The polymeric stability of the *Escherichia coli* F4 (K88) fimbriae enhances its mucosal immunogenicity following oral immunization

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ABSTRACT

Only a few vaccines are commercially available against intestinal infections since the induction of a protective intestinal immune response is difficult to achieve. For instance, oral administration of most proteins results in oral tolerance instead of an antigen-specific immune response. We have shown before that as a result of oral immunization of piglets with F4 fimbriae purified from pathogenic enterotoxigenic *Escherichia coli* (ETEC), the fimbriae bind to the F4 receptor (F4R) in the intestine and induce a protective F4-specific immune response. F4 fimbriae are very stable polymeric structures composed of some minor subunits and a major subunit FaeG that is also the fimbrial adhesin. In the present study, the mutagenesis experiments identified FaeG amino acids 97 (N to K) and 201 (I to V) as determinants for F4 polymeric stability. The interaction between the FaeG subunits in mutant F4 fimbriae is reduced but both mutant and wild type fimbriae behaved identically in F4R binding and showed equal stability in the gastro-intestinal lumen. Oral immunization experiments indicated that a higher degree of polymerisation of the fimbriae in the intestine was correlated with a better F4-specific mucosal immunogenicity. These data suggest that the mucosal immunogenicity of soluble virulence factors can be increased by the construction of stable polymeric structures and therefore help in the development of effective mucosal vaccines.

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1. Introduction

The default response against orally administered soluble antigens is the induction of oral tolerance [1,2]. However, oral administration of F4 fimbriae purified from F4⁺ enterotoxigenic *Escherichia coli* (ETEC) to piglets expressing an F4-specific receptor (F4R) on their intestinal epithelium, results in the induction of a protective mucosal immune response [3]. Therefore, this F4 model can be used to study the requirements of a soluble antigen to induce a mucosal immune response following oral administration [4].

ETEC bind with their fimbriae to specific receptors present in the intestine of man or animals, subsequently colonise the small intestine and induce diarrhoea in their host by secreting heat labile (LT) and heat stable (ST) enterotoxins [5]. The type of fimbriae expressed by ETEC bacteria determines their host specificity. Over twenty different fimbriae are identified on human isolates

[6], whereas porcine ETEC isolates express 5 different fimbriae of which F4 and F18 are most prevalent [7]. F4⁺ and F18⁺ ETEC induce neonatal and post-weaning diarrhoea in piglets worldwide. The interaction between the fimbriae and their specific receptor in the intestine is an essential step in the pathogenesis since piglets which do not express the F4-receptor (F4-receptor negative; F4R⁻) or the F18-receptor are resistant to F4⁺ or F18⁺ ETEC infections [8,9]. Binding of purified F4 fimbriae to the F4R is also important in its mucosal immunogenicity, since no F4-specific immune response is observed following oral administration of purified F4 fimbriae in F4R⁻ pigs [3]. However, binding of the fimbrial antigens to its intestinal receptor is not enough to induce an antigen-specific mucosal immune response. Oral immunization of F18R⁺ pigs with purified F18 fimbriae, even in the presence of a mucosal adjuvant or encapsulated to increase the stability in the gastro-intestinal tract, did not result in an (protective) immune response [10,11]. Moreover, it was shown that conjugation of antigen to cholera toxin B, an analogue of LT-B, enables them to bind to GM1 on the intestinal surface and dramatically reduces the amount of antigen needed for tolerance induction [12]. Therefore, additional factors other than enterocyte binding have to be involved in the mucosal

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immunogenicity of soluble non-replicating antigens like purified F4 fimbriae.

In most fimbrial systems, the adhesin is a highly conserved minor subunit present at the tip. The fimbrial structure is mainly composed of the less conserved major subunit, which may be a way to reduce cross-protection between different isolates since parenteral immunization with purified fimbriae showed mainly antibodies against the major subunit [13,14]. In contrast, the F4 fimbrial adhesin FaeG is also the major fimbrial subunit and is highly conserved within each of the three F4 serotypes ab, ac and ad [15,16]. So, this structural difference between F4 and most other fimbriae can be at least one of the causes of its mucosal immunogenicity. Indeed, purified F4 fimbriae of strain GIS26 (F₄_{GIS26}) are expected to reach the small intestine in a polymeric shape following oral administration since they pass the stomach before complete degradation will happen [17]. We have previously shown that oral immunization with non-polymeric F4 fimbrial FaeG adhesins was less efficient in the induction of a mucosal F4-specific immune response than immunization with polymeric F4 fimbriae but were unable to analyze the role of F4 fimbrial stability on its immunogenicity [18,19].

In a previous study with 22 F4⁺ ETEC isolates, purified F4 fimbriae from 21 isolates showed a characteristic ladder pattern of FaeG polymers on SDS-PAGE without heating the samples [16]. Only fimbriae purified from strain 5/95 (F₄_{5/95}) were less stable and appeared as FaeG monomers. The aim of this study was to identify the amino acids involved in the polymeric nature of F4 and to analyze whether the polymeric nature of the fimbriae was important in regard to its mucosal immunogenicity.

2. Materials and methods

2.1. Wild type strains

GIS26 and 5/95 are both F4ac⁺ ETEC strains (Table 1), but their F4 fimbriae have a different stability since they show a polymeric and monomeric band pattern respectively on a Coomassie stained SDS-PAGE without heat treatment of the samples. Interestingly, the FaeG amino acid sequence differs at 7 positions between both strains [16]. Five of these different amino acids present in FaeG of strain 5/95 (FaeG_{5/95}) were also found in the FaeG amino acid sequence of other F4⁺ *E. coli* field isolates with fimbriae showing a polymeric pattern on a Coomassie stained SDS-PAGE without heat treatment of the samples. However, 2 of them (K₉₇ and V₂₀₁)

were only detected in FaeG_{5/95} and their influence on the F4 polymeric nature is analyzed in the present study by mutagenesis. The bacteria expressing wild type and mutant fimbriae were cultured overnight at 37 °C in Luria Broth-media or on LB-agar plates with suitable antibiotic selection. For the villous adhesion assay or to purify F4 fimbriae, ETEC strains were cultured during 18 h in Tryptone Soya Broth (Oxoid, Basingstoke, Hampshire, England) at 37 °C and 85 rpm.

2.2. Construction of GIS26faeG::Cm

A mutant GIS26 ETEC strain, in which the F4 assembly is blocked by shutting down the gene encoding the major subunit FaeG, was created. Briefly, 4.1 kb fragment of GIS26 F4 operon was amplified by PCR (primers: 5'caaggatcctaaccgggtgacaaaagcac and 5'ccatctagaactcaatactaccggcagtg), a 3.6 kb Cm-cassette from pHP45 Ω cm [20] was cloned to SacI site in the middle of the *faeG* gene, and finally introduced into the suicide plasmid vector pGP704 [21] to construct pJJJ88 (Fig. 1A). The plasmid pJJJ88 containing the *faeG*::Cm was conjugated to GIS26 and the mutant allele was recombined into the chromosome by homologous recombination. Ter^RCm^RAmp^S colonies were screened to generate GIS26faeG::Cm. The presence of *faeG*::Cm was confirmed by DNA hybridization analysis. Briefly, 2.5 mg PstI-digested bacterial DNA was separated on agarose gel, blotted onto nitrocellulose membrane, and hybridized with dioxxygenin-labelled 0.8 kb *faeG* PCR-fragment (primers: 5'tggatgactggtgatttc and 5'tcagtaataagtattgctac) (Fig. 1B).

2.3. Complementation of GIS26faeG::Cm

The expression of F4 fimbriae in GIS26faeG::Cm was restored by introducing an expression vector containing an intact *faeG* gene (Table 1). Briefly, a 1.0 kb *faeG* fragment including the periplasm targeting signal sequence was amplified by PCR from ETEC strains GIS26 or 5/95 (primers: 5'caaggatcggatagttttacggaattcc and 5'ccaggatccttagtaataagtattgctacgt) and cloned into KpnI site in pBluescript II KS-(Stratagene, La Jolla, CA) under the control of the *lacZ*-promoter (pJJJ89 and pJJJ92 respectively). The expression vectors were introduced to GIS26faeG::Cm by conjugation under ampicillin selection. The amino acids K₉₇ and V₂₀₁ which have been detected only in FaeG_{5/95}, were introduced in FaeG_{GIS26} to analyze their influence on the polymeric nature of F4 fimbriae. Point mutations were introduced in pJJJ89 by using a QuickChange site-directed PCR mutagenesis kit according to manufacturer's

Table 1
Bacterial strains and plasmids

ETEC strains			
Name	Description		Selection
5/95	Wild type F4 ETEC strain with weak F4 fimbriae (O149:F4ac ⁺ LT ⁺ , STb ⁺)		Tet
GIS26	Wild type F4 ETEC reference strain (O149:F4ac ⁺ LT ⁺ , STa ⁺ , STb ⁺)		Tet
GIS26 <i>faeG</i> :: <i>Cm</i>	F4 deficient mutant GIS26		Tet, Cm
GIS26 <i>faeG</i> :: <i>Cm</i> (pJJJ89)	GIS26 <i>faeG</i> :: <i>Cm</i> transformed with pJJJ89 to complement F4 synthesis with wild type <i>faeG</i> sequence of strain GIS26		Tet, Cm, Amp
GIS26 <i>faeG</i> :: <i>Cm</i> (pJJJ92)	GIS26 <i>faeG</i> :: <i>Cm</i> transformed with pJJJ92 to complement F4 synthesis with wild type <i>faeG</i> sequence of strain 5/95		Tet, Cm, Amp
GIS26 <i>faeG</i> :: <i>Cm</i> (pHMM01)	GIS26 <i>faeG</i> :: <i>Cm</i> transformed with pHMM01 to complement F4 synthesis		Tet, Cm, Amp
GIS26 <i>faeG</i> :: <i>Cm</i> (pHMM02)	GIS26 <i>faeG</i> :: <i>Cm</i> transformed with pHMM02 to complement F4 synthesis		Tet, Cm, Amp
GIS26 <i>faeG</i> :: <i>Cm</i> (pHMM05)	GIS26 <i>faeG</i> :: <i>Cm</i> transformed with pHMM05 to complement F4 synthesis		Tet, Cm, Amp
Plasmids			
Name	Description	Selection	Host strain
pJJJ88	Suicide vector carrying <i>faeG</i> :: <i>Cm</i> and <i>pir</i> -dependent RK6 replicon	Cm, Amp	SM10λ <i>pir</i>
pJJJ89	FaeG _{GIS26} expression vector	Amp	TOP10F ⁺ (Invitrogen, Paisley, UK)
pJJJ92	FaeG _{5/95} expression vector	Amp	TOP10F ⁺
pHMM01	FaeG _{GIS26} I ₂₀₁ -V expression vector	Amp	TOP10F ⁺
pHMM02	FaeG _{GIS26} I ₂₀₁ -V, N ₉₇ -K expression vector	Amp	TOP10F ⁺
pHMM05	FaeG _{GIS26} N ₉₇ -K expression vector	Amp	TOP10F ⁺

Fig. 1. Generation of the F4-deficient GIS26faeG::Cm ETEC strain. (A) The F4 assembly was blocked by disrupting the gene encoding for major subunit FaeG with a Cm-gene cassette. (B) DNA hybridization analysis of PstI-digested DNA from GIS26faeG::Cm clones with faeG probe. Lanes 1–4, Cm^RAmp^R clones containing both faeG::Cm and WT faeG; lanes 5–11, Cm^RAmp^S clones containing only faeG::Cm lane 12, WT GIS26 faeG.

protocol (Stratagene, La Jolla, CA). FaeG_{GIS26} N₉₇ was changed to K (pHMM05) with primers 5'ctgtagtactcagaaacctgatggtgaaacta and 5'tagttccaccatcaggtttctgagtactacag and I₂₀₁ to V (pHMM01) with primers 5'gagtaaacgcaaatgttactctcttg and 5'caagagaagtaacatttgcgtttactc. A final plasmid was constructed with both mutations (pHMM02). The introduced mutations and the absence of unintended mutations were confirmed by sequencing.

2.4. Purification, gel- and ELISA analysis of F4 fimbriae

F4 fimbriae were purified from wild type and mutant strains as described previously [17]. The protein concentration of purified F4 was determined using the bicinchoninic acid reaction with bovine serum albumin as a standard (ICN Biomedicals, Belgium). SDS-PAGE (10%) was used to analyze the polymeric nature of F4 fimbriae since unboiled F4_{GIS26} results in a ladder pattern consisting of FaeG polymers and boiled F4_{GIS26} results only in the monomeric FaeG band [16]. The native gels (5%) and running buffer were prepared as before [22], but in the absence of SDS and FaeG-specific immunoblotting was performed as previously [23]. A sandwich ELISA based on the FaeG-specific monoclonal antibodies (MAb) IMM01 or IMM09 was used to confirm the monomeric or polymeric nature of purified F4 since MAb IMM01 recognizes both monomeric and polymeric F4 fimbriae [24] and the MAb IMM09 (also produced in our laboratory) only recognising polymeric F4 fimbriae.

2.5. Electron microscopy

Purified F4 fimbriae were treated by negative staining and visualized by transmission electron microscopy as described previously [25]. Briefly, suspension of equal amounts of fimbriae were brought

on carbon and pioloform coated grids, stained with 2% uranylacetate in water and visualized using a Technai Spirit transmission electron microscope (FEI, Eindhoven, The Netherlands) operating at 120 kV. Micrographs were made using a bottom-mounted 4 × 4 k Eagle camera (FEI).

2.6. In vitro villous adhesion and inhibition assay

The F4R⁺ status of the immunized piglets was confirmed by incubating small intestinal villi with F4⁺ ETEC strain GIS26 as described previously [17]. Adhesion of more than 5 bacteria per 250 μm villous length was noted as positive [26]. The F4R-binding capacity of wild type and mutant F4 fimbriae was analyzed by an *in vitro* villous adhesion inhibition assay [17]. The test was done with villi of three F4R⁺ and two F4R⁻ pigs. The percentage inhibition of adhesion was calculated for each sample by comparing with F4R⁺ villi incubated only with F4⁺ *E. coli*.

2.7. Stability of F4 fimbriae in gastro-intestinal conditions

Purified F4 fimbriae (0.5 mg/ml) were exposed to pepsin (Sigma P-7000, 0.16% (w/v)) digestion at pH 7.4, 4, 3, 2, or 1.5 for 30 min at 37 °C. The digestions were quenched by raising the pH over 8 by adding Na₂CO₃ to a final concentration of 45 mM, and 4.5 μg of F4 was analyzed on native-PAGE and FaeG-specific immunoblotting was performed as described previously [23].

Three piglets of 5 weeks (1 week following weaning) were euthanized and luminal content was sampled from the stomach, duodenum, jejunum, ileum and gall bladder. The samples were centrifuged (10 min, 500 × g, 18 °C) and the supernatant was collected. F4 fimbriae (10 μg/ml) were immediately incubated in the collected supernatant at 37 °C for 0, 30, 60, 120 and 240 min. Then, serial dilutions of these samples were made and directly analyzed in an F4-specific ELISA using the MAb IMM01 [18,24].

2.8. Oral immunization experiment

All experimental and animal management procedures have been approved by the animal care and ethics committee of the Faculty of Veterinary Medicine, Ghent University.

Eighteen, F4R⁺ and F4-seronegative conventionally bred pigs (Belgian Landrace × Piétrain) were weaned at the age of 3–4 weeks, transported to the experimental facilities at the faculty and subsequently housed in isolation units where they obtained water and food ad libitum. These piglets were treated orally with colistine (150,000 U/kg of body weight/day, Promycine pulvis, VMD, Berendonk, Belgium) from 2 days before till 3 days after weaning to prevent *E. coli* infections due to transport and handling.

One week post weaning, pigs were orally immunized on three successive days (days 0, 1 and 2) with 2 mg purified wild type F4 fimbriae (F4_{GIS26} group, *n* = 6) derived from strain GIS26 or 2 mg purified mutant F4 fimbriae (F4_{pHMM02} group, *n* = 6) derived from strain GIS26faeG::Cm(pHMM02) diluted in 10 ml PBS. Six animals received PBS (PBS group) and served as negative control group. All animals were deprived of food and water from 3 h before till 2 h after immunization.

One week following the oral immunization (day 8), three pigs of each group were euthanized by intravenous injection of pentobarbital (24 mg/kg; Nembutal, Sanofi Santé Animale, Brussels, Belgium) and subsequent exsanguination in order to determine the intestinal F4-specific immune response. F4-specific IgA and IgM antibodies were determined in duodenal, jejunal and ileal contents using the F4-specific ELISA and the F4-specific IgM, IgA and IgG antibody-secreting cells (ASCs) were enumerated in mesenteric lymph nodes (MLN), jejunal and ileal Peyer's patches (JPP and

IPP) and jejunal lamina propria (LP) [3]. Tissues and intestinal content were sampled following euthanasia of animals. Subsequently, monomorphonuclear cells (MC) were isolated from MLN, JPP and IPP as previously described [27]. In addition, MC were isolated from a mid jejunal intestinal segment without Peyer's patches (LP). After washing with PBS, the serosa and muscle layer of the intestinal fragment were removed. The tissue was cut into small pieces and incubated three times for 40 min in Hanks buffered salt solution with 0.94 mol/l DTT, 2.52 mol/l kestranal (EDTA), 100 µg/ml penicillin–streptomycin in a shaking incubator at 37 °C. The intestinal fragments were sieved, rinsed with Hanks buffered salt solution and incubated 30 min in RPMI with 5% FCS, 20 mM HEPES, 0.1 mg/ml DNase and 100 µg/ml penicillin–streptomycin. Subsequently, the tissue fragments were incubated 1 h in the RPMI solution with 0.36 mg/ml collagenase in a shaking incubator at 37 °C. Then, the cell clumps were removed by filtration through a 70 µm cell strainer. The obtained MC from the different tissues were washed and resuspended at 10^7 cells/ml [27].

Two weeks after the oral immunizations (day 15), the remaining animals received an intramuscular booster immunization with 100 µg purified F4_{GIS26} fimbriae in incomplete Freund's adjuvant as described previously [28]. Blood was taken from the vena jugularis at days 0, 7, 15, 20, 24 and 29 to analyze F4-specific antibodies using the F4-specific ELISA. At day 29, the remaining pigs were euthanized. Jejunal villi were isolated of all euthanized pigs to confirm the presence of F4R by performing the *in vitro* villous adhesion assay.

2.9. F4-specific antibodies and antibody secreting cells (ASC)

For detection of F4-specific antibodies, the previously described indirect ELISA was used [3]. The IgM, IgA and IgG cut-off values were calculated as the mean OD₄₀₅-value of all sera (dilution 1/10) at day 0, increased with 3 times the standard deviation. In case of intestinal contents, the IgA and IgM cut-off values were the OD₄₀₅-values of the dilution buffer, increased with 3 times the standard deviation. The F4-specific antibody titer was the inverse of the highest dilution that still had an OD₄₀₅ higher than the calculated cut-off value.

F4-specific IgM, IgA and IgG ASC were detected as described previously [3]. For each MC suspension, spots in 5 wells (10^6 MC/well) were counted with an ELISPOT reader (Immunospot, CTL) to obtain the number of isotype-specific ASCs per 5×10^6 MC.

2.10. Statistical analysis

Statistical analysis (SPSS 11) of antibody titers and F4-specific ASC was done using General Linear Model (Repeated Measures Analysis of Variance), adjusting for multiple comparisons by Bonferroni. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Mutations in *faeG*_{GIS26} reduce the F4 polymeric stability

The *faeG*_{GIS26} gene encoding the F4 fimbrial adhesin and major subunit FaeG in the F4⁺ ETEC reference strain GIS26 was disrupted with a *Cm*-gene cassette to block the assembly of F4 fimbriae (Fig. 1A). Replacement of *faeG*_{GIS26} with its *Cm*-disrupted counterpart was confirmed with DNA hybridization analysis to create GIS26*faeG::Cm* (Fig. 1B). Complementation of F4 biosynthesis in GIS26*faeG::Cm* with wild type *faeG* derived from GIS26 (pJJJ89) or 5/95 (pJJJ92) ETEC strains resulted in a similar F4 phenotype as their wild type F4 counterparts. Indeed, when purified fimbriae were analyzed on SDS-PAGE without heat treatment, F4_{GIS26} appeared as polymers while only FaeG monomers were detected with F4_{5/95}

Fig. 2. Immunoblot analysis of purified wild type and mutant F4 fimbriae under non-heat denaturing conditions detected with anti-FaeG rabbit serum. Lane 1, F4_{GIS26}; 2, F4_{5/95}; 3, F4_{GIS26faeG::Cm}; 4, F4_{GIS26faeG::Cm(pJJJ89)}; 5, F4_{GIS26faeG::Cm(pJJJ92)}; 6, F4_{GIS26faeG::Cm+(pHMM01)}; 7, F4_{GIS26faeG::Cm(pHMM02)}; and 8, F4_{GIS26faeG::Cm(pHMM05)}.

(Fig. 2). Purified F4_{GIS26} and F4_{5/95} are both recognized in Western blot (Fig. 3A) and ELISA (Fig. 3C) when using the FaeG-specific MAb IMM01 that recognizes both FaeG monomers and polymers, whereas only purified F4_{GIS26} are detected when using the FaeG-specific MAb IMM09 that recognizes FaeG polymers only (Fig. 3B and D).

Point mutations were introduced to *faeG*_{GIS26} to analyze which amino acids are involved in the reduced F4 polymeric stability of F4_{5/95}. Neither the change of the FaeG_{GIS26} I₂₀₁ to V (F4 purified from GIS26*faeG::Cm* (pHMM01)) nor the FaeG_{GIS26} N₉₇ to K (F4 purified from GIS26*faeG::Cm* (pHMM05)) had an influence on the F4_{GIS26} polymeric appearance on SDS-PAGE. However, when both mutations (F4 purified from GIS26*faeG::Cm* (pHMM02)); subsequently referred as F4_{pHMM02}) were present, the mutant F4_{pHMM02} fimbriae appeared only as FaeG monomers following SDS-PAGE since they are only recognized by MAb IMM01 in Western blot (Fig. 3A and B). Surprisingly, F4_{pHMM02} was recognized in ELISA by both Mabs IMM01 and IMM09 (Fig. 3C and D). Electron microscopy confirmed the presence of polymeric structures in samples of F4_{GIS26} as well as in samples of F4_{pHMM02} (Fig. 4). These results show that purified F4_{pHMM02} fimbriae have retained a polymeric nature but that their stability was reduced compared to the wild type F4_{GIS26} fimbriae since the FaeG intersubunit interaction in F4_{pHMM02} fimbriae was broken during SDS-PAGE migration.

3.2. Wild type F4 and mutant F4_{pHMM02} bind to the F4R

The capacity of purified F4_{GIS26} fimbriae (stable polymers) and F4_{pHMM02} fimbriae (unstable polymers) to bind the F4R was compared since this binding is necessary for induction of an F4-specific intestinal immune response following oral immunization. The results from an *in vitro* inhibition adhesion assay showed that both fimbriae have the same F4R binding profile, indicating that polymer stability of purified F4 does not significantly influence binding to the F4R *in vitro* (Fig. 5).

3.3. Wild type F4 and mutant F4_{pHMM02} are resistant to gastro-intestinal breakdown

A second *in vitro* assay revealed that there was no influence of the polymeric stability on its degradation in simulated gastric conditions. Both stable (F4_{GIS26}) and unstable (F4_{pHMM02}) F4 polymers survived the pepsin digestion at pH 2–4 (Fig. 6A), the pH condition of the stomach of weaned piglets [29]. They were only degraded at pH 1.5. F4_{5/95} are more susceptible to degradation since degradation starts at pH 2. Incubation of F4_{GIS26} and F4_{pHMM02} in

Fig. 3. Purified F4 fimbriae separated by SDS-PAGE and detected by Western blot using the FaeG-specific MAb IMM01 (A) or IMM09 (B) and interaction of different concentrations of purified F4 fimbriae with the F4-specific MAb IMM01 (C) or IMM09 (D) in ELISA. Lane (1) heat-denatured F4_{GIS26}; (2) non-heat-denatured F4_{GIS26}; (3) heat-denatured F4_{5/95}; (4) non-heat denatured F4_{5/95}; (5) heat-denatured F4_{pHMM02}; (6) non-heat-denatured F4_{pHMM02}.

luminal content freshly isolated at different locations of the gastro-intestinal tract revealed only degradation of the fimbriae when present in stomach content. Degradation was already observed for both fimbriae following 30 min incubation and was complete after 4 h (Fig. 6B and C). This experiment was performed with material from 3 animals and did not reveal differences in stability between F4_{GIS26} and F4_{pHMM02} in gastro-intestinal conditions (Fig. 6).

3.4. Reduced polymeric stability of F4 fimbriae reduces its mucosal immunogenicity

As we observed that stable F4 polymers (F4_{GIS26}) and unstable F4 polymers (F4_{pHMM02}) have a similar receptor-binding capacity and stability in gastro-intestinal conditions of newly weaned piglets, these fimbriae could be used to analyze the influence of the poly-

Fig. 4. Electron micrograph of purified F4_{pHMM02} fimbriae (A) and F4_{GIS26} (B). The scale bar represents 50 nm and the arrows are placed next to a fimbrial structure.

Fig. 5. Percentage inhibition of F4⁺ ETEC adhesion to F4R⁺ intestinal villi by concentration-dependent binding of F4_{GIS26} or F4_{pHMM02} to the F4R.

meric stability of this soluble, non-replicating fimbrial antigen on its *in vivo* mucosal immunogenicity. Newly weaned piglets were orally immunized with F4_{GIS26} or F4_{pHMM02} or received PBS as negative control. One week after immunization, the F4-specific immune response was analyzed in the intestinal tissues. Significantly higher numbers of F4-specific antibody secreting cells (ASC) were found in the ileal Peyer's patches ($p \leq 0.046$), mesenteric lymph nodes ($p \leq 0.046$) and the spleen ($p = 0.05$) of F4_{GIS26} immunized than PBS animals (Fig. 7). F4_{pHMM02} immunized animals had only significantly higher IgM ASC in the spleen, in comparison to the PBS animals (54 versus 10, $p = 0.05$). The number of IgA ASC was significantly higher in F4_{GIS26} immunized animals than in F4_{pHMM02} and PBS animals in the jejunal Peyer's patches and the mesen-

teric lymph nodes. F4-specific IgA ASC were also detected in the ileal Peyer's patches, peripheral blood and lamina propria, but these numbers were not significantly higher than those for the PBS animals. Low numbers of F4-specific IgG ASC were observed in peripheral blood, the spleen and mesenteric lymph nodes, but there was no significant difference between the groups. Furthermore, F4-specific IgM and IgA could be detected 1 week following oral immunization in the intestinal content of animals of the F4_{GIS26} group (maximum IgM titer 256 [$n = 2$], maximum IgA titer 16 [$n = 1$]) and of the F4_{pHMM02} group (maximum IgM titer 4 [$n = 2$], maximum IgA titer 2 [$n = 1$]), but the highest levels were found in the F4_{GIS26} group. In serum, a clear F4-specific IgA response was observed 1 week after oral F4_{GIS26} immunization, but it was not significantly higher than the antibody levels in the other groups (Fig. 8). These results show a significant intestinal immune response in F4_{GIS26} immunized animals, whereas there is only a weak mucosal priming in the F4_{pHMM02} immunized animals.

To confirm priming of the F4-specific immune response in the F4_{pHMM02} immunized animals, all animals were intramuscularly boosted with purified F4_{GIS26}. Five days later, the F4-specific IgM titer peaked in all 3 groups and was slightly higher in both F4 groups than in the PBS group (Fig. 8). However, at that moment IgA and IgG were also increasing in both F4 groups, whereas this was not the case in the PBS group resulting in a significant higher IgG titer in both F4 groups ($p < 0.02$). Furthermore, IgA and IgG already peaked 8 days post secondary immunization (dpi) whereas they were still increasing 15 dpi in the PBS group. These data clearly show a similar secondary immune response in the F4 groups and a primary antibody response in the PBS group.

Fig. 6. Stability of purified F4 fimbriae in gastro-intestinal conditions. (A) Purified F4 fimbriae incubated in simulated gastric fluid at pH 7, 4, 3, 2 and 1.5 were separated on a native-PAGE and detected by Western blotting using anti-FaeG rabbit serum. Incubation of purified F4_{GIS26} (B) and F4_{pHMM02} (C) in freshly isolated gastric content during 0, 30, 60, 120 and 240 min followed by detection of the fimbriae in ELISA using the F4-specific MAb IMM01.

Fig. 7. F4-specific ASC per 5×10^6 MC in blood (BL), mesenteric lymph nodes (MLN), spleen (SP), ileal and jejunal Peyer's patches (IPP and JPP) and lamina propria (LP) of weaned piglets immunized with PBS ($n = 3$), F4_{GIS26} ($n = 3$) or F4_{pHMM02} ($n = 3$) at 8 days post immunization. Bars with different numbers differ significantly ($p < 0.05$).

4. Discussion

The results of the present study show that a combination of two amino acids that are different in FaeG_{GIS26} and FaeG_{5/95} influence the F4 fimbrial stability. Analysis of the sequence and protein fold of FaeG revealed that the donor strand mechanism is involved in the biogenesis of F4 fimbriae [16,30]. Every fimbrial subunit has an immunoglobulin domain (Ig)-like fold lacking the C-terminal β -strand. The Ig-like structure is completed by inserting the N-terminal donor strand of the subsequent subunit in the fimbria [31]. This mechanism creates stable fimbriae with a tight interaction between subsequent subunits. Changing of amino acids asparagine and isoleucine at positions 97 and 201 in FaeG_{GIS26} to their FaeG_{5/95} counterparts lysine and valine resulted in a reduced stability of F4 fimbriae (F4_{pHMM02}). The two changed amino acids are located outside the conserved N- or C-terminal strands of FaeG what excludes a direct involvement in the donor strand mechanism. However, it is likely that the combination of both amino acid changes affects the global fold of the FaeG subunit and subsequently influences the subunit-subunit interaction since only monomers are detected in SDS-PAGE of purified mutant F4_{pHMM02} fimbriae. The presence of additional amino acid changes in F4_{5/95} will be necessary to further modify its folding, increase its accessibility to proteases or facilitate pH-driven denaturation, which could explain the observed inability of purified F4_{5/95} to interact in ELISA with the MAb IMM09 specific for F4 fimbrial polymers and its reduced stability in gastro-intestinal conditions [24].

In most fimbrial systems, the adhesin is located at the tip and contains a N-terminal lectin domain and a C-terminal pilin domain with an Ig-like folding connecting the lectin domain with the fimbrial structure [31,32]. The F4 fimbrial adhesin also functions as

Fig. 8. Mean F4-specific IgM, IgA and IgG serum antibody titers ($\pm$ S.E.M.) of weaned piglets immunized with PBS, F4_{GIS26} or F4_{pHMM02}. Black arrow, oral immunization; white arrow, intramuscular immunization (100 μ g F4_{GIS26} in IFA).

major subunit and exists as one large domain with an Ig-like folding [30]. Comparison of the amino acid sequences of the three different F4 serotypes and mutagenesis studies identified the regions containing amino acids 162–171 of FaeG as putative receptor-binding site [15]. The receptor-binding site of FaeG is probably located at the side of the domain [30] and would therefore be available in multiple positions throughout the F4 fimbria. Evaluation of the *in vitro* binding of F4_{GIS26} and F4_{pHMM02} to F4R⁺ villi indicated that substitution of both amino acids did not alter this binding. It was not expected that these amino acids would directly influence F4R binding since they are not located in the putative receptor-binding region.

The results of the present study show that oral immunization of weaned piglets with F4_{pHMM02} could induce an F4-specific immune response, but not as efficient as following oral immunization with purified F4_{GIS26}. This difference in mucosal immunogenicity between F4_{pHMM02} and F4_{GIS26} has to be related with their difference in polymer length stability since this was the only identified factor differing between both fimbriae. A similar resistance of both fimbriae in gastro-intestinal conditions suggests that both fimbriae will reach the intestinal tract in an identical form.

It is not yet clear how the difference in polymeric stability results in a different mucosal immunogenicity. Maybe the depolymerisation of F4_{pHMM02} fimbriae is faster in comparison to F4_{GIS26} fimbriae during transport of the fimbriae to the intestinal immune induction sites. This means that the antigen dose taken up by antigen presenting cells could be smaller with shorter F4_{pHMM02}

fimbriae than larger F4_{GIS26}. On the other hand, there can be a faster depolymerisation of F4_{pHMM02} fimbriae than F4_{GIS26} fimbriae inside antigen presenting cells, which could influence intracellular signalling. Cross-linking of receptors from antigen presenting cells is reported to have an effect on the regulation of immune responses [33,34] and is more efficient by molecules with a high polymeric nature. The use of antigenic repeats is indeed a known method to improve the immunogenicity of an antigen [35,36]. We have indications that incubation of porcine monocyte-derived dendritic cells with F4_{GIS26} fimbriae is more effective to mature these cells than F4_{pHMM02}, but this has to be further examined and will be part of further studies.

The obtained results show that the amino acids on position 97 and 201 in F4 fimbriae are important in the FaeG–FaeG interaction and consequently in the fimbrial polymeric character. Furthermore, the polymeric character of the F4 fimbriae is identified as one of the factors responsible for its mucosal immunogenicity. This information can be used to increase the mucosal immunogenicity of other soluble non-replicating antigens and stimulate the development of efficient mucosal vaccines against human and animal infectious diseases.

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