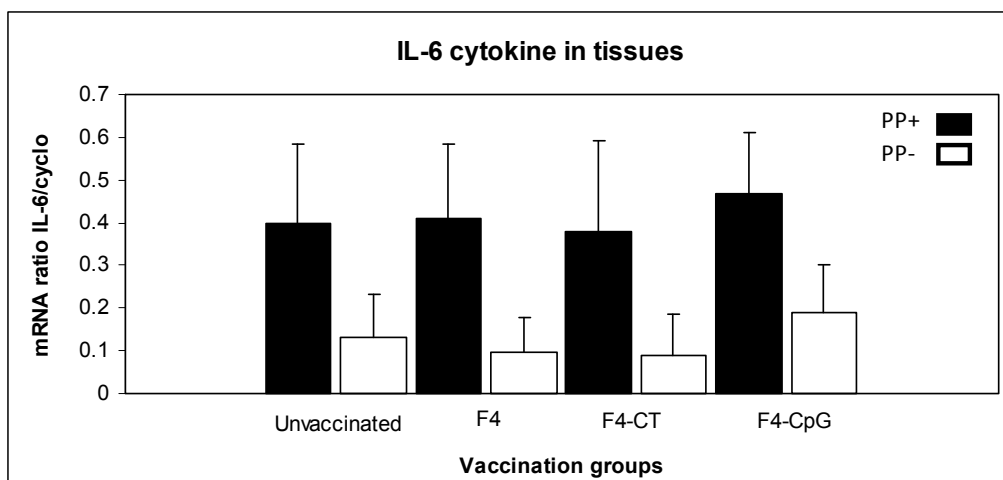
^a Significant ($P \leq 0.05$) up-regulation of cytokine gene expression compared to the F4 group,

^b Intestinal tissues lacking Peyer's patches,

^c Intestinal tissues containing Peyer's patches,

(-) indicates no significant difference in expression of cytokine gene when compared to the F4 group. General Linear Model (Repeated Measures Analysis of Variance) test was used.

n=5 pigs/group for vaccinated groups.



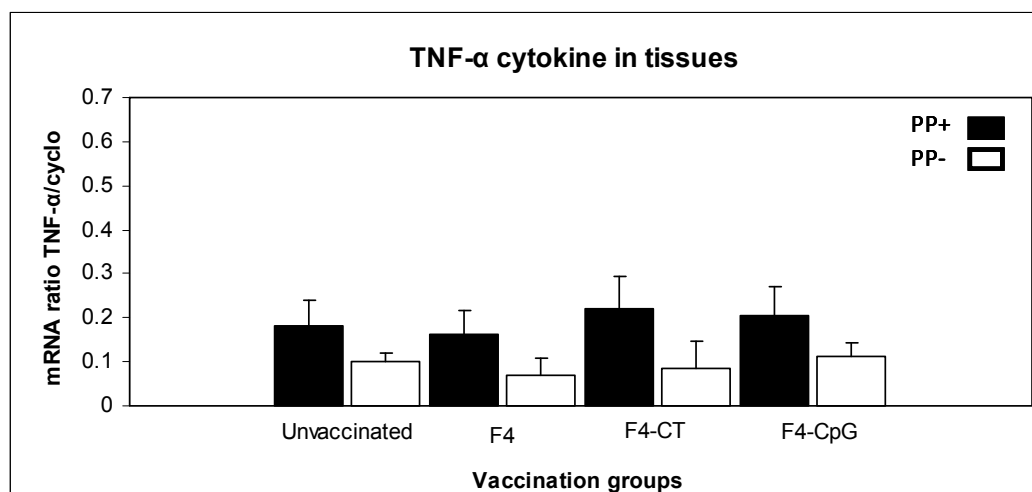


Figure 4. mRNA expression for cytokines IL-12p40, IL-6, IL-10 and TNF- α in intestinal tissues containing or lacking Peyer's patches (PP+ and PP- respectively) of pigs after oral administration of F4 fimbriae formulated with carboxymethyl high amylose starch (CMS) Unvaccinated group: CMS monolithic tablets (200 mg). Vaccinated groups: CMS monolithic tablets (200 mg) containing 500 μ g F4 fimbriae (F4 group), 500 μ g F4 fimbriae and 25 μ g complete cholera toxin (F4-CT group), 500 μ g F4 fimbriae and 50 μ g Cytosine-phosphate-Guanosine-Oligodeoxynucleotide (F4-CpG) group (n=5 pigs/group for vaccinated groups and n=7 pigs/group for unvaccinated group). Degree of substitution of CMS: 0.157.

For each cytokine, the amount of the RT-PCR product was normalized to the value of cyclophilin (cyclo), a constitutively expressed housekeeping gene used as standard for each sample.

DISCUSSION

CMS was proposed as an excipient where functionality depends on pH with the ability to protect F4 *fimbriae* in an oral vaccine against gastric acidity and pepsin, when formulated as monolithic tablet (9). Indeed, the F4 *fimbriae* formulated with CMS as monolithic tablets had a higher stability after incubation in simulated gastric fluid containing pepsin (32) than the non-protected F4 *fimbriae*, the latter being completely digested after 120 min of incubation (9). Moreover, the F4 *fimbriae* were liberated from CMS monolithic tablets during a period of up to 5 h of incubation in simulated intestinal fluid (32), a time interval that should be sufficient for F4 *fimbriae* to access F4-specific intestinal sites *in vivo* conditions. The present *in vivo* study confirmed the ability of F4 *fimbriae* in a CMS formulation to reach the specific sites of action in the pig intestine and to stimulate a specific mucosal immune response.

Different studies showed that, before the immunisation procedure with F4 *fimbriae* in pigs,

the gastric acidity has to be neutralized, in order to prevent possible alteration of fimbrial antigens during exposure to the gastric conditions. Sodium bicarbonate or other products, such as rabeprazole, a proton pump inhibitor, are generally used to neutralize or to decrease the production of stomach acid (33-35). This strategy allowed oral immunization of pigs deprived of food and water 3 h before and 2 h after gastric pH neutralization and immunisation with F4 *fimbriae* (administered as a solution). It was shown by Snoeck *et al.* (33) that oral vaccination with F4 *fimbriae* formulated as enteric-coated pellets was beneficial compared to F4 *fimbriae* in solution since a good gastric protection of F4 *fimbriae* preserves the *in vivo* receptor binding activity. As the non-formulated F4 *fimbriae* are not stable enough in gastric conditions (9, 33), the *in vivo* oral administration of the CMS monolithic tablets containing F4 *fimbriae* can further improve vaccination. Furthermore, the CMS formulation does not need a coating material, making the processes of matrix fabrication simple and less expensive since the amount to administered is markedly lower than that required for free-flowing F4 vaccine.

A significant adjuvant effect of CpG was shown for the first time in this *in vivo* study when the

adjuvant together with purified F4 *fimbriae* were formulated as monolithic tablets and orally administered to the pigs. This effect, manifested by an increased F4-specific IgM and IgG response in the intestinal contents, is beneficial for a vaccine designed to stimulate mucosal protection against the F4⁺ ETEC in pigs (5).

It has already been demonstrated that co-administration of a CpG with F4 *fimbriae* antigen to pigs by the parenteral route results in an increase of F4-stimulated PBMC proliferation *in vitro* and a reduction of faecal excretion of F4⁺ *E. coli*, although an effect on adaptive immunity was not shown (20). Our *in vivo* results complement well the *in vitro* data of Van der Stede *et al.* (19) who showed that CpG-A D19 induced IgM⁺ B-cell activation and increased expression of MHC-II molecules in lymphocytes, in cultured swine PBMC. In addition, Zhang *et al.* (21) reported that parenteral administration of CpG-A D19 to newly weaned pigs significantly stimulated the proliferation of PBMC and the production of IFN- γ in serum, thus enhancing the activation of the innate immune system.

The observation of a predominant IgM and IgG anti-F4 response in the present study is consistent with the relative antibody isotype response obtained in intestinal secretions of piglets in the first weeks of life (36). Snoeck *et al.* (8) showed a rapid and massive IgM response, as early as 14 days after intestinal immunization of pigs with F4 *fimbriae*, manifested by an increase in F4-specific IgM secreting cells in the PP+ tissues. This rapid response would be particularly important in the early critical period of susceptibility to ETEC in pigs after weaning (37) and of rapid bacterial colonization of the intestines (4). Our results further confirm those of Snoeck *et al.* (8) who found IgG secreting cells in the gut associated lymphoid tissue after oral administration of F4 *fimbriae* and jejunum exposure to F4 *fimbriae*, mainly at 21 days post-immunization of pigs of 53-56 days of age. Even if specific anti-F4 mucosal IgG is not recognized as a major class of immunoglobulin involved in mucosal anti-F4 defence, in part because of its susceptibility to proteolytic degradation in gut secretions (38), it may still play an early role in IgA antibody affinity tuning (39). It may also play a role in passive paracellular transfer and cell surface scavenger sampling of luminal antigens (40).

The finding of a predominance of F4-specific IgM antibodies in the intestinal contents is also consistent with other similar studies (34, 41) of F4 oral immunization. Bianchi *et al.* (41) found that IgM is more commonly associated with mucosal sites in pigs than in other animal species and is the preferentially expressed immunoglobulin in the first 12 weeks of age in specific pathogen-free pigs. Our results are also consistent with the predominance of IgM antibodies in intestinal secretions observed following infection with live F4⁺ ETEC during early development of animals (36).

In our study, the absence of specific anti-F4 IgA in the intestinal contents 26 days after initial immunization is not consistent with previous data, suggesting a delayed production of IgA F4-specific antibodies. Verdonck *et al.* (34) noted that the IgM and IgA induction and the switching from IgM to IgA isotype were slower following oral immunization with purified F4 *fimbriae* than following F4⁺ ETEC infection (4, 34). Similarly, few specific anti-F4 IgA secreting cells were found in the jejunum and ileum 21 days after oral immunization of pigs with F4 *fimbriae*, in relation to the overall high level of IgM production at the same points in time (8).

We also examined cytokine expression in regions of the small intestine with (+) or without (-) PP in an attempt to gain some insights into the mechanisms for the adjuvant effect of CpG. An interesting finding was an up-regulation of the IFN- γ (Th1-type cytokine) and of the IL-6 (Th2-type cytokine), these being hallmark cytokines of antibody production. Similarly, Yi *et al.* (42) observed an increase of IL-6 and, subsequently, of IgM antibodies in murine B cells stimulated with CpG and IFN- γ . Yamamoto *et al.* (43) reported that the production of IFN- γ was itself induced in T cells by the presence of CpG. In other studies, stimulation with CpG-A D19 resulted in an increase of the expression of MHC-II in swine PBMC (44) and in B cells (19), an up-regulation of co-stimulatory factors CD80/CD86 (45) and a high production of IFN- α , TNF- α and IL-12. The higher expression of Th1 cytokines, such as IFN- γ , IL-12, TNF- α and IL-1 β observed in our study, is consistent with the up-regulation of cytokine mRNA expression observed *in vitro* and *in vivo*, following stimulation with the same CpG-A D19 compound (16, 17, 21, 45). All of these observations tend to indicate that oral

administration of CpG - type A, particularly CpG-A D19, can stimulate innate and adaptive immune systems in pigs by generating a Th1/Th2 environment possibly involving both immune and non-immune cells in the intestinal mucosa. Our results fit well with other studies on CpG following parenteral administration or stimulation of immune cells in culture (21, 46, 47). Surprisingly, although the expression of IL-4 was increased in intestinal tissues both containing and lacking PP, the overall expression of this cytokine was relatively low compared to that of other cytokines in our study. Parenteral injection of CpG D19 has been reported to have no effect on IL-4 production (21, 48). In addition, CpG D19 administration was associated with an inhibition of IL-4 production involved in allergic responses, although it enhanced IFN- γ and IL-12 production (49) as reported in our study. The discrepancies concerning IL-4 production may be due to the different sites examined, various routes of vaccination or different experimental procedures used in these studies. The weak antibody and cytokine response following oral administration of F4 *fimbriae* alone underlines the need for an adjuvant to boost the humoral mucosal response. The difference between our results and those of Verdonck *et al.* (34), showing a significant anti-F4 antibody response following oral administration of F4 *fimbriae* without adjuvant, may be explained by the higher immunizing doses used in the latter study (2 mg *fimbriae* administered after the neutralization of acidic pH of the stomach). Other studies use even higher F4 *fimbriae* doses (4 mg *fimbriae* in PBS solution) to allow an optimal uptake of the antigen and to enhance the passage of unaltered ratio of *fimbriae* through the stomach (8). For different types of *fimbriae* (*i.e.* F18 *fimbriae*), even in the presence of a mucosal adjuvant, such as LT (R192G), higher doses of *fimbriae* (10 mg and, respectively, 30 mg) were used (50). Our combination of CpG-A D19 (50 μ g/dosage form) with relatively small amounts of F4 (500 μ g F4 *fimbriae*/dosage form) might provide a more cost-effective way of stimulating anti-F4 antibody production. The relatively small amounts of F4 used in our study (8 fold less than that used by Snoeck *et al.* (8)) stimulated significantly higher levels of mucosal anti-F4 antibody in the presence of CpG adjuvant (F4-CpG group) than in its absence (F4 group). In addition, the ability of CMS matrix to protect the F4 *fimbriae* during the gastric transit and

to deliver them to a specific absorption window probably contributed to the enhancement of this effect. Another beneficial effect of the CMS delivery system may be the resulting proximity between the F4 antigen and the adjuvant co-formulated in the same tablet, this being a prerequisite for the efficacy of the CpG adjuvant when delivered to the antigen presenting cells (51-52).

The lack of detectable specific anti-F4 antibodies in the sera of orally immunized pigs in our study may indicate that the systemic and small intestine mucosal responses are distinct when only the small intestine surface is exposed to F4 antigen. This may be due to differences in protein diffusion into villous capillaries, as hypothesized by Snoeck *et al.* (8) explaining lower serum specific antibody levels following colonic immunization with F4 *fimbriae*. In addition, since in other studies (5-7), F4 *fimbriae* were administered orally without a protecting and delivery system, it is possible that the immunogen would come into greater contact with pre-gut mucosal surfaces, such as buccal and laryngeal associated lymphoid tissues, thus generating a systemic antibody response. Another possible explanation for the lack of detectable serum anti-F4 antibodies observed in our study in the presence of adjuvants could be that the sows of the pigs used in our study had already been immunized with a commercial vaccine containing the F4 antigen.

Another surprising finding of our study was the lack of CT adjuvant effect on the mucosal antibody response to F4 *fimbriae*. This is unexpected, since complete CT is considered a potent mucosal adjuvant. Significantly higher salivary IgA and cellular F4 specific responses were observed following oral immunization of pigs with FaeG recombinant major adhesin of F4 (35) or with F4 *fimbriae* coupled to human serum albumin (53), when co-administered with CT. Furthermore, oral vaccination with non-replicating immunogens, such as subunit vaccines, has previously been associated with poor induction of serum antibodies (8, 54). A possible explanation for the decreased anti-F4 IgM levels when CT was co-administered with F4, could be a concomitant IgA isotype switching, as previously observed with CT (55). Our results are in agreement with data of Foss and Murtaugh (11) showing that the adjuvant effect of CT for heterologous proteins following oral administration

was variable, with no induction of antibody response when CT was co-administered. Although no CT-associated adjuvant effect was observed on anti-F4 antibody response in our studies, the pro-inflammatory cytokine expression was nevertheless stimulated. In particular, IFN- γ was up-regulated in both PP-containing and PP-lacking regions of the small intestine, suggesting that there was an inflammatory response and thus excluding the possibility of having provoked oral tolerance to F4 *fimbriae* by the immunization procedure. Oral immunization with CT in mice has been shown to influence protection against *Helicobacter pylori* through IL-18, a co-regulator of Th1 development associated with IFN- γ production in gastric tissues (56). Similarly, Th1-type cytokine IL-18, as well as IFN- α , were up-regulated in PP-lacking regions of the small intestine following co-administration of F4 *fimbriae* with CT in the present study. These findings provide more evidence of an immunomodulatory effect of CT, being different from that observed for CpG adjuvant.

In conclusion, our results suggest a potential use of CpG as mucosal immune adjuvant for oral immunization with subunit vaccines, and underline the role of a Th1/Th2 cytokine balance in its adjuvant effects. CMS is a promising excipient for the formulation of monolithic tablets to be used for oral immunisation of pigs, due to its capacity to afford gastric protection for both the F4 *fimbriae* active agent and specific adjuvants, delivering *fimbriae* to their specific sites in the small intestine. Thus, the F4 *fimbriae* formulated with CMS excipient (monolithic tablets) can retain their receptor binding activity, an essential condition for the obtention of a mucosal immune response.

Abbreviations

CMS, Carboxymethyl High Amylose Starch; cyclo, cyclophilin; CpG-A D19, Cytosine-phosphate-Guanosine-Oligodeoxynucleotide; CT, Cholera Toxin; F4⁺ ETEC, F4-positive Enterotoxigenic *Escherichia coli*; F4R, F4-specific Receptor; IFN, Interferon; Ig, Immunoglobulin; IL, Interleukin; MHC II, Major Histocompatibility Complex II; OD, Optical Density; PBMC, Peripheral Blood Mononuclear Cells; PBS, Phosphate Buffer Saline; PCR, Polymerase Chain Reaction; PP, Peyer's Patches; PWD, Post-Weaning Diarrhea; RT-PCR, Reverse Transcription Polymerase Chain Reaction; SDS-PAGE, Sodium Dodecyl Sulphate

Polyacrylamide Gel Electrophoresis; TNF, Tumour Necrosis Factor.

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