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DEVELOPMENT OF A REAL-TIME PCR ASSAY FOR THE RAPID DETECTION OF *netB* GENE IN *CLOSTRIDIUM PERFRINGENS*

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ABSTRACT: Necrotic enteritis (NE) has a huge economic impact in the poultry industry. Numerous studies have shown that necrotic enteritis toxin B (*netB*) is a key virulence factor in *Clostridium perfringens* strains that cause NE in chickens. Early detection of *netB* in *C. perfringens* is important to circumvent the spread of NE. In this study, we developed a novel combinatorial approach involving (i) a commercial 2-step DNA extraction kit and (ii) a real-time polymerase chain reaction (qPCR) for the detection of *netB*-positive *C. perfringens* in fecal samples. Melt curve analysis and specificity test demonstrated 100% specificity without any cross-reactivity in other bacterial species with a limit of detection of 10^2 cfu/g. Field validation was subsequently conducted on nine fecal composites collected from different layer houses at two commercial farms, leading to successful detection of four *netB* positive samples. The study presented a rapid diagnostic qPCR assay involving a 2-step DNA extraction protocol to screen for *C. perfringens* carrying *netB* gene in chicken fecal samples.

Keywords: *Clostridium perfringens;* Necrotic enteritis; *netB* gene; Poultry; Real-time PCR

INTRODUCTION

Necrotic enteritis (NE), an important enteric disease, has a huge economic impact on the poultry industry. While chronic cases typically result in a loss of productivity due to poor feed conversion ratio, a significant level of chicken mortality could be observed in acute cases (Lovland and Kaldhusdal, 2001). It is projected that NE in chickens can cost the global poultry production industry to an estimated of US$3 billion losses annually (Mcdevitt et al., 2006).

Although many predisposing factors play a role in the pathogenesis of the disease, the main causative agent for the dissemination of NE is the Gram-positive *Clostridium perfringens*. Toxicogenic classification of *C. perfringens* (Class A-E) is based on the production of four major toxin variants (alpha, beta, epsilon, and iota). It is well documented that NE is typically caused by the Class A isolates (Ezatkhah et al., 2016; Merati et al., 2017). Historically, a phospholipase C enzyme, alpha toxin, was long thought to be the important virulence factor in NE (Keyburn et al., 2008). However, the role of a novel beta-pore forming toxin, necrotic enteritis toxin B (*netB*), has been identified as a major pathogenicity factor in NE (Keyburn et al., 2008). A seminal study conducted by Keyburn et al. (2008) demonstrated that an alpha-toxin null mutant of a virulent NE isolate can still cause disease in chicken, signifying the non-essential role of alpha-toxin in NE. On the contrary, *C. perfringens* with mutated *netB* gene were avirulent while the pathogenicity of complemented strains was fully restored, demonstrating the critical role of *netB* in causing NE (Keyburn et al., 2008; Rood et al., 2016).

Since the discovery of *netB*, several studies have initiated the screening of the gene within a wide range of *C. perfringens* isolates. For instance, initial screening of a range of Australian poultry NE isolates found that majority of the strains (70%) were *netB* positive (Keyburn et al., 2010). In a separate study, *netB* was found in most of the *C. perfringens* isolated from chickens displaying clinical signs of necrotic enteritis (Rood et al., 2016). A study conducted in Canada by Chalmers et al. (2007) also showed that *netB* gene was predominantly detected in animals associated with NE. Similarly, Mwangi et al. (2019) also reported that 81% of the diseased chickens were *netB* positive.

To circumvent the spread of NE and better inform the stakeholders on the health status of the livestock, the ability to detect the presence of *netB* toxin is critical. For example, Lee et al. (2021) first reported the use of *netB*-specific mAb-based technique to determine the presence of native *netB* toxin in biological samples from NE-infected chickens.

Since the conventional polymerase chain reaction (PCR) was developed in 1983 for detection of DNA, the realm of molecular biology has been at the forefront for microbial diagnosis (Kralik and Ricchi, 2017). Compared to culture-based assays which typically require few days to completion, PCR-based detection method can be done rapidly with a higher sensitivity. An advancement from the conventional PCR technique, particularly quantitative PCR (qPCR), is often considered a method of choice for detection of microorganisms (Rinttilä et al., 2004). Given the high prevalence of *C. perfringens* in fecal samples (Hustá et al., 2020), this study embraced two main objectives; first, we developed a real-

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time qPCR probe-based assay that detects *netB* positive isolates from fecal samples; second, we also evaluated a commercial kit consisting of a 2-step protocol to concomitantly (i) extract genomic DNA from fecal samples and (ii) stabilize the DNA at room temperature post-extraction. This allows ease of transportation of the extracted DNA to a laboratory to conduct qPCR without the need for a cold chain facility. Furthermore, we applied this assay for selective detection of self-spiked *netB* positive *C. perfringens* in fecal samples and deployed this assay to two commercial layer farms for on-field evaluation.

MATERIALS AND METHODS

Ethical Approval

Approval to carry out this study was not required as no invasive method was involved.

Detection of *netB* gene toxin from *Clostridium perfringens* field isolates

Six *Clostridium perfringens* field isolates were screened for *netB* using conventional PCR with primers from Keyburn et al. (2008) (Table 1). Confirmatory tests such as iron-milk presumptive test, motility-nitrate test, and lactose-gelatin test were carried out for identification of the isolates. Briefly, DNA extraction of a single *C. perfringens* colony was carried out by resuspending the colony in 50 µL of sterile distilled water and heating the suspension at 98 °C for 10 minutes. The PCR amplification was conducted in a 25 µl volume consisting of 2 µl of DNA template, 0.3 µM of primers, 1x DreamTaq™ Green PCR Mastermix (Thermo Fisher Scientific™, USA) and Water, nuclease free (Thermo Fisher Scientific™, USA) on a thermocycler (miniPCR®, USA). The PCR conditions involved initial denaturation for 120 seconds at 95 °C, followed by 35 cycles of DNA denaturation for 15 seconds at 95 °C, annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds, with an additional extension for 5 minutes at 72 °C at the end of the 35th cycle. For amplicon visualization, 18 µl of amplified fragments were premixed with 2 µl of 10x BlueJuice™ Gel Loading Buffer (Invitrogen™, USA) and loaded into the wells of agarose gel containing SYBR™ Safe DNA Gel Stain (Invitrogen™, USA). Amplicons were observed using an electrophoresis visualization system, blueGel™ (miniPCR®, USA), with BenchTop 1 Kb DNA Ladder (Promega™, USA) for amplicon size estimation. The *netB* positive isolates were cryopreserved for subsequent downstream real-time PCR assay development.

Table 1 – Primers and probe used in conventional and real-time PCR for detection of *netB* gene

Primer or Probe	Sequence (5' – 3')	Size (bp)	Reference
AKP78 (Forward)[1]	CTTCTAGTGATACCGCTTCAC	383	Keyburn et al. (2008)
AKP79 (Reverse)[1]	CGTTATATTCACTTGTTGACGAAAG		
netB Fwd (Forward)[2]	TAATGGTGATAAAAATTTCACAGAT		
netB Rev (Reverse)[2]	TTAGCATTTTTAGGTGCTGTTA	105	-
netB Probe[2]	CTGGTGGATTTTCACCCAATATGGCTTTAG (FAM)		

[1]: Primer / Probe used in conventional PCR assay; [2]: Real-time PCR assay

Development of real-time PCR (qPCR) for detection of *netB* gene in *Clostridium perfringens*

Primer pair netB Fwd and netB Rev targeting 105 bp of the *netB* gene was designed using Primer3Plus (Untergasser et al., 2012) as shown in Table 1. The primer pair was designed using *Clostridium perfringens* strain EHE-NE18 plasmid pJIR3535 as the reference *netB* gene (Accession: CP025502.1). Basic Local Alignment Search Tool (BLAST®) was carried out to ensure homology of the primers to the *netB* gene sequence (Altschul et al., 1990). A temperature gradient was carried out to determine the optimal annealing temperature (55 °C) of the primer pair. To ensure the specificity of the primer pair, a fluorescent melt curve analysis was also performed. Synthetic *netB* fragment (Integrated DNA Technologies™, Singapore) was used as a positive control. The length of the fragment was 419 bp with coverage of nucleotide position from 30215 to 30633 of plasmid pJIR3535. A 20 µL reaction mixture containing synthetic *netB* DNA fragment, 0.3 µM of Fwd/Rev primers, 1x SensiFAST™ SYBR™ No-ROX Mix (Meridian Bioscience®, USA) and PCR Grade water was analyzed using Azure Cielo™ 3 (Azure Biosystems™, USA). The qPCR cycle used in this assay was as follows: Initial denaturation for 180 seconds at 95 °C, followed by 45 cycles of DNA denaturation for 5 seconds at 95 °C, annealing of primers and extension of template at 55 °C for 15 seconds.

Real-time TaqMan™ FAM probe targeting *netB* gene was designed using an online tool, Primer3Plus. Similarly, BLAST alignment was carried out to ensure homology of the probe designed. A 20 µL qPCR reaction mixture consisting of template, 0.3 µM of Fwd/Rev primer, 0.2 µM of probe (FAM), 1x SensiFAST No-ROX Probe Mix and PCR Grade water was analyzed using Azure Cielo™ 3 (Azure Biosystems™, USA). A total of three technical replicates along with corresponding positive/negative controls were included in the analysis.

The *netB* specificity test was further analyzed empirically. Common inhabitants found in the gut such as *Salmonella* spp*, Clostridium difficile, Clostridium butyricum, Campylobacter jejuni, Lactobacillus fermentum, Streptococcus suis, Enterococcus faecalis, Escherichia coli, and Bacillus subtilis* were tested for non-specific binding of *netB* primer pair and probe (Table 1). A *netB* negative *C. perfringens* ATCC® 13124 was also included in the analysis for potential cross-reactivity within the same species.

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Detection of *netB* positive *Clostridium perfringens* in fecal samples

Clostridium perfringens isolate carrying *netB* gene (M4.6) was cultured at 37 °C in Thioglycollate Medium USP (Oxoid™, UK) for 18 h. A ten-fold serial dilution was performed from the overnight culture to prepare a series of standards for spiking. The bacterial standards were plated on Perfringens Agar (Oxoid, UK) to quantify the bacterial load. Chicken fecal samples (4 g) were artificially spiked with the standards to a final concentration ranging from 10^1 to 10^6 cfu/g. Arcis Sample Prep Kit (Arcis Biotechnology, UK) was used for DNA extraction. Briefly, Reagent 1 (R1) containing lysis buffer and DNA stabilizer to rapidly disrupt cell membranes and protect the released DNA from degradation was added. Prior to qPCR process, an equal volume of Reagent 2 (R2) containing wash buffer was added to the extract. DNA sample was further purified from the R2-treated fecal samples using QIAamp® DNA Mini Kit (Qiagen®, Germany) according to the manufacturer's protocol. Prior to the qPCR, an exogenous internal control (Primerdesign, UK) was added to the reaction mix to check for potential PCR inhibition. In brief, the 20 µL qPCR reaction mixture contained template (sample and internal control), 0.3 µM of Fwd/Rev primer, 0.2 µM of probe (FAM), 1x internal control primer/probe mix (VIC), 1x SensiFAST No-ROX Probe Mix and PCR grade water.

Detection of naturally occurring *netB* positive *Clostridium perfringens* isolates in fecal samples

Validation study was conducted to evaluate the efficacy of this assay under field condition. Fecal samples were collected from two independent layer farms with a closed house system. A total of 50 g fecal composite was randomly collected from 3 collection points (front, middle and back) of the house. In this validation trial, a total of nine fecal composites were evaluated. Each of the fecal composite was homogenized using a disposable spatula, and 20 g of the composite was subsequently transferred to a sterile stomacher bag. An 80 ml of sterile Phosphate Buffered Saline was added to the stomacher bag and mixed homogenously. The content of the bag was left to stand for 10 minutes to allow sedimentation of debris particles. A 180 µL of the supernatant was added into a sterile microcentrifuge tube containing 300 µL of R1 (Arcis Biotechnology, UK). The stabilized extractants were delivered to a laboratory for qPCR analyses. Prior to qPCR process, an equal volume of R2 containing wash buffer was added to the fecal extract to remove nucleic acid chelation and enhance PCR reaction. QIAamp DNA Mini Kit was used subsequently for further purification and concentration of DNA according to the manufacturer's protocol. Real-time PCR analysis was then carried out for the purified samples.

RESULTS

Detection of *netB* gene toxin from *Clostridium perfringens* field isolates

Six *Clostridium perfringens* field isolates were cultured at 37°C in Thioglycollate Medium USP (Oxoid, UK) for 18 h. Subsequently, the cultures were plated on Perfringens OPSP Agar Base (Oxoid, UK) supplemented with Selective Supplements A and B (Oxoid, UK). The identity of all the field isolates was identified as *C. perfringens* through series of confirmatory tests. A conventional PCR amplification was subsequently deployed to screen for the presence of *netB* in *C. perfringens*. In this screening test, an expected *netB* amplicon size of 383 bp was observed for isolates S5.1 and M4.6 (Figure 1). On the other hand, *netB* was not detected for isolates S5.2, S5.3, M4.1, M4.3, and no template control (NTC) in the PCR assay. Isolates S5.1 and M4.6 were subsequently cryopreserved for subsequent downstream qPCR development.

Melt curve analysis

The melting curve analysis resulted in a single peak with an average melting temperature (Tm) of 76.6 °C to 77.2 °C. As shown in Figure 2, melting temperature of isolate S5.1 and M4.6 were close to the synthetic *netB* fragment, demonstrating the specificity of the primer pairs. No peaks were observed in the no template control (NTC).

Limit of detection (*netB* positive *Clostridium perfringens* isolate)

Limit of detection was determined as the lowest concentration of the isolate that can be detected using this assay. The range of detection spanned from 10^2 cfu/g to 10^6 cfu/g of *netB* positive *Clostridium perfringens* isolate. No significant difference was observed for the Cq value of the internal control for all the dilutions evaluated. As the assay was not able to detect fecal sample spiked at 10^1 cfu/g, the limit of detection of this assay was determined to be 10^2 cfu/g.

Specificity test

Specificity for *netB* real-time PCR assay was 100%. Expectedly, this assay was able to detect *Clostridium perfringens* isolate (S5.1) carrying *netB* with Cq value of 32.53. No cross reactivity was observed for other microorganisms tested, including *netB* negative *C. perfringens* ATCC 13124 strain in this test.

Field validation

Amongst the nine composites collected from different layer houses at two commercial farms (farm A and B), four of the samples were detected positive for *netB* gene (Table 3). An exogenous internal control was added into the reaction mixture to distinguish true target negatives from false negatives arising from potential PCR inhibition. As shown in Table 3, the Cq (VIC) values of the internal control across six samples were within the expected range of 28 ± 3, signifying no PCR inhibition in the test run.

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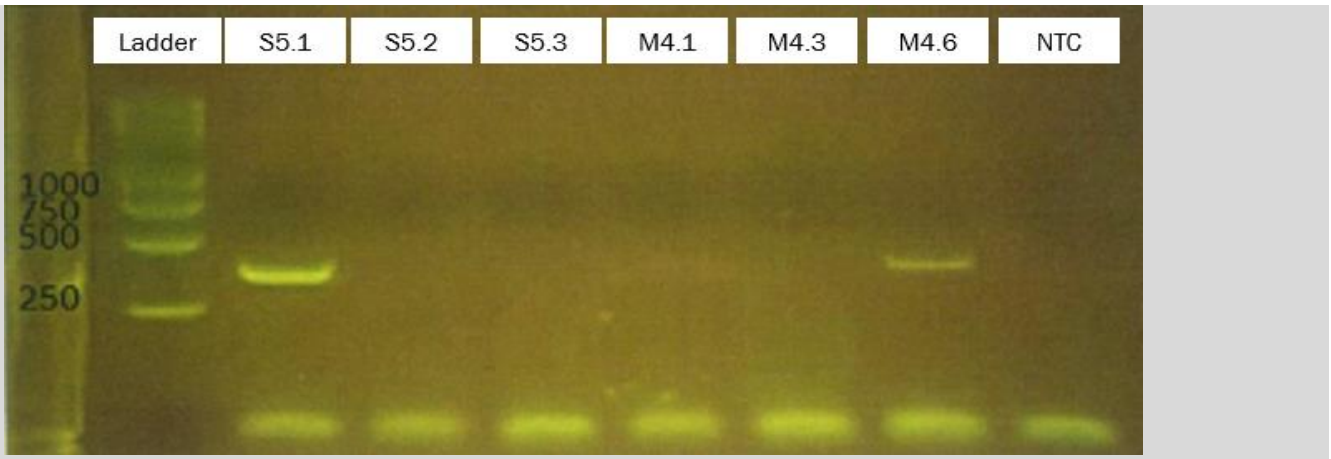

Figure 1 – Gel electrophoresis image of *Clostridium perfringens* isolates screening.

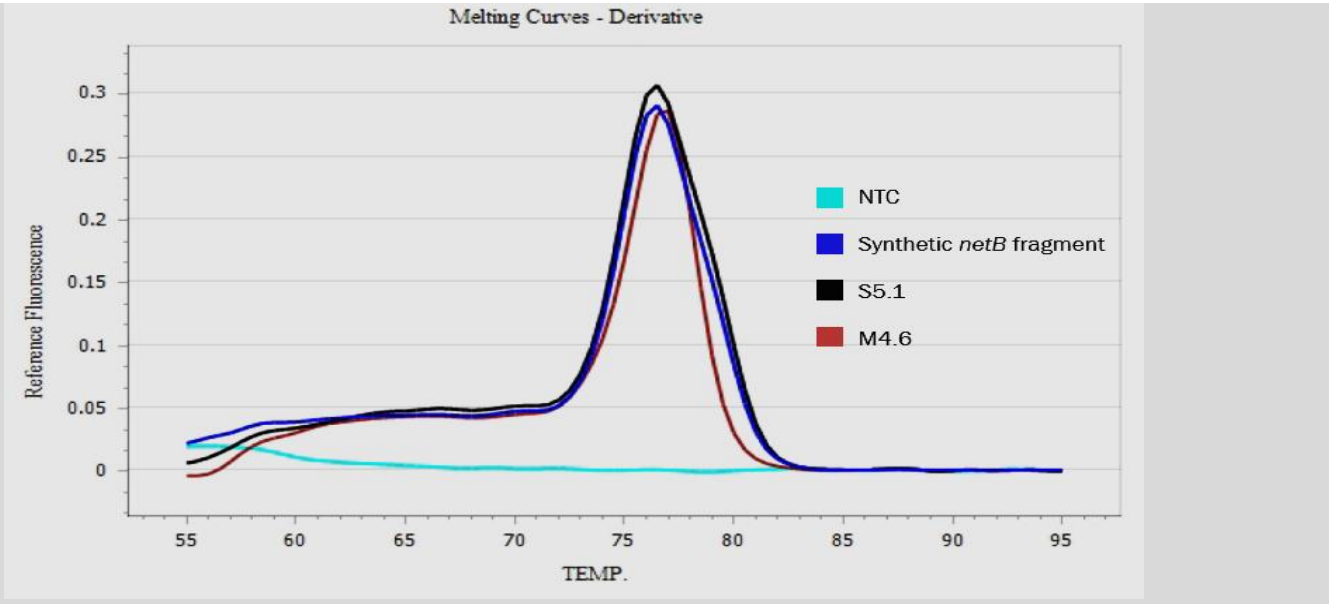

Figure 2 – Melt curve analysis of *netB* amplicons from real-time PCR.

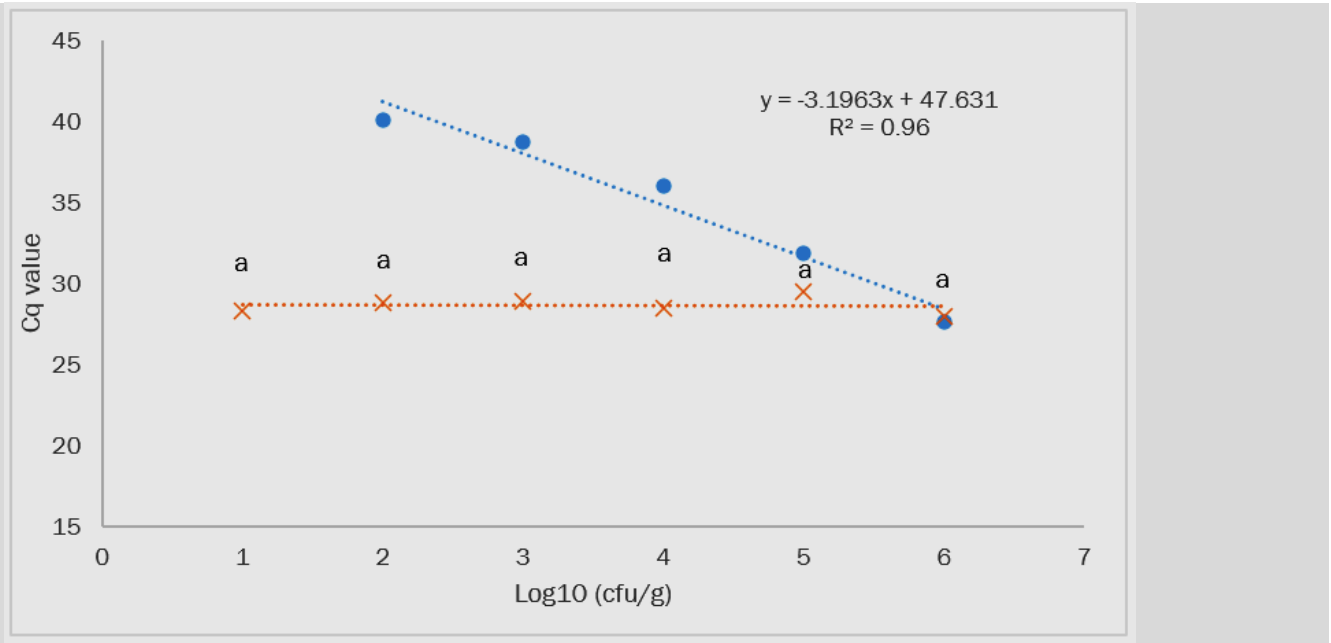

Figure 3 – Standard curve generated from fecal samples spiked with different levels of *netB* positive *Clostridium perfringens* standards (●). Cq values of internal control (×) were plotted to check for level of inhibition. At each dilution, the Cq value was plotted against the logarithm of the concentration of amplicon (cfu/g). Cq values of the internal control were not statistically different between the different dilutions ($p>0.05$) using One-Way ANOVA.

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Table 2 – Real-time PCR results on the specificity of the primer pair and probe on difference bacteria.

Bacteria	Strain	Cq (FAM)
Clostridium perfringens[1]	S5.1	32.53
Clostridium perfringens	ATCC® 13124	-
Salmonella spp.[2]	Feed Isolate	-
Clostridium difficile	ATCC® 9689	-
Clostridium butyricum	ATCC® 19398	-
Campylobacter jejuni	ATCC® 35918	-
Lactobacillus fermentum	ATCC® 23271	-
Streptococcus suis	ATCC® 99849	-
Enterococcus faecalis	NUS-EL 7/10 P4	-
Escherichia coli	ATCC® 25922	-
Bacillus subtilis	PB6	-

[1] *Clostridium perfringens* S5.1 was used as a positive control. [2] *Salmonella* spp. was isolated from feed sample and identified using Analytical Profile Index (API 20) E.

Table 3 – Field validation results of fecal composite samples from Farm A.

Sample	Cq (FAM)	Cq (VIC)
House 1	-	26.68
House 2	-	27.07
House 3	-	27.16
House 4	-	26.77
House 5	35.96	26.79
House 6	36.72	26.73
Positive Control	32.93	26.65
Negative Control	-	27.17
NTC	-	-

Table 4 – Field validation results of fecal composite samples from Farm B.

Sample	Cq (FAM)	Cq (VIC)
House 1	40.23	28.39
House 2	40.36	27.32
House 3	-	28.01
Positive Control	35.01	27.72
Negative Control	-	27.84
NTC	-	-

DISCUSSION

Necrotic enteritis is an important disease in the poultry industry. It has been well documented that the key contributing virulence factor of NE is a pore forming heptameric NetB toxin (Keyburn et al., 2008). In this study, we initially obtained two *netB*-positive *Clostridium perfringens* field strains through conventional PCR screening. The discovery of the bacterium carrying *netB* gene forms the experimental basis to further develop a *netB* real-time PCR diagnostic assay. We first designed a qPCR primer pair targeting 105 bp of the *netB* nucleotide sequence. A BLAST alignment was performed against the publicly available *netB* gene reference to ensure the coverage of the sequence of primers is within the consensus sequence of the target gene. Our melt curve analysis (Figure 2) and specificity test (Table 2) demonstrated the high specificity of our primers and FAM-based probe.

Preserving the integrity of DNA is critical for validation of different analyses, and this undertaking often requires different types of equipment for storage which may not be accessible in field (Groenenboom et al., 2019). To simplify the DNA extraction and storing process at a resource limited setting (e.g. farm), we coupled a simple 2-step DNA extraction process with our pre-established qPCR assay. This 2-step process incorporates two different reagents (R1 and R2) to (i). lyse bacterial cells, (ii) stabilize the genetic elements at field level, and (iii) enhance downstream PCR reaction through removal of inhibitors. With this rapid and convenient extraction method, we envision that any untrained farm personnel with limited resources will be able to collect, composite, and extract the DNA from fecal samples conveniently prior to sending the extracted sample to a scientific laboratory for real-time PCR analyses.

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To validate our combinatorial approach involving the 2-step DNA extraction and qPCR processes, first we artificially spiked layer fecal samples with different *C. perfringens* standards (10^1 to 10^6 cfu/g) to determine the limit of detection (LoD). We showed that our assay can be used to detect *C. perfringens* down to 10^2 cfu/g of bacterial load (Bustin et al., 2009). However, it is worth noting that we were not able to recover *netB* gene in fecal samples spiked with 10^1 cfu/ml of *netB* positive *C. perfringens*. It could be attributed to the high concentration of DNA from background bacteria which may reduce the PCR efficiency for low target load (Forootan et al, 2017; Pan et al., 2017) or sampling error due to low DNA concentration (Wilson, 1997; Tellinghuisen, 2020). At present, we are trying to improve the sensitivity of this assay through a combinatorial approach involving different extraction methods and chemicals.

For actual on-field evaluation, a total of nine fecal composites were collected from two independent layer farms. We utilized the 2-step extraction method involving R1 at initial stage to lyse the bacteria cells and stabilize the released DNA. Following that, the R1-treated samples were delivered to the laboratory for actual qPCR analyses. Four of the fecal composites were detected positive for *netB* (Table 3 and 4). As shown, the Cq value of our internal control across all nine samples were within the recommended range, suggesting no PCR inhibition in our assay. Altogether, our result demonstrated that qPCR assay could be used as a rapid environmental surveillance tool to detect the presence of *netB*-positive *C. perfringens* in fecal samples. Numerous studies have indicated that high load of *C. perfringens* carrying *netB* gene in poultry fecal is a strong indicator of NE occurrence in livestock (Chalmers et al., 2007; Mwangi et al., 2019; Thi et al., 2021). We propose that this real-time PCR assay can be deployed on field to evaluate pathogenicity potential of field isolates through detection of the virulence factor *netB* in *C. perfringens*.

CONCLUSION

The real-time PCR assay is a promising tool that can be deployed in the farm to detect presence of *netB*- positive *Clostridium perfringens* in chicken fecal samples. It can potentially be used as a routine surveillance system to diagnose the flocks' health conveniently. Fundamentally, detection of *netB*-positive *C. perfringens* in fecal samples may suggest potential field circulation of the pathogens amongst the flocks. As the *netB*-targeted approach is highly selective against pathogenic *C. perfringens*, this non-invasive assay could provide an alternative sampling technique for monitoring of potential NE outbreak in the farm segment.

DECLARATIONS

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Authors' contribution

G.H. Soo proposed the design of study, prepared the manuscript, and performed the laboratory analysis. J.W.H. Tan and H.C. Ong assisted in the design of study and reviewed the manuscript.

Conflict of interests

The authors declared that there is no conflict of interest in this study.

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