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Identification of five *Eimeria* species in broiler farms in southern Thailand using SYBR Green-based real-time polymerase chain reaction

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Introduction: Chicken coccidiosis is a globally significant poultry disease caused by *Eimeria* species, which are highly pathogenic protozoa that impair growth performance and contribute to high morbidity and mortality in the poultry industry. To identify specific *Eimeria* species, molecular techniques have been developed in several countries as alternatives to conventional diagnostic approaches, which are labor-intensive, time-consuming, and have low accuracy in detecting mixed infections.

Methods: This study aimed to develop a SYBR Green-based real-time polymerase chain reaction assay for the identification of *Eimeria* species in Thailand, using DNA from five reference species (*E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, and *E. tenella*) and 25 field samples from broiler farms in southern Thailand.

Results: The assay demonstrated high sensitivity, specificity, and reproducibility. Species-specific melting temperature profiles allowed reliable differentiation of *Eimeria* DNA from primer-dimers and potential contaminants. Field testing revealed a high prevalence of mixed infections, with *E. tenella*, *E. acervulina*, and *E. maxima* being the most common, whereas *E. brunetti* and *E. necatrix* were not detected.

Discussion: Compared with conventional gross examination, the SYBR Green-based real-time polymerase chain reaction assay proved to be a more accurate and efficient tool for diagnosing coccidiosis in commercial broiler farms, particularly in detecting subclinical and mixed-species infections.

KEYWORDS

broiler chicken, coccidiosis, *Eimeria* species, real-time polymerase chain reaction, SYBR Green, Thailand

1 Introduction

Coccidia are pathogenic intestinal protozoa that cause significant economic losses in the poultry industry worldwide, including in Thailand (Peek and Landman, 2011; Dorne et al., 2013; Del Cacho et al., 2016; Lin et al., 2017). Chicken coccidiosis is caused by *Eimeria* species, such as *E. acervulina, E. maxima, E. necatrix*, and *E. tenella* (Cerventes et al., 2020). Chickens are often infected with a mixture of *Eimeria* species, which leads to a range of clinical signs, including watery or bloody diarrhea, depression, poor growth performance, and mortality (Cerventes et al., 2020).

To prevent and control coccidiosis in broiler farms, anticoccidial drugs such as salinomycin, robenidine, and ionophores have been routinely added to feed programs for over 40 years (Peek and Landman, 2011; Kadykalo et al., 2018). However, the efficacy of these drugs has declined as a result of increasing resistance among *Eimeria* species (Chapman, 1984, 1986; Peek and Landman, 2003, 2006; Abbas et al., 2011; Sun et al., 2023). This resistance has led to widespread subclinical coccidiosis in broilers (Shirzad et al., 2011), which is a major concern, as it impairs growth performance and increases feed conversion ratios, resulting in financial losses for the poultry industry (Dalloul and Lillehoj, 2006; Blake et al., 2020).

Traditionally, the identification of *Eimeria* species has relied on clinical signs, characteristic gross intestinal lesions, and microscopic examination of oocyst morphology—methods that require highly skilled and experienced personnel (Carvalho et al., 2011b). In Thailand, coccidiosis surveillance in broiler farms is primarily conducted using these conventional approaches, especially gross lesion evaluation. This process is slow and delays the collection of epidemiological data. Moreover, subclinical lesions in medicated broiler farms often yield unreliable diagnostic information. Therefore, the application of molecular techniques for *Eimeria* species identification would significantly enhance the accuracy and efficiency of coccidiosis investigations.

With the increasing value of the global poultry market, the development of effective diagnostic methods to monitor and control chicken coccidiosis has become a priority for the poultry industry. Several studies have developed polymerase chain reaction (PCR) and real-time PCR assays to enhance the routine diagnosis and species-level identification of *Eimeria* (Schnitzler et al., 1998; Haug et al., 2007; Blake et al., 2008; Kawahara et al., 2008; Morgan et al., 2009; Vrba et al., 2010; Carvalho et al., 2011a, b; Gyorke et al., 2013; Lan et al., 2017; Brown Jordan et al., 2018; Geng et al., 2021; Pajic et al., 2023).

In Thailand, monitoring of chicken coccidiosis still relies on conventional techniques, including the evaluation of specific macroscopic lesions and oocyst morphology. This study is the first to apply a SYBR Green-based real-time PCR assay for the identification of *Eimeria* species and to investigate chicken coccidiosis outbreaks in southern Thailand.

2 Materials and methods

2.1 Positive control sample

A positive control sample containing *E. acervulina*, *E. maxima*, *E. tenella*, *E. brunetti*, and *E. necatrix* was provided by the pharmaceutical company HIPRA (Bangkok, Thailand). The average concentration of each species was approximately 300 sporulated oocysts per mL.

2.2 Sample collection and preparation

Fecal samples were collected from 25 small-scale broiler farms with closed-house systems in southern Thailand between 2022 and 2024. The chickens raised on these farms were commonly of the Cobb 500, Arbor Acres, or Ross 308 breeds, managed under an all-in/all-out system. The average age of the broilers was 25–35 days, with an average weight of 1.5–2.0 kg. Feed formulation and quantity met the nutritional requirements of each breed line, and water was provided *ad libitum*.

The selected farms met standardized criteria, including evidence of subclinical coccidiosis—defined as moderate to poor performance without overt clinical signs such as bloody diarrhea—and the use of anticoccidial shuttle programs in all flocks. These programs included standard commercial anticoccidial drugs commonly used in poultry farming in Thailand, such as ionophores (salinomycin, narasin, and monensin), synthetic compounds (nicarbazin, diclazuril, and robenidine), and chemical-ionophore combinations (nicarbazin-narasin). Shuttle programs typically involve rotation among different classes of anticoccidial drugs to reduce resistance while maintaining control of the parasite. None of the farms reported the use of coccidiosis vaccines.

To isolate coccidian oocysts, 5–10 birds were randomly selected from each farm. The birds were weighed and humanely euthanized using carbon dioxide. Each bird underwent necropsy, and gross lesions associated with specific *Eimeria* species were recorded according to the criteria of Johnson and Reid (1970), to compare with real-time PCR results (Supplementary Table S1).

Intestinal contents were equally collected by scraping the mucosal surface of the duodenum, jejunum, ileum, and cecum sections using forceps, obtaining approximately 10–20 g per bird. Samples from individual birds were processed separately. Each sample was placed in 2.5% (w/v) potassium dichromate in a Ziplock plastic bag and left overnight. The next day, the samples were washed with water through a mesh sieve into a beaker, then centrifuged at 1,500 rpm for 5 min to sediment the oocysts. The supernatant was discarded, and the resuspended pellets were incubated in 2.5% (w/v) potassium dichromate at 28°C for two days to allow sporulation, then stored at 4°C (Conway and

Mckenzie, 2007). The remaining suspension was examined for the presence of oocysts using a simple flotation method with saturated sodium chloride and visualized under a microscope.

2.3 DNA extraction

Genomic DNA from field samples was extracted from oocysts and other parasitic stages using the Quick-DNA Fecal/Soil Microbe Microprep Kit (Zymo Research, Orange, CA, USA), following the instructions of the manufacturer. Intestinal samples were directly added to ZR BashingBead™ Lysis Tubes and subjected to bead-beating using a Minilys homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at maximum speed for 1 min to disrupt the oocyst walls. Samples then underwent four cycles of freeze-thawing. Extracted DNA was subsequently analyzed by real-time PCR using species-specific primers targeting five *Eimeria* species, as summarized in Table 1.

2.4 Real-time PCR assay

Real-time PCR was performed using the CFX96 Touch Detection System (Bio-Rad, CA, USA). Each 20 µL reaction mixture contained 10 µL of 2x SensiFAST™ SYBR No-ROX Mix (Meridian Bioscience, OH, USA), 0.8 µL each of forward and reverse primers (400 nM), 7.4 µL of sterile DNase-free water, and 1 µL of DNA template.

The thermal cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 5 s; annealing at 51–59.5°C for 10 s (temperature depending on *Eimeria* species, as shown in Table 1); and extension at 72°C for 20 s. Distilled water was used as the template for the negative control. All reactions were performed in triplicate.

Fluorescence data were collected at every 0.1°C increment during the melting curve analysis. Melting temperature (Tm) was analyzed using Precision Melt Analysis Software (Bio-Rad, CA, USA) over a range of 65–95°C. Average quantification cycle (Cq) values, which reflect DNA copy number, were calculated using the Bio-Rad CFX software.

2.5 Serial DNA dilution

A 10-fold serial dilution of DNA-positive controls containing five *Eimeria* species was prepared and analyzed using real-time PCR. The highest genomic DNA concentration was quantified using a NanoDrop Lite spectrophotometer based on the A260/A280 absorbance ratio (Thermo Scientific, UK). The limit of detection was determined using serial dilutions, ranging from 7.5 ng/µL to 0.075 fg/µL of genomic DNA (10^0 to 10^{-8} dilution factor), along with corresponding Cq values (Bustin et al., 2009). Five dilution points (10^0, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were used for standard curve construction, as they consistently yielded reliable amplification within the detection range of the assay.

2.6 Statistical analysis

The prevalence of each *Eimeria* species was calculated as the percentage of positive samples among the total number of samples tested. Accordingly, 95% confidence intervals (CIs) were calculated using the Wilson score interval method (Wilson, 1927), which offers more accurate coverage than the standard Wald method, especially for small sample sizes and proportions near 0 or 1 (Newcombe, 1998). The Wilson score interval is computed using the following formula:

$$p + \frac{z^2}{2n} \pm z\sqrt{\frac{p(1-p)}{n} + \frac{z^2}{4n^2}}$$

TABLE 1 Specific primers for 5 *Eimeria* spp. and the annealing temperature.

Eimeria spp.	Primer	Oligonucleotides sequence (5′-3′)	PCR amplicon size (bp)	Annealing temperature (°C)	Target gene/ region	Reference
E. acervulina	EAF	GGCTTGGATGATGTTTGCTG	321	62	ITS-1 region of rDNA	Schnitzler et al., 1998
	EAR	CGAACGCAATAACACACGCT				
E. brunetti	EBF	GATCAGTTTGAGCAAACCTTCG	311	56	ITS-1 region of rDNA	Schnitzler et al., 1998
	EBR	TGGTCTTCCGTACGTCGGAT				
E. necatrix	ENF	TACATCCCAATCTTTGAATCG	384	51	ITS-1 region of rDNA	Schnitzler et al., 1998
	ENR	GGCATACTAGCTTCGAGCAAC				
E. tenella	ETF	AATTTAGTCCATCGCAACCCT	279	60	ITS-1 region of rDNA	Schnitzler et al., 1998
	ETR	CGAGCGCTCTGCATACGACA				
E. maxima	EMAF	GTGGGACTGTGGTGATGGGG	205	60	ITS-1 region of rDNA	Schnitzler et al., 1999
	EMAR	ACCAGCATGCGCTCACAACCC				

where p is the observed proportion (prevalence), n is the sample size, and z is the $1-\frac{\alpha}{2}$ quantile of the standard normal distribution (1.96 for a 95% CI). Fisher's exact test was used for pairwise comparisons between species, with Bonferroni correction applied to adjust for multiple comparisons. Statistical significance was set at $p < 0.05$.

Cq values for each *Eimeria* species were determined using SYBR Green-based real-time PCR. All reactions were performed in triplicate to ensure reliability. For each species in each sample, the mean Cq value and standard deviation were calculated (Supplementary Tables S1, S2). The corresponding 95% CIs for Cq values were calculated using the following formula:

$$CI_{95\,\%} = \bar{x} \pm t_{n-1,\alpha/2} \times \frac{s}{\sqrt{n}}$$

where $\bar{x}$ is the mean Cq value, s is the standard deviation, n is the number of replicates (3), and $t_{n-1,\alpha/2}$ is the critical t-value for n − 1 degrees of freedom at $\frac{\alpha}{2}$ (4.303 for n = 3, $\alpha = 0.05$).

One-way analysis of variance was performed to compare Cq values among the different *Eimeria* species, followed by Tukey's *post hoc* test for pairwise comparisons. Statistical significance was set at $p < 0.05$.

The reproducibility of the PCR assay was evaluated by calculating the coefficient of variation (CV) for each sample across three separate testing days. A lower CV indicates greater precision and reproducibility of the real-time PCR assay. The CV was calculated using the following formula:

$$CV(\,\%\,) = \frac{Standard\ Deviation}{Mean} \times 100$$

The standard deviation and mean were derived from the Cq values of triplicate measurements for each sample. Based on the CV, reproducibility was classified as excellent for CV values below 1%, good for values below 2%, acceptable for values below 5%, and poor for values ≥ 5% (Bustin et al., 2009).

Agreement and discrepancies between gross examination and real-time PCR results were assessed for each *Eimeria* species. The agreement rate was defined as the percentage of samples in which both methods produced the same result, whether positive or negative. The discrepancy rate referred to the percentage of samples for which the two methods disagreed. Discrepancies were further categorized as either Gross+/PCR− or Gross−/PCR+. McNemar's test was used to determine whether the detection rates of the two methods differed significantly.

Prevalence estimates, 95% CIs, and reproducibility analyses were performed using Python (version 3.8) with the NumPy and Pandas libraries. All statistical analyses were conducted using R (version 4.4.3).

3 Results

3.1 Sensitivity of the real-time PCR assay and limit of detection

The sensitivity of the real-time PCR assays for the five *Eimeria* species was assessed using a 10-fold serial dilution of genomic DNA. The limit of detection for all five species was determined to be at the 10^{-4} dilution (750 fg/μL), as Cq values remained detectable up to this dilution, whereas no amplification was observed at further dilutions (10^{-5} to 10^{-8}).

Standard curves were generated for each *Eimeria* species based on the 10-fold serial dilution. Cq values ranged from approximately 20 to 38 across the dilution series. The relationship between Cq values and the $\log_{10}$ of the dilution factors demonstrated strong linearity for all species, with R^2 values ranging from 0.993 to 0.999, indicating excellent correlation. The slopes of the standard curves ranged from −3.465 to −4.186, corresponding to real-time PCR efficiencies between 73.3% and 94.4%. *E. maxima* exhibited the highest PCR efficiency (94.4%) with a slope of −3.465, whereas *E. necatrix* showed the lowest efficiency (73.3%) with a slope of −4.186 (Figure 1).

3.2 Specificity of the real-time PCR assay

Agarose gel electrophoresis confirmed that each primer amplified only the expected target amplicon size for the corresponding *Eimeria* species DNA (Supplementary Figure S1). The primers used in this study were previously described (Schnitzler et al., 1998, 1999) and were designed based on the internal transcribed spacer 1 region of ribosomal DNA. This region is highly specific for *Eimeria* species identification because of its high interspecies variability and low intraspecies variability. The diagnostic specificity of these primers was previously validated by Schnitzler et al. (1999), who demonstrated that each primer pair specifically amplified DNA from its corresponding *Eimeria* species without cross-reactivity with other species or host DNA.

A single peak was observed in the Tm amplification curves for each *Eimeria* species when using the positive control (Figure 2), indicating specific amplification. Melting curve analysis further revealed distinct Tm profiles for all five species (Figure 3). *E. acervulina* exhibited the highest Tm range (87.0–87.5°C), followed by *E. tenella* (86.0–86.5°C), *E. brunetti* (81.0–81.5°C), *E. maxima* (79.5 ± 0.5°C), and *E. necatrix* (79.0°C). All species displayed characteristic peak patterns that were consistent across replicates.

The melting curves for *E. acervulina* and *E. tenella* showed sharp, well-defined peaks, whereas those for *E. maxima* and *E. necatrix* occasionally exhibited secondary peaks or shoulders. The fluorescence derivative (dF/dT) values varied by species, with *E. maxima* showing the highest peak amplitude (approximately 400 dF/dT) and *E. tenella* the lowest (approximately 140 dF/dT).

3.3 Cq values in the real-time PCR assay

No amplification was observed in the non-template controls for any of the *Eimeria* species in the real-time PCR assay (Supplementary Table S2). The mean Cq values and their 95% CIs were calculated for all five *Eimeria* species in both the positive control and field samples (Table 2).

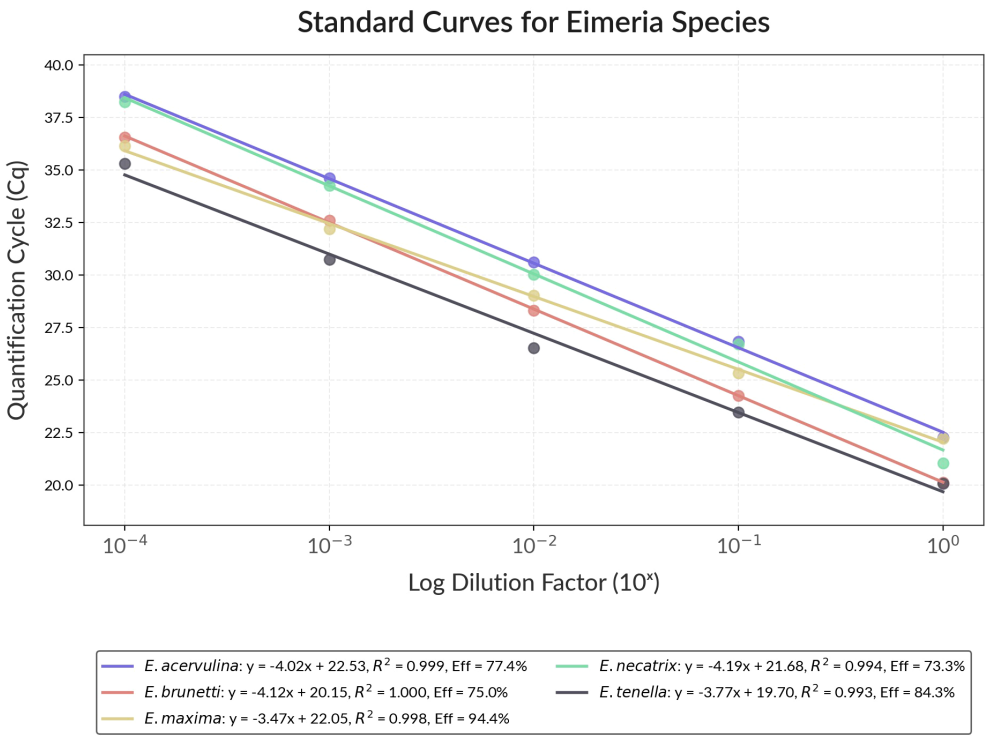

FIGURE 1
The graph displays standard quantification curves for five *Eimeria* species plotted as Quantification Cycle (Cq) versus Log Dilution Factor (10×). Each *species* is represented by a different colored line.

In the positive control, the mean Cq values were as follows: 25.56 ± 2.75 for *E. acervulina*, 22.12 ± 0.74 for *E. brunetti*, 24.22 ± 3.47 for *E. maxima*, 21.15 ± 1.19 for *E. necatrix*, and 22.89 ± 1.29 for *E. tenella*.

Among the field samples, *E. tenella* exhibited the lowest mean Cq value (23.45 ± 0.67), indicating the highest concentration of target DNA, followed by *E. acervulina* (27.32 ± 0.63). *E. maxima* had the highest mean Cq value (32.18 ± 1.19), suggesting the lowest DNA concentration among the detected species.

Statistical comparisons revealed that *E. tenella* had significantly lower Cq values than *E. acervulina* ($p = 0.015$), indicating a higher DNA load. In contrast, *E. maxima* had significantly higher Cq values than *E. acervulina* ($p < 0.001$), suggesting lower DNA levels. The most pronounced difference was observed between *E. tenella* and *E. maxima*, with *E. tenella* exhibiting significantly lower Cq values ($p < 0.001$), further confirming its higher target DNA concentration (Table 3).

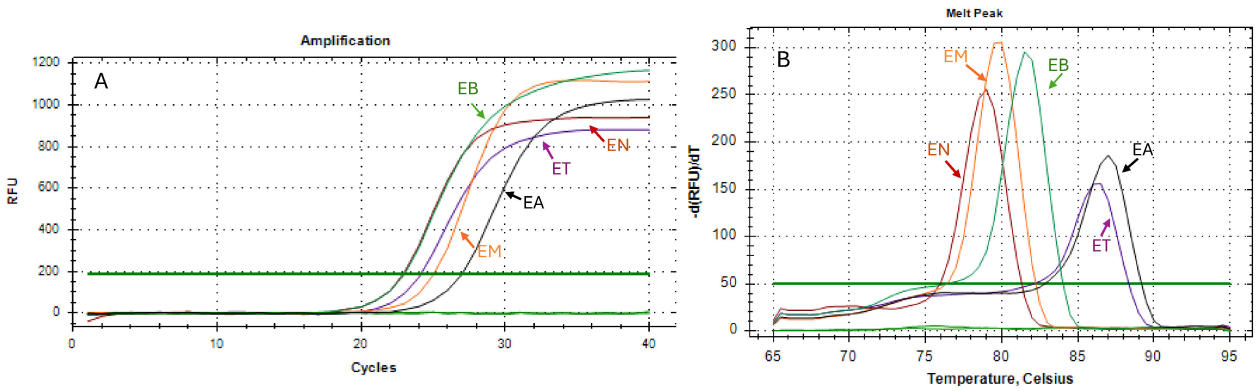

FIGURE 2
Real-time amplification of positive control **(A)** and Melting curve temperature **(B)**. EA, *Eimeria acervulina*; EB, *Eimeria brunetti*; EM, *Eimeria maxima*; EN; *Eimeria necatrix*; and ET, *Eimeria tenella*.

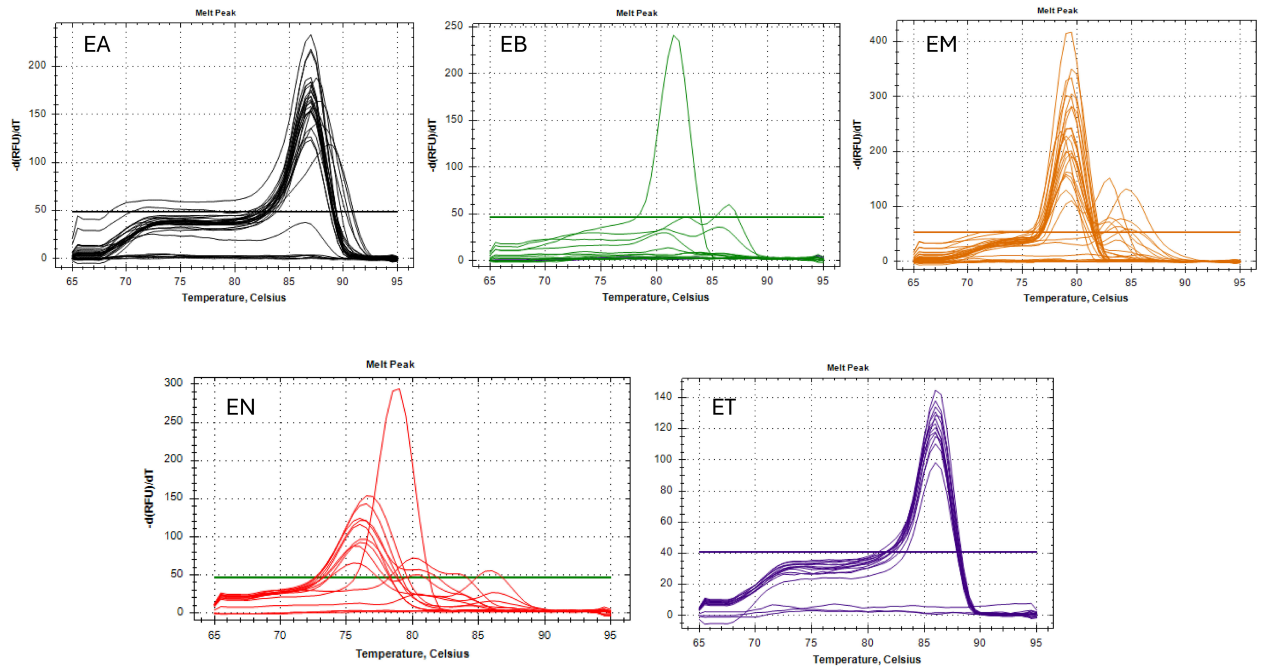

FIGURE 3
Melting curve analysis of 5 *Eimeria* spp. in field samples detected by Real-time PCR. EA, *Eimeria acervulina*; EB, *Eimeria brunetti*; EM, *Eimeria maxima*; EN, *Eimeria necatrix*; and ET, *Eimeria tenella*.

3.4 Prevalence of *Eimeria* species

E. tenella had the highest prevalence, detected in 96% of samples (95% CI: 80.46–99.29%), followed by *E. acervulina* at 92% (95% CI: 75.03–97.78%). *E. maxima* was detected in 68% of samples (95% CI: 48.41–82.79%), whereas *E. brunetti* and *E. necatrix* were not detected in any samples (0%; 95% CI: 0–13.32%; Table 3).

The half-width of the CI reflected the precision of the prevalence estimates. *E. brunetti* and *E. necatrix* had the narrowest intervals (6.66%), followed by *E. tenella* (9.42%) and *E. acervulina* (11.37%). In contrast, *E. maxima* showed the widest interval (17.19%), indicating greater uncertainty in its prevalence estimate compared with the other species.

Statistical analysis showed that *E. tenella, E. acervulina*, and *E. maxima* had significantly higher prevalence rates than *E. brunetti* and *E. necatrix* ($p < 0.001$). The difference in prevalence between *E. maxima* and *E. tenella* was also statistically significant ($p = 0.023$), whereas no significant difference was observed between *E. tenella* and *E. acervulina* ($p = 1.000$).

Analysis of infection patterns revealed that mixed infections were most common, as 64% of samples contained all three detected species (*E. acervulina, E. maxima*, and *E. tenella*). Dual infections involving *E. acervulina* and *E. tenella* were present in 28% of samples. Single-species infections were rare, with *E. maxima* and *E. tenella* each occurring alone in only 4% of cases (Figure 4).

3.5 Reproducibility analysis

The reproducibility of the real-time PCR assay was evaluated for all five *Eimeria* species using 25 samples per species, with measurements taken on three separate days (Table 4). *E. acervulina* showed the highest reproducibility, with a mean CV of 1.53%, followed by *E. maxima* (2.32%) and *E. tenella* (2.59%). No

TABLE 2 The Cq values with 95% confidence intervals for all *Eimeria* Species.

Species of *Eimeria*	Mean Cq of positive sample	95% CI of positive sample	Mean Cq of field samples	95% CI of field samples
E. acevulina	25.56 ± 1.11	22.80-28.32	27.36 ± 0.41[a]	19.13-34.49
E. brunetti	22.12 ± 0.30	21.37-22.87	N/D	N/A
E. maxima	24.22 ± 1.40	20.74-27.70	33.03 ± 0.80[b]	26.14-38.03
E. necatrix	21.15 ± 0.48	19.96-22.34	N/D	N/A
E. tenella	22.89 ± 0.52	21.60-24.18	25.08 ± 0.64[c]	14.45-33.49

N/D indicates species not detected in field samples, and N/A indicates that the information is unavailable. Different superscript letters (a, b, c) indicate statistically significant differences between species ($p < 0.05$).

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TABLE 3 The prevalence with 95% *confidence intervals* for eimeria Species.

Species	Positive Samples	Total Samples	Lower CI	Upper CI	Prevalence with CI	Statistical Group
E. acevulina	23	25	75.03389	97.77796	92.00% (75.03-97.78%)	a
E. brunetti	0	25	0	13.31923	0.00% (0.00-13.32%)	c
E. maxima	17	25	48.41027	82.79481	68.00% (48.41-82.79%)	b
E. necatrix	0	25	0	13.31923	0.00% (0.00-13.32%)	c
E. tenella	24	25	80.45594	99.29038	96.00% (80.46-99.29%)	a

Species sharing the same letter in the Statistical Group column are not significantly different from each other ($p > 0.05$, Fisher's exact test with Bonferroni correction).

valid data were available for *E. brunetti* and *E. necatrix*, as all Cq values for these species were negative.

Analysis of reproducibility categories revealed that *E. acervulina* had the highest proportion of samples with excellent reproducibility, as 43.5% of valid samples (10 out of 23) had a CV below 1%. *E. maxima* showed good reproducibility in 41.2% of valid samples (CV < 2%), whereas *E. tenella* had 45.8% of valid samples with acceptable reproducibility (CV < 5%).

3.6 Agreement and discrepancies between gross examination and real-time PCR results

The level of agreement between gross examination and real-time PCR varied considerably among the five *Eimeria* species (Table 5). *E. acervulina* showed an agreement rate of 44% and a discrepancy rate of 56%. *E. maxima* had a 72% agreement rate and a 28% discrepancy rate. *E. necatrix* and *E. brunetti* each demonstrated a 100% agreement rate, with no discrepancies observed. *E. tenella* exhibited a 56% agreement rate and a 44% discrepancy rate.

The nature of discrepancies also differed by species. For *E. acervulina*, all discrepancies (14 out of 25 samples) were cases where gross examination was negative, whereas real-time PCR was positive (Gross−/PCR+). For *E. maxima*, discrepancies included both Gross+/PCR− cases (two out of 25 samples) and Gross−/PCR+ cases (five out of 25 samples). For *E. tenella*, all discrepancies (11 out of 25 samples) were Gross−/PCR+ cases. No discrepancies were recorded for *E. necatrix* and *E. brunetti*, as all samples tested negative by both methods.

McNemar's test revealed a significant difference between the two methods, with real-time PCR detecting significantly more positive samples for *E. acervulina* ($p = 0.0005$) and *E. tenella* ($p = 0.0026$). However, no significant difference was found between the methods for *E. maxima* ($p = 0.4497$; Figure 5).

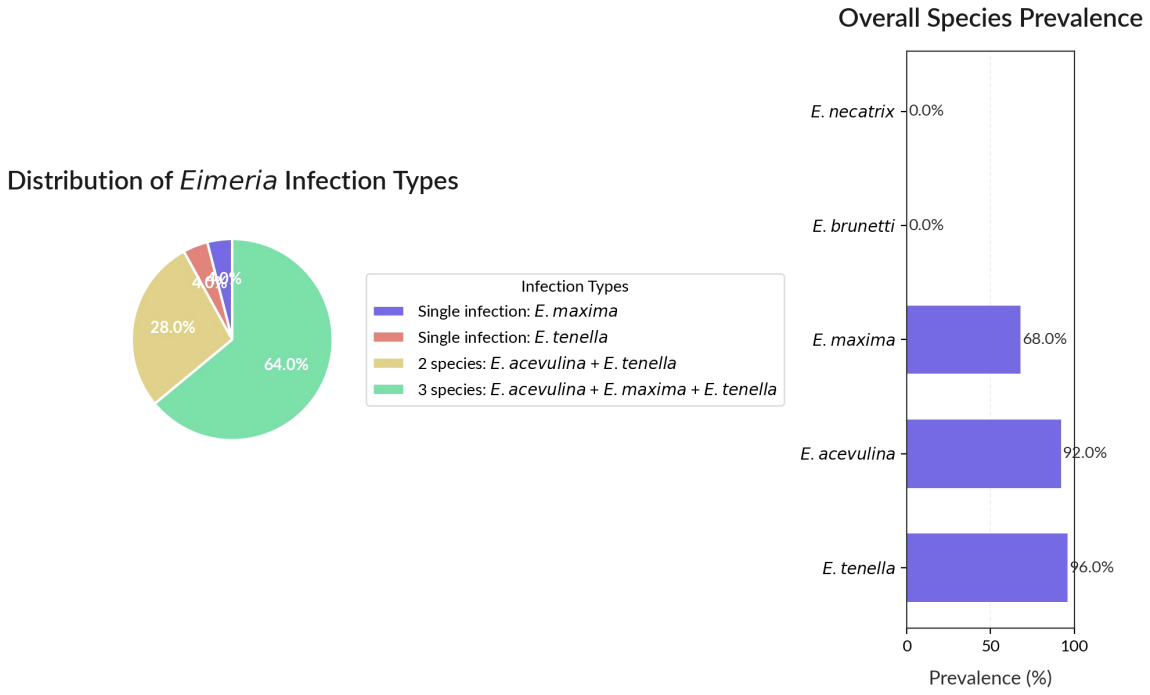

FIGURE 4
The pie chart demonstrates the distribution of infection types, the horizontal bar chart presents the overall prevalence of each *Eimeria* species, and the table provides the prevalence percentages.

TABLE 4 Reproducibility metrics for five *Eimeria* species.

Species	Mean CV (%)	Min CV (%)	Max CV (%)	Excellent (CV < 1%)	Good (CV < 2%)	Acceptable (CV < 5%)	Poor (CV ≥ 5%)	Insufficient data
E. acervulina	1.53	0.14	4.38	10	5	8	0	2
E. maxima	2.32	0.22	5.79	2	7	6	2	8
E. tenella	2.59	0.56	6.25	2	9	11	2	1
E. brunetti	NA	NA	NA	0	0	0	0	25
E. necatrix	NA	NA	NA	0	0	0	0	25

N/A indicates that the information is unavailable.

4 Discussion

In this study, SYBR Green-based real-time PCR was used to identify *Eimeria* species. Real-time PCR is recognized for its high specificity, sensitivity, and reproducibility in DNA detection (Bustin et al., 2009). In particular, SYBR Green offers a productive and cost-effective alternative to hybridization probe-based PCR for *Eimeria* identification (Kawahara et al., 2008). Efficient oocyst rupture and DNA extraction are critical for detecting protozoan cysts in fecal samples, as DNA yield and quality directly influence PCR performance. Incomplete rupture may cause false negatives, whereas excessive disruption can fragment DNA (Haug et al., 2007; Kumar et al., 2014; Reginato et al., 2020).

Our optimized protocol, which incorporates bead-beating in lysis tubes, improved rupture efficiency and reduced co-extraction of PCR inhibitors from fecal samples (Schrader et al., 2012). This method is particularly suitable for *Eimeria*, whose oocysts resist both chemical and mechanical disruption (Guy et al., 2004). By controlling bead size, quantity, and homogenization parameters, our mechanical disruption technique effectively breaks the robust oocyst wall while minimizing DNA damage, preserving genomic integrity for accurate PCR analysis (Hachimi et al., 2024).

The limit of detection for *Eimeria* DNA by real-time PCR was approximately 750 fg. Given that one sporozoite contains about 75 fg of DNA according to Cornelissen et al. (1984), this corresponds to the detection of approximately 1–1.2 sporulated oocysts in a mixed DNA sample of five species, or roughly two sporozoites per species. These results align with previous studies reporting detection thresholds between 1 and 13.6 sporozoites (Haug et al., 2007; Blake et al., 2008; Kawahara et al., 2008; Oliveira et al., 2011). In contrast, conventional PCR had a detection limit of 25 oocysts in earlier work (Schnitzler et al., 1998). However, because we did not conduct a direct sensitivity comparison between real-time and conventional PCR, no definitive conclusions can be drawn from our dataset.

Standard curves generated for all five *Eimeria* species demonstrated excellent linearity across five orders of magnitude ($R^2 > 0.99$), indicating strong quantification capability for diagnostic use (Bustin et al., 2009). According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines, optimal PCR efficiency ranges from 90% to 110%, with corresponding slopes between –3.1 and –3.6. In this study, efficiencies ranged from 73.3% to 94.4%, with *E. maxima* achieving the highest (94.4%) and *E. necatrix* the lowest (73.3%). The values for the remaining species ranged from 75.0% to 84.3%. This variability may be explained by several factors. Differences in genomic structure can affect primer binding and amplification success (Reid et al., 2014). High GC content can also hinder amplification efficiency; for example, *E. necatrix* has a higher GC composition than *E. maxima*, which may contribute to its lower efficiency (Haug et al., 2007; Opel et al., 2010). Furthermore, species-specific DNA secondary structures may impede polymerase activity and reduce amplification efficiency (Bustin and Huggett, 2017). Despite the lower efficiency observed with *E. necatrix*, consistent use of standardized protocols, transparent reporting of efficiency values, and appropriate standard curves

TABLE 5 Agreement and discrepancy analysis.

Species	Gross+/ PCR+	Gross+/ PCR-	Gross-/ PCR+	Gross-/ PCR-	Agreement_Rate (%)	Discrepancy_Rate (%)	McNemar's P-value
E. acervulina	9	0	14	2	44	56	0.005
E. maxima	12	2	5	6	72	28	0.4497
E. necatrix	0	0	0	25	100	0	NA
E. brunetti	0	0	0	25	100	0	NA
E. tenella	13	0	11	1	56	44	0.0026

McNemar's P-value <0.05 indicates a statistically significant difference between the detection rates of the two methods. Sensitivity and specificity were calculated using PCR as the reference method for comparison. NA, Not applicable due to the absence of positive samples.

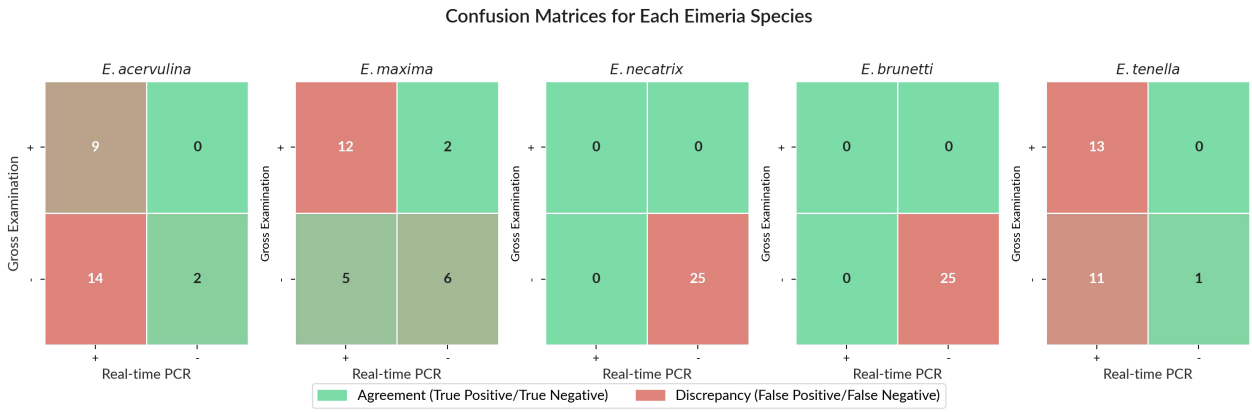

FIGURE 5
Confusion matrices showing the agreement and discrepancy between gross examination and real-time PCR for five Eimeria species. Each cell shows the number of samples in each category. Green cells represent agreement between the two methods (true positives in the upper left and true negatives in the lower right), while red cells represent discrepancy (false positives in the upper right and false negatives in the lower left).

support reliable and reproducible quantification—even when amplification performance is suboptimal (Bustin et al., 2009).

In this study, DNA from both positive controls and field samples was successfully amplified by SYBR Green-based real-time PCR, producing single, species-specific Tm peaks. The clear separation between high-Tm species (*E. acervulina* and *E. tenella*) and low-Tm species (*E. maxima* and *E. necatrix*) enabled reliable species differentiation without additional post-PCR processing. The higher Tm observed for *E. tenella* (85.5°C) and *E. necatrix* (84.5°C), compared with *E. acervulina* (83.5°C), *E. maxima* (82.5°C), and *E. brunetti* (81.5°C), reflects their higher GC content. This pattern aligns with established biophysical principles, as GC base pairs form three hydrogen bonds—more than the two in AT pairs—requiring greater thermal energy to denature (Santalucia, 1998; Ririe et al., 1997; Shchyolkina et al., 2000). Consequently, amplicons with higher GC content showed correspondingly higher Tm values, supporting their utility in species identification.

The occasional secondary peaks observed in *E. maxima* and *E. necatrix* samples may indicate sequence variation within the amplified regions. This is consistent with prior findings of substantial genetic diversity among *Eimeria* species, which can affect real-time PCR amplification patterns (Lew et al., 2003; Kumar et al., 2015; Prakashbabu et al., 2017). Alternatively, these peaks could result from primer-dimers or non-specific amplification (Lan et al., 2004). However, their consistent appearance at species-specific temperatures suggests that they likely represent genuine intraspecies variants. Morris and Gasser (2006) similarly showed that reproducible secondary peaks during melting curve analysis often reflect true genetic polymorphisms within the internal transcribed spacer 1 and other genomic regions, rather than technical artifacts. This phenomenon is particularly common in genetically diverse *Eimeria* species, such as *E. maxima* and *E. necatrix*. Although informative, these secondary peaks may introduce diagnostic ambiguity in field samples, where species identification often relies on melting temperature (Tm) profiles. Misinterpretation of secondary peaks may lead to false-positive or false-negative results, thereby compromising the accuracy of epidemiological assessments and the effectiveness of control strategies, particularly with common mixed infections (Scipioni et al., 2008).

Reproducibility analysis for *E. acervulina* detection yielded a CV of 1.53%, with 43.5% of samples demonstrating excellent reproducibility (CV < 1%). These values align with established precision standards for quantitative molecular diagnostics. The real-time PCR assays for *E. maxima* and *E. tenella* showed comparable reproducibility, with mean CVs of 2.32% and 2.59%, respectively—both within the acceptable threshold (CV < 5%) for reliable quantitative molecular assays (Bustin et al., 2009).

Cq values and their 95% CIs provided key insights into *Eimeria* species abundance and assay precision. *E. tenella* and *E. acervulina* exhibited lower Cq values in field samples, consistent with their higher prevalence and DNA concentration in infected birds. Measurement precision, reflected by CI width, was greater for *E. acervulina* (mean CI width: 0.86) than for *E. maxima* (mean CI width: 1.64), possibly owing to the same factors influencing real-time PCR efficiency (Blake et al., 2006). The wider CIs observed for *E. maxima* indicated greater variability in quantification, warranting cautious interpretation of infection intensity estimates.

The consistent detection of all five *Eimeria* species in positive controls confirmed assay specificity and sensitivity. However, the absence of *E. brunetti* and *E. necatrix* in field samples could not be evaluated for biological relevance because of their non-detection. Although Cq values generally correlated with oocyst load, real-time PCR cannot precisely quantify absolute oocyst numbers because of factors affecting DNA yield, such as the degree of sporulation, extraction efficiency, and infection stage at sampling (Haug et al., 2007; Morgan et al., 2009; Raj et al., 2013).

All field samples tested positive for chicken coccidiosis by real-time PCR. *E. acervulina* was detected in 23 of 25 samples (92%; 95%

CI: 75.03–97.78%), *E. maxima* in 17 samples (68%; 95% CI: 48.41–82.79%), and *E. tenella* in 24 samples (96%; 95% CI: 80.46–99.29%). The wider CI for *E. maxima* may reflect greater variability in its detection across samples. Mixed infections involving all three detected species were the most common among broiler farms in southern Thailand (64%). Neither *E. brunetti* nor *E. necatrix* was detected in any of the samples. To date, *E. brunetti* infection has not been reported in broiler farms in Thailand, whereas *E. necatrix* is typically observed in breeder flocks or older chickens (9–14 weeks), likely owing to its limited oocyst output in younger birds and greater susceptibility to anticoccidial drugs (Cerventes et al., 2020). Species-specific prepatent periods also influence detection rates, as *E. acervulina* (97–98 h) and *E. tenella* (115–120 h) have shorter prepatent periods than *E. maxima* (121–140 h) (Conway and Mckenzie, 2007; Cha et al., 2018). In addition, *E. acervulina* and *E. tenella* produce significantly more oocysts per infected cell than *E. maxima*, increasing their likelihood of detection (Williams, 2001). The absence of mucosal content collected from the rectum may have limited the detection sensitivity for *E. brunetti*, which primarily infects the lower intestinal tract (Cerventes et al., 2020). During the samples collection process, the efficiency of oocyst recovery may have differed among various *Eimeria* species. This study did not assess the uniformity of oocyst purification across different intestinal sections which may affect the detection sensitivity among species. These biological factors likely explain why *E. acervulina* and *E. tenella* were consistently the most prevalent species across broiler farms compared with *E. maxima*. All farms in this study used anticoccidial drugs in their feed programs, indicating that chicken coccidiosis remains widespread and may involve drug-resistant strains in this region. Notably, no positive samples were found for *E. mitis*, *E. precox*, *E. lata*, *E. nagambie*, and *E. zaria*—three of which are operational taxonomic units reported in Australia, India, the United States, and Europe (Jaramillo-Ortiz et al., 2023).

The perfect agreement between gross examination and real-time PCR results for *E. necatrix* and *E. brunetti* likely resulted from the absence of these species in the sample population. In the present study, all samples were collected exclusively from broiler farms. This observation aligns with previous research suggesting that *E. necatrix* and *E. brunetti* are uncommon in modern commercial broiler operations (Williams, 2005; Brown Jordan et al., 2018; Jaramillo-Ortiz et al., 2023). For the three detected species (*E. acervulina, E. maxima*, and *E. tenella*), agreement between methods ranged from moderate to poor. Most discrepancies involved gross examination failing to detect infections identified by real-time PCR. This is consistent with earlier research showing that PCR-based methods offer higher sensitivity than traditional diagnostic techniques (Haug et al., 2008; Vrba et al., 2010). The superior sensitivity of molecular techniques has important implications for coccidiosis surveillance in commercial broiler systems, as subclinical infections may remain undetected with gross examination alone, potentially leading to underestimated disease prevalence and avoidable production losses.

This study had several limitations. First, the sample size was relatively small (25 farms), which may limit the generalizability of the findings. Second, oocyst counting and histopathological examination were not performed, which could have provided additional insights into infection intensity and improved the accuracy of species identification. Third, although the real-time PCR assay is highly sensitive, it also has several limitations when applied in field conditions for detecting *Eimeria* infections in chickens. Real-time PCR may detect DNA from non-viable parasites or be inhibited by PCR inhibitors from an environment, potentially leading to an overestimation of true prevalence. It requires precise knowledge of infection timing, which is often difficult to determine field isolates, it cannot distinguish between different life stages of the parasite, potentially leading to misinterpretation if sampling isn't carefully timed. In addition, achieving standardized protocols across diverse field conditions is difficult but necessary for comparability (Nolan et al., 2015). Lastly, this study lacks performance indicators such as weight gain or feed conversion ratio (FCR), which would facilitate a more straightforward interpretation of the impact on growth performance based solely on *Eimeria* species detection.

Despite these limitations, our findings provide important comparative insights into the diagnostic performance of gross examination and real-time PCR for *Eimeria* detection. Mixed infections involving multiple *Eimeria* species present significant diagnostic challenges for conventional methods. They complicate lesion scoring, obscure subclinical lesions suppressed by anticoccidial drugs, and hinder the identification of distinct *Eimeria* subpopulations (Morgan et al., 2009; Sun et al., 2009; Vrba et al., 2010; Shirzad et al., 2011). Although newer technologies—such as probe-based quantitative PCR (qPCR), loop-mediated isothermal amplification (LAMP), and next-generation sequencing—have emerged, real-time PCR remains more economically viable in several countries (Arya et al., 2005; Pereira-Gómez et al., 2021) In the context of Thailand, the widespread use of advanced molecular diagnostics is often constrained by infrastructural, financial, and technical limitations. As such, real-time PCR serves as a feasible and scalable diagnostic option, effectively balancing diagnostic accuracy with the realities of local resource availability and laboratory capabilities.

In conclusion, this study successfully developed and validated a SYBR Green-based real-time PCR assay for the identification of *Eimeria* species in the poultry industry in Thailand. The assay demonstrated high sensitivity, specificity, and reproducibility. Distinct Tm profiles enabled reliable differentiation of *E. acervulina*, *E. brunetti*, *E. maxima, E. necatrix*, and *E. tenella*. Field sample analysis revealed a high prevalence of mixed *Eimeria* infections in commercial broiler farms, with *E. tenella*, *E. acervulina*, and *E. maxima* as the predominant species, whereas *E. brunetti* and *E. necatrix* were not detected. The molecular assay proved significantly more sensitive than gross examination, identifying infections that would have been missed using traditional methods. This increased sensitivity has important

implications for coccidiosis surveillance, as subclinical infections—often undetected by gross examination alone—can still lead to considerable production losses. The frequent occurrence of mixed *Eimeria* infections in medicated broiler farms, despite the use of anticoccidial shuttle programs, highlights the ongoing challenge of effective coccidiosis control in commercial poultry operations. Although real-time PCR is effective for *Eimeria* spp. detection, it exhibits limitations in the precise quantification of absolute *Eimeria* oocyst numbers. Its precision can be hindered by small sample sizes, PCR inhibitors in fecal samples, and detection of DNA from non-viable parasites, potentially overestimating prevalence.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal studies were approved by Standard of animal research, Research and development office, Prince of Songkla University (ethic document no. MHESI 68014/1779). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

BC: Investigation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. SP: Data curation, Supervision, Software, Validation, Writing – review & editing. KL: Investigation, Methodology, Writing – review & editing. PP: Investigation, Methodology, Writing – review & editing. AS: Investigation, Methodology, Writing – review & editing. AL: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fanim.2025.1533577/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Primer annealing time and DNA band pictures.

SUPPLEMENTARY TABLE 1
Gross lesion and real-time results table.

SUPPLEMENTARY TABLE 2
The Cq value and Tm of 5 *Eimeria* spp.

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