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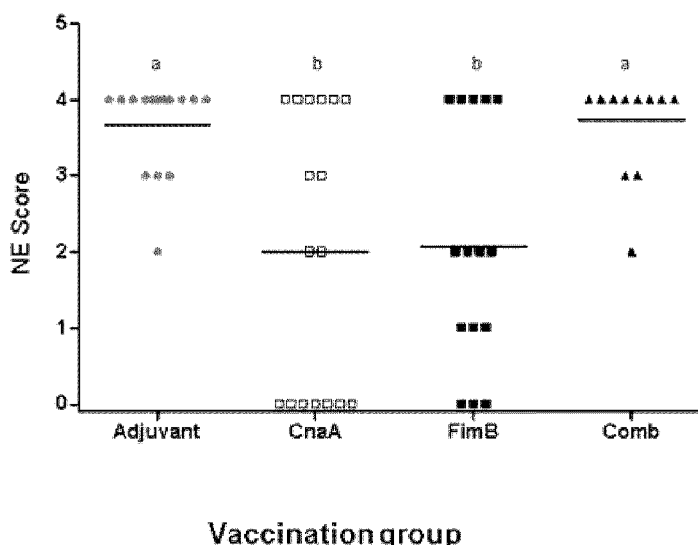

FIG. 6

(57) Abstract: An immunogenic polypeptide selected from an isolated Clostridium perfringens pilus polypeptide, a variant of the pilus polypeptide; a fragment of the pilus polypeptide; and a fragment of the variant, is useful for the preparation of a vaccine for the treatment or prevention of enteric necrosis in poultry. The isolated Clostridium perfringens pilus polypeptide includes an assembled pilus or the pilus subunits CnaA, FimA and/or FimB.

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VACCINE AGAINST NECROTIC ENTERITIS IN POULTRY

Background

[0001] The present application is directed to polypeptides useful in preparing a vaccine against necrotic enteritis in poultry. More specifically, the present application is directed to a *Clostridium perfringens* pilus polypeptide useful in preparing a vaccine against necrotic enteritis related to *Clostridium perfringens* infection in poultry.

[0002] Necrotic enteritis is an intestinal disease of poultry such as broiler chickens, that in 2015 was estimated to cost the poultry industry $US 6 billion in losses. Necrotic enteritis is caused primarily by certain Type A strains of *Clostridium perfringens* that produce the NetB pore-forming toxin, which overgrow and adhere to the intestinal mucosa, eventually causing the characteristic lesions of the disease. *Clostridium perfringens* is a normal inhabitant of the intestinal tract, and typically only those strains that carry the NetB toxin can cause necrotic enteritis. Necrotic enteritis is primarily controlled by application of in-feed antibiotics, a practice that is increasingly discouraged due to the potential spread of antimicrobial resistance, and which is currently being phased out of production in some countries. It is therefore important, from both a financial and public health perspective, to find alternative approaches to control necrotic enteritis, such as the development of a vaccine.

[0003] A *Clostridium perfringens* adherence genetic locus (VR-10B) has been recently identified through its association with NetB-positive strains (Lepp D, Gong J, Songer JG, Boerlin P, Parreira VR, Prescott JF. 2013. Identification of Accessory Genome Regions in Poultry *Clostridium perfringens* Isolates Carrying the netB Plasmid. Journal of Bacteriology 195: 1152-1166). The identified genetic locus was found to be present in 87% of *netB*-positive and 42% of *netB*-negative isolates, of 54 poultry isolates examined. This genetic locus (subsequently renamed the collagen adhesion (CA) locus) was later shown to be involved in collagen binding, and required for necrotic enteritis pathogenesis (Wade B, Keyburn AL, Haring V, Ford M, Rood JI, Moore RJ: The adherent abilities of *Clostridium perfringens* strains are critical for the pathogenesis of avian necrotic enteritis. Vet Microbiol 2016, 197: 53-61; Wade B, Keyburn AL, Seemann T, Rood JI, Moore RJ: Binding of *Clostridium perfringens* to collagen correlates with the ability to cause necrotic enteritis in chickens. Vet Microbiol 2015, 180: 299-303.).

[0004] A number of *Clostridium perfringens* proteins have previously been evaluated as vaccine candidates. However, these proteins offer at best partial protection against necrotic enteritis. In addition, many of these proteins are not specific to necrotic

enteritis-causing strains, and are not known to play a role in necrotic enteritis pathogenesis. Therefore, it is desirable to identify alternative *Clostridium perfringens* polypeptides which may be candidates for producing a vaccine against necrotic enteritis.

5 **Summary**

[0005] One aspect of the present invention provides an isolated *Clostridium perfringens* pilus polypeptide. In another aspect, the present invention provides an immunogenic polypeptide selected from an isolated *Clostridium perfringens* pilus polypeptide, a variant of the pilus polypeptide; a fragment of the pilus polypeptide; and 10 a fragment of the variant, wherein the pilus polypeptide, the variant, the fragment of the polypeptide and the fragment of the variant are immunogenic in poultry. In at least one embodiment, the pilus polypeptide is a CnaA polypeptide. In at least one embodiment, the pilus polypeptide is a FimA polypeptide. In at least one embodiment, the pilus polypeptide is a FimB polypeptide. In at least one embodiment, the pilus 15 polypeptide is an assembled pilus.

[0006] Another aspect of the present invention provides a polynucleotide comprising a sequence encoding an isolated *Clostridium perfringens* pilus polypeptide or an immunogenic polypeptide as described herein. In another aspect, the present application provides a vector comprising a polynucleotide having a sequence 20 encoding an isolated *Clostridium perfringens* pilus polypeptide or an immunogenic polypeptide as described herein, wherein the vector is configured for expression of the isolated *Clostridium perfringens* pilus polypeptide or immunogenic polypeptide in a host cell.

[0007] In another aspect, the present invention provides a vaccine for the treatment or 25 prevention of necrotic enteritis in poultry, wherein the vaccine comprises an immunogenic polypeptide as described herein. In another aspect, the present application provides a vaccine for the treatment or prevention of *Clostridium perfringens* infection in poultry, wherein the vaccine comprises an immunogenic polypeptide as described herein.

30 [0008] In another aspect, the present invention provides the use of an immunogenic polypeptide as described herein in the preparation of a medicament for the treatment or prevention of necrotic enteritis in poultry or for the treatment or prevention of *Clostridium perfringens* infection in poultry.

[0009] In another aspect, the present invention provides a method of treatment or 35 prevention of necrotic enteritis in poultry or for the treatment or prevention of

Clostridium perfringens infection in poultry, the method comprising administering an effective amount of an immunogenic polypeptide as described herein, or a vaccine thereof, to the poultry.

[0010] In another aspect, the present invention provides the use of an immunogenic polypeptide as described herein as a vaccine for the treatment or prevention of necrotic enteritis in poultry or for the treatment or prevention of *Clostridium perfringens* infection in poultry.

[0011] A further aspect of the present invention provides an antibody which binds selectively to an immunogenic polypeptide as described herein. In another aspect, the present invention provides a method of detecting *Clostridium perfringens* infection in poultry by obtaining a biological sample from the poultry and detecting in the biological sample the presence of an antibody which binds selectively to an immunogenic polypeptide as described herein. Yet another aspect of the present invention provides a method of detecting an immunogenic polypeptide as described herein comprising exposing the immunogenic polypeptide to an antibody which binds selectively to the immunogenic polypeptide and detecting binding of the immunogenic polypeptide to the antibody.

Brief Description of the Drawings

[0012] Further features of the present invention will become apparent from the following written description and the accompanying figures, in which:

[0013] Figure 1 is a diagrammatic representation of the 5.2 kilobase-pair *Clostridium perfringens* VR-10B chromosomal locus;

[0014] Figure 2A is a photograph illustrating separation of recombinant histidine-tagged pilus subunit polypeptide CnaA by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) visualized by Coomassie staining;

[0015] Figure 2B is a photograph illustrating separation of recombinant pilus subunit polypeptide FimA by SDS-PAGE visualized by Coomassie staining;

[0016] Figure 2C is a photograph illustrating separation of recombinant pilus subunit polypeptide FimB by SDS-PAGE visualized by Coomassie staining;

[0017] Figure 2D is a photograph illustrating visualization by SDS-PAGE and Coomassie staining of pooled fractions of CnaA, FimA and FimB following concentration and desalting;

[0018] Figure 3A is a graph illustrating serum IgY antibody response (absorbance at 405 nm) against CnaA recombinant polypeptide from chickens immunized with adjuvant alone, or with CnaA in a first vaccination trial. Each dot represents a single

individual and horizontal lines represent means. * indicates a significant difference from the pre-immune sample (d8) from each group at the $p < 0.05$ level, ** indicates a significant difference at the $p < 0.01$ level, and *** indicates a significant difference at the $p < 0.001$ level when measured by the Tukey's test (Tukey, J. "Comparing Individual Means in the Analysis of Variance". *Biometrics* (1949) **5**(2): 99–114);

[0019] Figure 3B is a graph illustrating serum IgY antibody response (absorbance at 405 nm) against FimA recombinant polypeptide from birds immunized with adjuvant alone, or with FimA in the trial of Figure 3A. Each dot represents a single individual and horizontal lines represent means. * indicates a significant difference from the pre-immune sample (d8) from each group at the $p < 0.05$ level, ** indicates a significant difference at the $p < 0.01$ level, and *** indicates a significant difference at the $p < 0.001$ level when measured by the Tukey's test;

[0020] Figure 4 is a graph illustrating necrotic enteritis (NE) lesion scores from groups of chickens immunized with adjuvant alone, or with CnaA or FimA in the trial of Figure 3A, followed by in-feed challenge with *Clostridium perfringens* strain CP1. Each dot represents a single individual and horizontal lines represent mean necrotic enteritis lesion score;

[0021] Figure 5A is a graph illustrating serum IgY antibody response (absorbance at 405 nm) against CnaA recombinant polypeptide from chickens immunized with adjuvant alone, with CnaA, or with a combination of CnaA, FimA and FimB (Comb), in a second vaccination trial. Each dot represents a single individual and horizontal lines represent means. * indicates a significant difference from the pre-immune sample (d7) from each group at the $p < 0.05$ level, ** indicates a significant difference at the $p < 0.01$ level, and *** indicates a significant difference at the $p < 0.001$ level when measured by the Tukey's test;

[0022] Figure 5B is a graph illustrating serum IgY antibody response (absorbance at 405 nm) against FimA recombinant polypeptide from chickens immunized with adjuvant alone or with a combination of CnaA, FimA and FimB (Comb), in the trial of Figure 5A. Each dot represents a single individual and horizontal lines represent means. * indicates a significant difference from the pre-immune sample (d7) from each group at the $p < 0.05$ level, ** indicates a significant difference at the $p < 0.01$ level, and *** indicates a significant difference at the $p < 0.001$ level when measured by the Tukey's test;

[0023] Figure 5C is a graph illustrating serum IgY antibody response (absorbance at 405 nm) against FimB recombinant polypeptide from chickens immunized with adjuvant alone, with FimB, or with a combination of CnaA, FimA and FimB (Comb), in

the trial of Figure 5A. Each dot represents a single individual and horizontal lines represent means. * indicates a significant difference from the pre-immune sample (d7) from each group at the p <0.05 level, ** indicates a significant difference at the p<0.01 level, and *** indicates a significant difference at the p<0.001 level when measured by the Tukey's test;

[0024] Figure 6 is a graph illustrating necrotic enteritis (NE) lesion scores from groups of chickens immunized with adjuvant alone, CnaA, FimB, or a combination of CnaA, FimA and FimB (Comb), in the trial of Figure 5A, followed by in-feed challenge with *Clostridium perfringens* CP1. Each dot represents a single individual and horizontal lines represent mean necrotic enteritis lesion score. Letters (a, b) denote significantly different groups (Tukey's; p<0.01);

[0025] Figure 7 is a graph illustrating necrotic enteritis (NE) lesion scores from groups of chickens following in-feed challenge with *Clostridium perfringens* strain CP1 or CP1 null-mutants of the pilus subunit genes *fimA* and *fimB* (CP1Δ*fimA*, and CP1Δ*fimB*). Lines represent mean necrotic enteritis lesion score;

[0026] Figure 8A is a photograph illustrating separation by SDS-PAGE of surface polypeptides extracted from *Clostridium perfringens* strain CP1 or CP1 null mutants of genes for each of the pilus subunits *cnaA*, *fimA* and *fimB* (CP1Δ*cnaA,* CP1Δ*fimA*, and CP1Δ*fimB*), visualized with Coomassie stain;

[0027] Figure 8B is a photograph illustrating a Western blot analysis of SDS-PAGE-separated surface polypeptides extracted from *Clostridium perfringens* strain CP1 or CP1 null mutants of genes for each of the pilus subunits *cnaA*, *fimA* and *fimB* (CP1Δ*cnaA,* CP1Δ*fimA*, and CP1Δ*fimB*), detected using anti-FimA antibodies obtained from chicken serum as the primary antibody;

[0028] Figure 8C is a photograph illustrating a Western blot analysis of SDS-PAGE-separated surface polypeptides extracted from *Clostridium perfringens* strain CP1 or CP1 null mutants of genes for each of the pilus subunits *cnaA*, *fimA* and *fimB* (CP1Δ*cnaA,* CP1Δ*fimA*, and CP1Δ*fimB*), detected using anti-FimA antibodies raised in rabbits as the primary anitibody;

[0029] Figure 9A is a photograph illustrating separation by SDS-PAGE of surface polypeptides extracted from various *Clostridium perfringens* strains visualized with Coomassie stain;

[0030] Figure 9B is a photograph illustrating a Western blot analysis of SDS-PAGE-separated surface polypeptides extracted from various *Clostridium perfringens* strains

visualized with anti-FimA antibodies obtained from chicken serum as the primary antibody; and

[0031] Figure 10 is a series of photographs obtained by transmission electron microscopy of cells of *Clostridium perfringens* strain CP1 or of the CP1 null mutants CP1Δ*fimA*, and CP1Δ*fimB* labeled with gold particles using rabbit anti-FimA antibody as a primary antibody and 6 nm immunogold-labelled goat anti-rabbit IgG as a secondary antibody.

Detailed Description

[0032] It has been found by the present applicants that the VR-10B genetic locus identified in strains of *Clostridium perfringens* associated with necrotic enteritis in poultry (Lepp D et al, *Journal of Bacteriology* (2013) 195: 1152-1166) contains six putative genes which have been found to encode an adhesive pilus: three genes (*cnaA*, *fimA* and *fimB*) encoding structural pilus subunits, and genes encoding two sortases and a signal peptidase presumably involved in pilus assembly. A diagrammatic representation of the VR-10B locus is shown in Figure 1.

[0033] A pilus is a hair-like structure that is present on the surface of many bacteria and is often involved in virulence. This type of pilus is composed of covalently-linked major and minor polypeptide subunits that form a cell surface structure having a length of approximately 1 μm. Pili have been extensively studied in Gram-negative bacteria, but several Gram-positive species, including *Corynebacterium diphtheriae*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes,* have more recently been shown to produce a specific type of pilus that is assembled by sortase enzymes. This type of adhesive Gram-positive pilus is assembled at the cell surface via covalent linkage of pilin subunits by housekeeping and pilin-specific sortase enzymes, and is eventually covalently linked to the cell wall peptidoglycan to form the assembled pilus.

[0034] Without being bound by theory, it is contemplated that the *Clostridium perfringens* pilus polypeptides described herein may be a viable and promising target for development of a vaccine against necrotic enteritis for a number of reasons. The gene locus is present mainly in necrotic enteritis-causing strains of *Clostridium perfringens*. Therefore, the immune response elicited by an immunogenic pilus protein is expected to specifically target strains of *Clostridium perfringens* that cause disease. In addition, pili are present on the surface of the bacterial cell, and are often involved in attachment to the host during the pathogenesis of bacterial infections, which can expose the pili to the host immune system. Furthermore, possibly because of their role

in disease and their location on the bacterial cell surface, pili have been successfully used to develop vaccines for a number of other infectious diseases.

[0035] Thus, one aspect of the present application provides an immunogenic polypeptide selected from an isolated *Clostridium perfringens* pilus polypeptide, a variant of the pilus polypeptide; a fragment of the pilus polypeptide; and a fragment of the variant, wherein the pilus polypeptide, the variant, the fragment of the polypeptide and the fragment of the variant are immunogenic in poultry.

[0036] As used herein, the term “poultry” is used to refer to species of birds or fowl which are raised agriculturally for products including but not limited to meat, eggs and feathers. Poultry include but are not limited to chickens, turkeys, ducks, geese, quail, ostriches, pheasants and other agriculturally relevant birds or fowl. Especially included are poultry which are susceptible to necrotic enteritis caused by *Clostridium perfringens* infection. In at least one embodiment, the poultry are broilers or chickens raised for meat production.

[0037] As used herein, the term “polypeptide” is intended to mean a compound containing two or more amino acid residues linked together by peptide bonds. Polypeptides include but are not limited to oligopeptides or polypeptides in which two or more amino acid residues are linked together sequentially by covalent peptide bonds to form a single polypeptide strand, and proteins comprising two or more polypeptide strands non-covalently associated with each other or linked with each other by covalent bonds other than peptide bonds, including but not limited to disulfide bonds and isopeptide bonds. As used herein, the term “isopeptide bond” is intended to mean an amide bond formed between an amino group of one amino acid and a carboxyl group of a second amino acid, wherein at least one of the amino group and the carboxyl group is located on the side chain of the respective amino acid.

[0038] As used herein, the term “*Clostridium perfringens* pilus polypeptide” is intended to mean a polypeptide which has the function of a pilus or a pilus subunit and which is encoded by one or more genes found in a strain of *Clostridium perfringens* associated with necrotic enteritis in poultry. In at least one embodiment, the gene is the *cnaA* gene, the *fimA* gene, or the *fimB* gene found in the VR-10B genetic locus identified in Lepp D et al, *Journal of Bacteriology* (2013) 195: 1152-1166, as diagrammatically represented in Figure 1.

[0039] As used herein, the term “variant” when used in reference to a polypeptide is intended to refer to a polypeptide which differs in its amino acid sequence from the sequence of a reference polypeptide to which the variant is being compared by one or more amino acid residues. The differences between the sequence of the variant and

the sequence of the reference polypeptide can include substitution of one or more amino acid residues with different amino acid residues, insertion of additional amino acid residues or deletion of amino acid residues. In certain embodiments, a variant can differ from a reference polypeptide by conservative substitution of one or more amino acid residues with replacement amino acid residues which may have similar properties, including but not limited to charge, size and hydrophilicity, to the amino acid residues which the new residues replace. In certain embodiments, variants may completely or partially retain one or more biological functions of the reference polypeptide, including but not limited to immunogenicity. In at least one embodiment, the reference polypeptide is an isolated *Clostridium perfringens* pilus polypeptide as described herein.

[0040] In at least one embodiment, the sequence of a variant can have at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99% or at least 99.9% identity to the sequence of a reference polypeptide. As used herein, the term "percent identity" or "% identity" when used in reference to the sequence of a polypeptide or a polynucleotide is intended to mean the percentage of the total number of amino acid or nucleotide residues, respectively, in the sequence which are identical to those at the corresponding position of a reference polypeptide or polynucleotide sequence. In at least one embodiment, when the length of the variant sequence and the length of the reference sequence are not identical, percent identity can be calculated based on the total number of residues in the variant sequence or based on the total number or residues in the reference sequence. Percent identity can be measured by various local or global sequence alignment algorithms well known in the art, including but not limited to the Smith-Waterman algorithm and the Needleman-Wunsch algorithm. Tools using these or other suitable algorithms include but are not limited to BLAST (Basic Local Alignment Search Tool) and other such tools well known in the art.

[0041] As used herein, the term "fragment" when used in relation to a polypeptide or a variant is intended to refer to a smaller polypeptide containing fewer amino acid residues than the polypeptide or variant and having a sequence which is identical to a portion of the sequence of the polypeptide or variant. In at least one embodiment, the fragment retains one or more biological activities of the polypeptide or variant, including but not limited to immunogenicity. In at least one embodiment, the fragment comprises an epitope of the polypeptide or variant. In at least one embodiment, the fragment is at least 6 amino acids in length or at least 7 amino acids in length, or at least 8 amino acids in length or at least 9 amino acids in length or at least 10 amino acids in length.

[0042] As used herein, the term “immunogenic” is intended to refer to an agent, including but not limited to a polypeptide or polynucleotide or a fragment thereof, which is capable of eliciting an immunoprotective response in a subject to which the immunogenic agent is administered. As used herein, the term “immunoprotective response” is intended to refer to an immune response that prevents, reduces or eliminates one or more of the symptoms of disease in an infected subject.

[0043] The present immunogenic polypeptide, including the present isolated *Clostridium perfringens* pilus polypeptide, the variant of the pilus polypeptide, the fragment of the pilus polypeptide and the fragment of the variant, are immunogenic in poultry. Thus, in at least one embodiment, poultry immunized with any one or more of the present isolated *Clostridium perfringens* pilus polypeptide, the variant of the pilus polypeptide, the fragment of the pilus polypeptide and the fragment of the variant will show an immunoprotective response to challenge with one or more of a *Clostridium perfringens* cell, an assembled *Clostridium perfringens* pilus, a *Clostridium perfringens* pilus polypeptide, a fragment of a *Clostridium perfringens* pilus polypeptide, or a portion of a *Clostridium perfringens* cell, including but not limited to a cell membrane or portion thereof, or a cell wall or a portion thereof, which bears one or more of an assembled *Clostridium perfringens* pilus, a *Clostridium perfringens* pilus polypeptide or a fragment of a *Clostridium perfringens* pilus polypeptide.

[0044] Another aspect of the present application provides a polynucleotide comprising a sequence encoding an isolated *Clostridium perfringens* pilus polypeptide or an immunogenic polypeptide as described herein. In at least one embodiment, the polynucleotide is messenger RNA (mRNA) having a sequence which can be translated to generate the isolated *Clostridium perfringens* pilus polypeptide or the immunogenic polypeptide. In at least one embodiment, the polynucleotide is DNA, at least one strand of which can be transcribed to produce mRNA which in turn can be translated to generate the isolated *Clostridium perfringens* pilus polypeptide or the immunogenic polypeptide. In at least one embodiment, the DNA can be expressed by a biochemical system, including but not limited to a cell, to produce the isolated *Clostridium perfringens* pilus polypeptide or the immunogenic polypeptide. In at least one such embodiment, the DNA can be incorporated into a vector configured for expression of the DNA in a host cell, as well known in the art.

[0045] In at least one embodiment, the polynucleotide can include a variant polynucleotide sequence which hybridizes to a polynucleotide comprising a sequence encoding an isolated *Clostridium perfringens* pilus polypeptide or an immunogenic polypeptide as described herein under at least moderately stringent conditions. By "at

least moderately stringent hybridization conditions" it is meant that conditions are selected which promote selective hybridization between two complementary nucleic acid molecules in solution. Hybridization may occur to all or a portion of a nucleic acid sequence molecule. The hybridizing portion is typically at least 15 (e.g. 20, 25, 30, 40 or 50) nucleotides in length. Those skilled in the art will recognize that the stability of a nucleic acid duplex, or hybrid, is determined by the melting temperature (T_m), which in sodium-containing buffers is a function of the sodium ion concentration ([Na^+]) and temperature (T_m = 81.5°C - 16.6 (Log_{10} [Na^+]) + 0.41(%(G+C) - 600/l), where %G+C is the percentage of cytosine and guanine nucleotides in the nucleic acid and l is the length of the nucleic acid in base pairs, or similar equation). Accordingly, the parameters in the wash conditions that determine hybrid stability are sodium ion concentration and temperature. In order to identify molecules that are similar, but not identical, to a known nucleic acid molecule, a 1% mismatch may be assumed to result in about a 1°C decrease in T_m. For example, if nucleic acid molecules are sought that have a >95% identity, the final wash temperature may be reduced by about 5°C. Based on these considerations those skilled in the art will be able to readily select appropriate hybridization conditions.

[0046] In some embodiments, stringent hybridization conditions are selected. By way of example the following conditions may be employed to achieve stringent hybridization: hybridization at 5x sodium chloride/sodium citrate (SSC)/5x Denhardt's solution/1.0% sodium dodecylsulfate (SDS) at T_m - 5°C based on the above equation, followed by a wash of 0.2x SSC/0.1% SDS at 60°C. Moderately stringent hybridization conditions include a washing step in 3x SSC at 42°C. It is understood, however, that equivalent stringencies may be achieved using alternative buffers, salts and temperatures. Additional guidance regarding hybridization conditions may be found in: Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 2002, and in: Sambrook et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, 2001 .

[0047] In at least one embodiment, the isolated *Clostridium perfringens* pilus polypeptide is a CnaA polypeptide. In at least one embodiment, the CnaA polypeptide has an amino acid sequence selected from SEQ ID NO:10 and SEQ ID NO:13. In at least one embodiment, the CnaA polypeptide is encoded by a polynucleotide having a sequence selected from SEQ ID NO:1, SEQ ID NO:4 and SEQ ID NO:7. In at least one embodiment, the CnaA polypeptide is encoded by a polynucleotide which hybridizes under at least moderately stringent conditions to a polynucleotide having a sequence selected from SEQ ID NO:1, SEQ ID NO:4 and SEQ ID NO:7. In at least

one embodiment, when the pilus polypeptide is a CnaA polypeptide, the variant has at least 75%, 80%, 85%, 90%, 95%, 99% or 99.9% sequence identity to an amino acid sequence selected from SEQ ID NO:10 and SEQ ID NO:13.

[0048] In at least one embodiment, the isolated *Clostridium perfringens* pilus polypeptide is a FimA polypeptide. In at least one embodiment, the FimA polypeptide has an amino acid sequence selected from SEQ ID NO:11 and SEQ ID NO:14. In at least one embodiment, the FimA polypeptide is encoded by a polynucleotide having a sequence selected from SEQ ID NO:2, SEQ ID NO:5 and SEQ ID NO:8. In at least one embodiment, the FimA polypeptide is encoded by a polynucleotide which hybridizes under at least moderately stringent conditions to a polynucleotide having a sequence selected from SEQ ID NO:2, SEQ ID NO:5 and SEQ ID NO:8. In at least one embodiment, when the pilus polypeptide is a FimA polypeptide, the variant has at least 75%, 80%, 85%, 90%, 95%, 99% or 99.9% sequence identity to an amino acid sequence selected from SEQ ID NO:11 and SEQ ID NO:14.

[0049] In at least one embodiment, the isolated *Clostridium perfringens* pilus polypeptide is a FimB polypeptide. In at least one embodiment, the FimB polypeptide has an amino acid sequence selected from SEQ ID NO:12 and SEQ ID NO:15. In at least one embodiment, the FimB polypeptide is encoded by a polynucleotide having a sequence selected from SEQ ID NO:3, SEQ ID NO:6 and SEQ ID NO:9. In at least one embodiment, the FimB polypeptide is encoded by a polynucleotide which hybridizes under at least moderately stringent conditions to a polynucleotide having a sequence selected from SEQ ID NO:3, SEQ ID NO:6 and SEQ ID NO:9. In at least one embodiment, when the pilus polypeptide is a FimB polypeptide, the variant has at least 75%, 80%, 85%, 90%, 95%, 99% or 99.9% sequence identity to an amino acid sequence selected from SEQ ID NO:12 and SEQ ID NO:15.

[0050] In at least one embodiment, the isolated *Clostridium perfringens* pilus polypeptide is an assembled pilus. In at least one embodiment, the assembled pilus comprises one or more subunits, each individually selected from a CnaA polypeptide, a FimA polypeptide, and a FimB polypeptide. In at least one embodiment, the one or more subunits are covalently bonded to each other. In at least one embodiment, the assembled pilus is a pilus isolated from a *Clostridium perfringens* cell, or a portion thereof including but not limited to a cell membrane or a portion thereof or a cell wall or a portion thereof. In at least one embodiment, the assembled pilus is a fragment of a pilus isolated from a *Clostridium perfringens* cell, or a portion thereof including but not limited to a cell membrane or a portion thereof or a cell wall or a portion thereof,

wherein the fragment comprises one or more subunits, each individually selected from a CnaA polypeptide, a FimA polypeptide, and a FimB polypeptide.

[0051] In at least one embodiment, the isolated *Clostridium perfringens* pilus polypeptide can be isolated from a culture of *Clostridium perfringens*. Thus, in at least one embodiment, the isolated *Clostridium perfringens* pilus polypeptide can be part of a preparation containing one or more portions of a *Clostridium perfringens* cell, including but not limited to a cell membrane or a portion thereof or a cell wall or a portion thereof, which bears the pilus polypeptide or a fragment thereof as described herein. In at least one embodiment, the isolated *Clostridium perfringens* pilus polypeptide can be recombinantly produced by expression in a suitable host cell of a vector comprising a polynucleotide having a sequence encoding the pilus polypeptide. In at least one embodiment, when the pilus polypeptide is an assembled pilus, the assembled pilus can be recombinantly produced by expression in a suitable host cell of a vector comprising a polynucleotide having a sequence encoding genes and other nucleotide sequences required for assembly of the assembled pilus. In addition, the isolated *Clostridium perfringens* pilus polypeptide can be at least partially purified after isolation or recombinant production. Suitable vectors and host cells, including but not limited to prokaryotic and eukaryotic host cells adapted for the production of recombinant polypeptides, and methods of isolating or recombinantly producing such polypeptides, including methods of at least partial purification of such polypeptides, are well known in the art or can be readily identified and selected by the skilled person with no more than routine experimental effort.

[0052] In another aspect, the present application provides a vaccine for the treatment or prevention of necrotic enteritis in poultry, or for the treatment or prevention of *Clostridium perfringens* infection in poultry, wherein the vaccine comprises at least one immunogenic polypeptide as described herein. As used herein, the term “vaccine” is intended to refer to an immunogenic preparation used to prevent, treat or reduce the effects of infection by *Clostridium perfringens*. Vaccine formulations typically contain an immunologically effective amount of an immunogenic agent, and may also contain an adjuvant or may be adjuvant-free. In the case of the present vaccine, the immunogenic agent can be an immunogenic polypeptide as described herein.

[0053] As used herein, the term “adjuvant” is intended to refer to an agent which is effective for enhancing an immune response against an immunogenic agent of a subject vaccinated with a vaccine comprising the immunogenic agent. Suitable adjuvants are well known in the art and include but are not limited to inorganic compounds including but not limited to alum, aluminum hydroxide, and other

aluminum-containing compounds; saponins including but not limited to Quil-A™; Freund's complete and incomplete adjuvants; lipid or mineral oil-containing adjuvants, including but not limited to oil-in-water emulsions; polysaccharide adjuvants; protein adjuvants; immunomodulators; adjuvants obtained from killed or attenuated bacterial
5 cells; and other suitable adjuvants known in the art.

[0054] Vaccines can be formulated in one or more pharmaceutically acceptable carriers. As used herein, the term "pharmaceutically acceptable" is intended to refer to molecular entities and compositions that are physiologically tolerable and do not typically produce untoward reactions when administered to an animal or a human.
10 Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals or humans. As used herein, the term "carrier" is intended to refer to a diluent, adjuvant, excipient, or vehicle with which a compound is administered. Suitable carriers are well
15 known in the art and, in at least one embodiment, are described in "Remington's Pharmaceutical Sciences" by E. W. Martin, 18th Edition, or other editions.

[0055] The vaccines can be formulated for administration by any convenient route known in the art, including but not limited to orally, rectally, nasally, transmucosally, transdermally, parenterally, intravenously, intramuscularly, subcutaneously, *in ovo*, or
20 by other known routes. In at least one embodiment, it is contemplated that the vaccine can be administered orally. Without being bound by theory, it is contemplated that oral vaccination can directly target gut-associated lymphoid tissues, at the site of infection by necrotic enteritis-associated strains of C*lostridium perfringens.* In at least one embodiment, it is contemplated that progeny can be immunized by vaccination of a
25 mother and subsequent transfer of maternal immunity, including but not limited to maternal antibodies, to the progeny.

[0056] In another aspect, the present invention provides the use of an immunogenic polypeptide as described herein in the preparation of a medicament for the treatment or prevention of necrotic enteritis in poultry or for the treatment or prevention of
30 *Clostridium perfringens* infection in poultry. The medicament can be a vaccine as described herein.

[0057] In another aspect, the present invention provides a method of treatment or prevention of necrotic enteritis in poultry or for the treatment or prevention of *Clostridium perfringens* infection in poultry, the method comprising administering an
35 effective amount of an immunogenic polypeptide or of a vaccine as described herein to the poultry. Administration can be by routes well known in the art, including but not

limited to orally, rectally, nasally, parenterally, intravenously, intramuscularly, subcutaneously or by other routes. In at least one embodiment, administration can be by subcutaneous injection. In at least one embodiment, administration can be oral. In at least one embodiment, the vaccine can be administered more than once to the poultry, to provide an initial immunization followed by one or more booster immunizations, as understood in the art. In at least one embodiment, one or more of the initial immunization and the one or more booster immunizations are administered to the poultry after the disappearance of maternal antibodies in the poultry. In at least one such embodiment, one or more of the initial immunization and the one or more booster immunizations are administered to the poultry no earlier than about 10 days after hatching.

[0058] In another aspect, the present invention provides the use of an immunogenic polypeptide as described herein as a vaccine for the treatment or prevention of necrotic enteritis in poultry or for the treatment or prevention of *Clostridium perfringens* infection in poultry.

[0059] A further aspect of the present invention provides an antibody which binds selectively to an immunogenic polypeptide as described herein. In at least one embodiment, the antibody is a poultry antibody. In at least one embodiment, the antibody can be a polyclonal antibody, a monoclonal antibody, a chimeric antibody, a single chain antibody or an antibody fragment that retains the property of selective binding to an immunogenic polypeptide as described herein. The term "antibody fragment" as used herein is intended to include but not be limited to Fab, Fab', $F(ab')_2$, scFv, dsFv, ds-scFv, dimers, minibodies, diabodies, and multimers thereof and bispecific antibody fragments. Antibodies can be fragmented using conventional techniques. For example, $F(ab')_2$ fragments can be generated by treating the antibody with pepsin. The resulting $F(ab')_2$ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. Papain digestion can lead to the formation of Fab fragments. Fab, Fab' and $F(ab')_2$, scFv, dsFv, ds-scFv, dimers, minibodies, diabodies, bispecific antibody fragments and other fragments can also be synthesized by recombinant techniques.

[0060] Methods of preparing and characterizing such antibodies and fragments thereof are well known in the art and can be readily carried out by the skilled person without undue effort. For example, polyclonal antisera or monoclonal antibodies can be made using standard methods. A mammal, (e.g., a mouse, hamster, or rabbit), bird (e.g. poultry) or other animal can be immunized with an immunogenic form of the present immunogenic polypeptide which elicits an antibody response in the mammal.

Techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. For example, the peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other
5 immunoassay procedures can be used with the immunogenic agent as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

[0061] To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by
10 standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art. Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with an immunogenic polypeptide as described herein and the monoclonal antibodies can be isolated. Therefore, the disclosure also contemplates hybridoma cells secreting
15 monoclonal antibodies with specificity for an immunogenic polypeptide as described herein.

[0062] Specific antibodies, or antibody fragments reactive against an immunogenic polypeptide as described herein may also be generated by screening expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria
20 with peptides produced from nucleic acid molecules as described herein. For example, complete Fab fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries.

[0063] In another aspect, the present invention provides a method of detecting infection of poultry by a strain of *Clostridium perfringens* associated with necrotic
25 enteritis, wherein the method includes obtaining a biological sample from the poultry and detecting in the biological sample the presence of an antibody which binds selectively to an immunogenic polypeptide as described herein. In at least one embodiment, the biological sample is a blood sample. In at least one embodiment, the sample is a fecal sample. In at least one embodiment, the detection includes
30 measurement of the amount or concentration of antibody present in the biological sample, using methods well known by those skilled in the art.

[0064] Yet another aspect of the present invention provides a method of detecting an immunogenic polypeptide as described herein comprising exposing the immunogenic polypeptide to an antibody which binds selectively to the immunogenic polypeptide
35 and detecting binding of the immunogenic polypeptide to the antibody. In at least one embodiment, the immunogenic polypeptide can be an isolated *Clostridium perfringens*

pilus polypeptide as described herein. In at least one embodiment, the immunogenic polypeptide can be an assembled pilus attached to the surface of a *Clostridium perfringens* bacterial cell. Such embodiments of the method can be useful for identifying and detecting strains of *Clostridium perfringens* which are capable of producing necrotic enteritis in poultry.

[0065] As used herein, the terms “about” or “approximately” as applied to a numerical value or range of values are intended to mean that the recited values can vary within an acceptable degree of error for the quantity measured given the nature or precision of the measurements, such that the variation is considered in the art as equivalent to the recited values and provides the same function or result. For example, the degree of error can be indicated by the number of significant figures provided for the measurement, as is understood in the art, and includes but is not limited to a variation of ±1 in the most precise significant figure reported for the measurement. Typical exemplary degrees of error are within 20 percent (%), preferably within 10%, and more preferably within 5% of a given value or range of values. Alternatively, and particularly in biological systems, the terms "about" and "approximately" can mean values that are within an order of magnitude, preferably within 5-fold and more preferably within 2-fold of a given value. Numerical quantities given herein are approximate unless stated otherwise, meaning that the term "about" or "approximately" can be inferred when not expressly stated.

[0066] As used herein, the term “substantially” refers to the complete or nearly complete extent or degree of an action, characteristic, property, state, structure, item, or result. For example, two substances which have “substantially” the same properties would have completely identical properties or would have properties which are so nearly completely the same that the differences are not measurable or significant. The exact allowable degree of deviation from absolute completeness may in some cases depend on the specific context. However, generally speaking the nearness of completion will be so as to have the same overall result as if absolute and total completion were obtained. The use of “substantially” is equally applicable when used in a negative connotation to refer to the complete or near complete lack of an action, characteristic, property, state, structure, item, or result.

EXAMPLES

[0067] Other features of the present invention will become apparent from the following non-limiting examples which illustrate, by way of example, the principles of the invention.

Example 1: Production of purified recombinant pilus-associated polypeptides from *Clostridium perfringens*

[0068] Coding regions for the three pilus subunits (*cnaA*, *fimA* and *fimB*) were codon-optimized and truncated to exclude the predicted N-terminal signal peptides and C-terminal cell wall sorting signal LPXTG transmembrane domains. The C-terminal domain is a hydrophobic region predicted to be removed during pilus assembly. The truncated codon-optimized coding regions were synthesized (Integrated DNA Technologies, Coralville, USA), cloned into the pET28a expression vector (MilliporeSigma, Etobicoke, Ontario, Canada) by In-Fusion™ cloning according to the manufacturer's instructions (Takara Bio USA, Mountain View, California, USA), sequence-verified, and then transformed into *E. coli* BL21 cells. Transformed colonies were grown at 37°C for 18h with shaking in 1 L LB broth supplemented with 50 µg/ml kanamycin and 1mM IPTG. The culture was pelleted and resuspended in 20 ml of binding buffer (20mM $NaPO_4$, 0.5M NaCl, 30mM imidazole) and lysed by sonication for 10 min on ice (10 s pulses, 20s pauses). The cell lysate was purified under native conditions on a HisTrap™ FF Crude column (GE Healthcare, Montreal, Canada) using a gradient of 50 to 500 mM imidazole on an ÄKTA™prime plus system. One ml fractions were collected, and fractions exhibiting a 280 nm peak were pooled and concentrated with Pierce™ Protein Concentrators (9K molecular weight cut-off) (Fisher Scientific, Unionville, Ontario, Canada) and desalted using Zeba™ Spin 7K molecular weight cut-off desalting columns (Fisher Scientific). Quantitation of the purified proteins was performed using the BCA (bicinchoninic acid) Protein Assay kit (Fisher) according to the manufacturer's instructions. Polypeptides were visualized by SDS-PAGE and Coomassie staining.

[0069] The truncated sequences resulted in high levels of expression of histidine (His)-tagged polypeptides, as evidenced by the SDS-PAGE gels shown in Figures 2A (CnaA), 2B (FimA), 2C (FimB) and 2D (pooled fractions of CnaA, FimA and FimB, following concentration and desalting). These increased levels of expression may be due to increased solubility of the resulting polypeptides.

[0070] Table 1 shows the sequences of the full-length, codon-optimized and truncated gene sequences, along with the sequences of the full-length subunit polypeptides and the expressed truncated and His-tagged subunit polypeptides.

Table 1: Sequences of pilus subunit genes and polypeptides

Sequence	cnaA	fimA	fimB
Full length gene	ATGAAAATAAATAAAAAAATTTTTAGCATGCTAT TTATGGTTATTGTACTTTTTACATGCATATCATC AAATTTTTCTGTTTCTGCTTCTTCTATTCAAAGA GGAAGAGATATCAGTAATGAGGTAGTTACAAGCC TAGTGGCTACTCCAAATAGTATAAATGATGGTGG AAACGTTCAGGTTCGTTTGGAATTTAAAGAAAAT CATCAAAGAAATATACAAAGTGGAGATACTATAA CTGTCAAATGGACAAATTCAGGGGAAGTATTTTT TGAAGGATATGAAAAAACAATTCCACTTTATATA AAAGACCAAAATGTTGGTCAAGCAGTAATAGAGA AAACAGGTGCAACACTTACATTTAATGATAAAAT AGATAAATTAGATGATGTTGGTGGATGGGCAACA TTTACTTTGCAAGGAAGAAACATTACCTCAGGTA ATCATGAACACACAGGAATAGCATATATTATATC TGGTTCAAAGCGGGCAGATGTAAATATAACCAAA CCAGAATCAGGTACAACTAGTGTATTCTATTATA AAACAGGTAGTATGTATACCAATGATACAAATCA TGTCAATTGGTGGTTACTGGTGAATCCAAGCAAG GTATATTCTGAAAAAAACGTTTATATTCAAGATG AAATCCAAGGCGGACAAACATTAGAACCTGATTC TTTTGAAATAGTAGTAACTTGGTATGATGGTTAT GTAGAAAAGTTTAAAGGAAAAGAAGCGATAAGGG AATTCCATAATAAATATCCAAATTCAAATATATC GGTATCAGAAAATAAAATAACAGTAAACATTTCA CAAGAGGATTCCACACAAAAGTTTATTAATATTT TTTATAAAACTAAGATTACAAATCCGAAACAAAA AGAATTCGTTAATAATACAAAAGCATGGTTTAAA GAGTATAATAAGCCAGCTGTAAATGGAGAATCCT TTAACCATAGCGTACAAAATATTAATGCAGATGC TGGAGTTAATGGAACTGTAAAAGGCGAATTAAAA ATCATAAAAACATTAAAAGATAAAAGTATTCCAA TTAAAGATGTTCAGTTTAAGATGAGAAGAGTTGA TAATACAGTTATCAAAGATGGTAAAAAAGAATTA TTACTAACAACTGATGATAAAGGTATTGCAAATG TAAAAGGTCTTCCTGTAGGAAAATATGAAGTAAA AGAGATTTCAGCTCCAGAATGGATTGCTTTTAAT CCTCTTATTGCACCAAAATTGGAATTCACAATAT CAGATCAGGACACAGAAGGCAAATTGTGGGCTGT TGAAAATGAATTAAAGACAATTTCAATTCCGGTT GAAAAGGTCTGGGTAGGACAAACTAGTGAACGAG CAGAAATCAAGCTTTTTGCAGATGGTATTGAAGT AGACAAAGTGATTTTAAATGCAGATAACAATTGG AAACACACATTTGAAAATAAACCTGAATATAATT CAGAAACAAAACAGAAAATCAATTATTCTGTGTC AGAGACAACTATTTCTGGATATGAAAGCAATATC ACAGGCGATGCTAAGAATGGTTTTATTGTAACCA ATACAGAACTTCCTGATTTGACTATTGGTAAAGA AGTTATAGGAGAATTGGGTGACAAGACGAAGGTA TTTAACTTTGAGCTTACATTAAAGCAAGCAGATG GAAAGCCTATCAATGGTAAATTTAATTACATTGG TAGTGTAGATGACAGGTACAAAAAAGAAAGCATA AAGCCTTCTGATGGTGAGATTACTTTTATAGAAG GAAAAGCAACTATAACTTTATCACATGGACAAGA GATTACAATCAAGGATTTACCATATGGGGTTACA TATAAAGTTATGGAAAAAGAAGCTAATGAAAATG GCTATTTAACTACCTATAATGGAAATAACGAAGT CACAACAGGTGAATTGAAACAGGATACAAAAGTA CAGGTAGTTAACAACAAAGAGTTTGTTCCAACAA CTGGTATATCAACCACAACAGAGCAAGGTACAAT GGTTGGAATGGTGATTTTTTCTATAGGAATACTT ATGGTCATGATTGTAGTTCTTTTACAATTGAATA AAGGACTGAAAAGATGA (SEQ ID NO:1)	ATGATAAACAAGAAAAAA TTAAGTGCATTATTATTA AGTGGAGCAATGTTTATG AGTATGAATACAAATGTA TTCGCATCAAATTTACCT TCTGGAGGGGTAGAAGGT ACAGAACAGAATCCTGCA AAAGCAACAATTACAAAG AATTTTGAATTTCCAGAA GGTATTAATACACCTAGT GCAACATTCAAGTTTACA GCAGAAAAAATAACTAAT GATGCGCCAGATGCAACA ATTGGAGATATTAATTAT ACACAAGGGGATAATGGA ACTTTATCAAATGGAAAA TATAGTGTAAAGAAAACA ACTGAAATTACTTTTGGA AATTTCCCACATGCAGGA GAATATGATTATAATGTA AAAGAAACGAATGAGGGA GTAGGTGGTATTACATAT GATACAAAAGAATACAAA GTTCATGTGTATGTTGCA AATAGTAACGCTATGGAT GGAAAAACTTATGTAAAA GCCATTACATCAGAAAAT GGAGGTGAAAAAGCTCCA ATTGAGTTTGTTAATACA TATAAAAAGGACACTTCT TTACTTATAGAAAAGAAT GTAATAGGAGATTTAGCT GACTTAACAAAACAGTTT GAGTTTCAGATTAATTTA AAAAAATCAGCAACATCT GACATAACAAAATTCGAA GGAAATATTATTAGAAAA GATGGTAAAATAGAGCCT GTAACATATACAGCTGAA AATACAGAAACTTTTAAA TTAGCAAATGGAGATAAA CTTAAGTTTGAAAGTATT CCAGCAGGAACAAAATAT GAAGTAAAAGAGATAGGT GCTAGTGATGGATATACA CCTTCTATAACAGTAATT GAAAATGGAAATGAGACT TCTAATAATCGTACGGTA GCTGAAAAAGATGGTATA TCATCTAAGTCAAATTCT AATGATAACTTAATTGGT GAAGGTGAAAACAAAGTA ACATTTACAAACACATAT AATGACAAACCTATCACA GGTATTGTTATGAATAAT ATTCCATTTATTCTAATG ATTAGTTTTGCTGTCCTT GGATTTGGTGCTTTAGCT ATTATAAAAAGACGTAAA ACTATAAGATAA (SEQ ID NO:2)	ATGGAAACAAAG AAAATAAGAAAC AAAATCCTTATG GCTATCGTAGCA TTGAGCTTTATA TTGCTTCCAAAC ACTAGAGTATAT GCTACTGAAAAT ACAGCAAATATT CCTTTGATAGTT AGACAGGAATTT AATGTATATACG AAAGATTCAAAA GCAATAGACATG ATTGGAAAATAC GAGCTAAAGGCA ATAAGTGAAAAT GCCCCTATGCCA GAAGAAAGTAAA AATGGAAGTTTT ATCTTTAATATA GATGGAAATGAT AAGCAGTTTACT ATTCCATTAGCT TATACACATGGT GGTGTGTATATC TATCAAATTCAA CAGATAACGCAA TCTAAAGATAAT TACATATATGAT AAAAATAGCTAT AAGATAACTGTA TATGTAAAAAAT GCAGAAAATAAT CATTTAATACCA CAAATTATTGTG AAAAATGAAAAT AATGAAAAATGT GAAGAAATATGT TTTTATAACATT TACAAACAAAAA AATAAAATTAAT GAGATTTCTAAA ACACCATATAAG CCAAATGGAATA AATGTTCCTAAA ACAGGCGATACC ACAAACATTGGA TTTTATATTGTA ATACTTATAATT TCACTTGGATTA CTTGTGGTATTG AAATGGAAAGAA TATAAAAAGAGA AAAAAAGAATAA (SEQ ID NO:3)

Sequence	cnaA	fimA	fimB
Codon-optimized gene	ATGAAGATCAACAAGAAGATCTTCAGCATGTTAT TTATGGTCATTGTGCTGTTCACCTGTATCAGCTC TAACTTCAGTGTGAGCGCGTCAAGCATCCAGCGC GGCCGGGACATCAGCAACGAGGTGGTGACATCGC TCGTAGCTACCCCGAATAGCATCAACGATGGTGG TAACGTCCAAGTGCGTCTGGAATTTAAAGAGAAT CACCAGCGGAACATTCAGTCCGGCGACACGATTA CGGTCAAATGGACTAACTCAGGTGAGGTCTTTTT TGAAGGCTACGAAAAAACCATCCCGCTGTATATC AAGGATCAGAACGTTGGCCAGGCGGTTATTGAAA AAACCGGTGCAACATTAACATTCAACGATAAGAT CGACAAATTAGATGATGTCGGCGGCTGGGCCACA TTCACGCTCCAGGGTCGCAATATTACTTCAGGAA ATCATGAGCATACTGGTATTGCGTACATTATCTC GGGTAGCAAACGTGCGGACGTTAACATCACAAAA CCTGAATCCGGAACAACGTCTGTGTTTTACTACA AGACGGGTTCGATGTACACCAATGACACAAATCA TGTGAATTGGTGGCTGCTGGTTAACCCGAGCAAA GTATACTCTGAGAAAAATGTCTATATTCAGGATG AAATTCAAGGCGGTCAGACCCTGGAGCCGGACAG TTTTGAAATCGTCGTTACATGGTACGATGGTTAT GTGGAAAAATTTAAAGGTAAAGAAGCGATCCGGG AGTTCCACAATAAATATCCGAATAGTAATATCTC GGTCAGTGAAAATAAAATCACGGTAAATATTTCG CAAGAAGATTCCACCCAAAAATTCATTAACATCT TTTACAAGACTAAAATCACCAACCCGAAGCAGAA AGAATTTGTAAACAACACCAAAGCCTGGTTCAAA GAGTACAATAAGCCGGCGGTTAACGGTGAAAGTT TTAATCACAGTGTGCAGAATATCAACGCAGATGC CGGGGTAAATGGTACTGTTAAAGGTGAATTGAAA ATTATCAAAACCCTGAAAGATAAAAGTATTCCGA TCAAGGATGTGCAGTTTAAGATGCGCCGCGTGGA TAATACCGTTATTAAAGACGGCAAGAAAGAGCTG CTGTTGACCACAGATGATAAAGGATTGCAAACG TGAAAGGTCTGCCAGTCGGGAAATACGAAGTCAA AGAAATCAGTGCGCCTGAGTGGATCGCCTTCAAT CCACTGATTGCGCCCAAACTTGAATTTACGATCA GCGATCAAGACACAGAGGGGAAATTATGGGCAGT GGAAAACGAACTCAAAACCATCTCGATTCCGGTC GAAAAAGTCTGGGTAGGTCAGACGAGTGAACGGG CGGAGATCAAACTGTTTGCGGATGGAATTGAAGT TGATAAGGTGATCCTGAACGCGGATAATAATTGG AAGCACACCTTTGAGAATAAACCCGAATATAACT CCGAGACTAAACAAAAAATCAACTATAGTGTGAG CGAAACTACCATCAGTGGCTATGAATCAAATATT ACTGGCGATGCGAAAAACGGATTTATTGTCACCA ACACAGAACTGCCTGATTTGACGATCGGGAAAGA GGTAATCGGCGAACTCGGCGATAAAACCAAGGTA TTCAACTTTGAACTGACACTTAAGCAGGCTGACG GAAAGCCCATTAACGGGAAATTTAACTATATTGG TTCGGTGGATGATCGTTATAAGAAGGAATCGATT AAGCCTAGCGATGGGGAAATTACGTTCATCGAGG GAAAAGCAACGATTACCCTCTCCCACGGACAAGA GATCACCATTAAGGACCTTCCGTATGGTGTGACC TATAAAGTCATGGAAAAAGAAGCCAACGAGAATG GATATTTAACCACTTACAACGGAAATAACGAAGT CACCACCGGGGAGTTGAAACAGGATACGAAAGTA CAAGTGGTTAATAATAAAGAATTCGTCCCGACAA CCGGGATCAGCACCACCACCGAACAGGGAACCAT GGTCGGGATGGTGATCTTTAGCATCGGTATTCTC ATGGTAATGATTGTCGTTCTGCTGCAGCTGAATA AAGGACTGAAACGC (SEQ ID NO:4)	ATGATTAATAAAAAGAAA CTGTCGGCGCTGCTCTTA AGCGGGGCCATGTTTATG AGCATGAACACGAATGTG TTCGCGTCTAACCTCCCA TCGGGTGGTGTGGAGGGC ACCGAACAAAACCCAGCG AAAGCGACAATCACGAAA AACTTCGAGTTTCCGGAA GGTATTAATACACCCAGC GCGACATTCAAATTTACC GCCGAAAAAATTACCAAC GATGCGCCGGATGCTACT ATTGGCGACATCAATTAT ACCCAAGGTGATAATGGG ACGTTAAGCAATGGCAAA TACAGTGTGAAAAAGACT ACCGAGATTACCTTCGGG AACTTCCCGCATGCTGGT GAGTATGATTATAACGTC AAAGAAACCAATGAAGGC GTGGGTGGCATTACTTAC GATACGAAAGAATATAAA GTTCATGTGTATGTGGCC AACTCAAATGCGATGGAC GGTAAGACATATGTTAAA GCGATTACTAGCGAAAAT GGCGGGGAAAAAGCACCG ATCGAATTCGTTAACACC TATAAAAAAGATACGTCG TTACTGATTGAAAAAAAT GTAATTGGCGATCTGGCA GACCTCACCAAACAGTTT GAGTTTCAAATCAACTTG AAAAAGAGCGCGACTAGT GATATTACCAAGTTTGAA GGTAACATTATTCGCAAA GACGGTAAGATTGAACCC GTGACCTATACCGCGGAA AATACCGAGACCTTTAAG TTAGCCAACGGAGACAAG TTAAAATTCGAGTCCATC CCCGCCGGTACAAAATAT GAAGTCAAGGAAATCGGG GCGAGCGATGGGTACACG CCCTCAATCACCGTTATC GAAAATGGCAACGAAACC TCAAATAACCGCACTGTA GCCGAAAAAGATGGAATC TCTAGCAAAAGCAACTCG AACGACAATTTAATCGGC GAAGGCGAAAATAAAGTG ACCTTTACCAATACGTAC AACGATAAACCAATCACG GGAATCGTAATGAATAAT ATTCCGTTCATTCTTATG ATTAGCTTTGCCGTTCTT GGCTTCGGTGCATTAGCG ATCATTAAACGCCGCAAA ACCATCCGCCCCATCGAT ACGCGT (SEQ ID NO:5)	ATGGCTATTGTT GCTTTGTCATTT ATCCTGCTCCCG AATACCCGGGTC TATGCGACGGAG AACACCGCTAAT ATCCCGTTAATT GTACGCCAAGAA TTTAATGTTTAC ACTAAAGATTCT AAAGCCATTGAC ATGATCGGAAAA TATGAATTAAAA GCCATTTCTGAG AACGCTCCCATG CCGGAGGAATCA AAAAATGGTAGC TTTATTTTTAAC ATCGACGGTAAT GATAAACAGTTT ACTATTCCGCTG GCGTACACTCAC GGTGGCGTCTAC ATCTATCAAATC CAGCAAATTACC CAGAGCAAGGAT AACTACATCTAC GATAAAAACAGC TATAAAATCACG GTATATGTCAAG AACGCAGAAAAC AATCATCTGATC CCGCAGATTATT GTAAAAAATGAG AACAATGAAAAA TGTGAAGAAATC TGCTTCTACAAT ATCTACAAACAG AAAAACAAGATC AATGAGATCTCT AAAACCCCCTAT AAGCCGAATGGT ATTAATGTCCCG AAAACGGGTGAT ACCACGAACATC GGATTCTACATT GTGATCTTGATT ATTTCCCTGGGC CTGCTGGTGGTC TTGAAGTGGAAA GAATATAAAAAA CGTAAGAAGGAA (SEQ ID NO:6)

Sequence	cnaA	fimA	fimB
Truncated gene	TCAAGCATCCAGCGCGGCCGGGACATCAGCAACG AGGTGGTGACATCGCTCGTAGCTACCCCGAATAG CATCAACGATGGTGGTAACGTCCAAGTGCGTCTG GAATTTAAAGAGAATCACCAGCGGAACATTCAGT CCGGCGACACGATTACGGTCAAATGGACTAACTC AGGTGAGGTCTTTTTTGAAGGCTACGAAAAAACC ATCCCGCTGTATATCAAGGATCAGAACGTTGGCC AGGCGGTTATTGAAAAAACCGGTGCAACATTAAC ATTCAACGATAAGATCGACAAATTAGATGATGTC GGCGGCTGGGCCACATTCACGCTCCAGGGTCGCA ATATTACTTCAGGAAATCATGAGCATACTGGTAT TGCGTACATTATCTCGGGTAGCAAACGTGCGGAC GTTAACATCACAAAACCTGAATCCGGAACAACGT CTGTGTTTTACTACAAGACGGGTTCGATGTACAC CAATGACACAAATCATGTGAATTGGTGGCTGCTG GTTAACCCGAGCAAAGTATACTCTGAGAAAAATG TCTATATTCAGGATGAAATTCAAGGCGGTCAGAC CCTGGAGCCGGACAGTTTTGAAATCGTCGTTACA TGGTACGATGGTTATGTGGAAAAATTTAAAGGTA AAGAAGCGATCCGGGAGTTCCACAATAAATATCC GAATAGTAATATCTCGGTCAGTGAAAATAAAATC ACGGTAAATATTTCGCAAGAAGATTCCACCCAAA AATTCATTAACATCTTTTACAAGACTAAAATCAC CAACCCGAAGCAGAAAGAATTTGTAAACAACACC AAAGCCTGGTTCAAAGAGTACAATAAGCCGGCGG TTAACGGTGAAAGTTTTAATCACAGTGTGCAGAA TATCAACGCAGATGCCGGGGTAAATGGTACTGTT AAAGGTGAATTGAAAATTATCAAAACCCTGAAAG ATAAAAGTATTCCGATCAAGGATGTGCAGTTTAA GATGCGCCGCGTGGATAATACCGTTATTAAAGAC GGCAAGAAAGAGCTGCTGTTGACCACAGATGATA AAGGGATTGCAAACGTGAAAGGTCTGCCAGTCGG GAAATACGAAGTCAAAGAAATCAGTGCGCCTGAG TGGATCGCCTTCAATCCACTGATTGCGCCCAAAC TTGAATTTACGATCAGCGATCAAGACACAGAGGG GAAATTATGGGCAGTGGAAAACGAACTCAAAACC ATCTCGATTCCGGTCGAAAAAGTCTGGGTAGGTC AGACGAGTGAACGGGCGGAGATCAAACTGTTTGC GGATGGAATTGAAGTTGATAAGGTGATCCTGAAC GCGGATAATAATTGGAAGCACACCTTTGAGAATA AACCCGAATATAACTCCGAGACTAAACAAAAAAT CAACTATAGTGTGAGCGAAACTACCATCAGTGGC TATGAATCAAATATTACTGGCGATGCGAAAAACG GATTTATTGTCACCAACACAGAACTGCCTGATTT GACGATCGGGAAAGAGGTAATCGGCGAACTCGGC GATAAAACCAAGGTATTCAACTTTGAACTGACAC TTAAGCAGGCTGACGGAAAGCCCATTAACGGGAA ATTTAACTATATTGGTTCGGTGGATGATCGTTAT AAGAAGGAATCGATTAAGCCTAGCGATGGGGAAA TTACGTTCATCGAGGGAAAAGCAACGATTACCCT CTCCCACGGACAAGAGATCACCATTAAGGACCTT CCGTATGGTGTGACCTATAAAGTCATGGAAAAAG AAGCCAACGAGAATGGATATTTAACCACTTACAA CGGAAATAACGAAGTCACCACCGGGGAGTTGAAA CAGGATACGAAAGTACAAGTGGTTAATAATAAAG AATTCGTCCCGACAACC (SEQ ID NO:7)	TCTAACCTCCCATCGGGT GGTGTGGAGGGCACCGAA CAAAACCCAGCGAAAGCG ACAATCACGAAAAACTTC GAGTTTCCGGAAGGTATT AATACACCCAGCGCGACA TTCAAATTTACCGCCGAA AAAATTACCAACGATGCG CCGGATGCTACTATTGGC GACATCAATTATACCCAA GGTGATAATGGGACGTTA AGCAATGGCAAATACAGT GTGAAAAAGACTACCGAG ATTACCTTCGGGAACTTC CCGCATGCTGGTGAGTAT GATTATAACGTCAAAGAA ACCAATGAAGGCGTGGGT GGCATTACTTACGATACG AAAGAATATAAAGTTCAT GTGTATGTGGCCAACTCA AATGCGATGGACGGTAAG ACATATGTTAAAGCGATT ACTAGCGAAAATGGCGGG GAAAAAGCACCGATCGAA TTCGTTAACACCTATAAA AAAGATACGTCGTTACTG ATTGAAAAAAATGTAATT GGCGATCTGGCAGACCTC ACCAAACAGTTTGAGTTT CAAATCAACTTGAAAAAG AGCGCGACTAGTGATATT ACCAAGTTTGAAGGTAAC ATTATTCGCAAAGACGGT AAGATTGAACCCGTGACC TATACCGCGGAAAATACC GAGACCTTTAAGTTAGCC AACGGAGACAAGTTAAAA TTCGAGTCCATCCCCGCC GGTACAAAATATGAAGTC AAGGAAATCGGGGCGAGC GATGGGTACACGCCCTCA ATCACCGTTATCGAAAAT GGCAACGAAACCTCAAAT AACCGCACTGTAGCCGAA AAAGATGGAATCTCTAGC AAAAGCAACTCGAACGAC AATTTAATCGGCGAAGGC GAAAATAAAGTGACCTTT ACCAATACGTACAACGAT AAACCAATCACG (SEQ ID NO:8)	ACGGAGAACACC GCTAATATCCCG TTAATTGTACGC CAAGAATTTAAT GTTTACACTAAA GATTCTAAAGCC ATTGACATGATC GGAAAATATGAA TTAAAAGCCATT TCTGAGAACGCT CCCATGCCGGAG GAATCAAAAAAT GGTAGCTTTATT TTTAACATCGAC GGTAATGATAAA CAGTTTACTATT CCGCTGGCGTAC ACTCACGGTGGC GTCTACATCTAT CAAATCCAGCAA ATTACCCAGAGC AAGGATAACTAC ATCTACGATAAA AACAGCTATAAA ATCACGGTATAT GTCAAGAACGCA GAAAACAATCAT CTGATCCCGCAG ATTATTGTAAAA AATGAGAACAAT GAAAAATGTGAA GAAATCTGCTTC TACAATATCTAC AAACAGAAAAAC AAGATCAATGAG ATCTCTAAAACC CCCTATAAGCCG AATGGTATTAAT GTCCCGAAAACG (SEQ ID NO:9)

Sequence	cnaA	fimA	fimB
Full-length polypeptide	MKINKKIFSMLFMVIVLFTCISSNFSVSASSIQR GRDISNEVVTSLVATPNSINDGGNVQVRLEFKEN HQRNIQSGDTITVKWTNSGEVFFEGYEKTIPLYI KDQNVGQAVIEKTGATLTFNDKIDKLDDVGGWAT FTLQGRNITSGNHEHTGIAYIISGSKRADVNITK PESGTTSVFYYKTGSMYTNDTNHVNWWLLVNPSK VYSEKNVYIQDEIQGGQTLEPDSFEIVVTWYDGY VEKFKGKEAIREFHNKYPNSNISVSENKITVNIS QEDSTQKFINIFYKTKITNPKQKEFVNNTKAWFK EYNKPAVNGESFNHSVQNINADAGVNGTVKGELK IIKTLKDKSIPIKDVQFKMRRVDNTVIKDGKKEL LLTTDDKGIANVKGLPVGKYEVKEISAPEWIAFN PLIAPKLEFTISDQDTEGKLWAVENELKTISIPV EKVWVGQTSERAEIKLFADGIEVDKVILNADNNW KHTFENKPEYNSETKQKINYSVSETTISGYESNI TGDAKNGFIVTNTELPDLTIGKEVIGELGDKTKV FNFELTLKQADGKPINGKFNYIGSVDDRYKKESI KPSDGEITFIEGKATITLSHGQEITIKDLPYGVT YKVMEKEANENGYLTTYNGNNEVTTGELKQDTKV QVVNNKEFVPTTGISTTTEQGTMVGMVIFSIGIL MVMIVVLLQLNKGLKR (SEQ ID NO:10)	MINKKKLSALLLSGAMFM SMNTNVFASNLPSGGVEG TEQNPAKATITKNFEFPE GINTPSATFKFTAEKITN DAPDATIGDINYTQGDNG TLSNGKYSVKKTTEITFG NFPHAGEYDYNVKETNEG VGGITYDTKEYKVHVYVA NSNAMDGKTYVKAITSEN GGEKAPIEFVNTYKKDTS LLIEKNVIGDLADLTKQF EFQINLKKSATSDITKFE GNIIRKDGKIEPVTYTAE NTETFKLANGDKLKFESI PAGTKYEVKEIGASDGYT PSITVIENGNETSNNRTV AEKDGISSKSNSNDNLIG EGENKVTFTNTYNDKPIT GIVMNNIPFILMISFAVL GFGALAIIKRRKTIR (SEQ ID NO:11)	METKKIRNKILM AIVALSFILLPN TRVYATENTANI PLIVRQEFNVYT KDSKAIDMIGKY ELKAISENAPMP EESKNGSFIFNI DGNDKQFTIPLA YTHGGVYIYQIQ QITQSKDNYIYD KNSYKITVYVKN AENNHLIPQIIV KNENNEKCEEIC FYNIYKQKNKIN EISKTPYKPNGI NVPKTGDTTNIG FYIVILIISLGL LVVLKWKEYKKR KKE (SEQ ID NO:12)
Expressed truncated polypeptide	MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGS EFSSIQRGRDISNEVVTSLVATPNSINDGGNVQV RLEFKENHQRNIQSGDTITVKWTNSGEVFFEGYE KTIPLYIKDQNVGQAVIEKTGATLTFNDKIDKLD DVGGWATFTLQGRNITSGNHEHTGIAYIISGSKR ADVNITKPESGTTSVFYYKTGSMYTNDTNHVNWW LLVNPSKVYSEKNVYIQDEIQGGQTLEPDSFEIV VTWYDGYVEKFKGKEAIREFHNKYPNSNISVSEN KITVNISQEDSTQKFINIFYKTKITNPKQKEFVN NTKAWFKEYNKPAVNGESFNHSVQNINADAGVNG TVKGELKIIKTLKDKSIPIKDVQFKMRRVDNTVI KDGKKELLLTTDDKGIANVKGLPVGKYEVKEISA PEWIAFNPLIAPKLEFTISDQDTEGKLWAVENEL KTISIPVEKVWVGQTSERAEIKLFADGIEVDKVI LNADNNWKHTFENKPEYNSETKQKINYSVSETTI SGYESNITGDAKNGFIVTNTELPDLTIGKEVIGE LGDKTKVFNFELTLKQADGKPINGKFNYIGSVDD RYKKESIKPSDGEITFIEGKATITLSHGQEITIK DLPYGVTYKVMEKEANENGYLTTYNGNNEVTTGE LKQDTKVQVVNNKEFVPTTVDKLAAALEHHHHHH (SEQ ID NO:13)	MGSSHHHHHHSSGLVPRG SHMASMTGGQQMGRGSEF SNLPSGGVEGTEQNPAKA TITKNFEFPEGINTPSAT FKFTAEKITNDAPDATIG DINYTQGDNGTLSNGKYS VKKTTEITFGNFPHAGEY DYNVKETNEGVGGITYDT KEYKVHVYVANSNAMDGK TYVKAITSENGGEKAPIE FVNTYKKDTSLLIEKNVI GDLADLTKQFEFQINLKK SATSDITKFEGNIIRKDG KIEPVTYTAENTETFKLA NGDKLKFESIPAGTKYEV KEIGASDGYTPSITVIEN GNETSNNRTVAEKDGISS KSNSNDNLIGEGENKVTF TNTYNDKPITVDKLAAAL EHHHHHH (SEQ ID NO:14)	MGSSHHHHHHSS GLVPRGSHMASM TGGQQMGRGSEF TENTANIPLIVR QEFNVYTKDSKA IDMIGKYELKAI SENAPMPEESKN GSFIFNIDGNDK QFTIPLAYTHGG VYIYQIQQITQS KDNYIYDKNSYK ITVYVKNAENNH LIPQIIVKNENN EKCEEICFYNIY KQKNKINEISKT PYKPNGINVPKT VDKLAAALEHHH HHH (SEQ ID NO:15)

Example 2: Preparation of *Clostridium perfringens* strain CP1 pilus subunit null-mutants

[0071] The three pilus subunit genes (*cnaA*, *fimA* and *fimB*) were each insertionally inactivated in the virulent *Clostridium perfringens* strain CP1 by ClosTron mutagenesis (Heap, J.T., et al, *Methods Mol. Biol.* (2010), 646: 165-182), essentially as described previously (Yu, Q., Lepp, D., Mehdizadeh Gohari, I., Wu, T., Zhou, H., Yin, X., Yu, H., Prescott, J.F., Nie, S.P., Xie, M.Y., Gong, J., 2017. The Agr-like quorum sensing system is required for necrotic enteritis pathogenesis in poultry caused by Clostridium perfringens. *Infection and Immunity* 85(6): e00975-16), to generate CP1 null-mutants for each of the pilus subunit genes (CP1Δ*cnaA*, CP1Δ*fimA*, and CP1Δ*fimB*). Briefly, ClosTron intron-targeting regions were designed to insert at the following gene

positions using the Perutka algorithm implemented at www.clostron.com: base-pair (bp) 183 of the *cnaA* sense strand, bp 231 of the *fimA* sense strand, and bp 273 of the *fimB* sense strand. The intron-targeting regions were synthesized and cloned into ClosTron plasmid pMTL007C-E2 by DNA 2.0 (Menlo Park, CA, USA). The resultant
5 plasmids were separately electroporated into CP1 as described previously with minor modifications (Jirásková A, Vítek L, Fevery J, Ruml T, Branny P. 2005. Rapid protocol for electroporation of Clostridium perfringens. J Microbiol Methods 62:125–127). Briefly, after growth at 37°C anaerobically overnight in 5 ml TGY broth (3% tryptone, 2% glucose, 1% yeast extract), CP1 was subcultured into 50 ml TGY and grown to
10 exponential phase (optical density at 600 nm [OD 600], 0.8). The cells were harvested by centrifugation at 6,000 g for 10 min at 20°C and washed once in 10 ml sucrose electroporation buffer (SEB) (272 mM sucrose, 1 mM $MgCl_2$, 5 mM Na_2HPO_4, pH 7.4) and then resuspended in 5 ml SEB. Aliquots (0.2 ml) were mixed with 2 µg concentrated plasmid DNA in prechilled cuvettes (0.2-cm gap), and plasmid DNA was
15 introduced into the cells by electroporation (1,000 V, 25F) using a Bio-Rad GenePulser Xcell apparatus (Bio-Rad, Hercules, CA, USA). Immediately after transformation, the mixture was transferred into 1 ml of TGY broth and incubated anaerobically at 37°C for 3 h, followed by plating onto TGY agar containing 15 µg/ml thiamphenicol anaerobically at 37°C overnight for selecting transformants. The
20 resulting colonies were subcultured onto TGY agar containing 10 µg/ml erythromycin for selecting integrants and then passaged for 10 consecutive days to cure the shuttle vector. Those clones resistant to erythromycin but sensitive to thiamphenicol were chosen for further analysis.

Example 3: Animal trials

25 [0072] Two vaccination trials were carried out to assess the ability of the three purified His-tagged recombinant pilus subunits to protect against necrotic enteritis (NE) in a chicken challenge model. Commercial day-old male White Plymouth Rock broiler chickens were randomly divided into experimental groups (n=15-17) and housed in separate rooms within an isolation unit. A summary of the trial designs is shown in
30 Table 2. In addition, the CP1Δ*fimA*, and CP1Δ*fimB* mutants were assessed for virulence in the same model.

Table 2: Summary of vaccination trial designs

Trial	Antigens tested	Vaccination days	Injection site	Serum collection days	CP1 challenge days	Day of euthanasia
1	CnaA, FimA	8, 20	i.m.	8, 20, 31	28, 29	31

Trial	Antigens tested	Vaccination days	Injection site	Serum collection days	CP1 challenge days	Day of euthanasia
2	CnaA, FimB, CnaA+FimA+FimB	7, 14, 19	s.c.	7, 19, 29	26, 27	29

Trial 1:

[0073] The first trial included three groups of 18 birds vaccinated with either adjuvant-only control, CnaA or FimA. Each bird was injected intramuscularly (i.m.) in the pectoral muscle with 200 µl phosphate-buffered saline (PBS) containing Quil-A™ adjuvant (50 µg) and recombinant pilus polypeptide (50 µg) at days 8 and 20, and birds were euthanized on day 31.

[0074] Serum was collected from five birds from each group at days 8 (prior to immunization), and at days 20 and 31 (after immunization). Serum IgY titres against CnaA and FimA were determined by ELISA (enzyme-linked immunosorbent assay). *C. perfringens* recombinant pilus polypeptides were diluted to 10 µg/ml in 50 mM carbonate/bicarbonate coating buffer at pH 9.6, and 100 µl was added to each well of a 96-well MaxiSorp™ Immuno plate (Fisher Scientific). Wells were coated for 1 h at 37°C, followed by overnight at 4°C, washed three times with wash buffer (PBS containing 0.05% Tween 20), and then blocked in wash buffer containing 1% bovine serum albumin (BSA) (Sigma) for 2 h at 37°C. Two-fold serial dilutions of each serum sample diluted in wash buffer containing 1% BSA (1/64 to 1/65,536) were incubated in separate wells for 2 h at 37°C and then washed three times in wash buffer. Wells were incubated with goat anti-chicken IgY horseradish peroxidase (HRP)-conjugated polyclonal antibody, diluted 1:5,000 in wash buffer for 1 h at room temperature, and then washed three times in wash buffer. Substrate solution (0.2mg/ml 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Sigma) in 1X ABTS buffer (Sigma)) was added to each well and incubated for 30 min at room temperature. After the reaction was stopped with 0.5% sodium dodecyl sulfate (SDS), the absorbance was measured in a BioTek™ plate reader at 405 nm. Titers were calculated as the $\log_2$ value of the lowest serum dilution with an absorbance greater than twice that of the background wells, in which PBS containing 1% BSA was used in place of serum. Statistical differences between pre-immune and post-immune titers for each antigen among the different vaccination groups were determined by one-way ANOVA followed by Tukey's post-hoc test.

[0075] The results are shown in Figures 3A and B, respectively. The average serum response against CnaA in the CnaA-immunized group was significantly higher at d31

compared to the pre-immune birds (d8), however the overall increase was small. In the FimA-immunized group, the average response against FimA did not significantly increase after immunization. However, two of the birds did exhibit a high titre by d31.

[0076] Birds were fed an antibiotic-free starter ration containing 20% protein until experimental induction of necrotic enteritis (NE). At day 27, birds were fasted for 24 h, and then switched to an antibiotic-free turkey starter ration (28% protein) containing *C. perfringens* CP1 culture at days 28 and 29 prior to euthanasia on day 31. The infected ration was prepared daily in the morning and afternoon by mixing with *C. perfringens* CP1 culture, grown in fluid thioglycollate (FTG) (Difco) medium at 37°C for 15 h or 24 h, respectively, at a 2:1 (v/w) ratio. Following euthanasia, the small intestine (duodenum to ileum) of the bird was examined grossly for necrotic enteritis lesions and scored blindly from 1 to 6 using the system described by Keyburn et al (Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, Parker D, Di Rubbo A, Rood JI, Moore RJ. 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens. PLoS Pathog. 4:e26) as follows:

0, no gross lesions;
1, thin or friable walls;
2, focal necrosis or ulceration (1–5 foci);
3, focal necrosis or ulceration (6–15 foci);
4, focal necrosis or ulceration (16 or more foci);
5, patches of necrosis 2–3 cm long;
6, diffuse necrosis typical of field cases.

[0077] Statistical differences between necrotic enteritis (NE) scores among groups were determined by one-way ANOVA (analysis of variance) followed by Tukey's post-hoc test. The results, shown in Figure 4, indicate that all groups had similarly high average lesion scores. The average necrotic enteritis scores for the adjuvant-only control, CnaA-immunized and FimA-immunized groups were 3.1, 3.0 and 3.3, respectively.

[0078] Without being bound by theory, it is contemplated that the immunization at day 8 may have been subject to interference from maternal antibodies, and there may not have been time for the immunization at day 20 to elicit sufficient immune response prior to challenge with *C. perfringens* CP1. Therefore, a second vaccination trial was carried out including an additional immunization prior to challenge with *C. perfringens* CP1.

Trial 2:

[0079] The second trial consisted of four groups of 18 birds vaccinated subcutaneously (s.c.) with either adjuvant-only control, CnaA, FimB or a combination of CnaA, FimA and FimB. In this trial, each bird was immunized subcutaneously with 50 µg of recombinant polypeptide combined with 50 µg of Quil-A™ adjuvant at days 7, 14 and 19, and serum was collected at days 7, 19 and 29 for measurement of antibody titres. Birds were challenged in-feed with *Clostridium perfringens* strain CP1 on days 26 and 27 as described for Trial 1, and on day 29, birds were euthanized and intestinal lesions were scored.

[0080] A significant ($p<0.001$) serum antibody (IgY) response was observed at both days 19 and 29 in all of the immunized groups compared to the pre-immune controls (with the exception of the group immunized with FimB at day 19), and the magnitude of response was also much greater than in Trial 1. The results are shown in Figures 5A (anti-CnaA serum response), 5B (anti-FimA serum response) and 5C (anti-FimB serum response).

[0081] In addition, as seen in Figure 6, both the CnaA- and FimB-immunized groups had significantly lower necrotic enteritis scores (2 and 2.06, respectively) compared to the adjuvant control (3.75), when measured and scored as in Trial 1, indicating these antigens offered at least partial protection against necrotic enteritis. For the FimB antigen, the number of birds with severe disease (necrotic enteritis score > 2) was 33.3% compared to 93.7% in the control. Immunization with the combined subunits did not appear to reduce the severity of disease (average necrotic enteritis score = 3.7), despite eliciting a strong serum response against all three subunits, as seen in Figures 5A-C.

Challenge of chickens with Clostridium perfringens strain CP1 pilus subunit null-mutants

[0082] Three groups of 18 birds in Trial 2 which had not been immunized were challenged in-feed twice daily on days 26 and 27 with CP1, CP1Δ*fimA* or CP1Δ*fimB* prepared as described in Example 2. On day 29, the birds were euthanized and necrotic enteritis lesions were scored as described in Example 3. As seen from the results presented in Figure 7, neither the CP1Δ*fimA* nor the CP1Δ*fimB* mutant strain caused disease in the challenged birds, indicating that a functional pilus appears to be required for necrotic enteritis pathogenesis.

Example 4: Characterization of *Clostridium perfringens* pilus surface polypeptides

Clostridium perfringens strain CP1 and CP1 pilus subunit mutants:

[0083] Surface polypeptides were extracted from *Clostridium perfringens* strain CP1
5 and the pilus subunit mutants CP1Δ*cnaA,* CP1Δ*fimA* and CP1Δ*fimB* described in
Example 3, using the method of Chang, C., Huang, I.-H., Hendrickx, A.P.A., Ton-That,
H. 2013. Visualization of Gram-positive Bacterial Pili, In: Delcour, H.A. (Ed.) Bacterial
Cell Surfaces: Methods and Protocols. Humana Press, Totowa, NJ, 77-95. Strains
were grown overnight in TGY medium (3% tryptone, 2% glucose, 1% yeast extract)
10 anaerobically at 37°C, subcultured 1:100 into 10 ml TGY medium and grown to an
OD_{600} ~ 1. Cells were pelleted at 6,000 x g for 5 min and washed once in SMM buffer,
pH 6.8 (0.5M sucrose, 10mM $MgCl_2$, 10mM maleate). The bacterial pellet was
resuspended in 1 ml SMM buffer, to which was added 60 µl of 5U/µl of mutanolysin
(Sigma) in muramidase buffer (2mM acetic acid, 48mM sodium acetate) and 10 µl of
15 0.1M phenylmethylsulfonyl fluoride (PMSF) (Sigma). Following at least 4 h incubation
at 37°C with constant rotation, protoplasts were pelleted at 20,000 x g for 5 min, and
the supernatant fraction containing cell wall proteins was removed. Proteins were
precipitated by addition of 81 µl 100% (w/v) trichloroacetic acid (TCA) (Sigma) per ml
and incubation at 4°C overnight. Following centrifugation at 20,000 x g at 4°C for 20
20 min, the protein pellet was washed with acetone and slowly resuspended in 50 µl
sample loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 4% β-
mercaptoethanol, 3M urea, 0.01% bromophenol blue) at room temperature for at least
15 min.

[0084] Surface protein extracts (5 µl) were loaded onto Novex™ NuPAGE™ 3-8%
25 Tris-Acetate gels (Fisher Scientific) and electrophoresed at 150V for 1 h. Gels were
stained with Bio-Safe™ Coomassie stain (BioRad), or were transferred onto a
polyvinylene difluoride (PVDF) membrane at 350V for 1h in 1X transfer buffer (48 mM
Tris, 39 mM glycine, 20% methanol, 0.1% SDS). Chemiluminescent detection was
performed with the WesternBreeze™ Chemiluminescent kit (Life Technologies)
30 according to the manufacturer's instructions, using chicken anti-FimA serum (1:200)
as primary antibody, and a goat anti-chicken IgY alkaline phosphatase (AP)-
conjugated secondary antibody (1:2,000). The serum used as primary Ab was
obtained at sacrifice from a FimA-immunized chicken from Trial 1 (Example 3) that
subsequently exhibited a high anti-FimA titer or polyclonal antibodies raised in rabbits
35 against the recombinant pilus polypeptides described in Example 1.The results are
shown in Figures 8A-C.

[0085] It is known that Western blot analysis of SDS-PAGE separated sortase-dependent pili can produce a high-molecular weight (HMW) ladder-like pattern reflecting different polymer lengths, reflective of the mechanism by which the pilus is assembled at the cell surface. Pilin subunits are covalently linked by housekeeping and pilin-specific sortase enzymes, resulting in a growing heteropolymeric structure, which is eventually covalently linked to the cell wall peptidoglycan. The termination of assembly, and hence polymer length, is variable, giving rise to a characteristic high molecular weight ladder-like pattern when these pili are visualized by Western blotting. As seen in Figures 8B and C,, a ladder-like pattern indicative of a pilus structure was observed in a Western blot of surface polypeptides extracted from *Clostridium perfringens* strain CP1 but not in a corresponding Western blot of surface polypeptides extracted from the mutant strains, whether visualized with antibodies obtained from chicken serum or raised in rabbits.

Various Clostridium perfringens strains:

[0086] Extraction of surface polypeptides from five *C. perfringens* isolates that originated from poultry (CP1, JGS4141 and JGS4120) or non-poultry (Strain 13, ATCC13124) sources was performed as described above. Surface protein extracts (5 µl) were loaded onto two Novex™ NuPAGE™ 3-8% Tris-Acetate gels (Fisher Scientific) and electrophoresed at 150V for 1 h. One gel was used for staining with Bio-Safe™ Coomassie stain (BioRad), and the second gel was transferred onto a polyvinylene difluoride (PVDF) membrane at 350V for 1h in 1X transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, 0.1% SDS). Chemiluminescent detection was performed with the WesternBreeze™ Chemiluminescent kit (Life Technologies) according to the manufacturer's instructions, using chicken anti-FimA serum (1:200) as primary antibody, and a goat anti-chicken IgY alkaline phosphatase (AP)-conjugated secondary antibody (1:2,000). The serum used as primary Ab was obtained at sacrifice from a FimA-immunized chicken that subsequently exhibited a high anti-FimA titer.

[0087] The results are shown in Figures 9A-B. The presence (+) or absence (-) of the genetic locus (VR-10B (CA) locus) for the pilus subunit genes *cnaA*, *fimA* and *fimB* in each *Clostridium perfringens* strain had previously been determined by both microarray analysis and polymerase chain reaction (PCR) methodology (Lepp D et al, *Journal of Bacteriology* (2013) 195: 1152-1166). As seen in Figures 9A-B, strains which carry the pilus genetic locus in their genomes (JGS4141 and CP1) showed the characteristic ladder-like pattern of a pilus structure in extracted surface polypeptides (indicated by the vertical line to the right of the gel image in Figure 9B), when the

Western blot was visualized with chicken anti-FimA antibody, while other strains which do not carry the pilus genetic locus in their genome do not show this pattern. Visualization of smaller molecular weight bands in the extracts are likely due to unrelated antibodies present in the crude chicken serum. None of the extracts showed a band corresponding to the FimA polypeptide itself, whose expected location is indicated by an arrow to the right of the gel image in Figure 9B. This is not surprising, as surface-associated proteins would not be expected to include the FimA monomer, which is only found within cells.

Immunogold labeling of Clostridium perfringens strain CP1 and CP1 pilus subunit mutants:

[0088] Cells of *Clostridium perfringens* strain CP1 or of the CP1 null mutants CP1Δ*fimA*, and CP1Δ*fimB* were labeled with gold particles using an immunogold technique including rabbit anti-FimA as a primary antibody and 6 nm Colloidal Gold-AffiniPure™ Goat Anti-Rabbit IgG (H+L) (min X Hu,Ms,Rat Sr Prot) (Cedarlane) as secondary antibody, and examined by transmission electron microscopy, essentially as described previously (Chang, C., Huang, I.-H., Hendrickx, A.P.A., Ton-That, H. 2013. Visualization of Gram-positive Bacterial Pili, In: Delcour, H.A. (Ed.) Bacterial Cell Surfaces: Methods and Protocols. Humana Press, Totowa, NJ, 77-95). As seen in Figure 10, cells of the native CP1 strain show the presence of a pilus structure on the cell surface, while cells of the CP1Δ*fimA*, and CP1Δ*fimB* mutants lack such structures.

[0089] The embodiments described herein are intended to be illustrative of the present compositions and methods and are not intended to limit the scope of the present invention. Various modifications and changes consistent with the description as a whole and which are readily apparent to the person of skill in the art are intended to be included. The appended claims should not be limited by the specific embodiments set forth in the examples, but should be given the broadest interpretation consistent with the description as a whole.

CLAIMS

1. An isolated *Clostridium perfringens* pilus polypeptide.

2. The isolated *Clostridium perfringens* pilus polypeptide according to claim 1, wherein the pilus polypeptide is a CnaA polypeptide.

3. The isolated *Clostridium perfringens* pilus polypeptide according to claim 2, wherein the CnaA polypeptide is selected from a polypeptide having an amino acid sequence selected from SEQ ID NO:10 and SEQ ID NO:13; a polypeptide encoded by a polynucleotide having a nucleotide sequence selected from SEQ ID NO:1, SEQ ID NO:4 and SEQ ID NO:7; and a polypeptide encoded by a polynucleotide which hybridizes under at least moderately stringent conditions to a polynucleotide having a sequence selected from SEQ ID NO:1, SEQ ID NO:4 and SEQ ID NO:7.

4. The isolated *Clostridium perfringens* pilus polypeptide according to claim 1 wherein the pilus polypeptide is a FimA polypeptide.

5. The isolated *Clostridium perfringens* pilus polypeptide according to claim 4, wherein the FimA polypeptide is selected from a polypeptide having an amino acid sequence selected from SEQ ID NO:11 and SEQ ID NO:14; a polypeptide encoded by a polynucleotide having a nucleotide sequence selected from SEQ ID NO:2, SEQ ID NO:5 and SEQ ID NO:8; and a polypeptide encoded by a polynucleotide which hybridizes under at least moderately stringent conditions to a polynucleotide having a sequence selected from SEQ ID NO:2, SEQ ID NO:5 and SEQ ID NO:8.

6. The isolated *Clostridium perfringens* pilus polypeptide according to claim 1 wherein the pilus polypeptide is a FimB polypeptide.

7. The isolated *Clostridium perfringens* pilus polypeptide according to claim 6, wherein the FimB polypeptide is selected from a polypeptide having an amino acid sequence selected from SEQ ID NO:12 and SEQ ID NO:15; a polypeptide encoded by a polynucleotide having a nucleotide sequence selected from SEQ ID NO:3, SEQ ID NO:6 and SEQ ID NO:9; and a polypeptide encoded by a polynucleotide which hybridizes under at least moderately stringent conditions to a polynucleotide having a sequence selected from SEQ ID NO:3, SEQ ID NO:6 and SEQ ID NO:9.

8. The isolated *Clostridium perfringens* pilus polypeptide according to claim 1 wherein the pilus polypeptide is an assembled pilus.

9. The isolated *Clostridium perfringens* pilus polypeptide according to claim 9 wherein the assembled pilus comprises one or more subunits each individually selected from a CnaA polypeptide, a FimA polypeptide and a FimB polypeptide.

10. An immunogenic polypeptide selected from an isolated *Clostridium perfringens* pilus polypeptide according to any one of claims 1 to 9, a variant of the pilus polypeptide; a fragment of the pilus polypeptide; and a fragment of the variant, wherein the pilus polypeptide, the variant, the fragment of the polypeptide and the fragment of the variant are immunogenic in poultry.

11. The immunogenic polypeptide according to claim 10 wherein the variant has at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99% or at least 99.9% sequence identity to the pilus polypeptide.

12. A polynucleotide comprising a sequence encoding an immunogenic polypeptide according to claim 10 or 11.

13. A vector comprising a polynucleotide according to claim 12, wherein the vector is configured for expression of the immunogenic polypeptide in a host cell.

14. A vaccine for the treatment or prevention of necrotic enteritis in poultry, the vaccine comprising at least one immunogenic polypeptide according to claim 10 or 11.

15. A vaccine for the treatment or prevention of *Clostridium perfringens* infection in poultry, the vaccine comprising at least one immunogenic polypeptide according to claim 10 or 11.

16. Use of an immunogenic polypeptide according to claim 10 or 11 in the preparation of a medicament for the treatment or prevention of necrotic enteritis in poultry.

17. Use of an immunogenic polypeptide according to claim 10 or 11 in the preparation of a medicament for the treatment or prevention of *Clostridium perfringens* infection in poultry.

18. A method of treatment or prevention of necrotic enteritis in poultry, the method comprising administering to the poultry an effective amount of an an immunogenic polypeptide according to claim 10 or 11 or an effective amount of a vaccine according to claim 14.

19. A method of treatment or prevention of *Clostridium perfringens* infection in poultry, the method comprising administering to the poultry an effective amount of an immunogenic polypeptide according to claim 10 or 11 or an effective amount of a vaccine according to claim 15.

20. Use of an immunogenic polypeptide according to claim 10 or 11 as a vaccine for the treatment or prevention of necrotic enteritis in poultry.

21. Use of an immunogenic polypeptide according to claim 10 or 11 as a vaccine for the treatment or prevention of *Clostridium perfringens* infection in poultry.

22. An antibody which binds selectively to an immunogenic polypeptide according to claim 10 or 11.

23. A method of detecting *Clostridium perfringens* infection in poultry by obtaining a biological sample from the poultry and detecting in the biological sample the presence of an antibody according to claim 22.

24. A method of detecting an immunogenic polypeptide according to claim 10 or 11 comprising exposing the immunogenic polypeptide to an antibody according to claim 22 and detecting binding of the immunogenic polypeptide to the antibody.

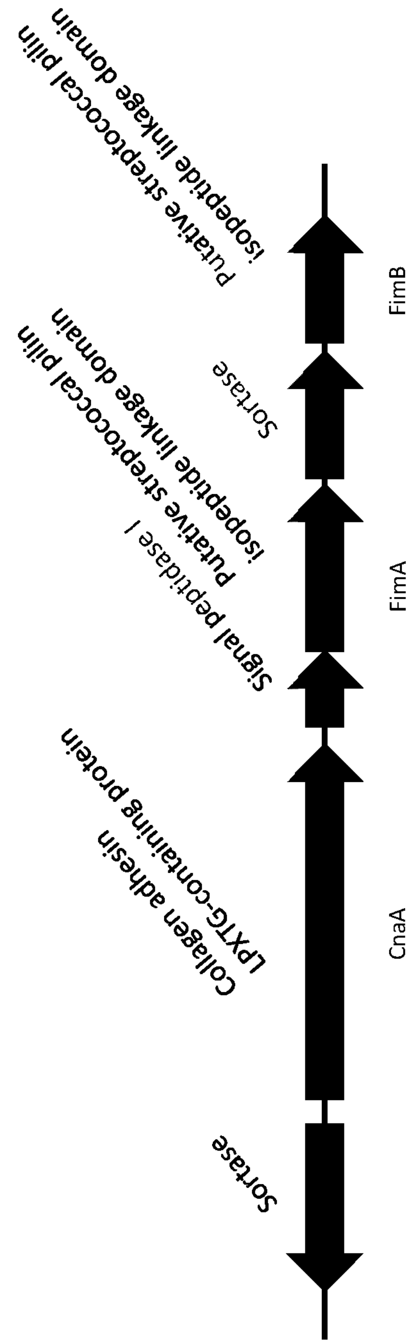

FIG. 1

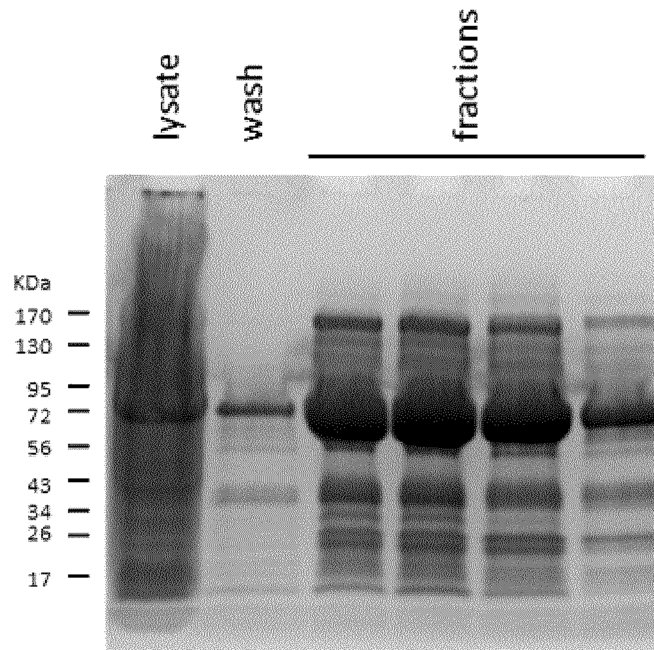

FIG. 2A

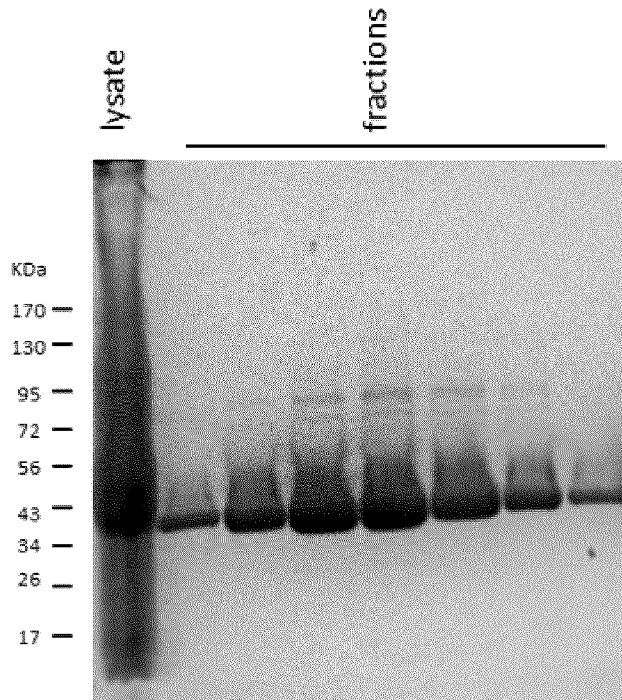

FIG. 2B

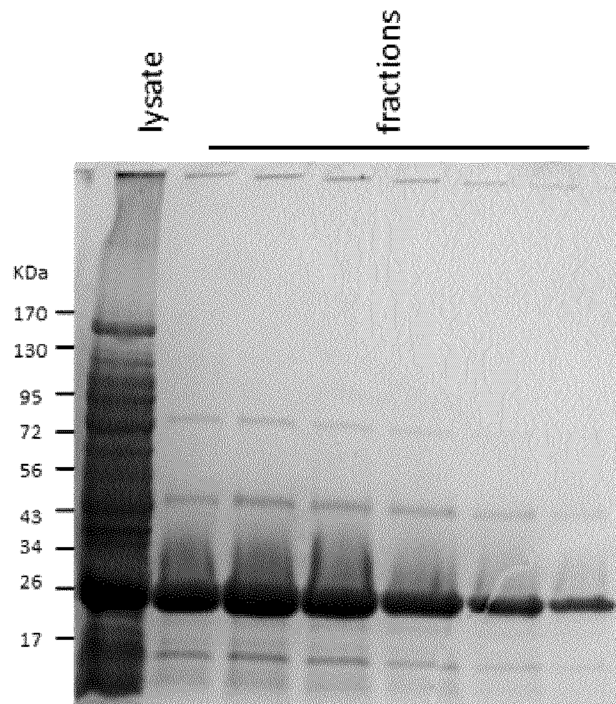

FIG. 2C

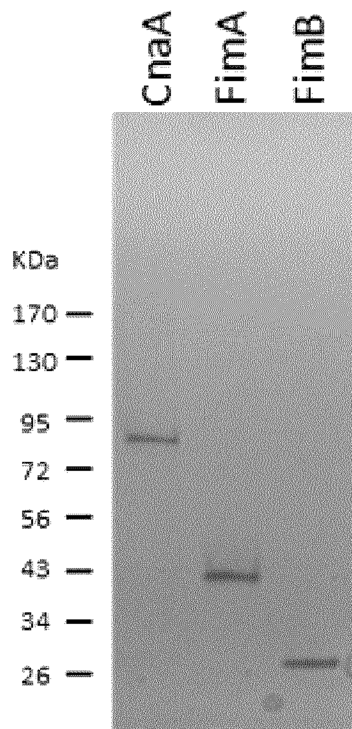

FIG. 2D

FIG. 3A

FIG. 3B

FIG. 4

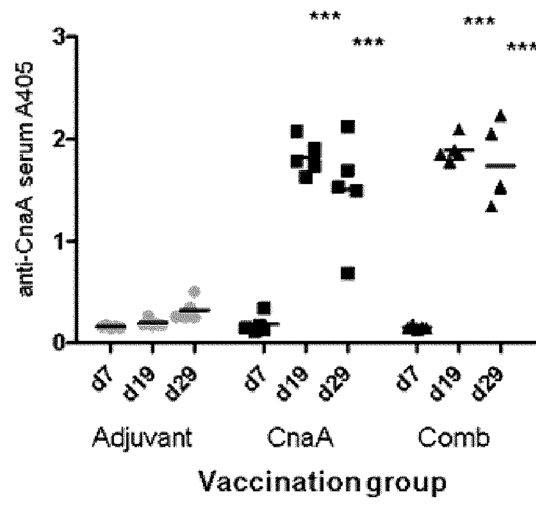

FIG. 5A

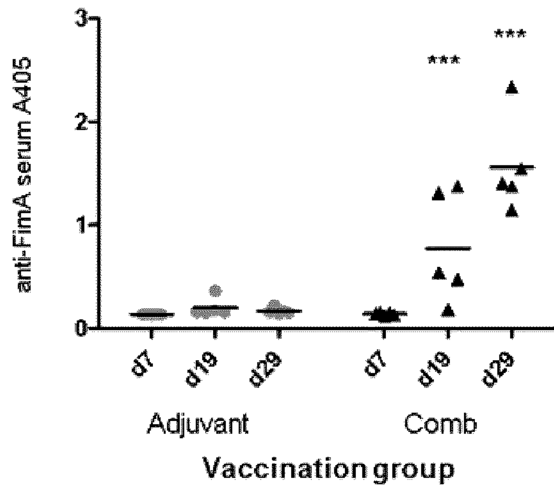

FIG. 5B

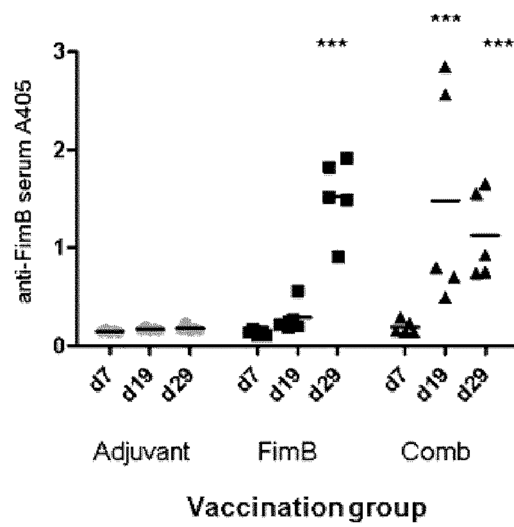

FIG. 5C

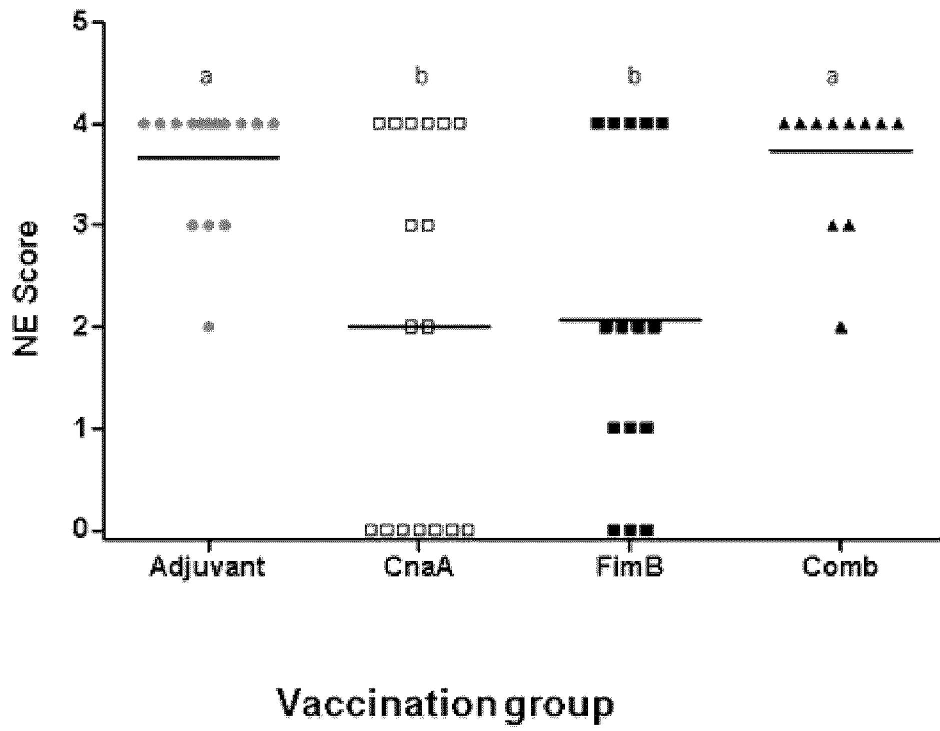

FIG. 6

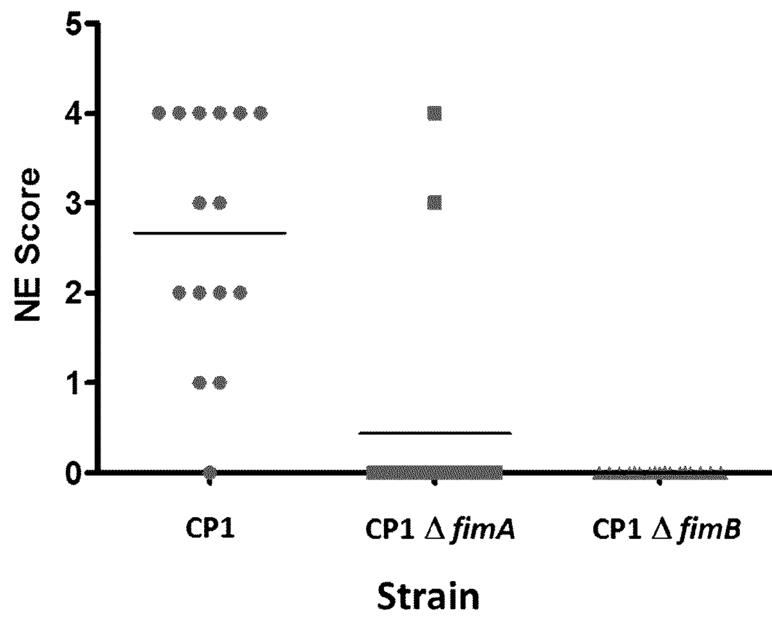

FIG. 7

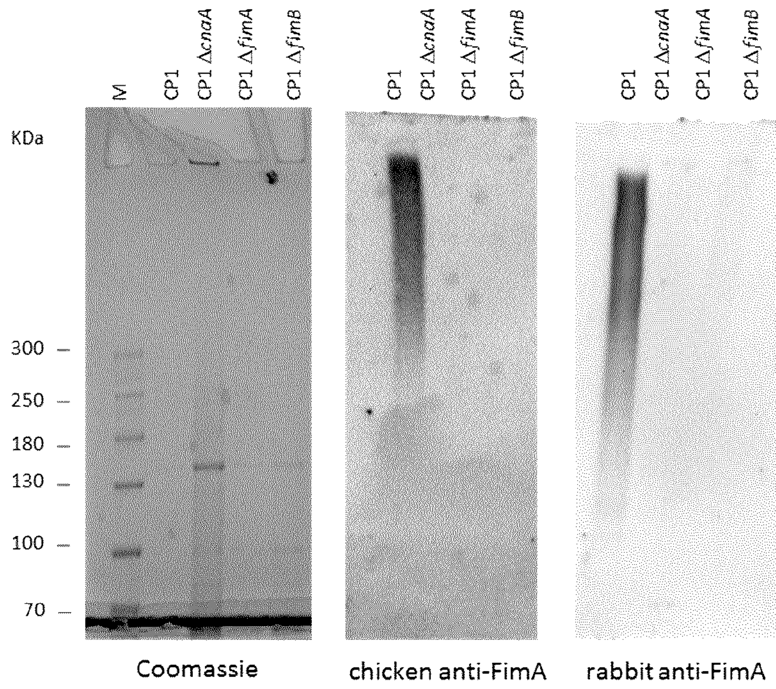

FIG. 8A **FIG. 8B** **FIG. 8C**

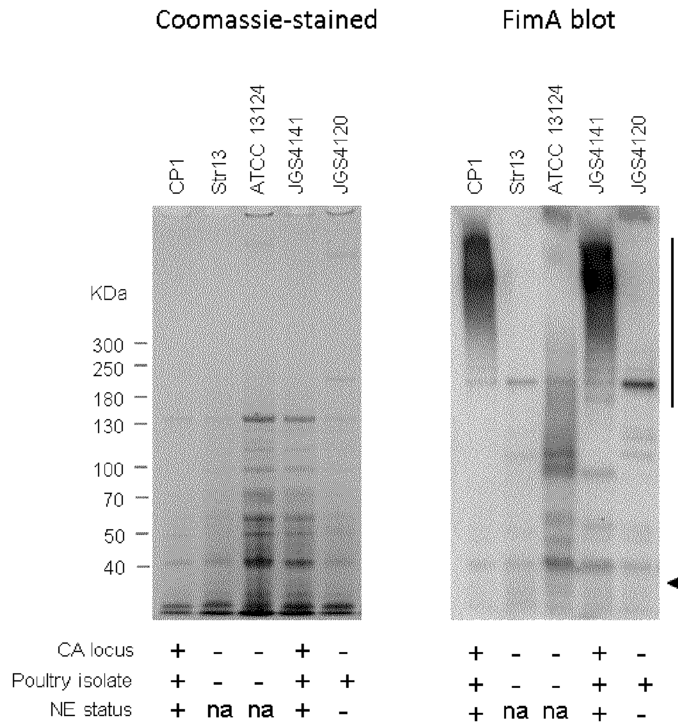

FIG. 9A **FIG 9B**

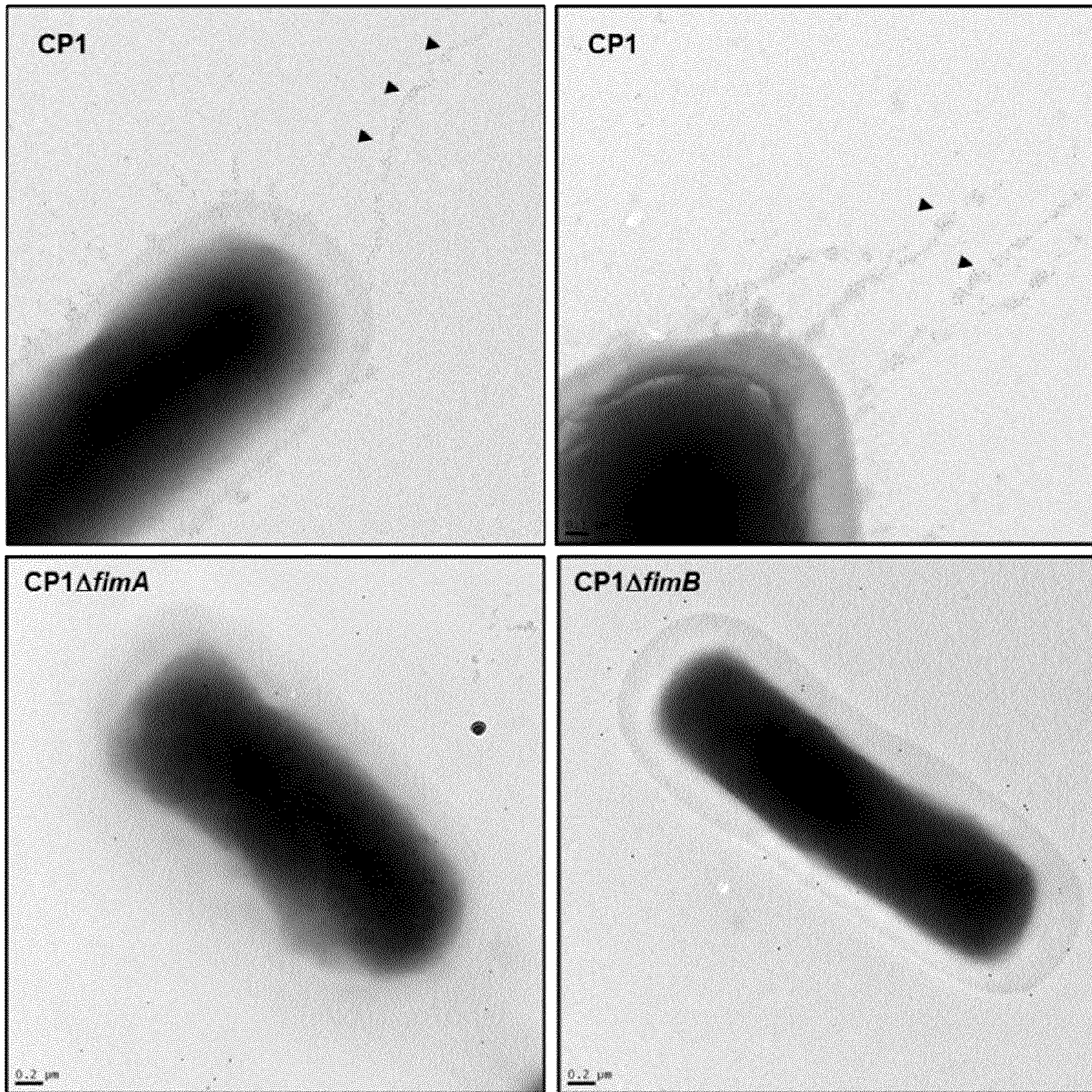

FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2018/050643

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C07K 14/33*** (2006.01) , ***A61K 39/08*** (2006.01) , ***A61P 31/04*** (2006.01) , ***A61P 37/04*** (2006.01) , ***C07K 16/12*** (2006.01) , ***C12N 15/31*** (2006.01) , ***C12N 15/85*** (2006.01) , ***G01N 33/569*** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Keywords used across the whole IPC.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Questel Orbit, USPTO, Espacenet, Canadian Patent Database, Pubmed, Google Scholar, GenomeQuest, keywords: Clostridium perfringens, pilus, polypeptide, CnaA, FimA, FimB, SEQ ID NOs: 1-15.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	"*Putative collagen adhesin [Clostridium perfringens]*". GenBank Protein Accession No. ALJ54440, 25 November 2015 (25-11-2015), [online] [retrieved on 23 July 2018 (23-07-2018)]. Retrieved from the Internet: https://www.ncbi.nlm.nih.gov/protein/ALJ54440 whole document	1-3, 8-13, 22 and 24
X	"*Hypothetical protein BXT94_03025 [Clostridium perfringens]*". GenBank Protein Accession No. AQW25808, 3 March 2017 (03-03-2017), [online] [retrieved on 23 July 2018 (23-07-2018)]. Retrieved from the Internet: https://www.ncbi.nlm.nih.gov/protein/AQW25808 whole document	1, 4, 5, 8-13, 22 and 24
X	"*Hypothetical protein BXT94_03035 [Clostridium perfringens]*". GenBank Protein Accession No. AQW25810, 3 March 2017 (03-03-2017), [online] [retrieved on 23 July 2018 (23-07-2018)]. Retrieved from the Internet: <https://www.ncbi.nlm.nih.gov/protein/AQW25810> whole document	1, 6-13, 22 and 24

☑ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 July 2018 (23-07-2018)	03 August 2018 (03-08-2018)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 819-953-2476

Authorized officer
Yolanda Romsicki (819) 639-7648

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PRESCOTT, J. F. et al., "*The pathogenesis of necrotic enteritis in chickens: what we know and what we need to know: a review*". Avian Pathology, 31 May 2016 (31-05-2016), Vol. 45, pp. 288-294, ISSN 1465-3338 whole document	1-24

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Isolated *Clostridium perfringens* pilus polypeptides are known in the art. Therefore, the claims lack unity *a posteriori* and are directed towards a plurality of inventive concepts as follows:
Invention 1
Claims 1-3 (all partly) and 8-24 (all partly) are directed towards an isolated *Clostridium perfringens* pilus polypeptide having SEQ ID NO: 10, a polynucleotide encoding said polypeptide, a vector comprising said polynucleotide, and vaccines, uses and methods related thereto.

Continued on extra sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☑ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of Box No. III

Invention 2

Claims 1-3 (all partly) and 8-24 (all partly) are directed towards an isolated *Clostridium perfringens* pilus polypeptide having SEQ ID NO: 13, a polynucleotide encoding said polypeptide, a vector comprising said polynucleotide, and vaccines, uses and methods related thereto.

Invention 3

Claims 1 (partly), 4 (partly), 5 (partly) and 8-24 (all partly) are directed towards an isolated *Clostridium perfringens* pilus polypeptide having SEQ ID NO: 11, a polynucleotide encoding said polypeptide, a vector comprising said polynucleotide, and vaccines, uses and methods related thereto.

Invention 4

Claims 1(partly), 4 (partly), 5 (partly) and 8-24 (all partly) are directed towards an isolated *Clostridium perfringens* pilus polypeptide having SEQ ID NO: 14, a polynucleotide encoding said polypeptide, a vector comprising said polynucleotide, and vaccines, uses and methods related thereto.

Invention 5

Claims 1 (partly) and 6-24 (all partly) are directed towards an isolated *Clostridium perfringens* pilus polypeptide having SEQ ID NO: 12, a polynucleotide encoding said polypeptide, a vector comprising said polynucleotide, and vaccines, uses and methods related thereto.

Invention 6

Claims 1 (partly) and 6-24 (all partly) are directed towards an isolated *Clostridium perfringens* pilus polypeptide having SEQ ID NO: 15, a polynucleotide encoding said polypeptide, a vector comprising said polynucleotide, and vaccines, uses and methods related thereto.

The claims must be limited to one inventive concept as set out in PCT Rule 13.