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ORIGINAL ARTICLE

Evaluation of a novel multiplex qPCR method for rapid detection and quantification of pathogens associated with calf diarrhoea

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Abstract

Aims: Diarrhoea is a common health problem in calves and a main reason for use of antimicrobials. It is associated with several bacterial, viral and parasitic pathogens, most of which are commonly present in healthy animals. Methods, which quantify the causative agents, may therefore improve confidence in associating a pathogen to the disease. This study evaluated a novel commercially available, multiplex quantitative polymerase chain reaction (qPCR) assay (Enterit4Calves) for detection and quantification of pathogens associated with calf-diarrhoea.

Methods and Results: Performance of the method was first evaluated under laboratory conditions. Then it was compared with current routine methods for detection of pathogens in faecal samples from 65 calves with diarrhoea and in 30 spiked faecal samples. The qPCR efficiencies were between 84%–103% and detection limits of 100–1000 copies of nucleic acids per sample were observed. Correct identification was obtained on 42 strains of cultured target bacteria, with only one false positive reaction from 135 nontarget bacteria. Kappa values for agreement between the novel assay and current routine methods varied between 0.38 and 0.83.

Conclusion: The novel qPCR method showed good performance under laboratory conditions and a fair to good agreement with current routine methods when used for testing of field samples.

Significance and impact of study: In addition to having fair to good detection abilities, the novel qPCR method allowed quantification of pathogens. In the future, use of quantification may improve diagnosis and hence treatment of calf diarrhoea.

KEYWORDS

Calf diarrhoea, calf scours, multiplex qPCR, quantification, detection

INTRODUCTION

Diarrhoea in calves is mainly caused by infectious agents. When diarrhoea occurs within the first 2 weeks of life, it is usually caused by either group A bovine rotavirus, bovine coronavirus, *Escherichia coli* F5 ($K99^+$) or *Clostridium perfringens* (Ngeleka et al., 2019; Rocha et al., 2017). The protozoan *Cryptosporidium parvum*, on the other hand, dominates in 2-3-week-old calves (Delafosse et al., 2015), while *Eimeria* species (coccidia) has been reported to be the predominant cause of the diarrhoea in older calves (Enemark et al., 2013; Forslid et al., 2015). *Salmonella* is likewise an important cause of calf diarrhoea, and this pathogen has a wider age span than the other pathogens, as it can be detected in calves from less than 1 week of age up to 15 weeks of age (Coura et al., 2014; Ngeleka et al., 2019). Each of the pathogens can cause disease on their own, but mixed infections are commonly reported and often lead to more severe disease (Abuelo et al., 2019; Cho et al., 2013; Izzo et al., 2011). Studies performed in European countries report prevalence of calf diarrhoea between 19.1% and 57.6% with mortality rates between 7.3% and 12.7% (Abuelo, 2016; Bartels et al., 2010; Klein-Jöbstl et al., 2014; Mahendran et al., 2017).

To avoid overuse of antimicrobials, it is important to determine the underlying cause of calf diarrhoea. Clinical examination cannot be used for this, and diagnosis needs to be supported by microbiological analysis. A complicating factor is that the causative agents are commonly found in healthy calves (Gulliksen et al., 2009), and simple positive or negative results from dichotomous diagnostic tests are not sufficient to establish causality. Quantitative PCR methods have been implemented to overcome this problem in other diseases, for example intestinal disease and respiratory diseases in pigs, where potentially pathogenic microorganisms with frequent occurrence of the pathogens in healthy animals is likewise the problem (Goecke et al., 2020). Recently such methods have also been developed and implemented for diagnosis of some of the pathogens associated with calf diarrhoea (Tsuchiaka et al., 2017). For example, a level of 2.6×10^5 *Cryptosporidium* oocysts per gram of faeces, or a *Cryptosporidium* Cq value of 31.4 by qPCR, has been reported as a diagnostic cut-off for a diarrhoea in calves (Operario et al., 2015).

A multiplex qPCR assay was recently developed to improve microbiological diagnosis in relation to calf-diarrhoea (https://dna-diagnostic.com/products/animal/enterit-4-calves/). This assay simultaneously detects and quantify *C. perfringens* (all types), *C. perfringens* type B and C, *Salmonella enterica* serovar Dublin (*S.* Dublin), *E. coli* F5, bovine rotavirus, bovine coronavirus, *C. parvum* and *Eimeria* spp. The aim of this study was to evaluate the performance of this assays based on laboratory testing and on the ability to detect and quantify pathogens in samples from calves with diarrhoea, including comparison with diagnostic methods currently used for microbiological diagnosis of calf diarrhoea.

MATERIALS AND METHODS

Enterit4Calves multiplex qPCR assay

The assay under evaluation is termed Enterit4Calves and is produced by the company DNA Diagnostic A/S (Risskov, Denmark). The Enterit4Calves kits consists of two sets of multiplex qPCR assays. The first assay, the Enterit4CalvesB kit (Assay A), contains primers and hydrolysis probes designed to amplify and detect DNA from *C. perfringens, C. perfringens* type B and type C, *S.* Dublin and *E. coli* F5. The second assay, the Enterit4CalvesV kit (Assay B), is a multiplex reverse transcription (RT)-qPCR assay containing primers and hydrolysis probes designed to amplify RNA of bovine rotavirus, bovine coronavirus, *C. parvum* and *Eimeria* species. The company has not released the primer and probe sequences.

In the use of this method, nucleic acids were extracted from faeces, broth cultures, and suspensions of DNA in phosphate-buffered saline (PBS) using a faecal extraction kit enclosed with the assays according to the manufacturer's instructions. Briefly, RNA and DNA were extracted from 0.1 g ± 0.03 g of material by homogenizing with pre-lysis buffer, the components of which has not been disclosed by the company. Cells were harvested by centrifugation at 5000×*g*, washed once with washing buffer, and then lysed in 120 µl of lysis buffer at 37°C for 20 min followed by incubation at 95°C for 15 min. Cell debris was pelleted by centrifugation. The obtained nucleic acids sample contained both DNA and RNA. Where relevant (Assay B), cDNA synthesis of RNA in the sample was immediately performed using cDNA master mix (DNA Diagnostic A/S) according to instructions from the manufacturer. The RT-reaction step consisted of incubation for 37°C for 60 min and then incubation at 70°C for 15 min. Lysate and cDNA samples were used immediately or stored at −20°C for later qPCR amplification.

The qPCR was performed using an Mx3005P qPCR System (Agilent Technologies, Glostrup, Denmark) with the following cycling parameters: 95°C for 1 min followed by 40 cycles of 95°C for 5 s and 60°C for 25 s. The machine was set to acquire fluorescence on the Cy5, ROX, HEX, FAM and ATTO425 channels corresponding to positive signals from the probes for detecting *C. perfringens* or bovine rotavirus, *C. perfringens* type B and C or bovine coronavirus, *S.* Dublin or *C. parvum*, *E. coli* F5 or *Eimeria* species and Internal Amplification Control (IAC) in the

two assays, respectively. The results were analysed using MxPro qPCR Software (Agilent Technologies, United States). According to the producer, samples with quantification cycle (Cq) of 37 cycles or less should be considered positive.

In vitro characterization of the performance of Enterit4Calves

For determining quantification range, qPCR efficiencies and repeatability of the assays, genomic DNA obtained from 10-fold serial dilutions of one strain of each of *E. coli* F5, *S.* Dublin, *Clostridium perfringens* type A and *Clostridium perfringens* Type C (strains marked in Supplementary Table S1) in the range of 10^6 to 10^0 copies. Strains of virus and parasites were not available for in vitro performance evaluation, and instead we used 10-fold serial dilutions *of* in vitro-transcribed RNAs of target sequences for virus and parasites (see below) in PBS. The performance of the multiplex qPCR was analysed in triplicate at each dilution to establish repeatability, and efficiency was determined by calculation of the assay coefficient of variation (CV) according to the formula: CV = (SD [Cq-value]/overall mean [Cq-value])×100, where SD indicates standard deviation (Holman et al., 2019). The PCR efficiency was calculated using the formula: E = 10(−1/slope) − 1 × 100 (Svec et al., 2015).

For the analysis described above, plasmids containing viral or parasitic target gene-sequences were transfected into *E. coli* DH5α (Life Technologies Europe BV, Denmark) and purified from here using the QIAGEN Plasmid Mini Kit (Qiagen, Germany) according to instructions from the manufacturer. Plasmids were linearized by digestion with XbaI restriction enzyme (Thermo Fisher Scientific), and subsequently they were transcribed into RNA using MEGAscript™ T7 Transcription Kit (Life Technologies) according to the protocol enclosed with the kit. DNA was removed with Turbo DNase (Life Technologies). The transcribed RNA was further purified using the MEGAclear™ Transcription Clean-Up Kit (Life Technologies) as instructed by the manufacturer. RNA quality and integrity were determined using a NanoDrop spectrophotometer (Saveen & Werner ApS). Plasmids were purchased from GenScript Biotech (Leiden, Netherlands) and supplied by the company producing Enterit4Calves, since primer sequences have not been disclosed.

In vitro sensitivity and specificity were determined on cultured strains of bacteria, consisting of *C. perfringens* ($n = 19$) including strains of *C. perfringens* type B ($n = 1$) and C ($n = 2$), *S. enterica* serovar Dublin ($n = 21$) and *E. coli* F5 ($n = 3$) (Supplementary Table S1) as well as 135 strains of nontarget bacteria (Supplementary Table S2).

Detection of pathogens in faecal samples from calves with diarrhoea using Enterit4Calves

Duplicate faeces samples were collected from 65 calves with diarrhoea on 14 Danish dairy farms during March to August 2019. One sample was used for detection and quantification of pathogens by Enterit4Calves, and one for detection of bacteria, virus and parasites by methods routinely used in relation to calf diarrhoea (see below). Calves were between 2 and 105 days old. Veterinarians who submitted the samples did not disclose the breed of the calves, but 70% of Danish dairy cows are Danish Holsteins, 13% are Jersey, 5% are Danish Red and the remaining 12% are other breeds or crosses. Sampling was done as part of routine veterinary services and farmers gave oral consent that samples could be used for research on condition that farm identity was not disclosed. The study protocol was approved by the Animal Ethics Institutional Review Board, Department of Veterinary and Animal Sciences, University of Copenhagen (AEIRB number 2021-08-VCM-010A).

The methods routinely used for microbiological diagnosis in relation to calf diarrhoea depended on the target organisms. For detection of *E. coli* F5, *S.* Dublin and *C. perfringens*, 1 g of faeces was diluted in 9 ml of sterile Buffered Peptone Water (BPW) and 1 µl of this was plated onto MacConkey agar (Oxoid CM015) and blood agar (Oxoid CM0055) with 5% of calf blood for isolation of *E. coli* and *C. perfringens*, respectively. For isolation of *S.* Dublin, 100 µl of the faecal solution were enriched in 9 ml of BPW overnight at 37°C, and 100 µl were then spotted onto Modified Semisolid Rappaport Vassiliadis agar (Oxoid CM0910). The MacConkey and Modified Semisolid Rappaport Vassiliadis plates were incubated aerobically at 37°C for 24 h, while the blood agar plates were incubated anaerobically at 37°C for 24–48 h. Typical colonies on MacConkey agar, Modified Semisolid Rappaport Vassiliadis agar, and blood agar were purified by streaking on MacConkey agar, Xylose Lysine Desoxycholate agar (Oxoid CM0469) or blood agar and incubated under the same growth conditions.

Further identification of the three bacteria was performed by PCR assays. Bacterial colonies were resuspended in 50 µl of distilled water, and DNA was obtained by heating for 10–20 min at 95°C followed by a quick centrifugation for 2 min. Polymerase chain reaction assays and detection of amplicons were performed according to previously published methods (Casey & Bosworth, 2009; Persson et al., 2012; van Asten et al., 2009).

Detection of viral pathogens and parasitic pathogens was made with an in-house High-Throughput qPCR system running on BioMark platform (Fluidigm,

AH-diagnostic, Tilst, Denmark). The primers and probes used in this study were adopted from a previously published study (Cho et al., 2010) and the high-throughput qPCR method was conducted with a few modifications from a previously published method (Goecke et al., 2020) used for detection of swine lung and intestinal pathogens. Briefly, target RNA was extracted from the sample using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The RNA was then reverse transcribed and pre-amplified on a T3 Thermocycler (Biometra) at 45°C for 20 min followed by enzyme inactivation at 95°C for 10 min followed by 24 cycles of 94°C for 15 s, 60°C for 45 s. For the parasite targets, the pre-amplification was performed with the following thermal cycling conditions: 95°C for 10 min, followed by 14 cycles at 95°C for 15 s, and 60°C for 4 min. The pre-amplified samples were then added to qPCR master mix and amplified in the high-throughput qPCR instrument BioMark (Fluidigm) with the following cycling conditions: 94 min at 94°C, followed by 40 cycles at 94°C for 15 s, at 60°C for 60 s. Data (Cq values and amplification curves) were acquired on the BioMark system and analysed using the Fluidigm Real-Time PCR Analysis software 4.1.3 (Fluidigm, USA).

Cryptosporidium parvum and *Eimeria* sp. were additionally detected by other methods commonly used routinely for detection of these pathogens. Faecal samples were analysed for *Eimeria* spp. using a modified McMaster technique with a sensitivity of 10 oocysts per gram (Roepstorff & Nansen, 2017). Oocysts were examined at 100x magnification and up to 50 oocysts per sample were identified to species level using the morphological criteria described by Deplazes et al. (2016).

DNA of *Cryptosporidium* species was extracted from faecal samples using the NucliSENS easyMag DNA extraction robot (BioMerieux, Ballerup, Denmark). One µL of DNA was amplified by a qPCR assay, which was modified from a previously published method (Stensvold & Nielsen, 2012; Verweij et al., 2004). In brief, amplification reactions were performed in 50 µl qPCR master mix and amplification comprised 15 min at 95°C, followed by 50 cycles of 15 s at 95°C and 30 s at 60°C and 30 s at 72°C. The method is referred to as Statens Serum Institut (SSI)-qPCR below.

Testing on spiked samples

No samples from calves were positive for *S*. Dublin, *E. coli* or *C. perfringens* type B and C (see Result section). To evaluate the ability of Enterit4Calves to detect these pathogens, 30 spiked samples were used. Faecal samples were collected on two Danish farms and proven negative for the pathogens by the routine methods applied in the study (see below). One gram of faeces was diluted in 9 ml of sterile BPW. Three *E. coli* F5 strains from the collection of clinical isolates at the Department of Veterinary and Animal Sciences, University of Copenhagen and three *S*. Dublin strains obtained from the National Food Institute of Denmark (Supplementary Table S1), all from calves, were cultivated aerobically in Luria-Bertani (LB) broth overnight at 37°C with shaking. CFU of the LB broths was determined by spotting 10-fold dilution made in BPW on LB agar and reading the number of colonies after overnight incubation at 37°C. Approximately 2×10^5 CFU of one *E. coli* and one *S*. Dublin strain were added into 10 ml faecal suspensions and mixed resulting in suspensions with approximately 2×10^4 CFU/mL of test bacteria. Aliquots of these suspensions were processed as natural samples. Genomic DNA, obtained from a strain of *C. perfringens* type B and a strains of *C. perfringens* type C were kindly provided by Statens Serum Institute, Denmark. First, 30 ng of this DNA was added directly to 10 ml faecal suspensions, and suspensions were processed as natural samples, however, this provided no positive samples. Therefore, in the final evaluation, the 30 ng DNA was added after the initial centrifugation step of the PCR methods.

Statistics

The diagnostic sensitivity and specificity with 95% confidence intervals and Coefficient of determination (R^2) between Cq values and $\log_{10}$ copy-numbers were calculated using DAG Stat spreadsheet (Mackinnon, 2000). The Cohen's kappa coefficient was interpreted as follows: Kappa = 0.00–0.20, slight agreement; Kappa = 0.21–0.40, fair agreement; Kappa = 0.41–0.60, moderate agreement; Kappa = 0.61–0.80, substantial agreement; Kappa = 0.81–1.00, almost perfect agreement (Landis & Koch, 1977).

RESULTS

Analytical performance of Enterit4Calves

Assay A detected all strains tested of *C. perfringens* of unknown toxin type ($n = 15$), *C. perfringens* type B ($n = 1$) and C ($n = 2$), *S. Dublin* ($n = 21$) and *E. coli* F5 ($n = 3$) (Supplementary Table S1). No positive reactions were observed from 135 nontarget bacteria (Supplementary Table S2), except for reaction to a strain of *S. enterica* serovar Naestved. This false positive reaction was in accordance with the declaration in the kit.

The analytical sensitivity of Assay A and B were determined based on 10-fold serial dilutions of genomic DNA of four target bacteria and in vitro transcribed RNA of target parasites and viruses. The limits of detection (LOD) were found to be between 100 to 1000 copies of nucleic acids per reaction depending on the assay (Tables 1 and 2, Figure 1). The assay CV within runs ranged from 0.20% to 1.40% for Assay A and from 0.28% to 2.23% for Assay B. The quantitative PCR efficiencies (E) ranged from 84% to 102% for Assay A (Table 1) and 92% to 103% for Assay B (Table 2). The coefficient of determination (R^2) between the Cq values and the copies of the nucleic acids per assay ranged from 0.97–0.99 for all assays.

Performance of Enterit4Calves on faecal samples from calves

Comparisons of test results between Enterit4Calves and different laboratory tests routinely used for detection of the same bacteria, virus and protozoan was evaluated on 65 samples from calves with diarrhoea. Results are summarized in Table 3 and details are shown in Supplementary Table S3. Enterit4Calves Assay A detected *C. perfringens* in 37 samples, which was 8 more than the number of positive samples identified by combined culture and colony-PCR method routinely used for detection of this pathogen. All other samples ($n = 28$) were negative for

TABLE 1 Performance of Assay A multiplex quantitative PCR assay for detection of DNA from target bacterial pathogens

Target	Copy number (DNA copies)	Mean cycle threshold (Ct)	*SD*	CV (%)	Positives/run	Slope	Efficiency (%)	R^2 value[a]
Clostridium perfringens	10^6	20.84	0.15	0.72	3/3	−3.274	102	0.9996
	10^5	23.88	0.05	0.20	3/3			
	10^4	27.22	0.10	0.38	3/3			
	10^3	30.5	0.17	0.54	3/3			
	10^2	33.9	0.47	1.40	3/3[b]			
	10^1	n.d.	n.a.	n.a.	0/3			
	10^0	n.d.	n.a.	n.a.	0/3			
C. perfringens type B and C	10^6	17.64	0.07	0.41	3/3	−3.387	97	0.9973
	10^5	20.89	0.04	0.20	3/3			
	10^4	24.08	0.05	0.20	3/3			
	10^3	28.18	0.19	0.68	3/3			
	10^2	30.93	0.13	0.41	3/3[b]			
	10^1	n.d.	n.a.	n.a.	0/3			
	10^0	n.d.	n.a.	n.a.	0/3			
Salmonella enterica serovar Dublin	10^6	18.88	0.06	0.32	3/3	−3.397	97	0.999
	10^5	22.49	0.13	0.58	3/3			
	10^4	25.74	0.10	0.40	3/3			
	10^3	29.42	0.09	0.29	3/3			
	10^2	32.4	0.27	0.83	3/3[b]			
	10^1	n.d.	n.a.	n.a.	0/3			
	10^0	n.d.	n.a.	n.a.	0/3			
Escherichia coli F5	10^6	21.08	0.09	0.42	3/3	−3.793	84	0.9943
	10^5	24.26	0.29	1.21	3/3			
	10^4	28.09	0.14	0.51	3/3			
	10^3	31.53	0.05	0.14	3/3[b]			
	10^2	36.41	0.49	1.35	2/3			
	10^1	n.d.	n.a.	n.a.	0/3			
	10^0	n.d.	n.a.	n.a.	0/3			

Abbreviations: n.a., not applicable; n.d., not detected.

[a]Coefficient of determination between Ct value and $\log_{10}$ copy-number. Analysis has performed on 10-fold dilutions of DNA of *C. perfringens*, *Salmonella enterica* serovar Dublin and *E. coli* F5.

[b]Limit of detection was defined as a lowest concentration at which a fluorescent signal could be detected in all replicates ($n = 3$).

TABLE 2 Performance of Assay B multiplex quantitative PCR for detection of DNA-copies of viral and parasitic pathogens

Target	Copy number (DNA copies)	Mean cycle threshold (Ct)	*SD*	CV (%)	Positives/run	Slope	Efficiency (%)	R^2 value[a]
Bovine coronavirus	10^6	17.32	0.11	0.64	3/3	−3.3583	99	0.9979
	10^5	20.80	0.26	1.24	3/3			
	10^4	24.30	0.04	0.19	3/3			
	10^3	27.40	0.17	0.60	3/3			
	10^2	30.31	0.25	0.84	3/3[b]			
	10^1	n.d.	n.a.	n.a.	0/3			
	10^0	n.d.	n.a.	n.a.	0/3			
Bovine rotavirus	10^6	20.38	0.09	0.45	3/3	−3.2417	103	0.9747
	10^5	24.75	0.15	0.62	3/3			
	10^4	27.54	0.22	0.79	3/3			
	10^3	32.17	0.58	1.80	3/3[b]			
	10^2	32.56	0.31	0.97	2/3			
	10^1	n.d.	n.a.	n.a.	0/3			
	10^0	n.d.	n.a.	n.a.	0/3			
C. parvum	10^6	17.96	0.22	1.21	3/3	−3.353	92	0.9995
	10^5	20.6	0.10	0.48	3/3			
	10^4	25.26	0.22	0.89	3/3			
	10^3	27.84	0.12	0.45	3/3			
	10^2	32.21	0.72	2.23	3/3[b]			
	10^1	n.d.	n.a.	n.a.	0/3			
	10^0	n.d.	n.a.	n.a.	0/3			
Eimeria spp	10^6	20.11	0.13	0.66	3/3	−3.4106	96	0.9918
	10^5	22.21	0.23	1.03	3/3			
	10^4	27.04	0.12	0.46	3/3			
	10^3	29.39	0.08	0.28	3/3			
	10^2	33.7	0.49	1.47	3/3[b]			
	10^1	n.d.	n.a.	n.a.	0/3			
	10^0	n.d.	n.a.	n.a.	0/3			

Abbreviations: n.a., not applicable, n.d., not detected.

[a]Coefficient of determination between Ct value and $\log_{10}$ copy-number. Analysis was performed on 10-fold dilutions of in vitro transcribed RNA of bovine coronavirus, bovine rotavirus, *C. parvum and Eimeria* spp.

[b]Limit of detection was defined as a lowest concentration at which a fluorescent signal could be detected in all replicates ($n = 3$).

C. perfringens by both assays. With the combined culture and PCR method as the gold standard, the sensitivity and specificity of Assay A for this target were 94% and 76%, respectively (Table 3 and Supplementary Table S4). The test agreement between Assay A and culture/colony-PCR was 83%, and the k value was 0.66. None of the samples were positive for *C. perfringens* types B and C, *S. enterica* serovar Dublin or *E. coli* F5 by Assay A or the combined culture PCR methods used for comparison. For this reason, only specificity (100%) could be determined for these pathogens based on this set of samples.

Enterit4Calves Assay B identified one more sample as positive for bovine coronavirus than the Fluidigm qPCR, whereas two Fluidigm qPCR-positive samples were negative for bovine coronavirus by Enterit4Calves. Using the Fluidigm qPCR methods as the gold standard, this corresponded to sensitivity and specificities of 33% and 98% for Enterit4Calves for this pathogen (Table 3 and Supplementary Table S4). Test agreement between the two assays was 95.4%, and k value was 0.38. Of the 65 samples, 28 were positive for bovine rotavirus by both Enterit4Calves Assay B and the Fluidigm qPCR assay used for comparison. In samples that were positive by both methods, the Cq values by Fluidigm qPCR were 5–12 values lower than results of Assay B (Supplementary Table S3). Six samples which were negative in the Fluidigm qPCR assay were

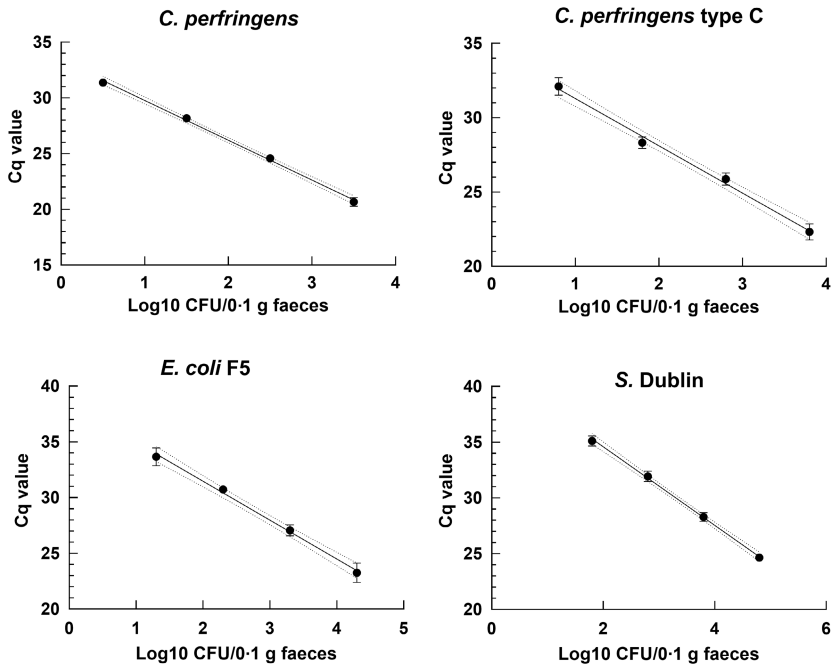

FIGURE 1 Relationship between $\log_{10}$CFU per 0.1 gram faeces and Cq values for *Clostridium perfringens*, *C. perfringens* type C, *Escherichia coli* F5 and *Salmonella enterica* serovar Dublin. Bacteria were spiked into pathogen free faecal samples in the numbers indicated and Cq valued in Enterit4Calves was determined. The correlation coefficients (R^2) of plots was 0.998, 0.9938, 0.9965 and 0.9989. Error bars show SD of Cq values and the dotted lines indicate the 95% confidence interval of the fitted line.

TABLE 3 Agreement between Assay A/B and reference methods for detection of pathogens in 65 faecal samples from calves diagnosed clinically with calf diarrhoea

		Assays A/B				% agreement (*K*)
Reference test		**Pos**	**Neg**	**Sensitivity**	**Specificity**	**(95% CI[a])**
Clostridium perfringens (Culture and colony-PCR)	Pos	29	3	91	76	83.0 (0.66)
	Neg	8	25			(0.48 to 0.84)
C. perfringens type B and C (culture + PCR)	Pos	0	0	ND[b]	100	100 (ND[b])
	Neg	0	65			
Escherichia coli F5 (PCR)	Pos	0	0	ND[b]	100	100 (ND[b])
	Neg	0	65			
Salmonella Dublin (PCR)	Pos	0	0	ND[b]	100	100 (ND[b])
	Neg	0	65			
Bovine coronavirus (Fluidigm)	Pos	1	2	33	98	95.4 (0.38)
	Neg	1	61			(−0.18 to 0.93)
Bovine rotavirus (Fluidigm)	Pos	22	6	79	84	81.5 (0.62)
	Neg	6	31			(0.43 to 0.82)
Cryptosporidium (SSI qPCR)	Pos	24	19	56	100	70.8 (0.46)
	Neg	0	22			(0.29 to 0.63)
Cryptosporidium (Fluidigm)	Pos	21	5	81	92	87.7 (0.74)
	Neg	3	36			(0.57 to 0.91)
Eimeria spp. (McMaster)	Pos	6	3	67	100	95.4 (0.78)
	Neg	0	56			(0.53 to 1.00)
Eimeria spp. (Fluidigm)	Pos	4	0	100	97	96.9 (0.78)
	Neg	2	59			(0.50 to 1.00)

Abbreviations: Neg, negative; Pos, positive.

[a]95% confidence interval.

[b]*C. perfringens* type B and C, *Escherichia coli* F5 and *Salmonella enterica* serovar Dublin were not detected in any of the faecal samples and thus estimates of sensitivity and kappa value were not calculated.

positive by Enterit4Calves, whereas 36 samples were negative for bovine rotavirus by both assays. Accordingly, the sensitivity and specificity of Enterit4Calves Assay B were calculated to be 79% and 84%, respectively, for this pathogen (for calculation of these, see Supplementary Table S4), the test agreement was found to be 81.5%, and the k value between the two assays to be 0.62.

Performance of Enterit4Calves Assay B for *C. parvum* was compared with two methods, the SSI-qPCR and the Fluidigm qPCR assays, which both detects other species of *Cryptosporidium* than *C. parvum*. Enterit4Calves detected *C. parvum* in 24 samples, which was 2 less than the number of samples identified as positive by Fluidigm qPCR and 19 less than the SSI-qPCR. Nineteen and five samples, which were positive by the SSI-qPCR and the Fluidigm qPCR, respectively, were negatives by Enterit4Calves, and 41 samples were negative by all three assays. Since Enterit4Calves and the methods used for comparison did not have identical range of target pathogens, the sensitivity could not be estimated for the ability of Enterit4Calves to detect *C. parvum*. Assuming that negative samples in the SSI-qPCR and Fluidime qPCR were true negative samples for any *Cryptosporidium* species, the specificity of Enterit4Calves in comparison with SSI-qPCR was 100% and the specificity in comparison with Fluidigm qPCR 92% (Table 3 and Supplementary Table S4).

Out of 65 samples, 6 samples tested positive for *Eimeria* spp. by Enterit4Calves, two of which were negative by Fluidigm qPCR. Nine samples were positive for *Eimeria* spp. by McMaster assay, and this assay categorized 56 samples as negative among the 59 negatives samples by Enterit4Calves. Of the 61 samples, 59 that were negative by Fluidigm qPCR was likewise negative by Enterit4Calves. This corresponded to sensitivity and specificities of 67% and 100% in comparison with the McMaster assay, and 100% and 97% in comparison with the Fluidigm qPCR (Table 3 and Supplementary Table S4). Test agreements were 95.4% and 96.9%, and k values were calculated to be 0.78 against both assays.

Assay performance on spiked faecal samples

The lack of positive samples for *S.* Dublin, *E. coli* F5 and *C. perfringens* type B and C hindered evaluation of the sensitivity of Enterit4Calves for these pathogens. To overcome this problem, 30 spiked samples were used (Table 4). The intention was to have 30 positive samples, since natural, negative samples had been tested already. However, *E. coli* F5 was only detected in 26 of the samples by Enterit4Calves, and the combined culture and PCR method used for comparison only detected *E. coli* F5 in 20 samples. *S.* Dublin was detected in 28 samples by Enterit4Calves, while the culture and PCR method used for comparison detected this bacterium in 23 samples. *C. perfringens* strains of toxin types B and C were not available and purified DNA was used. After spiking at the lysis step with 30 ng of DNA from each of these bacteria, Enterit4Calves was positive in 22 samples, and the reference PCR method was positive in 19 samples. Using the culture and PCR methods used for comparison as gold standards, this corresponded to sensitivities of 90% for *E. coli* F5, 100% for *S.* Dublin and 94.7% for *C. perfrigens* type B and/or C.

DISCUSSION

Real-time and quantitative PCR methods are increasingly used for microbiological diagnosis in veterinary medicine due to the possibility for high-throughput

TABLE 4 Agreement between Assay A and reference methods for detection of pathogens in 30 faecal samples from calves spiked with bacteria or purified DNA from calf diarrhoeal pathogens[a]

		Assay A				% agreement (K)
Reference test		**Pos**	**Neg**	**Sensitivity**	**Specificity**	**(95% CI[b])**
Escherichia coli F5 (PCR)	Pos	18	2	90.0	20.0	66.7 (0.12)
	Neg	8	2			(−0.21 to 0.45)
Salmonella Dublin (PCR)	Pos	23	0	100	28.6	83.3 (0.38)
	Neg	5	2			(−0.01 to 0.77)
Clostridium perfringens type B and C (PCR)	Pos	18	1	94.7	63.6	83.3 (0.62)
	Neg	4	7			(0.33 to 0.91)

[a]Each sample was spiked with one out of three *Escherichia coli* F5 strains, one out of three *S.* Dublin strains and 30 ng of DNA from *Clostridium perfringens* type B and type C.

[b]95% confidence interval.

analysis. However, it is still an expensive methodology, when multiple targets need to be identified and several single-plex qPCR assays must be run in parallel. Multiplex qPCR methods which allow the detection of several targets in a single or few tubes are therefore an attractive solution for diagnostic laboratories. The methods evaluated in the current study detect eight pathogens of relevance to calf diarrhoea in two tubes under the same qPCR conditions, and thus detection and quantification of all eight pathogens can be performed in two reaction tubes of a single run, facilitating rapid diagnosis.

The efficiencies of the qPCR assays in Enterit4Calves were found to be high, except the qPCR efficiency of the method used detecting *E. coli* F5 (84%). The acceptable range of PCR efficiency for quantitative multiplex assay is normally set at 90%–110% (Raymaekers et al., 2009), but can be adapted to 75%–110% according to Broeders et al. (2014). As the qPCR efficiency for detecting *E. coli* F5 was less than the qPCR efficiency for detecting other targets, this implied that the multiplex reaction is not optimal for *E. coli* F5 detection. This may be due to either DNA secondary structures in the target area of the genome, or suboptimal primers or thermo-cycling conditions. Detection of *E. coli* F5 under laboratory conditions was performed with a high coefficient of determination (R^2) value, indicating that, despite the relative low efficiency of this qPCR method, there was good coherence between detection by qPCR and culture method. The qPCR efficiencies, the coefficient of determination (R^2), and slopes of Enterit4Calves, Assay B was found to be like those previously published for multiplex qPCR assays for detection of bovine coronavirus, bovine rotavirus and single-plex qPCR for *Cryptosporidium* (Cho et al., 2010; Schroeder et al., 2012), suggesting that the qPCR methods perform at least as well as these methods from a technical point of view.

Analysis of the data using kappa coefficients showed a good agreement between Enterit4Calves Assay A and the traditional combination of culture and PCR methods used for the detection of *C. perfringens*. All faecal samples from calves with diarrhoea were negative for *C. perfringens* type B and C by both methods, suggesting that these types were not commonly involved as the causative agents for calf diarrhoea in the farms included in the current study. When faecal samples were spiked with DNA from these two types of bacteria, the agreement between Enterit4Calves and the methods used for comparison was good. Enterit4Calves had more samples positive than the methods used for comparison, suggesting that it was superior in detecting these two bacteria, however, spiking with DNA was done after the lysis step and this may have influenced the results.

The datasheet enclosed with Enterit4Calves emphasizes that the assay detects *S.* Naestved in addition to the *S.* Dublin because there is more than 99% nucleotide sequence homology between these two *Salmonella* serovars, and we confirmed this cross reaction. All calves were negative for *S.* Dublin and *E. coli* F5 by Enterit4Calves, and the methods used for comparison. Thus, the diagnostic sensitivity for these pathogens could not be evaluated on field samples. Based on spiked samples, however, the sensitivities were good (90% *E. coli* F5 and 100% *S.* Dublin). The product description does not disclose the target sequence for *E. coli* F5, but it may be a limitation for the assay if the primer sequences are directed against the fimbriae. As pointed out by Cho and Yoon (2014), other types of *E. coli* may be found in calves with diarrhoea, and these can be missed if the diagnosis focuses on *E. coli* F5 alone. On the other hand, a study has reported that significant histopathological changes were only observed in calves suffering from diarrhoea caused by toxin producing *E. coli* F5 (Ngeleka et al., 2019). In the current study, the traditional culture and PCR methods used for comparison were set up to detect many types of potentially pathogenic *E. coli*, and by this method we did not detect other types of *E. coli* as the cause of diarrhoea in any of the calves.

A fair to moderate agreement of test results between Enterit4Calves and Fluidigm qPCR methods were observed for the detection of bovine coronavirus and bovine rotavirus in faecal samples from calves. The Fluidigm qPCR method detected more samples positive for these two viruses than Enterit4Calves, and the Cq values were generally lower by Fluidigm qPCR method in positive samples than in Enterit4Calves method. This could indicate that the Fluidigm qPCR has a higher sensitivity than Enterit4Calves, possibly because the Fluidigm qPCR uses a pre-amplification step prior to the qPCR (Goecke et al., 2020). Samples with relatively high Cq values (Cq ≥ 22) by Fluidigm qPCR were often negative by Enterit4Calves (Supplementary Table S3). The bovine coronavirus and bovine rotavirus detected by Enterit4Calves likely originated from viral-infected cells in the faeces, because the first centrifugation at 5000 × *g*, which was performed prior to DNA/RNA extraction in Enterit4Calves, is likely to discard free viral particles with the supernatant. This may not be a problem. Viral infection causes intestinal villus atrophy, and infected cells are desquamated into the intestinal lumen. The exfoliated cells, which contain high loads of viral particles, are found in the faeces. In line with this, Enterit4Calves did not show any problems with detection of viral pathogens.

More samples were positive for *Cryptosporidium* by the qPCR methods used for comparison than by Enterit4Calves assay; however, this result is probably related to differences in the number of target

pathogens. The SSI-qPCR detects at least 12 species of *Cryptosporidium* (*C. parvum*, *C. hominis*, *C. meleagridis*, *C. felis*, *C. cuniculus*, *C. ubiquitum*, *C. canis*, *C. andersoni*, *C. bovis*, *C. xiaoi*, *C. viatorum* and *C. felis*), and the Fluidigm qPCR detects 4 species (*C. parvum*, *C. hominis*, *C. wrairi* and *C. meleagridis*), whereas Enterit4Calves was developed to detect only *C. parvum*. According to Delafosse et al. (2015), *C. parvum* is the relevant causative agent of the diarrhoea in calves up to 21 days of age, whereas *C. bovis*, *C. andersoni* and *C. ryanae* are found in older calves (Follet et al., 2011). Moreover, *C. hominis* was also identified in faecal samples from both young and older calves with and without diarrhoea (Razakandrainibe et al., 2018). Seven samples from calves between 50 and 105 days of age were positive for Cryptosporidium by the SSI-qPCR but negative by both Fluidigm qPCR and Enterit4Calves. It is reasonable to assume that such samples contained *C. bovis* and/or *C. andersoni* and thus did not contain *C. parvum*, the only species detectable by Enterit4Calves assay, but further testing is needed to confirm this. The differences in DNA preparation method and in materials and reagents use for qPCR can influence the efficiency of qPCR methods (Buzard et al., 2010; Dilhari et al., 2017). This may further explain the moderate agreement between Enterit4Calves and the SSI-qPCR assay.

Good agreements in the test results were observed between Enterit4Calves and the two methods used for comparison (McMaster method and Fluidigm qPCR assay) for identification of *Eimeria* spp. Discrepancies between the McMaster methods and Enterit4Calves were noted in a few samples, which contained low numbers of oocysts (≤70 OPG) (Supplementary Table S3). These samples were positive by the McMaster methods, but negative by Assay B and Fluidigm qPCR, suggesting that target gene sequences were below the detection level for these two methods. This may not disqualify the practical use of qPCR methods for detection of this protozoan, as the role of *Eimeria* in provoking diarrhoea in calves is considered only to be significant when OPG was ≥1000 (Enemark et al., 2013).

Overall, the Enterit4Calves assay evaluated in the current study showed fair to good agreement with methods currently used for detection of pathogens associated with calf diarrhoea. Among the 65 samples tested, absolute agreement on the diagnosis was observed for 29 samples, and in 20 samples the disagreement was related to the difference in target species among Cryptosporidium (see Supplementary Table S3). Thus, the Enterit4Calves disagreed with detection of one or more pathogens in 16 samples. In six of these samples, Enterit4Calves detected important pathogens that was overlooked by the traditional methods. Because we have used the traditional methods as Gold standard, this has been scored to the detriment of the kit; however, most likely it represents a better ability to detect the pathogens. However, it should be underlined that a relatively low number of samples have been tested, and that the methods used for comparison did not in all cases detect the same range of pathogens, making comparison of methods difficult. In addition, some of the pathogens, which Enterit4Calves were developed to detect and quantify, were not detected by either Enterit4Calves or the methods used for comparison, and as such no estimate of sensitivity under field conditions could be produced based on the analysed samples. In supplementary testing with spiked samples, the assays performed well for these pathogens, but detection of pathogens in spiked samples may be less demanding than detection in natural samples because the concentration of target sequences tend to be higher.

The results of microbiological diagnosis in veterinary and medical medicine will always be used in combination with clinical observations. In the current investigation, multiple pathogens were commonly observed in the same animal. Enterit4Calves allows quantification of the pathogens, which can help the practitioners to priorities between the pathogens. However, currently an understanding of the correlation between number of pathogens detected in faecal samples from calves and the degree of pathology observed in the intestine of the calves is lacking for most of the pathogens, and future studies should concentrate on establishing relevant cut off values for the assays.

In conclusion, the qPCR assay Enterit4Calves, which was developed for detection and quantification of eight important pathogens associated with calf diarrhoea, showed good performance in laboratory testing. A validation study was performed based on testing of 65 faecal samples from diarrhoeic calves and on 30 spiked samples. Fair to good agreement was observed between the assay under evaluation and standard methods used for detection of pathogens associated with calf diarrhoea.

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CONFLICT OF INTEREST

P. Pansri is an employee of the company DNA-diagnostic A/S, which produces and sells the assays evaluated in

the current study. The remaining authors declare that they do not have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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