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DESCRIPTION

Field of the Invention

[0001] The present invention relates to a method for detecting *C. perfringens* induced diseases in animals. More specifically, the present invention pertains to a method for determining whether or not an animal or an animal population suffers from *C. perfringens* induced diseases in clinical or sub-clinical state.

Background of the Invention

[0002] *Clostridium perfringens* is an ubiquitous pathogen that uses an arsenal of toxins to cause histotoxic and intestinal infections in animals and also in humans. *C. perfringens* is a Gram-positive, rod-shaped, spore forming, oxygen-tolerant anaerobe. Not all *C. perfringens* strains are virulent. The virulent C. *perfringens* strains are traditionally classified into five toxin types (A, B, C, D and E), based on the production of four suspected major toxins (alpha, beta, epsilon and iota). Depending on the toxins produced (major and additional toxins like NetB, Cpb2 and others), the *C. perfringens* sub-species specific syndromes/diseases can be induced in different host organisms [Rood, J. I. (1998) "Virulence genes of Clostridium perfringens"; Annual Review of Microbiology 52: 333-360]. The toxins are encoded by polynucleotide sequences located on the chromosome and/or on toxin plasmids [Popoff, M. R. and P. Bouvet (2013). "Genetic characteristics of toxigenic Clostridia and toxin gene evolution" Toxicon 75: 63-89].

[0003] As an animal pathogen, *C. perfringens* is responsible for several serious diseases including avian necrotic enteritis, which drains approximately US$ 6 billion/year from the global agricultural system [Wade, B., Keyburn, A.L. (2015), "The true cost of necrotic enteritis" World Poultry 31, 16-17].

[0004] Necrotic enteritis (NE) is an enteric disease of poultry that was first described in 1961. NE in chickens manifests as an acute or chronic enterotoxaemia. The acute disease results in significant levels of mortality due to the development of necrotic lesions in the gut wall, whereas the chronic disease leads to a significant loss of productivity and welfare. Early studies on NE suggested that the main virulence factor involved in the disease was the alpha-toxin (known as Cpa or Plc), which has phospholipase C and sphingomyelinase activity [Keyburn, A. L. et al. (2006) "Alpha-toxin of Clostridium perfringens is not an essential virulence factor in necrotic enteritis in chickens", Infection and Immunity 74(11): 6496-6500]. All *C. perfringens* strains harbor the gene encoding the alpha toxin [Rood, J. I. (1998) "Virulence genes of Clostridium perfringens", Annual Review of Microbiology 52: 333-360; Titball, R. W., et al. (1999) "The Clostridium perfringens α-toxin." Anaerobe 5(2): 51-64]. Recent studies however showed that alpha-toxin seems not to be an essential virulence factor since alpha

toxin mutant strains were capable of causing NE, which questions the role of alpha-toxin in the disease in general. In more recent studies, the novel pore forming toxin, NetS, has been suggested to play a major key role in the development of this disease [Keyburn, A. L. et al. (2008) "NetS, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens" PLoS Pathogens 4(2)].

[0005] NE is known to affect broilers, laying hens, turkeys, and quail. The clinical form is most commonly seen in two to five week-old broilers. Typically, this is also near the time that diets are switched from starter feed to grower feed and near the transition period from the maternal immune system to the adaptive immune system, respectively, so opportunistic *C. perfringens* may take advantage of this transitional period in the intestinal environment and proliferate [Timbermont, L. et al. (2011) "Necrotic enteritis in broilers: An updated review on the pathogenesis." Avian Pathology 40(4): 341-347].

[0006] Apart from the mere association of the virulence factors per se to the incidence of NE, there is still a great burden to show the mechanistic link between these factors to the establishment of the disease in birds or of other *C. perfringens* induced diseases in other (farm) animals.

[0007] Moreover, relating subclinical NE infection to any of these virulence factors is extremely difficult to achieve since birds are void of signs to warrant their examination earlier before slaughter. Therefore the need for a method for early detection of NE, and most importantly, the subclinical forms of NE, remains imperative. Similar considerations apply for other *C. perfringens* induced diseases of animals, in particular of animals in life stock production such as enteritis in pigs, horses, foals, goats, rabbits, lambs, dogs and cattle; diarrhea in pigs; enterotoxaemia in sheep, goat and cattle; necrotizing enteritis in piglets, lambs, foals and calves (neonatal); typhlocolitis in horse; fatal canine hemorrhagic gastroenteritis; fatal foal necrotizing enterocolitis; gas gangrene (*clostridial myonecrosis*) in sheep, cattle, horses and other species [Rood, J. I. (1998) "Virulence genes of Clostridium perfringens" Annual Review of Microbiology 52: 333-360].

[0008] Bovine enterotoxaemia caused by Clostridium perfringens is a sudden death syndrome with necro-hemorrhagic lesions in the small intestine, which mainly affects suckling calves and veal calves [Goossens, E. et al. (2014), "Clostridium perfringens strains from bovine enterotoxaemia cases are not superior in in vitro production of alpha toxin, perfringolysin O and proteolytic enzymes" BMC Veterinary Research 10; Lebrun M, Mainil JG, Linden A: "Cattle enterotoxaemia and Clostridium perfringens: description, diagnosis and prophylaxis" Vet Rec 2010, 167(1):13-22]. In veal calves, predominantly beef cattle breeds are affected. The syndrome accounts for approximately 20% of the mortalities in these calves, compared to 4% in dairy and mixed breed veal calves [Pardon B, De Bleecker K, Hostens M, Callens J, Dewulf J, Deprez P: "Longitudinal study on morbidity and mortality in white veal calves in Belgium" BMC Vet Res 2012, 8:26].

[0009] Current methods are based on the examination of individualized animals and make use

of necropsy and subsequent histopathology or tests to identify the pathogen. However, monitoring of tissue biomarkers or blood biomarkers is, because of its invasive nature, time-consuming and impractical, when large numbers of samples are involved like for farm animals.

[0010] Recently published European Patent Application EP 3 112 474 A1 describes a method for detecting avian NE by isolating microvesicles from an avian sample and subsequently determining the presence and/or the level of RNA markers indicative for NE. The avian sample may be a bodily fluid or a bodily excrement and the marker indicative for NE is selected from specific *C. perfringens* and host sequences, homologues and fragments thereof. However, the microvesicles have to be isolated and lysed prior to analysis and quantification of the RNA markers.

[0011] It was thus a remaining need to provide a fast and reliable, non-invasive *ante mortem* method for determining whether or not an animal population suffers from diseases induced by *C. perfringens* that can be performed at low cost and with minimal effort.

Summary of the Invention

[0012] Accordingly, one object of the present invention is to provide a method for detecting *C. perfringens* induced diseases in an avian flock, the method comprising:

1. a) collecting sample material of the avian flock at consecutive points in time;
2. b) determining the amount of a first marker and a second marker contained in the sample material; and
3. c) determining the ratio of the first marker to the second marker contained in the sample material;

wherein the first marker comprises a polynucleotide sequence being specific for the *C. perfringens* sub-species inducing the targeted disease;

and the second marker comprises a polynucleotide being specific for the species *C. perfringens;* and

wherein an increase in the ratio of the first marker to the second marker in the analyzed sample material over time is an indication of the targeted disease.

[0013] Embodiments of the method of the invention are disclosed in the appended claims.

[0014] In the following, the crucial aspects of the present invention are described in detail.

Detailed Description of the Invention

Detection of C. perfringens induced diseases in animals

[0015] The present inventors have unexpectedly found that the ratio of a first marker polynucleotide to a second marker polynucleotide in animal excrements correlates with the manifestation of diseases being induced by the bacterium *C. perfringens.* More specifically, the present inventors have found that an increase in the ratio of the above-mentioned markers over time is an indication of the targeted disease.

[0016] Accordingly, the present invention pertains to a method for detecting *C. perfringens* induced diseases in an avian flock, the method comprising:

1. a) collecting sample material of the avian flock at consecutive points in time;
2. b) determining the amount of a first marker and a second marker contained in the sample material; and
3. c) determining the ratio of the first marker to the second marker contained in the sample material;

wherein the first marker comprises a polynucleotide sequence being specific for the *C. perfringens* sub-species inducing the targeted disease;

and the second marker comprises a polynucleotide being specific for the species *C. perfringens;* and

wherein an increase in the ratio of the first marker to the second marker in the analyzed sample material over time is an indication of the targeted disease.

[0017] As used herein, the term "polynucleotides" refers to DNA or RNA. In a particularly preferred embodiment, the term "polynucleotides" refers to DNA.

[0018] As used herein, the term "disease" refers to any abnormal condition in an animal that interferes with its vital physiological processes, caused by pathogenic *C. perfringens* strains. The term "disease" corresponds to an increase of pathogenicity factors and thus also includes early stages of the targeted disease as well as a certain risk that the targeted disease will break out. In accordance therewith and as used in the context of the present invention, the term "targeted disease" refers to the specific *C. perfringens* induced disease (occurring in specific animal species) for which the animal sample is to be analyzed.

Markers

[0019] The **first marker** comprises a polynucleotide sequence being specific for the *C.*

perfringens sub-species inducing the targeted disease. That is, the first marker represents a specific and conserved region determining the virulence and pathogenicity of the selected *C. perfringens* sub-species. In a preferred embodiment, the first marker constitutes a virulence factor for the targeted disease.

[0020] The first marker may be either a marker gene encoding a specific toxin or homologues or fragments thereof, or, alternatively, a pathogenicity island. In the context of the present invention, the term "homologue" refers to a polynucleotide sequence having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the respective polynucleotide or marker gene. The term "fragment" refers to a polynucleotide sequence being truncated by not more than 100 or 80, preferably by not more than 70, especially by not more than 60, most preferably by not more than 50 nucleotides compared to the respective polynucleotide or marker gene.

[0021] As used herein, the term "pathogenicity island" (PAI) refers to discrete genetic units carrying genes encoding for one or more virulence factors [Hacker et al. (1997) "Pathogenicity islands of virulent Bacteria: structure and impact on microbial evolution", Molecular Microbiology 23(6): 1089-1097]. The PAI may either contain genes to regulate the virulence genes encoded on the PAI or it may contain genes to regulate genes outside of the PAI. Pathogenicity islands are incorporated in the genome (chromosomally or extrachromosomally) of the pathogenic *C. perfringens* strain.

[0022] The first marker may be located on a toxin plasmid of *C. perfringens* and/or on the *C. perfringens* chromosome.

[0023] As an example, the *cpe* gene may be used as a first marker gene. Said *cpe* gene may be found in a variable region on the chromosome in some *C. perfringens* strains and on large plasmids in other strains [Petit et al. (1999) "Clostridium perfringens: toxinotype and genotype", Trends in Microbiology Vol. 7, No. 3, 104-110].

[0024] In a particularly preferred embodiment, the marker gene is located on a toxin plasmid of *C. perfringens.*

[0025] *C. perfringens* strains can carry multiple large toxin and antibiotic resistance plasmids, whereby these plasmids are closely related sharing up to 35 kb of almost identical sequences [Wisnieswski et al. (2017) "The Tcp conjugation system of Clostridium perfringens", Plasmid 91, 28-36]. Plasmid-encoded toxins are for example the beta-toxin, the beta2-toxin, the epsilon-toxin, the iota-toxin, NetS and TpeL [Li, J., et al. (2013); "Toxin plasmids of Clostridium perfringens"; Microbiology and Molecular Biology Reviews 77(2): 208-233].

[0026] The main diseases associated with C. *perfringens* in animals and the key toxins are listed in the following table [Popoff, M. R. (2014) "Clostridial pore-forming toxins: Powerful virulence factors." Anaerobe 30: 220-238; Rood, J. I. (1998) "Virulence genes of Clostridium perfringens" Annual Review of Microbiology 52: 333-360; Gohari, I. M. et al. (2015) "A novel

pore-forming toxin in type A Clostridium perfringens is associated with both fatal canine hemorrhagic gastroenteritis and fatal foal necrotizing enterocolitis" PLoS ONE 10(4); Keyburn, A. L., et al. (2008) "NetS, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens" PLoS Pathogens 4(2)]:

Disease	Host	Virulence factor	Reference
gas gangrene (clostridial myonecrosis)	Human	Perfringolysin (Theta-Toxin), Alpha toxin (Cpa)	Rood, 1998
food poisoning (food borne poisoning), sporadic diarrhea	Human	Enterotoxin (Cpe)	Popoff, 2014
Diarrhea	Pig	Enterotoxin (Cpe)	Popoff, 2014
enteritis necroticans (pigbel; Darmbrand)	Human	Beta-toxin (Cpb)	Rood, 1998
Enterotoxemia	Sheep, goat, cattle	Epsilon toxin (Etx)	Popoff, 2014
Unidentified	Human, animals	Delta toxin	Popoff, 2014
Necrotizing enteritis	Piglet, calve	Beta-toxin (Cpb)	Popoff, 2014
Enterotoxemia (struck)	Sheep	Beta-toxin (Cpb)	Popoff, 2014
Necrotizing enteritis	Piglet	Beta2-toxin (Cpb2)	Popoff, 2014
Typhlocolitis	Horse	Beta2-toxin (Cpb2)	Popoff, 2014
fatal canine hemorrhagic gastroenteritis	Canine	NetF	Gohari, 2015
fatal foal necrotizing enterocolitis	Foal	NetF	Gohari, 2015
avian necrotic enteritis	Avians	NetB	Keyburn, 2008

[0027] Accordingly, the first marker gene may also be selected from the group consisting of *cpe,* beta-toxin (*cpb*), beta2-toxin (*cpb2*), epsilon-toxin (*etx*), *netF* and *netB* and homologues and fragments of these genes. Alternatively, polynucleotide sequences comprising one of the aforementioned genes may also be used. Preferably, these polynucleotide sequences are being elongated by not more than 100 base pairs or not more than 80 base pairs, preferably by not more than 70 base pairs or by not more than 60 base pairs, especially by not more than 50 base pairs or not more than 40 base pairs and most preferably by not more than 30 base pairs compared to the respective marker genes.

[0028] In a preferred embodiment, the gene *netB* is used as the first marker.

[0029] The **second marker** comprises a polynucleotide sequence being specific for the species *C. perfringens* in general and represents a conserved and specific region on the *C. perfringens* genome (chromosomally or extrachromosomally).

[0030] The second marker may be a polynucleotide sequence or a specific marker gene located on a plasmid of *C. perfringens* or on the *C. perfringens* chromosome. A marker gene located on the *C. perfringens* chromosome is particularly preferred.

[0031] Preferably, the second marker gene is selected from the group consisting of the genes *cpa, 16S rDNA, virR, virS, pfoA* and homologues and fragments of these genes. Alternatively, polynucleotide sequences comprising one of the aforementioned genes may also be used.

[0032] The gene *cpa* is particularly preferred as the second marker.

[0033] The *C. perfringens* induced disease may be selected from the group consisting of avian necrotic enteritis; enteritis in dogs, pigs, horses, foals, goats, rabbits, lambs and cattle; diarrhea in pigs; enterotoxemia in sheep, goat and cattle; necrotizing enteritis in piglets, lambs, foals and calves (neonatal); typhlocolitis in horse; fatal canine hemorrhagic gastroenteritis; fatal foal necrotizing enterocolitis; gas gangrene (*clostridial myonecrosis*) in sheep, cattle, horses and other spp. or in humans and human *enteritis necroticans.* The above method is particularly suitable for detecting avian necrotic enteritis.

[0034] Moreover, the method according to the present invention is particularly suitable for detecting *C. perfringens* induced diseases being in sub-clinical or latent state. In such sub-clinical or latent forms of the *C. perfringens* infections, no overt clinical signs are present and usually there is no peak mortality. In a particularly preferred embodiment, the above method is used for detecting avian necrotic enteritis is sub-clinical or latent state.

Determination of C. perfringens induced diseases in individual animals

[0035] In a related aspect of the disclosure that does not form part of the invention, the method of the present invention may be used for determining whether or not an individual animal suffers from a *C. perfringens* induced disease. In that case, the sample material originates from an individual animal.

[0036] The individual animal may for example be a pet or domestic animal, a farm animal as occurring in life stocks, a wild-living animal or a zoo animal. Further, animal individuals being transported for slaughter or for re-location may be examined using the above method.

[0037] As an example, sample material originating from an individual dog collected at consecutive points in time can be analyzed in accordance with the above method in order to

determine whether or not the dog suffers from fatal canine hemorrhagic gastroenteritis. In this specific case, *netF* is a suitable first marker and *cpa* is a suitable second marker.

[0038] The sample material is selected from the group consisting of dust samples, swab samples, litter samples, liquid manure samples, fur samples, feather samples, skin samples and samples of bodily excrements and solutions or suspensions thereof. Bodily excrements are urine, fecal or cecal excrements. In a preferred embodiment, the sample material is feces.

[0039] In general, the term "litter" is to be understood as a mixture of animal excrements with the bedding material.

[0040] As used in the context of this aspect, the term "litter samples" refers to excremental droppings from an individual animal. Further, in the context this embodiment, the term "liquid manure samples" refers to an excremental sample containing feces and urine from an individual animal.

[0041] Samples from individual animals can be taken either directly from the animal, e.g. with swabs.

[0042] Alternatively and especially in case of single-housed animals, the sample material can be collected from the floor of the pen, cage or slat. The sample material has to be assignable to the investigated animal.

[0043] Suitable sample volumes are, for example, 0.05 ml to 20 ml or 0.1 to 20 ml, in particular 0.2 to 10 ml, preferably 0.5 to 5 ml. Suitable sample masses are, for example 0.05 g to 20 g or 0.1 to 20 g, in particular 0.2 to 10 g, preferably 0.5 to 5 g.

Determination of C. perfringens induced diseases in animal populations

[0044] The inventive method is used for determining whether or not an animal population suffers from a *C. perfringens* induced disease.

[0045] As used herein, the term "animal population" refers to a group of animal individuals belonging to the same species. The animal population may for example be a group of pets or domestic animals as occurring in animal breeding, a group of farm animals as occurring in life stock production or in life stock breeding, or a group of wild-living animals or zoo animals.

[0046] The animal population according to the invention is an avian flock. In a preferred embodiment, the animal population is an animal flock as occurring in life stock production processes. In other aspects not covered by the present invention, the animal population or the animal flock can be a flock of sheep, goat or cattle, a flock of horses or a flock of pigs.

[0047] The method of the present invention is particularly suitable for determining the health

status of an animal population via bulk testing. As used herein, the term "bulk testing" refers to a test method, wherein the sample material is a pooled sample of an animal population. A "pooled sample" is to be understood as a composite sample from randomly selected separate samples, one sample taken with one or several moistened fabric swabs or pooled samples made up of separate samples of fresh samples taken at random from a number of sites in the house or space in which the animal population or the animal flock is kept. The pooled samples reflect the amount of pathogen marker polynucleotides or marker genes present in the animal population.

[0048] The sample material is selected from the group consisting of dust samples, litter samples, liquid manure samples, fur samples, feather samples, skin samples, swab samples and samples of bodily excrements and solutions or suspensions thereof. Bodily excrements are urine, fecal or cecal excrements. In a preferred embodiment, the sample material is feces.

[0049] As used in the context of this embodiment, the term "litter samples" refers to mixed excremental droppings in the pen cage or slat. Further, in the context this embodiment, the term "liquid manure samples" refers to mixed excremental samples containing feces and urine.

[0050] These litter samples can, for example, be collected from an animal population using the overshoe method or using litter grabs at different places in the pen.

[0051] Boot swabs being sufficiently absorptive to soak up moisture are particularly suitable for collecting pooled animal samples. Tube gauze socks are also acceptable.

[0052] Suitable sample volumes are, for example, 0.1 to 20 ml, in particular 0.2 to 10 ml, preferably 0.5 to 5 ml. Suitable sample masses are, for example 0.1 to 20 g, in particular 0.2 to 10 g, preferably 0.5 to 5 g.

[0053] The animal population is an avian flock. The avian flock according to the invention is preferably poultry. Preferred poultry according to the invention are chickens, turkeys, ducks and geese. The poultry can be optimized for producing young stock. This type of poultry is also referred to as parent and grandparent animals. Preferred parent and grandparent animals are, accordingly, (grand)parent broilers, (grand)parent ducks, (grand)parent turkeys and (grand)parent geese.

[0054] The poultry according to the invention can also be selected from fancy poultry and wild fowl. Preferred fancy poultry or wild fowl are peacocks, pheasants, partridges, guinea fowl, quails, capercaillies, goose, pigeons and swans. Further preferred poultry according to the invention are ostriches and parrots. Most preferred poultry according to the invention are broilers.

[0055] In order to perform the above method, the animal samples are collected in consecutive points in time.

[0056] Preferably, the samples are taken on a weekly, daily or hourly basis. Collecting the animal samples at consecutive days is particularly preferred.

[0057] In a particularly preferred embodiment, the first and the second marker are detected on DNA level, i.e. the polynucleotides are DNA-polynucleotides.

[0058] The marker polynucleotides may be isolated from the animal samples prior to quantification. Polynucleotide isolation can, for example, be performed via extraction using the Cetyltrimethylammoniumbromid (CTAB) method or by diverse commercial nucleic acid extraction kits, in which cell lysis is achieved either through chemical lysis and/or by mechanical cell disruption and nucleic acid is captured on silica matrices or on silica-cladded magnetic beads. Commercial extraction kits specialized on fecal material or harsh material are particularly suitable.

[0059] The marker genes may be detected and/or quantified by commonly known methods such as sequencing, hybridization or various PCR techniques known in the art.

[0060] In an alternative embodiment, the marker genes contained in the animal sample can be directly quantified, for example via PCR, qPCR, sequencing or hybridization techniques.

[0061] The present invention further provides a diagnostic kit comprising a set of oligonucleotides (primers and probes) for amplifying and/or quantifying at least one polynucleotide encoding at least one of the marker genes. In a preferred embodiment, the diagnostic kit comprises PCR primers and probes for both, the first marker and the second marker. More specifically, the diagnostic kit comprises PCR primers and probes for detecting both, the first marker and the second marker, wherein the first marker comprises a polynucleotide sequence being specific for the *C. perfringens* sub-species inducing the targeted disease; and the second marker comprises a polynucleotide being specific for the species *C. perfringens.* The kit may further comprise buffer solutions, such as PCR buffer; magnesia salts; deoxy nucleotide triphosphates (dNTPs). The kit may also include elements such as sample collection tubes, reagents to isolate the nucleic acids and/or instructions for its use.

Detection of avian necrotic enteritis

[0062] The applicants have unexpectedly found that the occurrence of (sub-clinical) necrotic enteritis can be determined by monitoring the development of the *netB*/*cpa* ratio in avian samples over time.

[0063] In accordance thereto, the present invention provides a method for detecting avian necrotic enteritis, the method comprising:

1. a) collecting samples of a specific avian population at consecutive points in time;

2. b) determining the amount of a first marker and a second marker contained in the sample material; and
3. c) determining the ratio of the first marker to the second marker contained in the sample material;

wherein the first marker is *netB* or homologues or fragments thereof;

the second marker is *cpa* or homologues or fragments thereof;
and

wherein an increase in the ratio of the first marker to the second marker in the analyzed sample material over time is an indication of avian necrotic enteritis.

[0064] As an alternative, the first marker may also be a polynucleotide sequence comprising *netB* and/or the second marker may be a polynucleotide sequence comprising *cpa.* Preferably, these polynucleotide sequences are being elongated by not more than 100 base pairs or not more than 80 base pairs, preferably by not more than 70 base pairs or by not more than 60 base pairs, especially by not more than 50 base pairs or not more than 40 base pairs and most preferably by not more than 30 base pairs compared to the genes *netB* and *cpa.*

[0065] The term "necrotic enteritis" refers to NE both in clinical and in sub-clinical/latent state.

[0066] Therefore, in a particularly preferred embodiment, the present invention provides a method for detecting avian necrotic enteritis, the method comprising:

1. a) collecting samples of a specific avian population at consecutive points in time;
2. b) determining the amount of a first marker and a second marker contained in the sample material; and
3. c) determining the ratio of the first marker to the second marker contained in the sample material;

wherein the first marker is *netB* and the second marker is *cpa;*
and

wherein an increase in the ratio of the first marker to the second marker in the analyzed sample material over time is an indication of avian necrotic enteritis.

[0067] The polynucleotide sequences of *netB* and *cpa* are known in the art. However, for the sake of clarity and completeness, the consensus sequence of *netB* is indicated under SEQ ID NO. 1 and the consensus sequence of *cpa* is indicated under SEQ ID NO. 2.

[0068] The alpha toxin gene (*cpa*) is present on the chromosome of all *C. perfringens* strains (pathogenic and non-pathogenic), meaning that the concentration of *cpa* should have similar

levels in excremental samples of healthy and NE-infected animals and indicates the presence of *C. perfringens* only in general.

[0069] The above method may be used for determining whether or not an individual avian subject suffers from (sub-clinical/latent) necrotic enteritis, or, in an alternative embodiment, for determining whether or not an avian population suffers from (sub-clinical/latent) necrotic enteritis ("bulk testing").

[0070] In case an **individual avian subject** is to be examined, the sample material may be selected from the group consisting of dust samples, swab samples, litter samples, liquid manure samples, feather samples and samples of bodily excrements and solutions or suspensions thereof. Bodily excrements are urine, fecal or cecal excrements. In a preferred embodiment, the sample material is feces.

[0071] In general, the term "litter" is to be understood as a mixture of animal excrements with the bedding material.

[0072] As used in the context of this embodiment, the term "litter samples" refers to excremental droppings from an individual avian subject. Further, in the context this embodiment, the term "liquid manure samples" refers to an excremental sample containing feces and urine from an individual animal.

[0073] Samples from individual avian subjects can be taken either directly from the avian, e.g. with swabs. Alternatively and especially in case of single-housed avians, the sample material can be collected from the floor of the pen, cage or slat. The sample material has to be assignable to the investigated animal.

[0074] Suitable sample volumes are, for example, 0.05 ml to 20 ml or 0.1 to 20 ml, in particular 0.2 to 10 ml, preferably 0.5 to 5 ml. Suitable sample masses are, for example 0.05 g to 20 g or 0.1 to 20 g, in particular 0.2 to 10 g, preferably 0.5 to 5 g.

[0075] In case the health status of an **avian population** is to be determined, a pooled sample of the avian population is examined ("bulk testing"). A "pooled sample" is to be understood as a composite sample from randomly selected separate samples, one sample taken with one or several moistened fabric swabs or pooled samples made up of separate samples of fresh samples taken at random from a number of sites in the house or space in which the avian population is kept. The pooled samples reflect the amount of pathogenic *netB* marker genes present in the animal population.

[0076] The sample material may be selected from the group consisting of dust samples, litter samples, liquid manure samples, feather samples, swab samples and samples of bodily excrements and solutions or suspensions thereof. Bodily excrements are fecal or cecal excrements. In a preferred embodiment, the sample material is feces.

[0077] As used in the context of this embodiment, the term "litter samples" refers to mixed excremental droppings in the pen, cage or slat. These litter samples can, for example, be collected from a population using the overshoe method or using litter grabs at different places in the pen. Further, in the context this embodiment, the term "liquid manure samples" refers to mixed excremental samples containing feces and urine.

[0078] Boot swabs being sufficiently absorptive to soak up moisture are particularly suitable for collecting pooled avian samples. Tube gauze socks are also acceptable.

[0079] The animal population is an avian flock. The avian flock according to the invention is preferably poultry. Preferred poultry according to the invention are chickens, turkeys, ducks and geese. The poultry can be optimized for producing young stock. This type of poultry is also referred to as parent and grandparent animals. Preferred parent and grandparent animals are, accordingly, (grand)parent broilers, (grand)parent ducks, (grand)parent turkeys and (grand)parent geese.

[0080] The poultry according to the invention can also be selected from fancy poultry and wild fowl. Preferred fancy poultry or wild fowl are peacocks, pheasants, partridges, guinea fowl, quails, capercaillies, goose, pigeons and swans. Further preferred poultry according to the invention are ostriches and parrots. Most preferred poultry according to the invention are broilers.

[0081] Suitable sample volumes are, for example, 0.1 to 20 ml, in particular 0.2 to 10 ml, preferably 0.5 to 5 ml. Suitable sample masses are, for example 0.1 to 20 g, in particular 0.2 to 10 g, preferably 0.5 to 5 g.

[0082] The samples are generally to be taken and analyzed on a weekly, daily or hourly basis. In a preferred embodiment, the animal samples are collected at consecutive days. Preferably, sample collection and sample analysis are started before day 14. For example, sample collection and sample analysis is started from day 1, from day 5, from day 10 or from day 13 on a daily basis.

[0083] The marker genes *netB* and *cpa* may be isolated from the animal samples prior to quantification. Polynucleotide isolation can, for example, be performed via extraction using the Cetyltrimethylammoniumbromid (CTAB) method or by diverse commercial nucleic acid extraction kits, in which cell lysis is achieved either through chemical lysis and/or by mechanical cell disruption and nucleic acid is captured on silica matrices or on silica-cladded magnetic beads. Commercial extraction kits specialized on fecal material or harsh material are particularly suitable.

[0084] The marker genes may be detected and/or quantified by commonly known methods such as sequencing, hybridization or various PCR techniques known in the art.

[0085] In an alternative embodiment, the marker genes contained in the animal sample can be

directly quantified, for example via PCR, qPCR, sequencing or hybridization techniques.

[0086] Intestinal lesions caused by sub-clinical or clinical necrotic enteritis were scored using a scale from 0 to 6 [Keyburn et al. (2006) "Alpha toxin of *Clostridium perfringens* is not an essential virulence factor in necrotic enteritis in chickens", *Infect. Immun.* **74**:6496-6500]. In accordance therewith, the lesions in the small intestine (duodenum to ileum) were scored as follows: score 0 = no gross lesions; 1 = congested intestinal mucosa; 2 = small 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. Lesion scores 5 to 6 correspond to clinical necrotic enteritis; lesion scores 1 to 4 correspond to the sub-clinical state.

[0087] The inventors have found that ratio of *netB*/*cpa* marker genes in avian excrement samples at a level of 0.5 already show traces of congested intestinal mucosa.

[0088] Accordingly, the present disclosure also provides a method for detecting necrotic enteritis, the method comprising

1. a) collecting an avian sample
2. b) determining the amount of marker genes *netB* and *cpa* in this avian sample, and
3. c) determining the ratio of *netB*/*cpa* marker genes in the avian samples,

wherein a *netB*/*cpa* ratio being greater than 0.5 constitutes an indication of necrotic enteritis.

[0089] In addition, the present disclosure also provides new PCR primers and probes suitable for detecting and/or quantifying the *netB* and *cpa* marker genes present in the excrements of animals infected with necrotic enteritis. As used herein, the term "(PCR) probe" refers to an oligonucleotide sequence that increases the specificity of quantitative PCR.

[0090] The oligonucleotide primers and probes described in the following turned out to be particularly effective. Accordingly, the present disclosure provides oligonucleotides selected from the group consisting of

1. a) oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the polynucleotide as depicted in SEQ ID NO:3;
2. b) oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the polynucleotide as depicted in SEQ ID NO: 4;
3. c) oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the polynucleotide as depicted in SEQ ID NO: 5;
4. d) oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the polynucleotide as depicted in SEQ ID NO: 6;
5. e) oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the polynucleotide as depicted in SEQ ID NO: 7;
6. f) oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the polynucleotide as depicted in SEQ ID NO:8;

7. g) oligonucleotides being complementary to the oligonucleotides according to (a) to (f)
8. h) oligonucleotides comprising any one of the oligonucleotides according to (a) to (g) and being elongated by not more than 5 nucleotides compared to the oligonucleotides according to (a) to (g).

[0091] Therein, the polynucleotide as depicted in SEQ ID NO: 3 is a PCR primer (fwd) for detecting *netB.* The polynucleotide as depicted in SEQ ID NO: 4 is a PCR primer (rev) for detecting *netB.* The polynucleotide as depicted in SEQ ID NO: 5 is a PCR probe for detecting *netB.*

[0092] Further, the polynucleotide as depicted in SEQ ID NO: 6 is a PCR primer (fwd) for detecting *cpa.* The polynucleotide as depicted in SEQ ID NO: 7 is a PCR primer (rev) for detecting *cpa.* The polynucleotide as depicted in SEQ ID NO: 8 is a PCR probe for detecting *cpa.*

[0093] These oligonucleotides are preferably used as primers and/or probes in one of the above-described methods for detecting necrotic enteritis.

[0094] Accordingly, the oligonucleotides selected from the group consisting of

1. a) oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the polynucleotide as depicted in SEQ ID NO:3;
2. b) oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the polynucleotide as depicted in SEQ ID NO: 4;
3. c) oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the polynucleotide as depicted in SEQ ID NO: 5;
4. d) oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the polynucleotide as depicted in SEQ ID NO: 6;
5. e) oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the polynucleotide as depicted in SEQ ID NO: 7;
6. f) oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the polynucleotide as depicted in SEQ ID NO:8;
7. g) oligonucleotides being complementary to the oligonucleotides according to (a) to (f);
8. h) oligonucleotides comprising any one of the oligonucleotides according to (a) to (g) and being elongated by not more than 5 base pairs compared to the oligonucleotides according to (a) to (g);

are used in the above-described method as a PCR primer and/or as a PCR probe.

[0095] The present disclosure further provides a diagnostic kit for determining whether or not an avian population, such as an avian flock, suffers from (sub-clinical) necrotic enteritis. Said kit comprises a set of oligonucleotides for amplifying and/or quantifying at least one polynucleotide encoding at least one of the marker genes *netB* and/or *cpa.* Preferably, the

diagnostic kit comprises PCR primers and probes for both, *netB* and *cpa.* The kit may comprise one or more oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to one or more of the polynucleotides as depicted in SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7 and/or SEQ ID NO: 8; oligonucleotides being complementary to the oligonucleotides according to SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7 and/or SEQ ID NO: 8; oligonucleotides comprising any one of the aforementioned oligonucleotides and being elongated by not more than 5 base pairs compared to the oligonucleotides according to SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7 and/or SEQ ID NO: 8. Preferably, the kit comprises at least two primers or at least four primers. A kit comprising two primers and one probe or a kit comprising for primers and two probes is particularly preferred. The kit may further comprise buffer solutions, such as PCR buffer; magnesia salts; deoxy nucleotide triphosphates (dNTPs). The kit may also include elements such as sample collection tubes, reagents to isolate the nucleic acids and/or instructions for its use.

[0096] In addition to the above, the present inventors have unexpectedly found that an increased level of *netB* marker gene, homologues and fragments thereof, in comparison to a non-infected control and an increase in the level of *netB* over time, respectively, is an indication of necrotic enteritis.

[0097] Accordingly, the present disclosure also pertains to a method for detecting avian necrotic enteritis, the method comprising

1. a) collecting excremental samples of a specific avian or of a specific avian population at consecutive points in time; and
2. b) monitoring the level of marker gene *netB* and/or homologues and fragments thereof in these samples;

wherein an increase in the level of the marker gene constitutes an indication of avian necrotic enteritis.

[0098] In a preferred aspect of the aforementioned method, the present disclosure also pertains to a method for detecting avian necrotic enteritis, Method for detecting avian necrotic enteritis, the method comprising

1. a) collecting excremental samples of a specific avian or of a specific avian population at consecutive points in time; and
2. b) monitoring the level of marker gene *netB* in these samples;

wherein an increase in the level of *netB* constitutes an indication of avian necrotic enteritis.

[0099] In that regard and in accordance with common understanding, an increase of about 0.2 to 0.3 log (i.e. factor two to five) is to be considered relevant.

[0100] The inventors have found that an amount of *netB* of at least 10^7 copies/g feces

constitutes an indication of necrotic enteritis.

[0101] Accordingly, the present disclosure also pertains to a method for detecting avian necrotic enteritis, the method comprising

1. a) collecting excremental samples of a specific avian or of a specific avian population at consecutive points in time; and
2. b) monitoring the level of marker gene *netB* in these samples;

wherein an amount of *netB* of at least 10^7 copies/g feces constitutes an indication of necrotic enteritis.

[0102] The above methods are suitable for detecting both, clinical and sub-clinical/latent states of necrotic enteritis.

[0103] Surprisingly, the inventors also have found that the amount of *netB* marker gene correlates with the extent of intestinal lesions of the corresponding avian subject and thus with the grade of necrotic enteritis. Accordingly, the present disclosure is also directed to a method for determining the grade of avian necrotic enteritis, the method comprising determining the amount of *netB* marker gene and/or homologues and fragments thereof, in avian excremental samples, wherein the amount of said marker gene indicates the grade of necrotic enteritis.

[0104] In the above methods, the oligonucleotide primers and probes described in the following turned out to be particularly effective.

[0105] Accordingly, oligonucleotides selected from the group consisting of

1. a) oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the polynucleotide as depicted in SEQ ID NO:3;
2. b) oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the polynucleotide as depicted in SEQ ID NO: 4;
3. c) oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to the polynucleotide as depicted in SEQ ID NO: 5;
4. d) oligonucleotides being complementary to the oligonucleotides according to (a) to (c);
5. e) oligonucleotides comprising any one of the oligonucleotides according to (a) to (d) and being elongated by not more than 5 base pairs compared to the oligonucleotides according to (a) to (d);

may be used in one of the above-described methods for detecting avian necrotic enteritis as a PCR primer and/or as a PCR probe for detecting *netB.*

[0106] The polynucleotide as depicted in SEQ ID NO: 3 is a PCR primer (fwd) for detecting *netB.* The polynucleotide as depicted in SEQ ID NO: 4 is a PCR primer (rev) for detecting *netB.* The polynucleotide as depicted in SEQ ID NO: 5 is a PCR probe for detecting *netB.*

[0107] The present disclosure further provides a diagnostic kit for determining whether or not an avian population, such as an avian flock, suffers from (sub-clinical) necrotic enteritis. Said kit comprises a set of oligonucleotides for amplifying and/or quantifying the polynucleotide encoding the *netB* marker gene. In a preferred embodiment, the kit comprises one or more oligonucleotides having a sequence identity of at least 80%, preferably at least 85, 90 or 95%, most preferably 100%, to one or more of the polynucleotides as depicted SEQ ID NO:3, SEQ ID NO:4 and/or SEQ ID NO:5; oligonucleotides being complementary to the oligonucleotides according to SEQ ID NO:3, SEQ ID NO:4 and/or SEQ ID NO:5; oligonucleotides comprising any one of the aforementioned oligonucleotides and being elongated by not more than 5 base pairs compared to the oligonucleotides according to SEQ ID NO:3, SEQ ID NO:4 and/or SEQ ID NO:5. Preferably, the kit comprises two primers. A kit comprising two primers and one probe is particularly preferred. The kit may further comprise buffer solutions, such as PCR buffer; magnesia salts; deoxy nucleotide triphosphates (dNTPs). The kit may also include elements such as sample collection tubes, reagents to isolate the nucleic acids and/or instructions for its use.

[0108] The present invention provides the above-described non-invasive methods for detecting avian necrotic enteritis and for determining the grade of necrotic enteritis which can be performed *ante mortem.* This enables the farmer to efficiently treat the avian population suffering from necrotic enteritis, if necessary, subsequently after having performed the above-mentioned test. Alternatively or additionally, the farmer may also administer health-promoting substances, such as zootechnical feed additives.

[0109] Preferably, the therapeutic agent or the health-promoting substance is selected from the group consisting of antibiotic agents, probiotic agents, praebiotic agents, organic/fatty acids, bacteriophages and bacteriolytic enzymes.

[0110] In accordance therewith, the present disclosure is also directed to antibiotic agents for use in the treatment of necrotic enteritis, wherein the necrotic enteritis is detected or evaluated by performing one of the methods indicted above. Moreover, the present disclosure is directed to the use of probiotic agents, praebiotic agents organic/fatty acids, bacteriophages and bacteriolytic enzymes for ameliorating the health status of an animal or an animal population.

[0111] Applications of the methods according to the invention are for example (i) aiding in the diagnosis and/or prognosis of *C. perfringens* induced diseases, such as avian necrotic enteritis, (ii) monitoring the progress or reoccurrence of these diseases, (iii) aiding in the evaluation of treatment efficacy for an animal population undergoing or contemplating treatment, or (iv) controlling (therapeutic) vaccination efficiency against *C. perfringens* induced diseases.

[0112] Applications of the invention in particular help to avoid loss in animal performance like weight gain and feed conversion.

[0113] In the following, the invention is illustrated by non-limiting examples and exemplifying

embodiments.

Examples

1. In vivo model

[0114] A sub-clinical *in vivo* model for necrotic enteritis was used. In this model Ross 308 broilers (female and male) were grouped with 27 birds per group. They were fed a wheat/rye (43%/7.5%) based diet, with soybean meal as protein source until day 16. From day 17 onwards, soybean meal was replaced by fishmeal (30%) as protein source. At day 4 and 9 the broilers were orally inoculated with Poulvac Bursa Plus (Zoetis) as immunosuppressant. At day 14 and 16, the broilers were orally inoculated with a ten-fold dose of Hipracox.

[0115] The birds of 5 groups (P.A - P.E) were three times per day challenged orally with approximately 4×10^8 cfu *netB*-positive, pathogenic *C. perfringens* (late-log culture) bacteria (*Cp56*) for two consecutive days (day 18 and 19). Two groups of birds (P.F and P.G) were challenged only one time per day, to induce milder necrotic lesions than the previous groups challenged three times per day.

[0116] The chickens were challenged for two consecutive days. All animals were euthanized at day 20. Intestinal lesions in the small intestine (duodenum to ileum) were scored as follows: 0 = no gross lesions; 1 = congested intestinal mucosa; 2 = small 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. Non-infected control birds were treated in the same manner except for the inoculation with *C. perfringens.* These control birds were inoculated with sterile bacterial growth medium (BHI-broth).

[0117] None of the negative control birds, receiving sterile bacterial medium (BHI) instead of the pathogenic *C. perfringens* culture developed necrotic enteritis. Also the birds challenged with a non-pathogenic *C. perfringens* strain were free of necrotic lesions. In the other groups, challenged with pathogenic *Cp56,* most birds developed necrotic enteritis. With a high amount of birds showing severe lesions.

[0118] Summary of the *in vivo* model:

	D4	D9	D14	D16	D17	D18	D19	D20
Poulvac Bursa Plus	x	X						
Hipracox x10			x	X				
Feed + fishmeal					x	x	x	
Inoculation *C.*						x	x	

	D4	D9	D14	D16	D17	D18	D19	D20
perfringens								
Scoring								x

[0119] Fresh cloacal samples (i.e. samples of individual animals) were collected immediately after death and snap frozen in liquid N_2 prior to storage at -80°C.

[0120] Cloacal samples of individualized animals:

- Infected birds (different lesion scores)
- non-infected control groups
- birds inoculated with *netB*-negative *C. perfringens*

[0121] Litter samples (=mixed fecal droppings in the pen, i.e. pooled samples of the avian flock) were collected from each group using the overshoe method (only day 19) and using litter grabs at 5 different places in the pen from the day before inoculation (day 17) until day 19.

[0122] Types and amounts of samples collected:

SAMPLE		AMOUNT OF SAMPLES
CLOACAL SAMPLES	Non-infected	10
	Infected with *netB*-negative *C. perfringens* Score 0	10
	Score 2	10
	Score 3 or 4	10
	Score 5 or 6	8
LITTER SAMPLES	Non-infected (BHI)	
	Before inoculation	1 litter grab
	After first inoculation	1 litter grab
	After second inoculation	1 litter grab + 1 shoe cover sample
	Infected with Non-pathogenic *C. perfringens*	
	Before inoculation	2 litter grabs
	After first inoculation	2 litter grabs
	After second inoculation	2 litter grabs + 2 shoe cover samples
	Infected with *netB*-positive *C. perfringens*	
	Before inoculation	7 litter grabs
	After first inoculation	7 litter grabs
	After second inoculation	7 litter grabs + 6 shoe cover samples

[0123] Shoe cover samples were collected only at day 19. Litter samples were collected on day 17 until day 19. The samples were homogenized and split in three portions.

2. DNA extraction and quantification

[0124] DNA was extracted from the samples using the CTAB method. Therefore, 100 mg of cloacal material and 200 mg of litter grab or shoe cover sample was used as starting material. The total DNA was eluted in 100 µl water. The DNA concentration was determined with a Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and adjusted to a final concentration of 50 ng/µl.

[0125] qPCR was performed using SYBR-green 2× master mix (Bioline, Brussels, Belgium) in a Bio-Rad CFX-384 system. Each reaction was done in triplicate in a 12 µL total reaction mixture using 2 µL of the DNA sample and 0.5 µM final qPCR primer concentration. The qPCR conditions used were the same for both genes: 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and stepwise increase of the temperature from 65° to 95 °C (at 10s/0.5°C). Melting curve data were analyzed to confirm the specificity of the reaction. For construction of the standard curve, the PCR product was generated using the standard PCR primers listed below and DNA from *C. perfringens* strain CP56. After purification (MSB Spin PCRapace, Stratec Molecular, Berlin, Germany) and determination of the DNA concentration. The concentration of the linear dsDNA standard was adjusted to 1×10^7 to 1×10^1 copies per µL with each step differing by 10 fold. The copy numbers of samples were determined by reading off the standard series with the Ct values of the samples.

[0126] The detection limit of the qPCR technique is dependent on the DNA concentration obtained after CTAB extraction and was around 10^5 gene copies / g material.

[0127] Primers used in the PCR assay:

PCR			
cpa	AGT CTA CGC TTG GGA TGG AA	(fwd)	SEQ ID NO:9
	TTT CCT GGG TTG TTC ATT TC	(rv)	SEQ ID NO:10
netB	TGA TAC CGC TTC ACA TAA AGG T	(fwd)	SEQ ID NO:11
	ACC GTC CTT AGT CTC AAC AAA T	(rv)	SEQ ID NO:12
qPCR			
cpa	GTT GAT AGC GCA GGA CAT GTT AAG	(fwd)	SEQ ID NO:13
	CAT GTA GTC ATC TGT TCC AGC ATC	(rv)	SEQ ID NO:14
netB	TCA ATT GGT TAT TCT ATA GGC GGT A	(fwd)	SEQ ID NO:15
	ATA TGA AGC ATT TAT TCC AGC ACC A	(rv)	SEQ ID NO: 16

3. Aggregate testing: Correlating the amount of marker genes in cloacal samples of individualized animals

[0128] In these Examples, the above-mentioned cloacal samples were investigated.

[0129] At the day of necropsy the alpha toxin gene (*cpa*) was present in cloacal samples from all groups (both uninfected and infected birds) and also the *netB* gene was present in all cloacal samples.

3.1 Correlation of the amount of *netB* with lesion score

[0130] Only in cloacal samples from birds challenged with a pathogenic, *netB* positive *C. perfringens* strain, the mean *netB* signal is above 10^7 copies / g feces.

	***netB* [copies/g sample]**	***netB* [Log copies/g sample]**	**standard deviation**
Non-infected (BHI)	3,16E+06	6,50	0,37
Non-path. Cp	3,92E+06	6,59	0,32
Score 0	1,16E+08	8,06	0,54
Score 2	4,96E+08	8,70	0,73
Score 3-4	7,18E+08	8,86	0,79
Score 5-6	4,83E+09	9,68	0,64

[0131] This Example shows that the amount of *netB* gene in the analyzed samples directly correlates with the intestinal lesion score of the analyzed bird, i.e. with the grade of necrotic enteritis of the infected bird.

[0132] The correlation between the lesion scores from the birds infected with the pathogenic *C. perfringens strain* and the amount of the genes (*netB*/*cpa*) present in the feces was calculated by spearman correlation. The spearman r-value can range from 0 to 1: r=1 → perfect correlation, r=0 → no correlation. The presence of all tested genes show a correlation with the disease severity, with the highest correlation with the netB gene.

	copies/g sample		**Log copies/g sample**		**standard deviation**	
	netB	***cpa***	***netB***	***cpa***	***netB***	***cpa***
Non-infected (BHI)	3,16E+06	3,72E+08	6,50	8,57	0,37	0,87
Non-path. Cp	3,92E+06	6,59E+07	6,59	7,82	0,32	0,95
Score 0	1,16E+08	2,81E+08	8,06	8,45	0,54	0,48
Score 2	4,96E+08	4,44E+08	8,70	8,65	0,73	0,77
Score 3-4	7,18E+08	1,33E+09	8,86	9,12	0,79	0,62

	copies/g sample		Log copies/g sample		standard deviation	
	netB	***cpa***	***netB***	***cpa***	***netB***	***cpa***
Score 5-6	4,83E+09	1,93E+09	9,68	9,29	0,64	0,75

Spearman-Calculation:

[0133]

Parameter	*cpa*	*netB*
Number of XY Pairs	38	38
Spearman r	0,4195	0,6110
95% confidence interval	0.1055 to 0.6574	0.3534 to 0.7825
P value (two-tailed)	0,0088	P<0.0001
P value summary	**	***
Exact or approximate P value?	Gaussian Approximation	Gaussian Approximation
Is the correlation significant? (alpha=0.05)	Yes	Yes

[0134] The Spearman correlation provides mathematical prove on the correlation between the amount of *netB* in the cloacal sample and the grade of necrotic enteritis.

3.2 Correlation *netB*/cpa ratio with lesion score

[0135] The ratio of *netB* gene versus the *cpa* gene in the cloacal samples of non-infected birds (BHI), birds challenged with the non-pathogenic strain (Non-path) and birds challenged with the pathogenic C. *perfringens* strain were investigated.

[0136] The birds showed different degrees of disease severity (score 0 to score 5 to 6):

	Mean ratio ***netB/cpa***
Non-infected (BHI)	0,04
Non-path.	0,09
Cp	
Score 0	0,83
Score 2	1,85
Score 3-4	1,27
Score 5-6	2,69

4. Bulk testing: Monitoring the amount of marker genes in excremental samples of the

avian population over time

[0137] In these Examples, the litter samples indicated above were investigated.

4.1 Monitoring the amount of *netB* over time

[0138] It was found that the amount of *netB* gene in the litter samples significantly increased starting from the day following the day of inoculation (day 18) with the pathogenic *C. perfringens* strain:

	netB			
	copies/g sample	Log sample	copies/g	standard deviation
Non-infected (BHI) - day 17	1,97E+05	5,29		
Non-infected (BHI) - day 18	2,53E+05	5,40		
Non-infected (BHI) - day 19	1,12E+05	5,05		
Non-infected (BHI) - day 19 - BS	8,46E+04	4,93		
Non-path. Cp - day 17	6,92E+04	4,84		0,09
Non-path. Cp - day 18	4,17E+04	4,62		0,05
Non-path. Cp - day 19	8,01E+04	4,90		
Non-path. Cp - day 19 - BS	4,16E+04	4,62		0,39
NE-group - day 17	3,21E+05	5,51		0,93
NE-group - day 18	7,03E+07	7,85		0,74
NE-group - day 19	1,11E+08	8,05		0,24
NE-group - day 19 - BS	2,63E+08	8,42		0,95

4.2 Monitoring the netblcpa ratio over time

[0139] It was found that the ratio of *netB* gene versus the *cpa* gene in the litter samples collected at days 17 to 19 significantly increased starting from the day following the day of inoculation (day 18) with the pathogenic *C. perfringens* strain:

	netB/*cpa*
Non-infected (BHI) - day 17	0,00
Non-infected (BHI) - day 18	0,01
Non-infected (BHI) - day 19	0,00
Non-infected (BHI) - day 19 - BS	0,00
Non-path. Cp - day 17	0,00
Non-path. Cp - day 18	0,00
Non-path. Cp - day 19	0,00
Non-path. Cp - day 19 - BS	0,00
NE-group - day 17	0,08
NE-group - day 18	1,27
NE-group - day 19	2,45
NE-group - day 19 - BS	2,63

[0140] The above experiments show that an increase in the amount of *netB* gene and in the ratio of *netB*/*cpa* genes, respectively, in pooled animal excremental samples collected in consecutive points in time, correlates with the development or the progression of necrotic enteritis. Therewith, it is proven that the methods of the present invention allow an assessment of the health status of a whole animal flock regarding *C. perfringens* induced diseases.

REFERENCES CITED IN THE DESCRIPTION

Cited references

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Patentkrav

1. Fremgangsmåde til påvisning af C. perfringens-inducerede sygdomme i en fugleflok, hvilken fremgansgmåde omfatter:
a) indsamling af prøvemateriale fra fugleflokken på hinanden følgende tidspunkter;
b) bestemmelse af mængden af en første markør og en anden markør, der er indeholdt i prøvematerialet; og
c) bestemmelse af forholdet mellem den første markør og den anden markør, der er indeholdt i prøvematerialet;
hvor den første markør omfatter en polynukleotidsekvens, der er specifik for C. perfringens-underarten, der inducerer den målrettede sygdom;
og den anden markør omfatter et polynukleotid, der er specifikt for arten C. perfringens; og
hvor en stigning i forholdet mellem den første markør og den anden markør i det analyserede prøvemateriale over tid er et tegn på den målrettede sygdom.

2. Fremgangsmåde ifølge krav 1, hvor den første markør enten er et markørgen, der koder for et specifikt toksin, eller en patogenicitetsø.

3. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor den første markør er placeret på et toksinplasmid i C. perfringens, og den anden markør er placeret på C. perfringens-kromosomet.

4. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor den første markør er valgt fra gruppen, der består af cpe, beta-toksin (cpb), beta2-toksin (cpb2), epsilon-toksin (etx), netF og netB og homologer og fragmenter deraf.

5. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor den anden markør er cpa.

6. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor prøvematerialet er en puljet prøve valgt fra

gruppen, der består af støvprøver, strøelsesprøver, væskeformige gødningsprøver, belægningsprøver, fjerprøver, hudprøver og prøver af kropsekskrementer og opløsninger eller suspensioner deraf.

7. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor prøvematerialet er puljet fæces.

8. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor prøvemassen er 0,1 g til 20 g.

9. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor prøverne tages og analyseret ugentlig, dagligt eller hver time, fortrinsvis på hinanden følgende dage.

10. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor markørgenerne, der er indeholdt i prøvematerialet, påvises og kvantificeres ved hjælp af PCR, fortrinsvis qPCR.

11. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor den C. perfringens-inducerede sygdom er i subklinisk eller latent stadie.

12. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor den C. perfringens-inducerede sygdom er nekrotisk enteritis, fortrinsvis i subklinisk eller latent stadie.