Evaluation of a mPCR for *Clostridium perfringens* type A and rotavirus type A

Research project Veterinary medicine University Utrecht

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Abstract

Introduction. *C. perfringes* type A, *E. coli* and Rotavirus type A (RVA) are the most important pathogens causing diarrhea in suckling piglets. These three pathogens may be present in feces or enteric content simultaneously. Therefore it is hard to determine the causative agent of piglet diarrhea in some cases based on bacterial culture or PCR. The general objective of this project is to determine a cut-off value for the combined "diarrhea package PCR test" of the Animal Health Service with respect to diagnosing diarrheic cases. The objective is broken down into several research questions,

1) At which value for alpha toxin, beta 2 toxin, or RVA is the specificity of the qPCR higher than 90%.
2) Is there interference between RVA, *C. perfringens* type A and *E. coli* in qPCR.
3) Which toxin, alpha or beta 2, is preferred to infer conclusions over *C. perfringens* type A.

Material & methods. An observational retrospective cross sectional study was conducted.
Data were collected at the Animal Health Service (GD Deventer) from submitted samples. The data consist of quantitative PCR (qPCR) results for RVA and alpha/beta 2 toxin genes for *C. perfringens* and a qualitative PCR for *E. coli* fimbriae genes. In addition, veterinarians and pathologists decided based on clinical data which pathogen was the probable cause of diarrhea. A comparison was made between the veterinarian's interpretation and the qPCR results.

Results

1) ROC curves showed a specificity of 90% at values of $10^{4.50}$ Cfu/ml for alpha toxin, $10^{4.02}$ Cfu/ml for beta 2 toxin and $10^{5.66}$ copies/ml for RVA.
2) A significant lower mean for beta 2 toxin was detected in samples that tested positive for a combination of RVA and *E. coli* when compared to samples that tested only positive for *C. perfringens.*
3) *C. perfringens* type A detected by qPCR only *(C. perfringens*-qPCR) results showed a larger deviation between alpha toxin and beta 2 toxin when compared to *C. perfringens* what was detected by qPCR and veterinarians (*C. perfringens*-AD). This was supported by the correlation coefficients of 0.572 for *C. perfringens*-qPCR and 0.896 for *C. perfringens*-AD. Besides, beta 2 toxin had a higher area under the curve (AUC) compared to alpha toxin.

Discussion. 1) High viral loads (>$10^{5.66}$ copies/ml) of RVA or high copy numbers for alpha (>$10^{4.50}$ Cfu/ml) and beta 2 toxin (>$10^{4.02}$ Cfu/ml) give a good indication for the identification of the causative agents of diarrhea. Furthermore, qPCR test for RVA has a higher analytic sensitivity compared to the commonly used fast-antigen test. Conversely, qPCR tests detecting alpha and beta 2 toxin should be used with a certain caution, since *C. perfringens* type A is part of the normal microbiome of the piglet intestine.
2) The presented data indicates interference between pathogens in qPCR test in the group where all pathogens are simultaneously present. However, it is unclear whether the interference is test, pathogen or piglet related.
3) This study shows that beta 2 toxin may be a preferred gene for identifying *C. perfringens* type A in cases of piglet diarrhea if one toxin has a low copy number value.

Introduction

Piglet diarrhea leads to a reduced growth in piglets and results in economic loss for the piglet industry. Nowadays, detection of causative agents of diarrhea can be performed using multiple tests. Therefore, GD Deventer developed a qPCR test to simultaneously detect and quantify the presence of three diarrhea-associated pathogens, rotavirus type A (RVA), *Clostridium perfringens* type A and *Escherichia coli.* First, some background information of the pathophysiology and the detection methods now used for these agents will be introduced and the aim of this thesis is provided.

Rotavirus type A

Rotavirus type A (RVA) is an RNA-virus that belongs to the *Reoviridae* family and is a non-enveloped virus with a triple-layered capsule. Based on antigenicity, four serotypes are described (A, B, C and E). From these four serotypes, RVA comprises for 90% of all rotavirus diarrheic cases in piglets (1).
RVA diarrhea occurs in piglets from birth till seven days post weaning, but mainly develops in five till twenty-one day old piglets. The primary infection route is fecal-oral. Piglets develop watery diarrhea and depression. In some cases, fever develops within 12-48 hours after birth. RVA diarrhea may occur if colostrum intake or quality is too low, leading to an insufficient maternal antibody concentration.
Cases of RVA infection in suckling piglets result in yellowish pasty feces. Within two or three days, a more grey coloured pasty feces develops and persists for three to five days. Pigs may also become gaunt and rough-haired. Morbidity is 20%, while mortality, due to dehydration is 15% (2). Morbidity and mortality will increase if there is a co-infection with *E. coli* which results in a more severe diarrhea (2). In recent studies RVA prevalence in diarrheic samples was found to be up to 67%, while in older studies it varies from 10% to 70 %(3, 4).

Several methods are possible to diagnose RVA as the causative agent for diarrhea. Post mortem examinations may reveal a thin-walled small intestine in combination with liquid feces in the cecum and colon. Milk in the stomach is another indication for RVA as the cause of diarrhea (5). Furthermore, villous atrophy can be seen in the jejunum by microscopy.

Detection of RVA can be done by several techniques, such as an antigen enzyme-linked immunosorbent assay (antigen-ELISA), virus isolation, latex agglutination and polymerase chain reaction (PCR). RVA can be detected in feces and jejunal content of healthy piglets as well. Therefore it is possible that RVA is not the causative agent of diarrhea. This means that detection of RVA in the piglet intestine alone is not enough to conclude that it caused piglet diarrhea. In addition, titer measurements of IgA and IgM antibodies raised against RVA are used to determine the piglet's immune status. Recent infection of RVA can thus be detected using anti-RVA IgM levels in blood (6). Additionally, correlation exists between high viral loads of RVA and piglet diarrhea. However, the implications of this correlation remain inconclusive (6).

Clostridium perfringens type A

Clostridia are anaerobic, Gram-positive, spore-forming rods-shaped bacteria which produce several toxins (Table 1). These toxins cause enteric infections in all kinds of animals.
Clostridium perfringens (*C. perfringens*) type A (which can cause neonatal diarrhea) produces alpha and beta 2 toxins. The exact role of beta 2 toxin is still poorly understood.

***C. perfringens* class**	**Secreted toxins**	**Associated pathologies**
A	α; β2	Muscle necrosis, food poisoning, necrotisec enteritis (foal), enteric toxaemia (cattle), enteritis (piglet), equine colitis, gastro enteritis (dog)
B	α; β1; ε	Dysenteric, chronic enteritis(lamb) bloody enteritis (calve, foal, sheep)
C	α; β1; ε	Necrotic enteritis(foal), haem or necrotic enteric toxaemia (piglet, lamp, calve, goat, foal, human)
D	α; ε	Enteric toxaemia (lamb, calve) enteric colitis (goat)
E	α; ι	Enteric toxaemia (calve, lamb) enteritis (rabbit)
A-E	Enterotoxin	Enteritis in several species

Table 1. Overview of *Clostrida perfringens* types, their toxins and toxin-associated pathologies (7).

Infection with *Clostridium perfringens* type A leads to a pasty or creamy diarrhea, which can occur within 48 hours after birth and can last for five to six days. Mucous and sometimes even pink feces is seen, with perianal fecal staining. Besides diarrhea, a rough coat may be observed (8). Most piglets will recover. However, they may be behind in growth compared to their unaffected littermates. Macroscopically, the small intestine is thin walled, filled with gas or a watery content. However, mucosa inflammation is mild with some necrotic material attached. The large intestine may be distended with pasty content but lesions are not common (9).
Histologic lesions include villous tip necrosis and fibrin accumulation. However, villi can also be remain unaffected. The ileum and jejunum may be colonized with *C. perfringens*, although it is not uncommon to find large numbers of *C. perfringens* in the intestinal lumen. Unlike infection with *C. perfringens* type C, there is in most cases no haemorrhage and capillaries can be dilated in cases of *C. perfringens* type *A*. Gram positive rods may be detected in villous epithelial cells of ileum and jejunum.

Presence of *C. perfringens* type A is determined by bacterial culturing and genotyping of alpha and beta 2 toxin by qPCR. However, *C. perfringens* is a common inhabitant of the pig's gut (9). Quantitative testing for *C. perfringens* is becoming available, but it is unknown if and how quantitative results can be associated to the probability of causing diarrhea.

Escherichia coli

Escherichia coli (*E. coli)* is an important causative agents of various pig diseases, such as neonatal diarrhea (ND), post-weaning diarrhea (PWD) and edema disease (ED). ND results in economic losses due to morbidity, mortality, decreased weight gain and costs of treatment. *E. coli* can be classified by somatic (O), capsular (K), flagella (H) and fimbriae (F) antigens (10). For this report only F genes are considered as an indicator to classify *E. coli.* Fimbriae are involved in the attachment of bacteria's to the intestinal wall. Over 20 F antigens are recognized. Important F antigens for ND or PWD are shown in Table 2. Although shiga toxin Stx2e is involved in ED, its importance is also described for PWD. Shiga toxin (Stx2E) causes vascular damage in target organs when it is absorbed by the help of F18 (10).

Form of *E. coli* enterotoxaemia	**Fimbriae antigens**
Neonatal enterotoxaemia	F4, F5, F6, F41
Post weaning enterotoxaemia	F4, F18, Stx2e (shigatoxine)
Edema disease	F4, F18, STx2e (shigatoxine)

Table 2. Types off enterotoxaemia in piglets and their associated fimbriae antigens (10).

Neonatal diarrhea

ND caused by Enterotoxigenic *Escherichia coli (*ETEC) is mostly observed in piglets that are zero to four days old. The route of infection is fecal-oral. There is direct contact between piglets and feces of the sow when piglets are born. This direct contact means that there is a higher risk for ND in conditions of poor hygiene or in a management system without an all in all out principle. Therefore morbidity in most farms differs between 30-40% while mortality may reach 70% in those affected litters. ETEC with virulence factor F4 colonizes the lumen of jejunum and ileum, while *E. coli* with F5, F6 or F41 mostly colonize the posterior of the jejunum and the ileum. Piglets are more susceptible for infections with F5 and F6 in the first days of life because of a reduction of receptors of the intestinal epithelial cells later in life. ETEC adheres at the intestinal mucosa and produces enterotoxins, such as Stex2, which alters the small intestine electrolyte and water flow (10).

Clinical diarrhea may be mild with no sign of dehydration or could be clear, watery and profuse with extensive dehydration. Feces colour varies from clear to various shades of brown. Furthermore, vomiting and weight loss may be seen which can result in dehydration. Acute cases of ND due to ETEC result in dead piglets while chronic or less acute cases may recover provided that piglets keep drinking and are cared for. In these chronic cases inflamed anus and perineum could be seen from alkaline faecal material.
Macroscopic lesions are general dehydration and dilation of the stomach containing undigested milk. The greater stomach curvature may contain venous infarcts with some congestion on the small curvature.
Histologically, *E. coli* can be observed adhering to the mucosal epithelial cells of the jejunum and ileum in cases of F4. Furthermore, vascular congestion with haemorrhages and numbers of neutrophils and macrophages may be seen in the lamina propria. Detection of *E. coli* is possible by bacterial culturing and genotyping of O, K, H, or F genes by PCR (10).

Prevalence of co-infections causing diarrhea in piglets

C. perfringes type A*, E. coli* and RVA alone or combined are the most important pathogens causing diarrhea in piglets (11). These three pathogens are usually present in the environment and the pig's intestine, it is hard to determine which pathogen is the causative agent. The aim of this research was to evaluate the qPCR results for the above-mentioned pathogens in feces samples. The results should provide better guidance for veterinarians on how to interpret qPCR results, especially in case multiple pathogens are detected to be present in fecal samples simultaneously.

Aim of the study and research questions

The general objective of this project is to determine a cut-off value for the new combined "diarrhea package" of the Animal Health Service with respect to conclude on the causation for diagnosing diarrheic cases. The objective is broken down into several research questions.

1) At which qPCR value of *C. perfringens, E. coli* and RVA is the probability of a false positive low enough?
2) Is there interference between alpha toxin and other pathogens when detected together with other pathogens? The same question also applies for beta 2 toxin and RVA.
3) Which toxin, alpha or beta 2, is preferred to infer conclusion over *C. perfringens* type A.

Materials and methods

Study design

In the period of December 2017 to March 2018 an observational retrospective cross sectional study was performed.

Inclusion criteria

Results from qPCR tests on feces were compared to pathology of piglets which were submitted by veterinarians or by pathomorphological examinations of diarrheic piglets, which were not submitted for post mortem examination.
All these test results (qPCR, pathology and bacterial culture) were collected from the GD database from March 2017 to January 2018. Veterinarians were consulted by telephone when cases lacked data. Piglets that were diagnosed with Porcine Epidemic Diarrhea by PCR were excluded from the study, because it had no additional relevance for this research.

Data and variables

Several types of data were collected.

1. Microbiological data on fecal samples. In total 240 results from diarrheic piglets were submitted by veterinarians. Of these 240 samples, 83 piglets were submitted for post-mortem examination and 157 feces swabs of both rectal as well as feces samples from the pen floor were submitted to GD for PCR analysis. At post mortem examination at GD, swabs were taken from affected parts from the intestine. All these 240 swab samples were tested by the combined PCR package for the presence of *E. coli*, RVA and *C. perfringens* type A. This PCR test included a quantitative PCR test for alpha toxin genes, beta 2 toxin genes and RVA as well as a qualitative PCR test for several *E. coli* fimbriae types. For details about these PCR tests, please refer to the next section ("Detection of *Escherichia coli*, detection of rotavirus type A and detection of *Clostridium perfringens* type A"). Results will be divided into several groups which were based on what there was detected by the combined PCR package. These groups were further divided in to two groups (Figure 1), associated disease by PCR (qPCR) and associated disease by PCR and veterinarians (AD) (Table 3).
2. Pathological diagnosis was made by a certified veterinary pathologist with expertise in piglets. Judgement was done by several markers which were shown in Table 4.
3. Clinical case data. Veterinarians were contacted by telephone to obtain for additional anamnestic information and their judgement on the specific case to determine which pathogen was the causal agent. Meaning that all results of this research should be looked at with a certain caution.

Group	**Definition**
- *C. perfringens*-AD - RVA-AD - *E. coli*-AD	Associated Disease: Pathogen was detected positive by qPCR and was afterwards confirmed by veterinarians
- *C. perfringens*-qPCR - RVA-qPCR - *E. coli*-PCR	Pathogen was only detected positive by PCR

Table 3. Distribution of groups which were detected by PCR.

Flowchart of group sampling

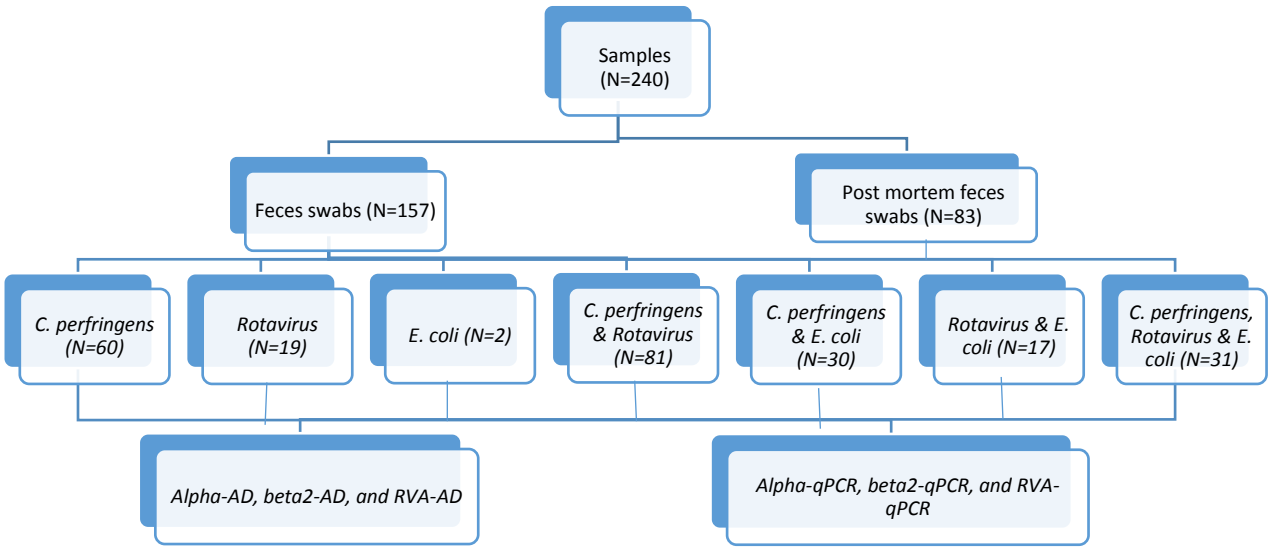


Figure 1. Flow chart of how groups were constructed.

Pathologic findings	**Pathogen**
- Thin-walled small intestine with watery feces in cecum and colon - Food in stomach - Villus atrophy in jejunum.	Rotavirus type A
- Thin-walled with small intestine, gas-filled, with watery content - Mild mucosal inflammation with some necrotic material - Pasty content in large intestine - Villous tip necrosis and fibrin accumulation	*C. perfringens* type *A*
- Dilation of the stomach containing milk - Venous infarcts or congestion in curvatures - Neutrophils and macrophages in lamina propria may be observed	*E. coli*

Table 4. Pathological findings of RVA, *C. perfringens* type A and *E. coli* (6, 9,10).

Detection of Porcine Rotavirus type A by qPCR

For Rotavirus type A, a qPCR specific for the detection of the NSP3 gene was used. The detection limit was between 2.75 and $2.75x10^1$ copies/ml and the measurement limit was between 2.75×10^1 copies/ml and 2.75×10^4 copies/ml (12). In this research, qPCR values of RVA were transformed to $\log_{10}$ copies/ml to enhance a normal distribution.

Detection of *C. perfringens* toxins by qPCR

For *C. perfringens* a quantitative PCR was used for the detection of alpha (encoded by the *Cpa* gene) and beta 2 (encoded by the *Cpb2* gene) toxins (Table 5). In this research, qPCR values were transformed to $\log_{10}$ Cfu/ml to enhance a normal distribution.

Gen	Detection limit (Cfu/ml)	Measurement reach (Cfu/ml)
Cpa	Between 1.5×10^4 and 1.5×10^5	$1.5 \times 10^4 – 1.5 \times 10^8$
Cpb2	Between 1.5×10^4 and 1.5×10^5	$1.5 \times 10^4 – 1.5 \times 10^8$

Table 5. Detection limit and measurement limit of several *Clostridia* type A toxins (13).

Detection of *E. coli* fimbriae by PCR

For *E. coli* a qualitative PCR was used which was based on fimbriae genes with different detection and measurement limits (Table 6).

Virulence factor	Detection limit	Measurement range
F4	Between 1.5×10^5 and 1.5×10^6	1.5×10^6 - 1.5×10^8
F5	Between 1.5×10^3 and 1.5×10^4	1.5×10^4 - 1.5×10^8
F6	Between 5.3×10^0 and 5.3×10^1	5.3×10^1 - 5.3×10^5
F18	Between 1.5×10^5 and 1.5×10^6	1.5×10^5 - 1.5×10^8
F41	Between 5.2×10^1 and 5.2×10^2	5.2×10^2 - 5.2×10^6
Stx2e	Between 1.5×10^5 and 1.5×10^6	1.5×10^6 - 1.5×10^8

Table 6. Detection limit and measurement limit of several fimbriae for *E. coli* (14).

Statistical analysis

Descriptive analysis was performed by dividing all qPCR results of each pathogen in to two groups (Table 3).

1) For determining a cut-off value for alpha toxin, beta 2 toxin and RVA a ROC curve was made. The optimal cut-off value was determined using the most extreme point in the top left-hand corner of the curve (15). Another approach was to choose a cut-off value that provided what was regarded as the optimal combination of sensitivity and specificity for the scenario of interest. In this research “Animal Health Service” considered a specificity of 90% high enough to keep the probability on a false positive as low as possible.
2) To see if there was interference between pathogens. Boxplots and independent t-test were performed on all groups to see if there was a higher qPCR value of alpha toxin, beta 2 toxin, or RVA when detected combined with other pathogens compared to when detected alone (Figure 1). These groups were tested for normal distribution by a Shapiro-Wilk test.
3) To determine which toxin can preferentially be used to identify *C. perfringens* type A, boxplots were made of alpha and beta 2 toxin PCR results for the two groups of qPCR results (*C. perfringens*-qPCR results and *C. perfringens*-AD results). In addition, scatterplots were made and Pearson correlation was performed to see if there was correlation between results of alpha and beta 2 toxin in the groups *C. perfringens*-AD and *C. perfringens*-qPCR. Also, the area under the curve (AUC) of the ROC curve was used, meaning that if an AUC value was closer to 1, a test was a more appropriate way to identify the pathogen (>0.9 very good, >0.8 good, >0.7 reasonable and >0.6 moderate) (15).

Results

Descriptive analysis

In total 240 samples were analysed. In 84% of the samples *C. perfringens* type A was detected by qPCR, while RVA was detected in 61.2% of the samples. This indicates that there were cases of diarrhea where different pathogens were present at the same time. However, this did not mean that the pathogen detected by qPCR is the causative agent of diarrhea (Table 7). Besides, in 80% of all cases where *E. coli* was detected, *E. coli* was confirmed as the diarrhea-causing agent by veterinarians.

Clinical (AD or qPCR) / **qPCR positive results**	*C. perfringens*-qPCR	*C. perfringens*-AD	RVA-qPCR	RVA-AD	*E. coli*-PCR	*E. coli*-AD	Total
C. perfringens (all results together)	76	126	NA	NA	NA	NA	202
RVA (all results together)	NA	NA	56	92	NA	NA	148
*E. coli (*all results together)	NA	NA	NA	NA	16	64	80

Table 7. Overview in absolute numbers of all qPCR findings per group, these groups are shown in table 3.

In cases where *C. perfringens* type A was detected alone, 91.6% was confirmed by veterinarians as a *C. perfringens*-AD case. However, not more than 56% was confirmed as *C. perfringens*-AD in cases where other pathogens were present in combination with *C. perfringens* type A (Table 8).

In 89.5 % of the cases where RVA was detected, veterinarians indicated that the samples were submitted from RVA-AD cases. Furthermore, 62.3% was confirmed as RVA-AD when RVA was detected together with *C. perfringens* type A while it was 50% when RVA was detected in all cases with *E. coli* (Table 8).

Furthermore, if *E. coli* was detected with RVA, veterinarians always (100 %) identified the diarrhea as *E. coli*-AD. This number decreased to 83.3% in all cases where *E. coli* and *C. perfringens* type A were detected together and to 70.9% in cases where all pathogens were simultaneously present (Table 8). This indicates that in cases of piglet diarrhea more pathogens may be present at the same time and that these pathogens may interfere with each other in the diagnostic process.

Clinical (AD or qPCR / **qPCR positive results**	*C. perfringens-qPCR*	*C. perfringens-AD*	RVA-qPCR	RVA-AD	*E. coli-qPCR*	*E. coli-AD*	Total
C. perfringens	5	55	NA	NA	NA	NA	60
RVA	NA	NA	2	17	NA	NA	19
C. perfringens / RVA	35	46	30	51	NA	NA	81
RVA / *E. coli*	NA	NA	8	9	0	17	17
C. perfringens / *E. coli*	13	17	NA	NA	5	25	30
C. perfringens / *E. coli* / RVA	23	8	16	15	9	22	31
E. coli	NA	NA	NA	NA	2	0	2

Table 8. Overview in absolute numbers of combinations of qPCR findings per group, these groups are shown in table 3.

1) ROC curves

Next section ROC curves were presented to determine a cut-off value for RVA, alpha- and beta 2 toxin

Rotavirus type A

The optimal cut-off value for RVA was reached at a cut-off value of $10^{3.60}$ copies/ml with both a specificity and sensitivity of 80%. For purpose of this research a specificity around 90% was calculated to minimalize the chance of a false positive. So a specificity of 90.3 % was reached at a cut-off value of $10^{5.66}$ copies/ml that resulted in an accompanying sensitivity of 61.1%. This means that there was a probability of 10% that there was a false positive outcome at a value higher than $10^{5.66}$ copies/ml (Figure 2).

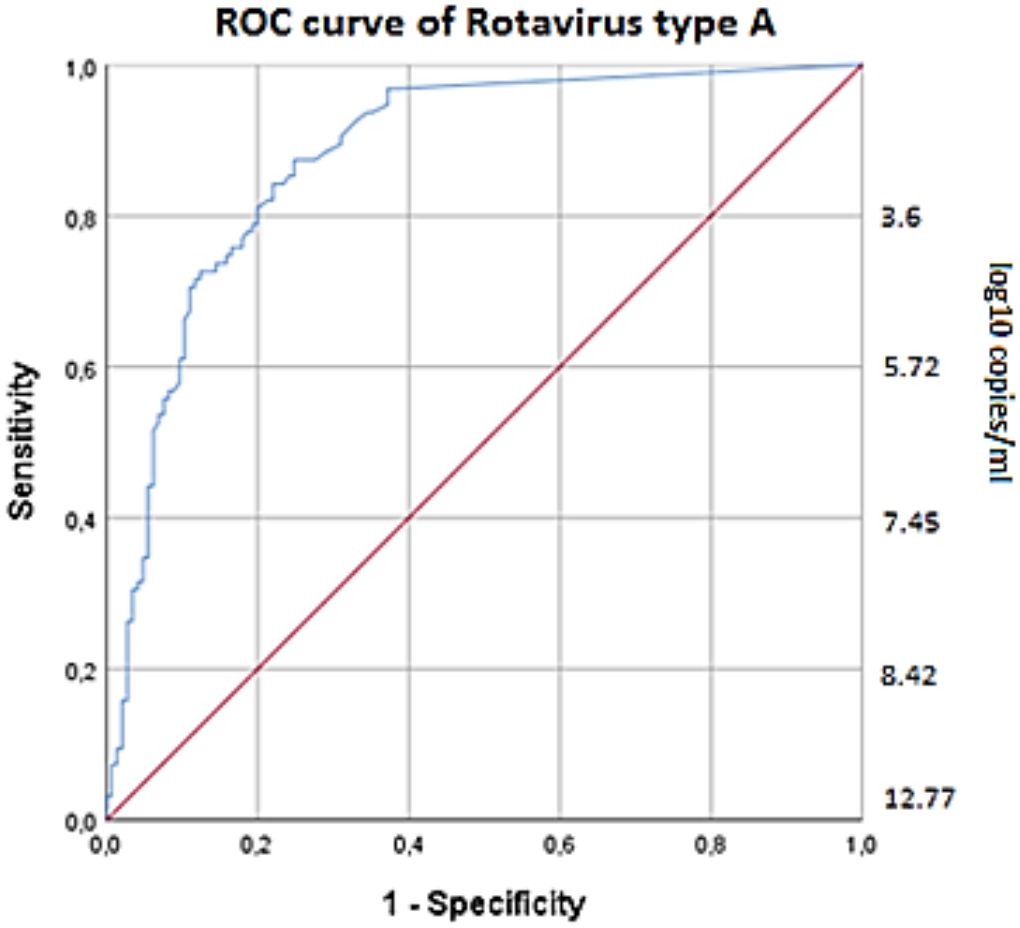


Figure 2. ROC Curve of RVA with an AUC of 0.879 N= 240, coordinates are listed in the supplementary Table 1.

C. perfringens alpha and beta 2 toxin

The optimal cut-off value for alpha toxin was given at a cut-off value of $10^{3.17}$ Cfu/ml with a specificity of 67.3% that will result in an accompanying sensitivity of 84.1%. However, to minimalize the chance of a false positive outcome, a cut-off value of $10^{4.50}$ Cfu/ml was taken with a specificity of 89.7% that will result in an accompanying sensitivity of 43.7%. Meaning that a value higher than $10^{4.50}$ Cfu/ml gave a probability of 10% on a false positive outcome (Figure 3).

For beta 2 toxin the optimal cut-off value was obtained at a value of $10^{2.80}$ Cfu/ml with a specificity of 71.7 % and a sensitivity of 88.1%.
However, to minimalize the chance of a false positive outcome, a cut-off value of $10^{4.02}$ Cfu/ml was taken with a specificity of 90.3% and that will result in an accompanying sensitivity of 60.0 %. This means that a value higher than $10^{4.02}$ Cfu/ml gave a hazard of 10% on a false positive outcome (Figure 3).

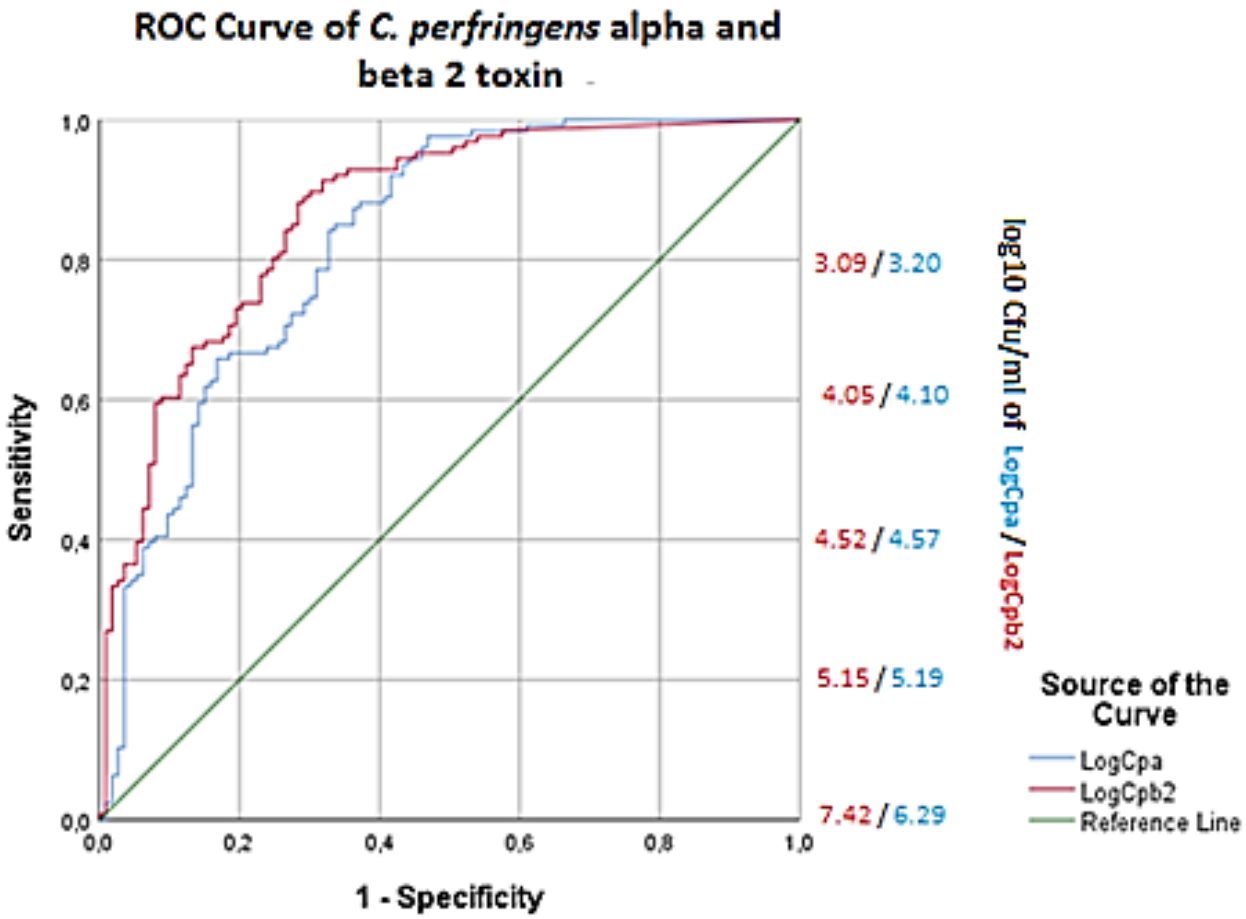
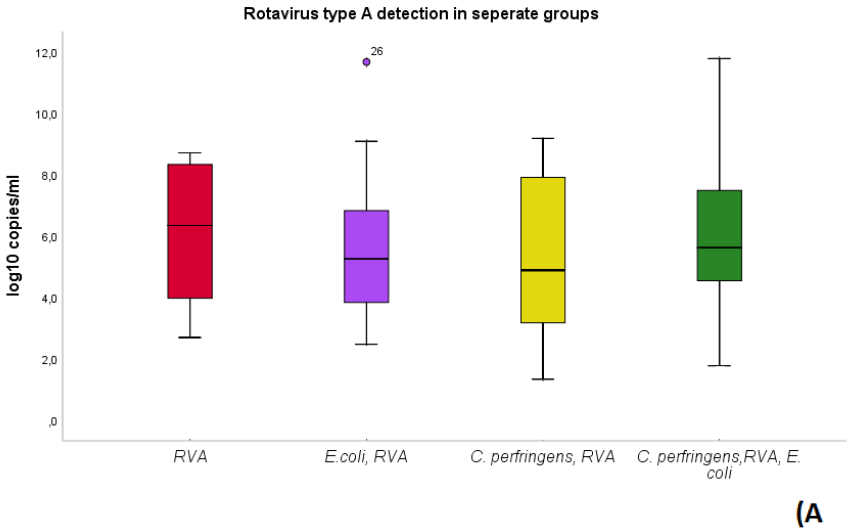


Figure 3. ROC Curve of *C. perfringens* alpha (blue line) and beta 2 (red line) toxin with an AUC of 0.828 for alpha toxin and an AUC of 0.866 for beta 2 toxin. N=240, coordinates are listed in the supplementary info table 2 and 3.

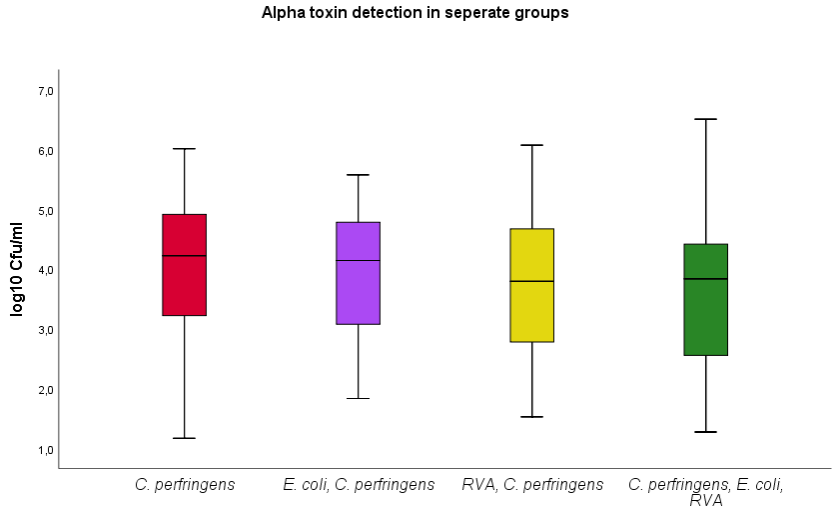
2) Interference between pathogens

Boxplots for RVA qPCR results indicated that the median of RVA qPCR results was higher in group RVA in comparison with all other groups where RVA was detected in combination with other agents (Figure 4A). However, an independent t- test showed no significant difference between means of RVA (Table 8).

Furthermore, both medians of *C. perfringens* toxins alpha and beta 2 results seems to be higher if *C. perfringens* was detected alone when compared to groups where *C. perfringens* was detected combined with other agents (Figure 4B and C). Nevertheless, independent t-test analysis showed that only the mean of beta 2 toxin was significant lower in results of *C. perfringens* with *E. coli* and RVA when compared to results where *C. perfringens* was detected alone (Table 9) (P<0.05).



(A



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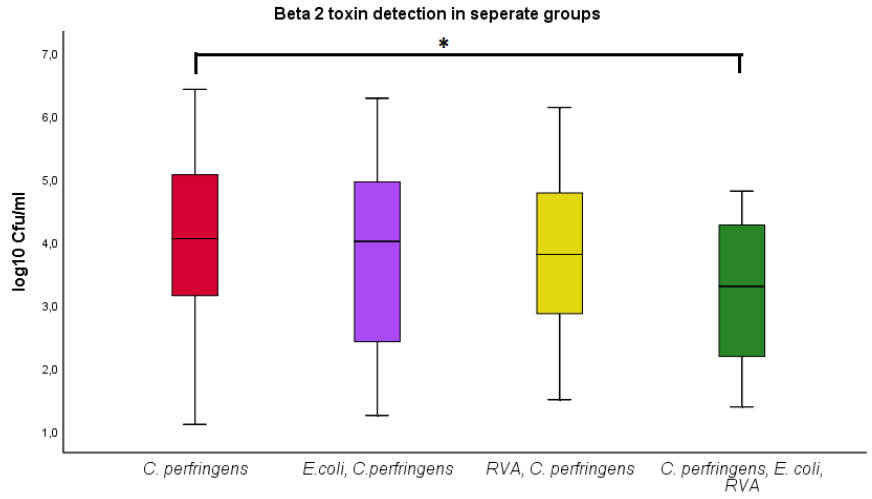

C)

Figure 4. Boxplots of RVA, alpha- and beta 2 toxin. X-axis, groups divided by PCR results. Y-axis, log_{10} Cfu/ml or log_{10} copies/ml.

A) Log10 copies/ml findings of RVA in different groups.

B) log_{10} Cfu/ml findings of *C. perfringens* alpha toxin in different groups.

C) log_{10} Cfu/ml findings of *C. perfringens* beta 2 toxin in different groups (*P<0.05).

Group / **Toxin /Virus**	*C. perfringens*	RVA	*C. perfringens* and RVA	*E. coli* with either RVA or *C. perfringens*	*C. perfringens*, *E. coli* and RVA
Alpha toxin $\log_{10}$ Cfu/ml	4.06	NA	3.93	4.82	3.58
Beta 2 toxin $\log_{10}$ Cfu/ml	3.96*	NA	3.61	3.59	2.71*
RVA $\log_{10}$ copies/ml	NA	6.12	5.43	5.75	5.86

Table 8 Copies/ml means of Rotavirus type A and Cfu/ml means of *C. perfringens* alpha and beta 2 toxin in all groups (*Significant different from each other).

3) Correlation between alpha toxin and beta 2 toxin

For alpha toxin, qPCR values between $10^{1\text{-}6.5}$ Cfu/ml were detected in both *C. perfringens*-qPCR as well as *C. perfringens*-AD, groups. However, most qPCR results in *C. perfringens*-AD were detected between $10^{3.5\text{-}5.0}$ Cfu/ml, while this was $10^{2.2\text{-}4.0}$ Cfu/ml for *C. perfringens*-qPCR (Figure 5). Beta 2 toxin showed qPCR values between $10^{0.0\text{-}6.5}$ Cfu/ml in both groups. However, in *C. perfringens*-AD most qPCR results were between $10^{3.2\text{-}5.0}$ Cfu/ml, while it was $10^{2.0\text{-}3.8}$ Cfu/ml for *C. perfringens*-qPCR (Figure 6).

Scatterplots show that *C. perfringens*-AD contained a qPCR value of $10^{0.0}$ Cfu/ml for beta 2 toxin while for alpha toxin qPCR values were detected upward of a value of $10^{1.0}$ Cfu/ml (Figure 7). However, *C. perfringens*-qPCR showed more cases of beta 2 toxin with a value of $10^{0.0}$ Cfu/ml when compared to *C. perfringens*-AD. Cases of *C. perfringens*-qPCR showed a higher value of $\log_{10}$ Cfu/ml for alpha toxin at a lower value of beta 2 toxin, meaning that there is a larger deviation between alpha and beta 2 toxin in *C. perfringens*-qPCR in comparison with *C. perfringens*-AD (Figure 7). This deviation difference between those two groups was supported by the correlation coefficients between alpha- and beta2 toxin of 0.572 for *C. perfringens*-qPCR and 0.896 for *C. perfringens*-AD (Table 9). In other words in feces from *C. perfringens*-AD pigs the association between the quantity of two toxins is better than in samples from other samples.

qPCR/AD	**Correlation coefficient**
C. perfringens-qPCR	0.572
C. perfringens-AD	0. 896

Table 9. Correlation coefficient between alpha and beta toxin in cases of *C. perfringens*-qPCR or *C. perfringens*-AD.

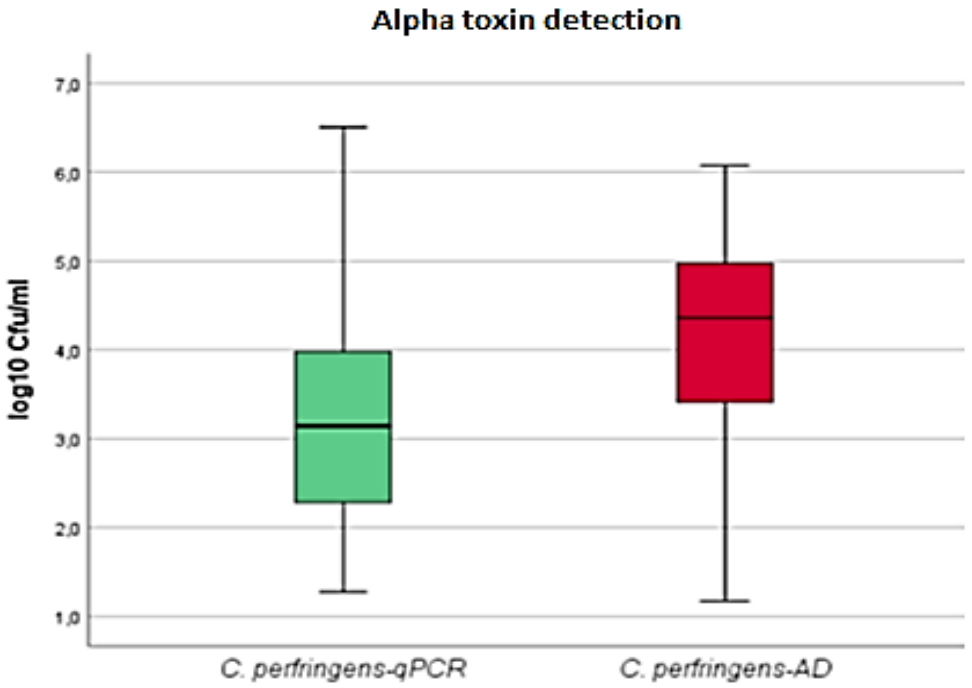

Figure 5. Alpha toxin detection in different groups, *C. perfringens*-qPCR (N=76), *C. perfringens*-AD (N=126).

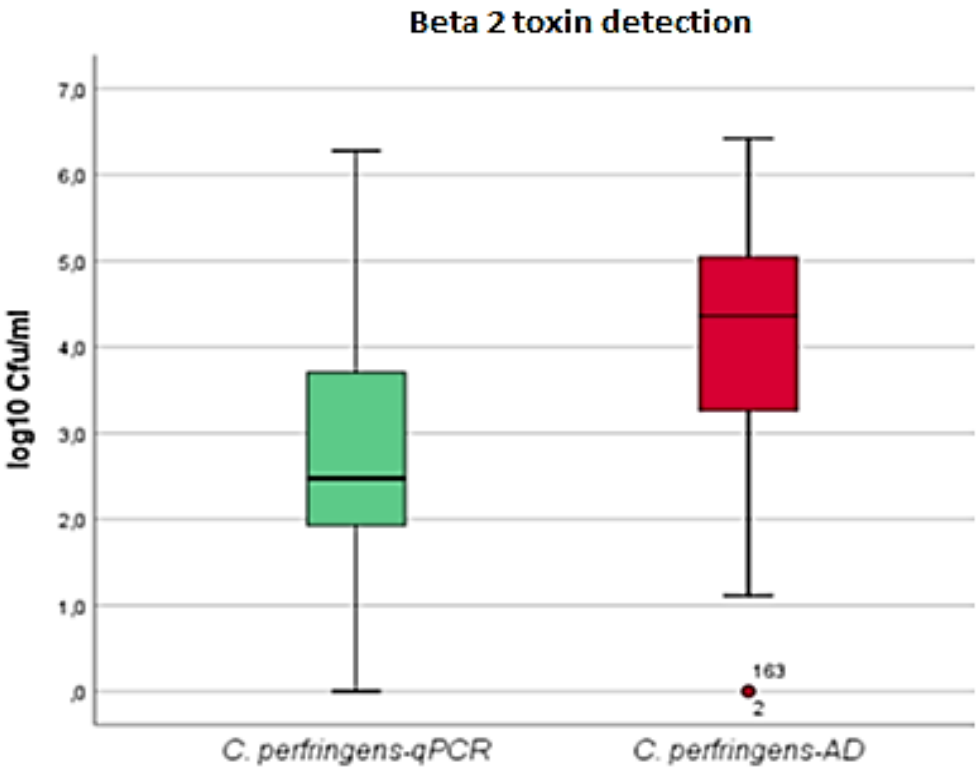

Figure 6. Beta 2 toxin detection in different groups. *C. perfringens*-qPCR (N=76*), C. perfringens*-AD (N=126).

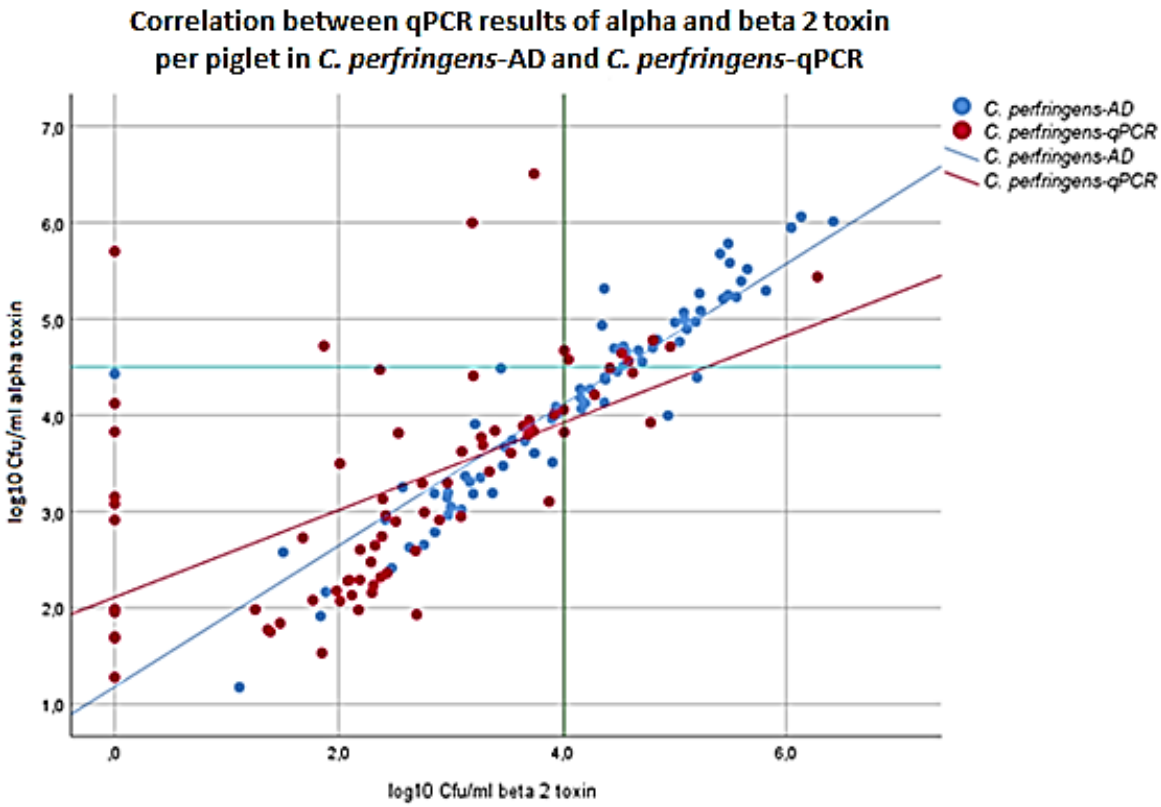

Figure 7. Correlation of alpha and beta 2 toxin qPCR results in *C. perfringens*-AD (N=126) in blue and *C. perfringens*-qPCR (N=76) in red with the cut-off value of alpha toxin (horizontal light blue line) and beta 2 toxin (vertical green line).

Discussion

Diarrhea as a result of enteric infections with bacteria and viruses is a serious problem during the first couple of weeks of a piglet's life. It often remains a challenge for veterinarians to identify the etiological cause of this diarrhea. The general objective of this project was to determine a cut-off value for veterinarians of the new combined "diarrhea package" of the Animal Health Service with respect to conclude on the causation for diagnosing diarrheic cases. Cut-off values of $10^{5.41}$ copies/ml for RVA, $10^{4.50}$ Cfu/ml for alpha toxin and $10^{4.02}$ for beta 2 toxin are determined. Several considerations for using the qPCR are demonstrated below.

1) Cut-off values of Rotavirus type A and *C. perfringens* alpha and beta 2 toxin.

In this study a cut-off value of $10^{3.60}$ copies/ml gives a specificity of 80% for RVA, while it is 89.7% at a cut-off value of $10^{5.41}$ copies/ml. Other studies consider feces samples containing RVA viral loads of $10^{5.40\text{ -}11.63}$ copies/g feces as 'substantially high' whereas a viral load of up to $10^{8.86}$ copies/g feces was considered as a 'high' viral load (16). It is unclear what the specificity is for those viral loads in the study of Theuns et al (16).
Furthermore, our research demonstrates that qPCR tests can detect RVA from a level of $10^{0.34}$ copies/ml, while in earlier research RVA infections could not be detected when only a fast-antigen detection test is used (16). This implies that the qPCR test of RVA has a higher sensitivity and specificity compared to a fast-antigen detection test. Admittedly, that research did not give the number of copies when a fast antigen detection can detect RVA (16).

This study demonstrates for alpha toxin a specificity of 80% at a cut-off value of $10^{3.86}$ Cfu/ml and for beta 2 toxin a cut-off value of $10^{3.39}$ Cfu/ml is necessary to reach the same specificity of 80%. A specificity of around 90% is reached at a cut-off value of $10^{4.50}$ Cfu/ml for alpha toxin and $10^{4.02}$ Cfu/ for beta 2 toxin.
In contrast, Smith and Jones found that *C. perfringens* type A reach $10^{8.2}$ Cfu/g in the stomach and $10^{8.3}$ Cfu/g in the intestine in healthy piglets twelve hours after birth (17). These numbers have fallen to $10^{2.0}$ Cfu/g in the stomach and $10^{2.7}$ Cfu/g in the intestine after seven days (18). This is all part of the normal colonization process of *C. perfringens* type A in the small intestine and stomach.
So this in line with the diagnosis of diarrhea caused by *C.* perfringens while in qPCR results of this research values below $10^{8.0}$ Cfu/g are correlated with diarrhea in piglets of three days old.

In short, a specificity of around 90% is reached at a cut-off value of $10^{5.66}$ copies/ml for RVA, $10^{4.50}$ Cfu/ml for alpha toxin and $10^{4.02}$ Cfu/ml for beta2 toxin. According to previous research the ROC curves of *C. perfringens* in this study should be used with a certain substantiation of the veterinarian.

2) Interference between pathogens

This study shows that lower qPCR numbers of RVA and *C. perfringens* type A are detected when found together with other pathogens in comparison when detected alone (Figure 4). However, only the mean of *C. perfringens* beta 2 toxin is significant lower in group *C. perfringens, E. coli* and RVA in comparison to when *C. perfringens* type A is detected alone.

However, previous other *in vitro* research suggest that synergism between bacteria and RVA involves certain biologic pathways which may be involve in the attachment or the invasion by co-infecting pathogens through an upregulation of certain receptors.
For example, no pathogenic effects of *E. coli* infection alone is detected while the risk of diarrhea is increased in the occurrence of RVA. Alternatively, the inflammatory response caused by RVA is likely

to damage epithelium, thereby altering the mucosal structure facilitating the attachment and invasion of other co-infecting agents (18). During inflammation, there is a release of mucin, fluid and cellular debris, which may contain nutrients for bacterial agents. Furthermore, the gut microbiota could be altered by secretion of antimicrobials, which allows agents to occupy the commensal gut flora (18).

It is given that healthy pigs have $10^{1.1}$ Cfu/g more *C. perfringens* type A in comparison with diarrheic piglets, namely $10^{6.5}$ Cfu/g against $10^{5.4}$ Cfu/g (19). This may indicate that the normal flora is affected by other pathogens.

In conclusion, previous research suggest synergism between pathogens (18,19). In contrast, this study shows according beta 2 toxin only interference between pathogens when *C. perfringens* is detected with *E. coli* and RVA. That only one interference between pathogens is detected may be happened by a low sample size (N<20) in the groups RVA and RVA with *E. coli*.
Furthermore, interference may also occur in the PCR reaction, as in this multiplex PCR more probes are used simultaneously for one sample that may interfere with the efficiency of the individual PCR reactions.

3) *C. perfringens* alpha and beta 2 toxin gen detection

According to the ROC curves, beta 2 toxin has a higher specificity at the same cut-off value in comparison with alpha toxin. However, the AUC values of both toxins are above 0.820 meaning that they are both good indicators for detecting *C. perfringens* type A. Earlier research shows that no significant difference is detected regarding the quantity of beta 2 toxin in fecal samples from diarrheic piglets compared to healthy piglets (20,21). In our research, a significant difference is detected between *C. perfringens*-qPCR and *C. perfringens*-AD.

Furthermore, our research shows in cases of *C. perfringens*-AD that the results of both toxins do not differ much from each other, whereas in cases of *C. perfringens*-qPCR high results of alpha toxin are detected in combination with low results of beta 2 toxin. This deviation is supported by the correlation coefficients of *C. perfringens*-AD and *C. perfringens*-qPCR. However, further research should be done to determine which toxin is more important for finding the cause of piglet diarrhea when either the qPCR values of alpha or beta 2 toxin is low in cases of *C. perfringens* type A.

In conclusion, our research suggests that the detection of beta 2 toxin may be of higher diagnostic value in detecting *C. perfringens* type A infection in cases of piglet diarrhea if one toxin has a low value, while other research suggest the opposite.

Conclusion

Overall, this study suggests that high viral loads (>$10^{4.50}$ copies/ml) of RVA provides a good indication for diarrhea caused by RVA, while fewer copies of RVA are necessary to play a role in piglet diarrhea when other agents are present ($p>0.05$).

This research shows that high values of *C. perfringens* alpha toxin (>$10^{4.50}$ Cfu/ml) and beta 2 toxin (>$10^{4.02}$ Cfu/ml) toxin are an indicator for the presence of diarrhea caused by *C. perfringens* type A. However, all opinions of veterinarians are based on the results of the qPCR tests, which may or will have interfered with their judgement on the case. Furthermore, veterinarians treat piglets against RVA while *C. perfringens* type A is also detected by qPCR which indicates that veterinarian's opinions are also based on outcome of treatment. Therefore, more research should be conducted using a different golden standard than veterinarian's opinions to evaluate the qPCR results. Furthermore, more research is necessary to determine which toxin has a higher positive predictive value if one toxin has a low qPCR value of $\log_{10}$ Cfu/ml.

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Supplementary information

Rotavirus type A

LogRota	Sensitivity	1-specificity	LogRota	Sensitivity	1-specificity
-1,00	1	1	6,26	0,558	0,083
0,67	0,968	0,386	6,33	0,558	0,076
1,56	0,968	0,379	6,36	0,547	0,076
1,89	0,968	0,372	6,40	0,537	0,076
2,15	0,947	0,372	6,45	0,537	0,069
2,34	0,937	0,352	6,52	0,526	0,069
2,43	0,937	0,345	6,63	0,516	0,062
2,54	0,926	0,331	6,77	0,505	0,062
2,64	0,905	0,31	6,85	0,484	0,062
2,69	0,895	0,31	6,88	0,474	0,062
2,74	0,884	0,29	6,92	0,463	0,062
2,81	0,874	0,276	6,98	0,453	0,062
2,92	0,874	0,262	7,02	0,442	0,062
3,07	0,874	0,248	7,15	0,442	0,055
3,16	0,863	0,248	7,30	0,432	0,055
3,20	0,853	0,248	7,39	0,421	0,055
3,25	0,853	0,241	7,45	0,4	0,055
3,34	0,842	0,234	7,48	0,379	0,055
3,41	0,842	0,228	7,51	0,368	0,055
3,45	0,842	0,221	7,53	0,358	0,055
3,48	0,832	0,221	7,55	0,347	0,055
3,52	0,821	0,221	7,58	0,347	0,048
3,56	0,821	0,214	7,64	0,337	0,048
3,58	0,811	0,2	7,69	0,316	0,048
3,60	0,8	0,2	7,75	0,316	0,041
3,63	0,789	0,2	7,80	0,305	0,041
3,75	0,789	0,193	7,86	0,305	0,034
3,85	0,779	0,193	7,92	0,295	0,034
3,87	0,779	0,186	7,96	0,284	0,034
3,88	0,768	0,179	7,99	0,274	0,034
3,92	0,758	0,179	8,07	0,263	0,034
3,98	0,758	0,172	8,16	0,263	0,028
4,04	0,758	0,166	8,22	0,242	0,028
4,14	0,747	0,166	8,33	0,221	0,028
4,23	0,747	0,159	8,41	0,211	0,028
4,28	0,737	0,159	8,42	0,2	0,028
4,31	0,737	0,152	8,46	0,189	0,028
4,37	0,737	0,145	8,50	0,168	0,028
4,42	0,726	0,145	8,51	0,158	0,028
4,48	0,726	0,138	8,59	0,158	0,021
4,56	0,726	0,131	8,68	0,147	0,021
4,63	0,726	0,124	8,75	0,126	0,021
4,71	0,716	0,124	8,81	0,116	0,021
4,82	0,716	0,117	8,84	0,105	0,021
4,92	0,705	0,117	8,86	0,095	0,021
4,96	0,705	0,11	8,88	0,095	0,014
5,07	0,695	0,11	8,91	0,084	0,014
5,22	0,684	0,11	8,94	0,074	0,014
5,33	0,674	0,11	8,95	0,074	0,007
5,41	0,663	0,103	9,00	0,063	0,007
5,45	0,653	0,103	9,06	0,053	0,007
5,52	0,642	0,103	9,10	0,042	0,007
5,57	0,632	0,103	9,15	0,032	0,007
5,61	0,611	0,103	10,26	0,032	0
5,66	0,611	0,097	11,50	0,021	0
5,72	0,6	0,097	11,72	0,011	0
5,84	0,589	0,097	12,77	0	0
5,99	0,579	0,097			
6,08	0,568	0,09			
6,16	0,568	0,083			

Table 1. ROC curve coordinates of RVA with the thereby belonging specificity and sensitivity

C. perfringens alpha toxin gen

LogCpa	Sensitivity	1-specificity	LogCpa	Sensitivity	1-specificity	LogCpa	Sensitivity	1-specificity
-1,00	1	1	3,30	0,786	0,31	4,50	0,437	0,097
0,59	1	0,664	3,31	0,778	0,31	4,53	0,429	0,097
1,23	0,992	0,664	3,34	0,77	0,31	4,55	0,421	0,097
1,41	0,992	0,655	3,36	0,762	0,31	4,55	0,413	0,097
1,61	0,992	0,646	3,39	0,754	0,31	4,56	0,405	0,097
1,72	0,992	0,637	3,41	0,746	0,31	4,57	0,405	0,088
1,76	0,992	0,628	3,45	0,746	0,301	4,58	0,405	0,08
1,81	0,992	0,619	3,49	0,738	0,301	4,61	0,397	0,08
1,88	0,992	0,611	3,50	0,738	0,292	4,65	0,397	0,071
1,92	0,984	0,611	3,56	0,73	0,292	4,66	0,389	0,071
1,94	0,984	0,602	3,61	0,722	0,292	4,67	0,389	0,062
1,97	0,984	0,593	3,61	0,722	0,283	4,68	0,381	0,062
1,98	0,984	0,584	3,64	0,722	0,274	4,69	0,373	0,062
1,98	0,984	0,575	3,66	0,714	0,274	4,70	0,365	0,062
2,03	0,984	0,566	3,68	0,706	0,274	4,71	0,357	0,062
2,08	0,984	0,558	3,71	0,706	0,265	4,71	0,349	0,062
2,10	0,984	0,549	3,74	0,698	0,265	4,71	0,349	0,053
2,14	0,984	0,54	3,75	0,69	0,265	4,72	0,341	0,053
2,16	0,984	0,531	3,77	0,683	0,265	4,74	0,341	0,044
2,17	0,976	0,531	3,77	0,683	0,257	4,77	0,333	0,044
2,21	0,976	0,522	3,79	0,675	0,257	4,78	0,333	0,035
2,26	0,976	0,513	3,81	0,675	0,248	4,78	0,325	0,035
2,28	0,976	0,504	3,82	0,675	0,239	4,78	0,317	0,035
2,29	0,976	0,496	3,82	0,667	0,239	4,81	0,31	0,035
2,30	0,976	0,487	3,83	0,667	0,23	4,86	0,302	0,035
2,34	0,976	0,478	3,83	0,667	0,221	4,91	0,294	0,035
2,37	0,976	0,469	3,84	0,667	0,212	4,93	0,286	0,035
2,40	0,968	0,469	3,86	0,667	0,204	4,94	0,278	0,035
2,44	0,96	0,469	3,89	0,667	0,195	4,96	0,27	0,035
2,52	0,96	0,46	3,90	0,667	0,186	4,96	0,262	0,035
2,57	0,952	0,46	3,92	0,659	0,186	4,97	0,254	0,035
2,58	0,944	0,46	3,93	0,659	0,177	4,98	0,246	0,035
2,60	0,944	0,451	3,95	0,659	0,168	4,99	0,238	0,035
2,61	0,944	0,442	3,96	0,651	0,168	5,00	0,23	0,035
2,64	0,937	0,442	3,98	0,643	0,168	5,03	0,222	0,035
2,65	0,937	0,434	4,00	0,635	0,168	5,07	0,214	0,035
2,67	0,929	0,434	4,01	0,627	0,168	5,10	0,206	0,035
2,71	0,921	0,434	4,02	0,627	0,159	5,15	0,198	0,035
2,73	0,921	0,425	4,04	0,619	0,159	5,20	0,19	0,035
2,74	0,921	0,416	4,06	0,619	0,15	5,21	0,183	0,035
2,75	0,913	0,416	4,07	0,611	0,15	5,21	0,175	0,035
2,77	0,905	0,416	4,08	0,603	0,15	5,22	0,167	0,035
2,78	0,897	0,416	4,10	0,595	0,15	5,24	0,159	0,035
2,84	0,889	0,416	4,12	0,595	0,142	5,25	0,151	0,035
2,90	0,889	0,407	4,13	0,587	0,142	5,28	0,143	0,035
2,91	0,881	0,407	4,16	0,579	0,142	5,29	0,135	0,035
2,91	0,881	0,398	4,19	0,571	0,142	5,30	0,127	0,035
2,93	0,881	0,389	4,20	0,563	0,142	5,31	0,119	0,035
2,95	0,881	0,381	4,22	0,563	0,133	5,35	0,111	0,035
2,96	0,881	0,372	4,23	0,556	0,133	5,41	0,103	0,035
2,98	0,873	0,372	4,25	0,548	0,133	5,48	0,103	0,027
3,01	0,873	0,363	4,27	0,54	0,133	5,53	0,095	0,027
3,03	0,865	0,363	4,28	0,532	0,133	5,56	0,087	0,027
3,05	0,857	0,363	4,30	0,524	0,133	5,58	0,079	0,027
3,06	0,849	0,363	4,32	0,516	0,133	5,63	0,071	0,027
3,09	0,849	0,354	4,35	0,508	0,133	5,69	0,063	0,027
3,12	0,849	0,345	4,36	0,5	0,133	5,71	0,063	0,018
3,14	0,849	0,336	4,38	0,492	0,133	5,75	0,056	0,018
3,15	0,841	0,336	4,39	0,484	0,133	5,86	0,048	0,018
3,17	0,841	0,327	4,40	0,476	0,133	5,96	0,04	0,018
3,19	0,833	0,327	4,42	0,476	0,124	5,98	0,032	0,018
3,19	0,825	0,327	4,43	0,468	0,124	5,99	0,024	0,018
3,19	0,817	0,327	4,44	0,46	0,124	6,00	0,024	0,009
3,20	0,81	0,327	4,44	0,46	0,115	6,04	0,016	0,009
3,23	0,802	0,327	4,45	0,452	0,115	6,07	0,008	0,009
3,26	0,794	0,327	4,46	0,444	0,115	6,29	0	0,009
3,28	0,786	0,327	4,48	0,444	0,106	7,51	0	0
3,29	0,786	0,319	4,49	0,437	0,106			
3,29	0,786	0,319	4,49	0,437	0,106			

Table 2. ROC curve coordinates of *C. perfringens* alpha toxin with the thereby belonging specificity and sensitivity

C. perfringens beta 2 toxin gen

LogCpb2	Sensitivity	1-specificity	LogCpa	Sensitivity	1-specificity	LogCpb2	Sensitivity	1-specificity
-1,00	1	1	3,20	0,778	0,239	4,48	0,421	0,062
0,56	0,984	0,575	3,21	0,778	0,23	4,50	0,413	0,062
1,18	0,976	0,575	3,22	0,77	0,23	4,52	0,405	0,062
1,31	0,976	0,566	3,22	0,762	0,23	4,53	0,397	0,062
1,38	0,976	0,558	3,24	0,754	0,23	4,53	0,397	0,053
1,43	0,976	0,549	3,26	0,746	0,23	4,54	0,389	0,053
1,49	0,976	0,54	3,27	0,738	0,23	4,55	0,381	0,053
1,59	0,968	0,54	3,28	0,738	0,221	4,56	0,373	0,053
1,73	0,968	0,531	3,32	0,738	0,212	4,58	0,365	0,053
1,81	0,968	0,522	3,36	0,738	0,204	4,61	0,365	0,044
1,85	0,96	0,522	3,39	0,73	0,204	4,65	0,365	0,035
1,86	0,96	0,513	3,42	0,73	0,195	4,69	0,357	0,035
1,88	0,96	0,504	3,46	0,722	0,195	4,74	0,349	0,035
1,93	0,952	0,504	3,48	0,714	0,195	4,78	0,341	0,035
2,00	0,952	0,496	3,51	0,706	0,195	4,80	0,341	0,027
2,01	0,952	0,487	3,54	0,706	0,186	4,81	0,333	0,027
2,05	0,952	0,478	3,55	0,698	0,186	4,82	0,333	0,018
2,09	0,952	0,469	3,60	0,69	0,186	4,84	0,325	0,018
2,11	0,952	0,46	3,66	0,69	0,177	4,84	0,317	0,018
2,14	0,952	0,451	3,68	0,683	0,177	4,87	0,31	0,018
2,17	0,944	0,451	3,70	0,683	0,168	4,90	0,302	0,018
2,18	0,944	0,442	3,71	0,683	0,159	4,93	0,294	0,018
2,19	0,944	0,434	3,72	0,683	0,15	4,95	0,286	0,018
2,21	0,944	0,425	3,74	0,675	0,15	4,95	0,278	0,018
2,25	0,937	0,425	3,74	0,675	0,142	4,96	0,27	0,018
2,28	0,929	0,425	3,75	0,675	0,133	4,97	0,27	0,009
2,29	0,929	0,416	3,78	0,667	0,133	4,99	0,262	0,009
2,31	0,929	0,407	3,82	0,659	0,133	5,03	0,254	0,009
2,32	0,929	0,398	3,86	0,651	0,133	5,06	0,246	0,009
2,35	0,929	0,389	3,89	0,651	0,124	5,08	0,238	0,009
2,37	0,929	0,381	3,91	0,643	0,124	5,09	0,23	0,009
2,38	0,929	0,372	3,92	0,635	0,124	5,10	0,222	0,009
2,39	0,929	0,363	3,93	0,635	0,115	5,11	0,214	0,009
2,41	0,929	0,354	3,95	0,627	0,115	5,15	0,206	0,009
2,42	0,921	0,354	3,95	0,619	0,115	5,19	0,198	0,009
2,43	0,921	0,345	3,97	0,611	0,115	5,20	0,19	0,009
2,45	0,921	0,336	4,00	0,603	0,115	5,21	0,183	0,009
2,49	0,913	0,336	4,01	0,603	0,106	5,22	0,175	0,009
2,52	0,913	0,327	4,02	0,603	0,097	5,23	0,167	0,009
2,55	0,913	0,319	4,03	0,603	0,088	5,25	0,159	0,009
2,60	0,905	0,319	4,05	0,595	0,088	5,27	0,151	0,009
2,66	0,897	0,319	4,06	0,595	0,08	5,35	0,143	0,009
2,69	0,897	0,31	4,10	0,587	0,08	5,42	0,135	0,009
2,71	0,897	0,301	4,12	0,579	0,08	5,44	0,127	0,009
2,74	0,889	0,301	4,14	0,571	0,08	5,46	0,119	0,009
2,75	0,889	0,292	4,16	0,563	0,08	5,48	0,111	0,009
2,76	0,881	0,292	4,16	0,556	0,08	5,49	0,103	0,009
2,80	0,881	0,283	4,17	0,548	0,08	5,52	0,095	0,009
2,85	0,873	0,283	4,17	0,54	0,08	5,58	0,087	0,009
2,86	0,865	0,283	4,19	0,532	0,08	5,63	0,079	0,009
2,88	0,857	0,283	4,23	0,524	0,08	5,73	0,071	0,009
2,90	0,849	0,283	4,26	0,516	0,08	5,82	0,063	0,009
2,93	0,849	0,274	4,28	0,508	0,08	5,88	0,056	0,009
2,97	0,841	0,274	4,32	0,508	0,071	5,99	0,048	0,009
2,97	0,841	0,265	4,36	0,5	0,071	6,07	0,04	0,009
2,98	0,833	0,265	4,37	0,492	0,071	6,09	0,032	0,009
2,99	0,825	0,265	4,37	0,484	0,071	6,10	0,024	0,009
3,01	0,817	0,265	4,38	0,476	0,071	6,12	0,016	0,009
3,05	0,81	0,265	4,38	0,468	0,071	6,21	0,008	0,009
3,09	0,81	0,257	4,38	0,46	0,071	6,35	0,008	0
3,10	0,802	0,257	4,39	0,452	0,071	7,42	0	0
3,12	0,802	0,248	4,41	0,444	0,071			
3,15	0,794	0,248	4,43	0,444	0,062			
3,18	0,786	0,248	4,45	0,437	0,062			
3,20	0,786	0,239	4,46	0,429	0,062			

Table 3. ROC curve coordinates of *C. perfringens* beta 2 toxin with the thereby belonging specificity and sensitivity