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
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 (F4GIS26) 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 (F45/95) 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 (FaeG5/95) were also found in the FaeG amino acid sequence of strain GIS26 [16]. Only F4 fimbriae purified from strain 5/95 (F45/95) 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.

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<tr>
<td>5/95</td>
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<tr>
<td>GIS26</td>
<td>Wild type F4 ETEC reference strain (O149:F4ac’LT’, Sta’, Stb’)</td>
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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, a 4.1 kb fragment of GIS26 F4 operon was amplified by PCR (primers: 5’caaggatcctacgaggtacaaagcac and 5’cccagcatctaaatcgggcaaggagctg), a 3.6 kb Cm-cassette from pH4512 cm [20] was cloned to SacI site in the middle of the faeG gene, and finally introduced into the suicide plasmid vector pG7P04 [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. Tet’Cm’ Amp’ 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 dioxygenin-labelled 0.8 kb faeG PCR-fragment (primers: 5’ctgtgctgctgtggcag and 5’ctgtaagtaatgctgagctc) (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’caaggatcctacgaggtacaaagcac and 5’cccagcatctaaatcgggcaaggagctg) 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 construct GIS26faeG::Cm by conjugation under ampicillin selection. The amino acids K97 and V201 which have been detected only in FaeG5/95, were introduced in FaeGGIS26 to analyze their influence on the polymeric nature of F4 fimbriae. Point mutations were introduced in pJJJ89 by using a QuickChange site-directed PCR mutation kit according to manufacturer’s
immunoblotting was performed as previously [23]. A sandwich and polymeric F4 fimbriae [24] and the MAb IMM09 (also produced as before [22], but in the absence of SDS and FaeG-specific polymers since unboiled F4GIS26 results in a ladder pattern consisting of FaeG polymers and boiled F4GIS26 results only in the monomeric FaeG). SDS-PAGE (10%) was used to analyze the polymeric nature of F4 fimbriae (F4GIS26 group, lanes 5–11, CmR AmpS clones containing only faeE::Cm lane 12, WT GIS26 faeG.

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). Purified mutant F4 fimbriae (F4pHMM02 group, n=6) derived from strain GIS26 or 2 mg purified wild type F4 fimbriae (F4GIS26 group, n=6) derived from strain GIS26∥faeG::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 ileal lymph nodes (ILN), jejunal and ileal lymph nodes (JLNs) and cecal lymph nodes (CLNs) using flow cytometry.

2.6. In vitro villous adhesion and inhibition assay

The F4R status of the immunized pigs 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, 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 pigs 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 (F4GIS26 group, n=6) derived from strain GIS26 or 2 mg purified mutant F4 fimbriae (F4pHMM02 group, n=6) derived from strain GIS26∥faeG::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.

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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 pioform 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).
analyzed on SDS-PAGE without heat treatment, F4GIS26 appeared wild type F4 counterparts. Indeed, when purified fimbriae were 5/95 (pJJJ92) ETEC strains resulted in a similar F4 phenotype as their indirect ELISA was used\[3\]. The IgM, IgA and IgG cut-off values were 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 OD_{405} 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 OD_{405}-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 OD_{405} 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 Ellispot reader (Immunosopt, 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 GIS26faeG::Cm (Fig. 1B). Complementation of F4 biosynthesis in GIS26faeG::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). Purified F4_{GIS26} and F4_{5/95} are both recognized in Western blot (Fig. 3A) and ELISA (Fig. 3C) when using the FaeG-specific MAbs 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 GIS26faeG::Cm (pPHMM01)) nor the Faeg_{GIS26} N97 to K (F4 purified from GIS26faeG::Cm (pPHMM05)) had an influence on the F4_{GIS26} polymeric appearance on SDS-PAGE. However, when both mutations (F4 purified from GIS26faeG::Cm (pPHMM02); subsequently referred as F4_{pPHMM02}) were present, the mutant F4_{pPHMM02} 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_{pPHMM02} 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_{pPHMM02} (Fig. 4). These results show that purified F4_{pPHMM02} 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_{pPHMM02} fimbriae was broken during SDS-PAGE migration.

3.2. Wild type F4 and mutant F4_{pPHMM02} bind to the F4R

The capacity of purified F4_{GIS26} fimbriae (stable polymers) and F4_{pPHMM02} 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 F4 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_{pPHMM02} 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_{pPHMM02}) 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_{pPHMM02} in

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3. Luminal content freshly isolated at different locations of the gastrointestinal 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 F4GIS26 and F4pHMIM02 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 (F4GIS26) and unstable F4 polymers (F4pHMIM02) 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-
meric stability of this soluble, non-replicating fimbrial antigen on its in vivo mucosal immunogenicity. Newly weaned piglets were orally immunized with F4GIS26 or F4pHMM02 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 F4GIS26 immunized than PBS animals (Fig. 7). F4pHMM02 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 F4GIS26 immunized animals than in F4pHMM02 and PBS animals in the jejunal Peyer's patches and the mesenteric 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 F4GIS26 group (maximum IgM titer 256 [$n = 2$], maximum IgA titer 16 [$n = 1$]) and of the F4pHMM02 group (maximum IgM titer 4 [$n = 2$], maximum IgA titer 2 [$n = 1$]), but the highest levels were found in the F4GIS26 group. In serum, a clear F4-specific IgA response was observed 1 week after oral F4GIS26 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 F4GIS26 immunized animals, whereas there is only a weak mucosal priming in the F4pHMM02 immunized animals.

To confirm priming of the F4-specific immune response in the F4pHMM02 immunized animals, all animals were intramuscularly boosted with purified F4GIS26. 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 F4GIS26 (B) and F4pHMM02 (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. 5. Percentage inhibition of F4⁺ ETEC adhesion to F4R⁺ intestinal villi by concentration-dependent binding of F4GIS26 or F4pHMM02 to the F4R.
Fig. 7. F4-specific ASC per 5 x 10⁶ 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), F4GIS26 (n = 3) or F4pHM02 (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 FaeGIS26 and FaeG5/95 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 FaeGIS26 to their FaeG5/95 counterparts lysine and valine resulted in a reduced stability of F4 fimbriae (F4pHM02). 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 F4pHM02 fimbriae. The presence of additional amino acid changes in F45/95 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 F4pHM02 to interact in ELISA with the MAb IMM09 specific for F4 fimbrial polymers and its reduced stability in gastrointestinal 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 a 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 F4GIS26 and F4pHM02 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 F4pHM02 could induce an F4-specific immune response, but not as efficient as following oral immunization with purified F4GIS26. This difference in mucosal immunogenicity between F4pHM02 and F4GIS26 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 F4pHM02 fimbriae is faster in comparison to F4GIS26 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 F4pHM02
fimbriae than larger F4(GIS26). On the other hand, there can be a faster F4pHMM02, 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 Faec–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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