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Morphological and Molecular Characterization of Coccidiosis in Local Chickens of Mekong Delta in Vietnam

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ABSTRACT

Eimeria species are causative pathogens considered as a tremendous threat to the poultry industry in Vietnam. Sufficient assessment of the coccidiosis prevalence is critical for improving the prophylaxis strategies to control the disease. The objective of the current study was to investigate the prevalence of coccidiosis in local chickens (Noi breed) in the Mekong Delta, Vietnam. A total of 1200 fecal samples were collected from 20 various farms in Ben Tre and Hau Giang provinces. Using a microscopic examination, the prevalence rates of coccidiosis in local chickens in Ben Tre and Hau Giang provinces were 65.83% and 68.50%, respectively. *Eimeria* species identification was performed based on morphological characteristics and molecular methods. PCR analysis was conducted by targeting the internal transcribed spacer-1 (ITS-1) region of the *Eimeria* genome. Four *Eimeria* species, namely *E. mitis, E. acervulina, E. maxima,* and *E. tenella* were found in both methods. By applying PCR, the infection rates of *E. mitis, E. acervulina, E. maxima,* and *E. tenella* were 20%, 61.67%, 66.66%, and 83.33%, respectively. The findings can be considered as the first attempt to identify *Eimeria* species at the molecular level in local chickens (Noi breed) in the Mekong Delta, Vietnam that paves the way for improving the prophylaxis strategies for avian coccidiosis.

Keywords: *Eimeria* spp., ITS-1, Local chicken, Noi breed, PCR, Vietnam

INTRODUCTION

Poultry production is a crucial role in economic and social development in Vietnam. Intensive poultry husbandry farms are mainly located in urban areas near the two large delta regions, namely the Red River (North Vietnam) and the Mekong Delta (South Vietnam). The local chicken breeds occupied more than 70% of the total poultry population in Vietnam (Lan Phuong et al., 2015). Among them, the Noi breed is one of the most favorite breeds because of its high meat quality and being easily raised in many different environments, especially in Mekong Delta.

Coccidiosis, caused by the obligatory intracellular protozoan *Eimeria* spp., is one of the most common worldwide parasitic diseases, and it affects poultry production every year. Through the high morbidity, decreased efficiency of feed conversion, reduced egg production, diarrhea, and mortality (Allen and Fetterer 2002; Shirley et al., 2005; Blake and Tomley, 2014), coccidiosis causes a global economic loss of up to 3 billion dollars per year (Blake and Tomley, 2014). Furthermore, eradicating infectious oocysts shedding in the surrounding environment is challenging because the oocysts are of high persistency (Blake and Tomley, 2014) leading to reinfections in poultry. In the poultry industry, seven species of *Eimeria*, including *E. tenella, E. maxima, E. necatrix, E. praecox, E. mitis, E. acervulina,* and *Eimeria brunetti* can frequently appear in infected chickens, causing intestinal lesions with different severity. The infection rate of *Eimeria* was recorded in broilers in Red River Delta (North of Vietnam), Bac Giang, Hue city was 80.56% (Tran et al., 2014), 33.17% (Doan et al., 2014), and 50.92% (Huynh, 2017), respectively. However, there have been no reports on the prevalence of coccidiosis in the local chickens of Noi breed in the Mekong Delta, Vietnam. The current study, therefore, focused on evaluating the epidemiology of coccidiosis in Ben Tre and Hau Giang provinces in Mekong Delta by applying morphological and molecular techniques.

MATERIALS AND METHODS

Ethical approval

This current study was designed and performed based on Can Tho University regulations.

Sample size

The sample size was calculated based on the formula of Thrusfield (2007), $N = \frac{1.96^2 \times pq}{d^2}$ where, N presents the

required sample size, p signifies expected prevalence, q refers to 1-p, and d explains desires absolute precision. The expected prevalence is assumed 50% because there has been no study on the prevalence of coccidiosis in local chickens of Noi breed in Ben Tre and Hau Giang provinces. With 5% absolute precision at a 95% confidence level and the expected prevalence at 50%, the required samples were 384. To increase the precision of the current study, 600 samples per province were collected. A total of 1200 samples were collected from 20 Noi breed chicken farms (free-ranging system, chickens aged 3-7 weeks) located in Ben Tre and Hau Giang provinces, Vietnam.

Vaccination against coccidiosis has not been administered to the surveyed chickens. The age and breed were informed by the owners. Fresh feces samples were randomly collected throughout the studied farms.

Parasites preparation and microscopic examination

Oocysts from fecal samples were collected by flotation method using saturated sodium chloride solution (Conway Donal and McKenzie, 2007). Clean oocysts were further identified based on oocysts morphometry (shape, size, color, the presence or absence of micropyle) and the sporulation time to differentiate *Eimeria* species, which were described by Levine (1985). All measurements were performed in 100 sporulated oocysts per species. The oocysts were visualized under microscope ECLIPSE Nikon 200. Then, oocysts and incubated in 2.5% potassium dichromate solution ($K_2Cr_2O_7$) for 24-48 hr at room temperature to observe the sporulation of oocysts. The number of coccidial oocysts in 1 g feces (OPG) was quantified by the McMaster method. The intensity of infection was classified into 4 levels based on OPG, namely 1+ (less than 1000 oocysts), 2+ (1000-5000 oocysts), 3+ (5000-20000 oocysts), and 4+ (more than 20000 oocysts)

Identification *of Eimeria* species by the polymerase chain reaction

DNA extraction was performed on *Eimeria* spp positive farms (5 samples per each). In this regard, 100 mg of chicken feces were subjected to DNA extraction, using DNA Stool Kit (Qiagen, Germany) and following the instructions of the manufacturer.

PCR amplification

PCR targeting on internal transcribed spacer ITS-1 regions of rDNA was performed to detect different *Eimeria* species. Table 1 provides more detail about primer sequences, annealing temperature, and PCR product size. The single PCR reaction was performed, consisting of 12.5 µl Master mix, 0.5 µl of primer, 0.5 µl dNTP (10 µM), DNA/ nuclease-free water up to 25 µl. The thermal reaction cycling consists of initial denaturation with 96°C for 5 minutes, followed by 30 cycles of 95°C denaturations for 1 minute, 59-73°C annealing for 1 minute (depending on the *Eimeria* species); 72°C extensions for 1 minute 30 seconds, and a final extension 72°C for 7 minutes (Schnitzler et al., 1998; Lew et al., 2003). After amplification, PCR products were loaded on 1.5% (w/v) agarose gel in 1X TAE 120V in 30 min with a DNA ladder of 100 bp. The gel was stained by ethidium bromide and visualized under UV light (Biorad UV 2000)

Table 1. Species-specific primer sets for detecting *Eimeria* spp. by PCR

	Eimeria spp	**Primer sequences (5´-3´)**	**Annealing temperature (°C)**	**PCR product size (bp)**
1	*E. tenella*	ETF: ATTTTAGTCCATCGCACCCCT ETR: CGAGGGCTCTGCATAGGACA	59	278
2	*E. acervulina*	EAF: GGCTTGGATGATGTTTGCTG EAR: CGAACGCAATAACACACGCT	62*	321
3	*E. mitis*	EMi5FA: CGGAGCTGGGGTTTTCTTTC EMi5RA: CTGCATATCCACAGTTCGAACATAC	60*	193
4	*E. maxima*	EMFA2: GCGGTTTCATCATCCATCATCG EMRA2: CGTTGTGAGAAG/AACTGA/GAAGGG	60*	145
5	*E. necatrix*	ENF: TACATCCCAATCTTTGAATCG ENR: GGCATACTAGCTTCGAGCAAC	59	383
6	*E. praecox*	EPRA: AAAAGCAACAGCGATTCAAG EPRA: CCAAGCGATTTCATCATTCGGGGAG	61	116
7	*E. brunetti*	EBF: GATCAGTTTGAGCAAACCTTCG EBR: TCTTCCGTACGTCGGAT	73	311

* Modified annealing temperature in the current study (Schnitzler et al., 1998; Lew et al., 2003).

Phylogenetic analysis

The results of ITS-1 sequences were aligned using ClustalW Multiple alignments. Phylogenetic analysis was conducted using software MEGA X (The Molecular Evolution Genetics Analysis) and explained by the Maximum Likelihood method and Tamura-Nei model (Lew et al, 2003). Reference ITS-1 sequences of *Eimeria* from Sweden, Australia, India, and China isolates were accessed through GenBank (Table 2) and used in phylogenetic analysis.

Table 2. Sequences of ITS-1 used to perform phylogenetic analysis

GenBank accession no.	Species strain	Original
AF446062.1	*E. mitis*	Australia
AF026384.1	*E. acervulina*	Sweden
AF446060.1	*E. maxima*	Australia
KY117143.1	*E. tenella*	China
GQ856312.1	*E. acervulina*	India
GQ153626.1	*E. mitis*	China
GQ856293.1	*E. maxima*	India
AF446074.1	*E. tenella*	Australia

Data analysis

The variations of infection prevalence and intensity among different age groups were determined by Chi-Square ($\chi 2$) test and Tukey test using Minitab software (version 16). The significant difference was considered at p value < 0.05.

RESULTS

The prevalence of coccidiosis in survey areas in the Mekong Delta, Vietnam

The overall infection rate of coccidiosis in local chickens (Noi breed) in Ben Tre and Hau Giang provinces was 67.82% and 68.50%, respectively (Tables 3 and 4). In Ben Tre province, the prevalence of coccidiosis in local chickens in Ben Tre province tended to increase by age. The highest infection rate and intensity (4+) were recorded at 6-week-old chickens with 100% as 84.2%, respectively. There was a statistically significant infection rate among age groups in Ben Tre ($\chi 2 = 260.26$, df = 4, $p < 0.05$) and in Hau Giang ($\chi 2 = 77.51$, df = 4, $p < 0.05$). The infection rate of coccidiosis in Hau Giang province also increased from the third week to the fourth week; however, the infection rate significantly declined in next following week ($p < 0.05$). At the fourth week, the peak of infection was recorded with an infection rate of 96.67% and the intensity (4+) with 25.86%, resulting in clinical symptoms of coccidiosis, such as anorexia, foamy feces, and bloody droppings.

Morphological and molecular identification

The morphological and molecular identification was carried on 60 samples from 12 positive farms (5 samples/ farm). Based on the morphological features of *Eimeria* spp. oocysts and sporulation time, the characteristics of each *Eimeria* species were described in detail (Table 5 and figure 1). In the current study, five different *Eimeria* species *E. mitis E. necatrix, E. acervulina*, *E. maxima,* and *E. tenella,* were found in fecal samples of chickens in the Mekong Delta. However, we determined four *Eimeria* species except for *E. necatrix* by PCR method. *E. praecox* and *E. brunetti* were not detected from tested samples in both methods. By applying molecular technique, *E. acervulina, E. maxima, E. mitis, and E. tenella* were successfully amplified with corresponding amplicons 321 bp, 145bp, 193 bp, and 278bp (Figure 2*).* The prevalence rates of *E. mitis, E. acervulina*, *E. maxima,* and *E. tenella* were 20%, 61.67%, 66.66%, and 83.33%, respectively

Phylogenetic analysis

The Maximum Likelihood method was used to generate the phylogenetic tree, based on the ITS-1 sequences from *Eimeria* species from local chicken (Noi breed) in the current study and other ITS-1 sequences of *Eimeria* species available in the GenBank database from Australia, Sweden, China, and India (Table 2). BLAST analysis of nucleotide sequences of *E. mitis, E. acervulina*, *E. maxima,* and *E. tenella* in the current study showed high homology (95.54% to 98.97%) with corresponding *Eimeria* strains deposited in GenBank (GQ153626.1, AF026384.1, AF446060.1, KY117143.1) The phylogenetic tree demonstrated that *E. tenella* had close relationships to *E. mitis* and *E. acervulina* (Figure 3). However, *E. maxima* sequences were clustered into two different clades. *E. maxima* isolate in Vietnam and Australia formed one small cluster and separated to GQ85629 *E. maxima* isolate from India.

Table 3. The infection rate and infection intensity of coccidiosis in Ben Tre province, Vietnam, by microscopic examination

Age (week)	Infection rate			Intensity of infection and percentage			
				1+	2+	3+	4+
	Samples	Positive samples	Percentage (%)	(%)	(%)	(%)	(%)
3	120	25	20.8[a]	60	40	0	0
4	120	51	42.5[b]	52.9	39.2	5.88	1.96
5	120	100	83.3[c]	14	25	37	24
6	120	120	100[d]	0	5.8	10	84.2
7	120	111	92.5[e]	36	25.2	12.6	26.1
Total	600	407	67.83	40.73	27.04	16.37	34.06

[a,b,c,d,e]Values in the same column with different superscripts differ significantly ($p < 0.05$).

Table 4. The infection rate and infection intensity of coccidiosis in Hau Giang province, Vietnam, by microscopic examination

Age (week)	Infection rate			Intensity of infection and percentage			
				1+	2+	3+	4+
	Samples	Positive samples	Percentage (%)	(%)	(%)	(%)	(%)
3	120	67	55.83[a]	58.21	22.39	7.46	11.94
4	120	116	96.67[b]	24.14	26.72	23.28	25.86
5	120	89	74.17[c]	30.34	30.34	22.47	16.85
6	120	81	67.5[c]	38.27	33.33	18.52	9.88
7	120	58	48.33[a]	58.62	15.52	17.24	8.62
Total	600	411	68.5	41.92	25.66	17.79	14.63

[a,b,c,d]Values in the same column with different superscripts differ significantly ($p < 0.05$).

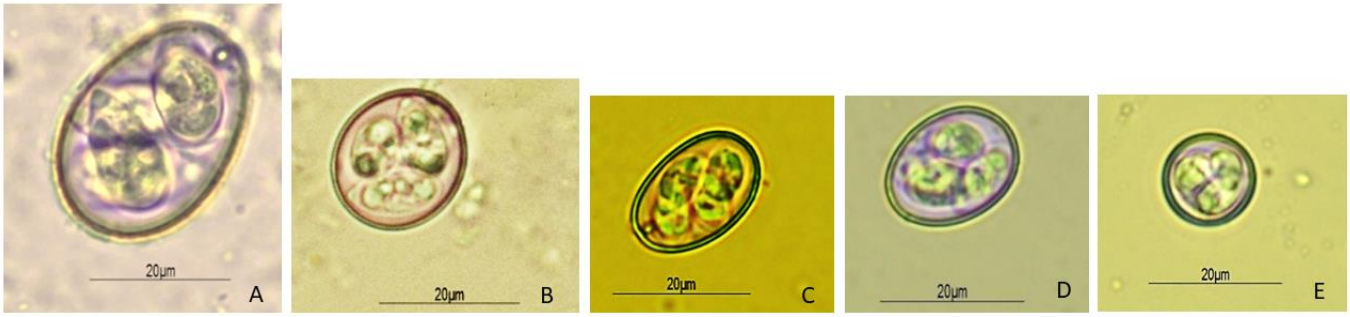

Figure 1. Microscopic images of *Eimeria* species. **A:** *E. maxima*, **B:** *E. tenella*, **C:** *E. necatrix*, **D:** *E. acervulina*, **E:** *E. mitis*

Table 5. Morphological characteristics of *Eimeria* species

Oocyst characteristics	Length (µm) Mean ± SE	Width (µm) Mean ± SE	Sporulation time (h)	Species
Oocyst ovoid or ellipsoid, smooth wall, no micropyle	18.08 ± 0.641	14.24 ± 0.78	16-28	*E. acervulina*
Oocyst ovoid, yellowish, slightly rough surface wall, without micropyle	31.83 ± 1.145	21.29± 0.99	24-42	*E. maxima*
Oocyst spherical, smooth and colourless wall, no micropyle	16.07 ± 0.883	15.43 ± 0.889	14-36	*E. mitis*
Oocyst ovoid, smooth and colourless wall, no micropyle	23.48 ± 0.83	19.29 ± 0.75	18-26	*E. tenella*
Oocyst ovoid or spherical, smooth wall, no micropyle	18.84 ± 1.41	16.40 ± 1.54	20-32	*E. necatrix*

Measurement was conducted in 100 oocysts for each *Eimeria* species. SE: Standard errors

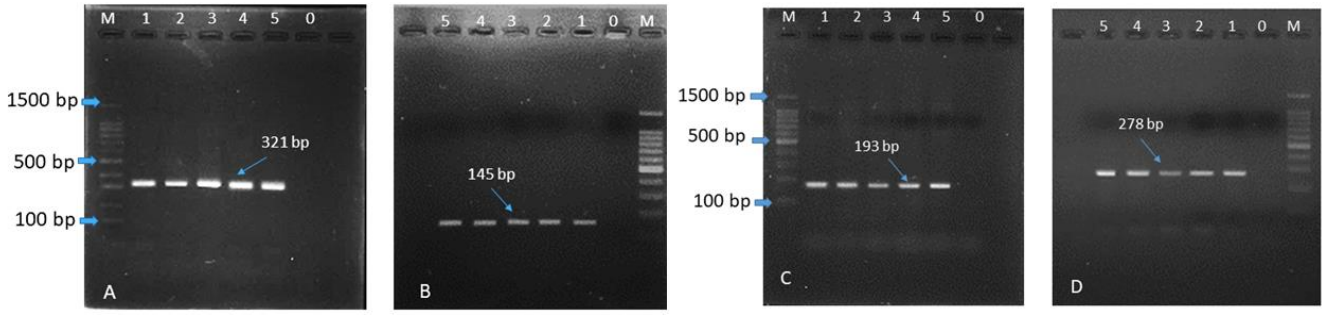

Figure 2. PCR results of the *Eimeria* species-specific ITS-1 region, followed by agarose gel 2 % electrophoresis. M: Marker 100 bp, Lane 0: negative control, and lanes 1, 2, 3, 4, 5 samples. **A:** *E. acervulina* 321 bp, **B:** *E. maxima* 145 bp, **C:** *E. mitis* 193 bp, **D:** *E. tenella* 278 bp.

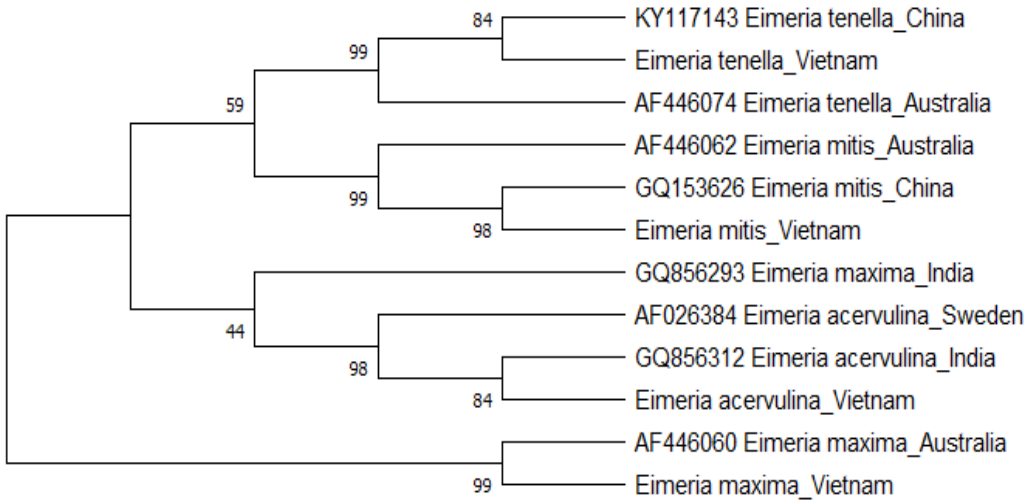

Figure 3. Phylogenetic analysis based on ITS-1 nucleotide sequences of *Eimeria* spp. using the Maximum Likelihood method (Tamura-Nei model) with bootstrap 1000 replications.

DISCUSSION

Chickens are a crucial source of food supplies in Vietnam. However, the avian coccidiosis causes dramatic economic loss due to high mortality, reduced nutrient absorption, growth retardation, and decreased productivity of poultry. Besides commercial broilers, local chickens also play an important role to supply meat for local markets but the coccidiosis in local chickens especially in the Noi breed is neglected. The overall infection rate of coccidiosis in the local chicken- Noi breed was higher than studies in broilers in Bac Giang province 33.17% (Doan et al., 2014) and Thua Thien Hue province 50.92% (Huynh, 2017). The current study showed that the prevalence of coccidiosis in the local chicken- Noi breed was influenced by age. The high infection rate was seen in chickens from weeks 4 to 6. This might be explained due to the dramatic decrease of maternal immunity against coccidiosis in during 31-45 days (Kaboudi et al., 2016). Moreover, the accumulation of oocysts in the poultry litter on the ground created good opportunities for reinfection. These findings are accordant to studies as indicating that poultry had a high prevalence of coccidiosis during 31-45 days (Sharma et al., 2015) (Kaboudi et al., 2016). Moreover, the high prevalence also revealed that the prophylaxis strategies of coccidiosis in surveyed farms were not adequately concerned. Noi breed chickens were reared in a free-ranging system and were not vaccinated for coccidiosis. The farmers just gave anticoccidial drugs as observing the typical symptoms such as anorexia, foamy feces, and bloody droppings. This situation was also recorded in many farms in Hau Giang province, resulting in a significant decrease in the prevalence of coccidiosis from the fourth to fifth week. However, the findings of the present study were lower than studies in Red River Delta, Vietnam as 80.56% (Tran et al., 2014), in An Hui, China, as 87.75% (Huang et al.,

2017), and in Columbia as 92.8% (Mesa et al., 2021). The possible explanation can be related to risk factors, such as age, breed, husbandry system, and disease management that can result in the various prevalence rates of coccidiosis.

E. tenella, *E. maxima*, *E. acervulina*, and *E.mitis* were found in both morphological and molecular methods. *E. necatrix* has been found by morphological identification in the current study, but it was not confirmed by molecular technique. It was a challenge in the morphological identification of *E. necatrix* due to the overlap in morphological characteristics between *E. tenella* and *E. necatrix* that was also mentioned in the study of Matsubayashi et al., (2020). Moreover, the mixed infection with many *Eimeria* species occurred more frequently than a single infection (Carvalho et al., 2011) that also hindered the accurately morphological identification. The current study revealed that it is necessary to apply molecular techniques in *Eimeria* species identification. In fact, the precise identification of *Eimeria* species plays an important role in diagnosing disease and developing effective treatments, or prophylaxis in live vaccines (Aarthi et al., 2010) and biological studies (Lee et al., 2010). Thereby, the molecular identification based on many gene targets, such as small subunit rRNA (Tsuji et al., 1997), ITS-1 (Schnitzler et al. 1998; Lew et al., 2003; Kumar et al., 2014) has been established to differentiate species of *Eimeria* to overcome the limitations of morphological identification. Among them, ITS-1 sequences have been considered as a genetic marker for the identification of species, including *Eimeria* species due to high heterogeneity among different species. By PCR targeting ITS-1, the prevalence of E. *tenella*, *E. maxima*, *E. acervulina*, and *E. mitis* was 83.33%, 66.66%, 61.67%, and 20.00%, respectively. These findings are in line with many earlier studies in Bac Giang 78.16% (Doan et al., 2014), in Red River Delta in Vietnam 90% (Tran et al., 2014) indicating that *E. tenella* is the predominant *Eimeria* spp. in domestic fowl in Vietnam and worldwide. Besides, *E. tenella* is one of the most pathogenicity leading to clinical symptoms in chickens. Thus, it is necessary to have appropriate prophylaxis strategies in this survey area to minimize economical loss.

The phylogenetic analysis of the ITS-1 sequences from *Eimeria* species showed a clear species-specific cluster, regardless of the geographical distribution for all ITS-1 sequences of *E. tenella*, *E. acervulina*, and *E. mitis*. However, *E. maxima* in Vietnam and Australia formed a cluster and was separated from *E. maxima* in India. This could be explained due to the diversity of intra-species ITS-1 lineages of *E. maxima*, which was reported in previous studies (Lew et al., 2003; Cantacessi et al., 2008; Kumar et al., 2015). Understanding the occurrence of *Eimeria* species in specific regions using molecular detection methods is useful for choosing an appropriate vaccine to prevent coccidiosis.

CONCLUSION

The high prevalence of coccidiosis in local chickens (Noi breed) in the Mekong Delta revealed that the disease management was not adequately performed. Therefore, the farmers should pay attention to improving environmental hygiene and prophylaxis strategies in local chickens to prevent avian coccidiosis outbreaks in Vietnam and minimize economic loss. In addition, the study characterized the molecular information about four *Eimeria* species in the Noi breed, including *E. tenella*, *E. acervulina*, *E. maxima*, and *E. mitis*. The findings can be of great contribution to future studies for establishing appropriate prophylaxis strategies, especially in developing the coccidiosis vaccine in Vietnam. This study was one of the first attempts to apply molecular techniques to characterize *Eimeria* species in the local chicken breed (Noi) in Mekong Delta, Vietnam.

DECLARATION

Authors' contribution

Hung Nguyen Huu, and Tran Nguyen-Ho-Bao designed the study, data analysis, wrote the manuscript with the contribution from Trung Van Le, Tien Ai Lu. All authors established experiment protocols.

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Competing interests

The authors declare that they have no competing interests

Ethical considerations

Ethical issues including data fabrication and/ or falsification, plagiarism, consent to publish, double submission, redundancy) have been carefully checked by the authors.

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