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# Improvement DNA Extraction for Molecular Identification of *Eimeria* species in Different Age-groups and Breeding Conditions of Chicken in Bacgiang Province, Vietnam

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**Abstract:** This study aimed to describe the improvement of DNA extraction of coccidial oocysts then DNA extraction used for Polymerase Chain Reaction amplification to identify *Eimeria* sp. infection in chickens in Bacgiang province of Vietnam. A total of 126 faecal samples were collected from households, semi-commercial and commercial poultry farms in different age-groups. These chickens had no history of vaccination against coccidiosis and had never been given coccidiostat drugs. Oocysts were collected from the microscopically positive samples and DNA was isolated for the PCR (Polymerase Chain Reaction) amplification. The results showed that 87 (77.68%) were positive. The infection rate of *E. tenella*, *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis* and *E. necatrix* were 78.16, 70.11, 58.62, 43.68, 28.74 and 26.44%, respectively. *Eimeria praecox* was not found in this study. So, far in Vietnam, the application of PCR techniques in diagnosis diseases in animal in general and in parasitic diseases in particularly has not been common yet, throughout this study, an important step will be opened the new tendency in improvement of DNA extraction and molecular diagnosis animal diseases in stead of conventional methods.

**Key words:** Improvement, *Eimeria* sp., chicken, bacgiang province, vietnam

## INTRODUCTION

Coccidiosis is caused by protozoan parasite which causes mortality, morbidity and weight loss in chicken (Jensen *et al*., 2000). It is one of the most commonly prevalent and economically important parasitic diseases in poultry industry all over the world (Conway and Mckenzie, 2007; Intervet, 2009). Nine different species of *Eimeria* have been identified (Permin and Hansen, 2005; (Conway and Mckenzie, 2007). Out of them *E. acervulina*, *E. necatrix*, *E. tenella*, *E. maxima* and *E. brunetti* are the major species infected in chickens. The infection of *Eimeria* sp. usually mixed due to ingestion of Oocysts (Fayer, 1980). These infections lead to disorders digestion resulting from damage to the intestinal epithelium, mal-absorption of nutrients, changes in protein metabolism after absorption, reduced efficiency of feed conversion and reduction in weight gain (Conway *et al*., 1993; Shirley *et al*., 2005). Mortality and economic losses, especially in cases of outbreaks are frequent (Morris and Gasser, 2006). Avian coccidiosis is a very common disease and is considered one of the diseases causing major damage in livestock. Whooping make poor growth, reduced weight gains and egg production, stunting, emaciation and anaemia as well as mortality (Intervet, 2009). Every year, the cost for the vaccine up to $90 million in the U.S in Italy 20 million Lira, Hungary is 15 million forint in China the annual expenditure is $30-60 million due to in-feed medication for the control of Eimeria (Hao *et al*., 2007).

Poultry farming is quite common in Vietnam in general and Bacgiang province in particular. Bacgiang is a mid-high land in the Northern of Vietnam here has been developing the breeding chickens with various systems, contributing the majority of income for the rural population at large scale. In the earlier investigations

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of the infection of *Eimeria* species in chicken in Vietnam, 5 species (*Eimeria tenella*, *Eimeria necatrix*, *Eimeria maxima*, *Eimeria acervulina* and *Eimeria mitis*) were found in the Northern provinces of Vietnam by morphological methods and 2 species (*Eimeria tenella* and *Eimeria necatrix*) were most pathogenic. However, these methods still relied on prevalence estimates or individual species identification.

DNA extraction is a beginning and important technique for molecular studies. The most commonly used protocol for molecular identification of *Eimeria* species is based on homogenate grinding of oocysts combined with classical phenol-chloroform DNA extraction (Al-Idreesi *et al*., 2013; Keller and Manak, 1989).

This protocol is both labour and time-consuming. The bottleneck for an effective diagnostic procedure is not the PCR amplification of the genomic coccidial DNA, since that has been shown to be highly sensitive but rather the preparation of the DNA from oocysts (Fernandez *et al*., 2003).

Recent year, PCR assay has been applied for the diagnosis of coccidial parasites of man and animals. A number of approaches have proved to be both specific and highly sensitive for analyses either of parasites grown *in vitro* or present in tissue samples and clinical material (Patra *et al*., 2010).

The development of molecular techniques has allowed precise diagnosis of *Eimeria* species, investigation of the genetic variability of these pathogens and a search for molecular characteristics associated with phenotypical characteristics that may constitute the use of molecular markers (Costa *et al*., 2001; Schnitzler *et al*., 1999). Molecular techniques may also contribute to the development of new vaccines and selection of anti-coccidial drugs to be used in control programs (Lee *et al*., 2010; Morris and Gasser, 2006; Sun *et al*., 2009).

In this study, researchers reported the improvement of DNA extraction for further studies (molecular diagnosis, cloning, sequencing, etc.) and analyzed the molecular prevalence and identification of the *Eimeria* species infection on chickens on different age-groups and breeding conditions of chicken in Bacgiang province, Vietnam and also looked at the *Eimeria* species preponderance.

## MATERIALS AND METHODS

**Study area and sampling methods:** The study area was located at Bacgiang province, one local with hot-humid climatic condition of Vietnam. The average temperature of Bacgiang is 22.5°C, ranging between 10.4 and 37.2°C. The 42 households with scavenging chickens, 42 households with semi-commercial and 42 commercial farms were randomly selected for sampling. A minimum of 30 birds including apparently healthy and sicky chickens were again randomly sampled per household without consideration for age or sex. The sampling was done between the months from September, 2012 to August, 2013. Fresh faecal droppings were collected in sterile universal bottles and carcasses were collected in polythene leather bags and transported to the laboratory immediately for processing.

**Laboratory examination:** Laboratory examination was done by wet mount smears of the faecal droppings as described by Fleck and Moody (1993). Concentration technique was also used for counting of oocyst as described by Brown and Neva (1983) and examined under the microscope using x10 objective. Oocysts were collected from microscopically positive samples (Daugschies *et al*., 2002) and sporulation was performed at 24-26°C in a 2.5% aqueous solution of potassium dichromate ($K_2Cr_2O_7$). The sporulated oocyst were concentrated by centrifugation and stored in potassium dichromate at 4°C.

**DNA extraction:** Coccidial oocysts was first purified described by Zhao *et al*. (2001) follow by extraction from sporulated oocysts glass beads to the oocyst suspension and then vortex until the glass-bead grinding ruptures the oocysts (Al-Idreesi *et al*., 2013; MacPherson and Gajadhar, 1993; Molloy *et al*., 1998; Procunier *et al*., 1993; Shirley, 1975). Then, the DNA was transferred to purify using DNA extraction spins protocol (TaKaRa Biotech Science) as manufacturer's instruction (Fig. 1). Briefly, the DNA was placed a Miniprep column into a 2 mL microfuge tube (provided). Discard the filtrate from the 2 mL microfuge tube. Return the Miniprep column the 2 mL microfuge tube and add 500 µL Buffer W1, then washed with Buffer W2 two times. Transfer the Miniprep column into a clean 1.5 mL microfuge tube (provided). To elute the DNA, add 25-30 µL of Eluent Buffer or de-ionized water to the center of the membrane. Let it stand for 1 min at room temperature. Centrifuge at 12,000x g for 1 min. The present *Eimeria* species in each mixture of oocyst was tested by PCR using published species primer sequences (Table 1).

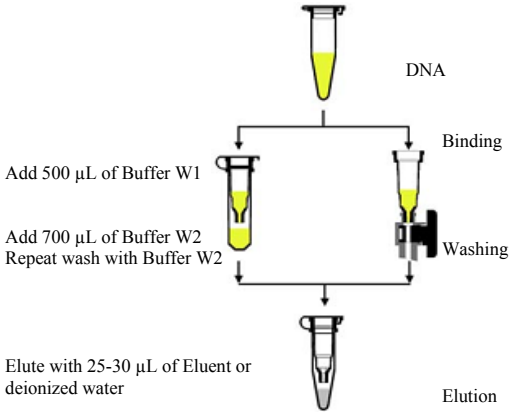


Fig. 1: DNA extraction spins steps

Table 1: Primers sequences, annealing temperatures and predicted sizes of products for PCR amplification of *Eimeria* sp.

| *Eimeria* species | Primer | PCR product sequence (5'-3') | Annealing temperature (°C) | Size (nt) |
|---|---|---|---|---|
| *E. acervulina* | EaF | GGCTTGGATGATGTTTGCTG | 60 | 321 |
| | EaR | CGAACGCAATAACACACGCT | | |
| *E. brunetti* | EbF | GATCAGTTTGAGCAAACCTTCG | 45 | 310 |
| | EbR | TGGTCTTCCGTACGTCGGAT | | |
| *E. maxima* | EmaF | CGTTGTGAGAARACTGRAAGGG | 51 | 144 |
| | EmaR | GCGGTTTCATCATCCATCATCG | | |
| *E. mitis* | EmiF | TATTTCCTGTCGTCGTCTCGC | 54 | 306 |
| | EmiR | GTATGCAAGAGAGAATCGGGA | | |
| *E. necatrix* | EnF | GTCAGCTTTTTGCCTGGGTG | 55 | 285 |
| | EnR | ACAGACCGCTACACAACACG | | |
| *E. praecox* | EpF | CATCATCGGAATGGCTTTTTGA | 54 | 368 |
| | EpR | AATAAATAGCGCAAAATTAAGCA | | |
| *E. tenella* | EtF | AATTTAGTCCATCGCAACCCT | 60 | 271 |
| | EtR | CGAGCGCTCTGCATACGACA | | |

**PCR identification:** The extracted DNA was submitted to PCR and the volume of 25 μL was used for PCR amplification. Thermo-cycling condition reaction were as follows: 1 cycle at 95°C for 7 min; 35 cycles at 95°C for 20 sec, 44 to 60°C for 30 sec, 72°C for 1 min; 1 cycle at 72°C for 5 min (Gautam *et al*., 2010; Jenkins *et al*., 2006a, b). 200 nM dNTP (Amersham, Piscataway, NJ), 20 mM Tris pH 8.4, 50 mM KCl, 3.0 mM $MgCl_2$, 1 U rTaq polymerase (New England Biolabs, Ipswich, MA) in a PTC200 Mini-cycler™ (MJ Research, Watertown, VA) (Haug *et al*., 2007; Schnitzler *et al*., 1999, 1998).

PCR products were separated by 1.0% agarose gel electrophoresis (Bio-metra, Gottingen, Germany). The gels were stained in an aqueous ethidium bromide solution (0.5 μg $mL^{-1}$) and DNA bands were visualized under UV light (transillumi-nator; UV wavelength, 254 nm; TFX-20 M, Vilber Lourmat, France) and photographed by a digital camera (CSE-0028, Cybertech, Berlin, Germany) (Jenkins *et al*., 2006a, b; Tsuji *et al*., 1997).

## RESULTS

The bands showed in Fig. 2 indicated that this DNA extraction method was high efficiency (only one smear appeared in each lane) meanwhile the impurities in DNA samples might be reduced. In the tested 126 samples of households, 39 faecal samples did not yield positive PCR amplification and 87 (77.68%) were positive for *Eimeria* sp. (Fig. 2).

The results of the PCR in case of different age-groups revealed that *E. tenella* was identified with high infection as 78.16% followed by *E. acervulina*, *E. maxima*, *E. brunetti*, *E. mitis* and *E. necatrix* (Table 2). The same trend was also found in case of different breeding conditions *E. tenella* was most prevalent followed by *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis* and *E. necatrix* (Table 3) and *E. praecox* was not found.

In different age-groups chickens, the infection rate of *Eimeria* species was recorded as highest in week 4; 5 and 6 (94.44; 100 and 100%) in case of lowest in week 2 (33.33%). This study also showed that the infection rate of *Eimeria* species begin infection from week 2, highest in week 4; 5 and 6 and then decreased in week 7 and 8.

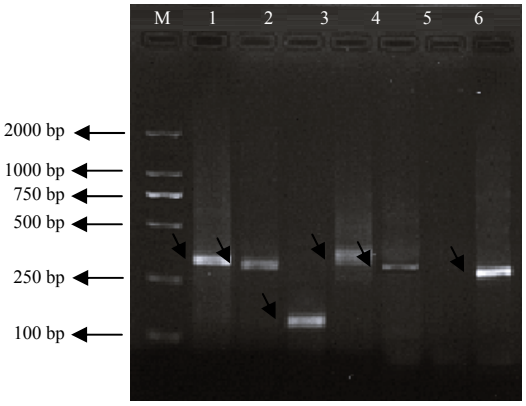


Fig. 2: Identification of *Eimeria* species in chickens in Bacgiang province of Vietnam by agarose gel electrophoresis (1%) of PCR products; Lane M 100 bp DNA ladder; Lane 1 *E. acervulina* primers (+, 321 bp); Lane 2 *E. brunetti* primers (+, 310 bp); Lane 3 *E. maxima* primers (+, 144 bp); Lane 4 *E. mitis* primers (+, 306 bp); Lane 5 *E. necatrix* primers (+, 271 bp); Lane 6 *E. praecox* primers (-, 368 bp); Lane 7 *E. tenella* primers (+, 285 bp)

In different breeding conditions chickens, the highest infection rate of *Eimeria* species was conducted in commercial chicken farm (78.57%) followed by semi-commercial (69.05%) and lowest in households (59.52%). This indicated *Eimeria* species infected popularly in commercial farm. All positive samples had multiple infections with 2-6 species of *Eimeria*. None of them had infections with single species of *Eimeria* or all seven species.

Table 2: Identification of *Eimeria* species by PCR amplification in different age-groups

| Age-groups | No. of examined samples | Microscopically positive (%) | Molecular identification by PCR (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | *E.a* | *E.b* | *E.ma* | *E.mi* | *E.n* | *E.p* | *E.t* |
| Week 2 | 18 | 6 (33.33) | 4 (22.22) | 1 (5.56) | 3 (16.67) | 0 | 0 | 0 | 4 (22.22) |
| Week 3 | 18 | 10 (55.56) | 5 (27.78) | 3 (16.67) | 5 (27.78) | 1 (5.56) | 0 | 0 | 7 (38.89) |
| Week 4 | 18 | 17 (94.44) | 11 (61.11) | 8 (44.44) | 9 (50.00) | 4 (22.22) | 2 (11.11) | 0 | 13 (72.22) |
| Week 5 | 18 | 18 (100) | 14 (77.78) | 8 (44.44) | 10 (55.56) | 6 (33.33) | 4 (22.22) | 0 | 13 (72.22) |
| Week 6 | 18 | 18 (100) | 13 (72.22) | 9 (50.00) | 11 (61.11) | 7 (38.89) | 5 (27.78) | 0 | 14 (77.78) |
| Week 7 | 18 | 11 (61.11) | 9 (50.00) | 6 (33.33) | 8 (44.44) | 4 (22.22) | 7 (38.89) | 0 | 10 (55.56) |
| Week 8 | 18 | 7 (38.38) | 5 (27.78) | 3 (16.67) | 5 (27.78) | 3 (16.67) | 5 (27.78) | 0 | 7 (38.89) |
| Total | 126 | 87 (77.68) | 61 (70.11) | 38 (43.68) | 51 (58.62) | 25 (28.74) | 23 (26.44) | 0 | 68 (78.16) |

E.a: , *Eimeria acervulina*; E.b:, *Eimeria brunetti*; *E.ma*: *Eimeria maxima*; *E.mi*: *Eimeria mitis*; *E.n*: *Eimeria necatrix*; *E.p*: *Eimeria praecox*; *E.t*: *Eimeria tenella*

Table 3: Identification of *Eimeria* species by PCR amplification in different breeding conditions

| Breeding conditions | No. of examined samples | Microscopically positive (%) | Molecular identification by PCR (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | *E.a* | *E.b* | *E.ma* | *E.mi* | *E.n* | *E.p* | *E.t* |
| House-hold | 42 | 25 (59.52) | 14 (33.33) | 9 (21.43) | 12 (28.57) | 7 (16.67) | 5 (11.90) | 0 | 16 (38.10) |
| Commercial farm | 42 | 33 (78.57) | 26 (61.90) | 17 (40.48) | 20 (47.62) | 10 (23.81) | 9 (21.43) | 0 | 27 (64.29) |
| Semi-commercial farm | 42 | 29 (69.05) | 21 (50.00) | 12 (28.57) | 19 (45.24) | 8 (19.05) | 9 (21.43) | 0 | 25 (59.52) |
| Total | 126 | 87 (77.68) | 61 (70.11) | 38 (43.68) | 51 (58.62) | 25 (28.74) | 23 (26.44) | 0 | 68 (78.16) |

*E.a*, *Eimeria acervulina*; *E.b*, *Eimeria brunetti*; *E.ma*, *Eimeria maxima*; *E.mi*, *Eimeria mitis; E.n*, *Eimeria necatrix; E.p*, *Eimeria praecox*; *E.t*, *Eimeria tenella*

## DISCUSSION

In this study, six *Eimeria* sp. were identified in chickens. The overall prevalence of *Eimeria* sp. was 77.68% (87 of 126 birds). This showed that the infection of *Eimeria* species in Bacgiang province of Vietnam was prevalent.

Some reported results of some researchers about prevalence of coccidian parasite such as the *Eimeria* species infecting chickens mostly in Ethiopia showed *E. tenella*, *E. acervulina*, *E. maxima*, *E.brunetti*, *E. mitis* and *E. necatrix* (Gari *et al*., 2008). In another study of the prevalence of *E. tenella* was 80% among the house reared chicks in Saudi Arabia (Al-Quraishy *et al*., 2009) besides in Korea, *E. tenella* was the highest infection rate (78%) and *E. necatrix* has the lowest infection rate (36%) among the *Eimeria* sp. (Heo *et al*., 2004). In case of the prevalence and preponderance of *Eimeria* species sp. among chickens in India were showed that *Eimeria necatrix* infecting chickens was the highest rate (100%) (Aarthi *et al*., 2010). The most recent result of the major *Eimeria* species *E. tenella* was the most prevalent species (24%) followed by *E. acervulina* (18%), *E. necatrix* (12%) and *E. maxima* (10%) (Hadipour *et al*., 2011).

In current research, *Eimeria tenella* was the most prevalent species (78.16%). Six species of *Eimeria* (*E. tenella*, *E. acervulina*, *E. maxima*, *E. brunetti*, *E. mitis* and *E. necatrix*) were identified and the rate of infection *Eimeria* sp. among different age-groups chicken was different, highest in week 4; 5 and 6, decreased from week 7 and 8 (Fig. 3). Hence, coccidiosis just occurred and damaged strongest from week 3-7.

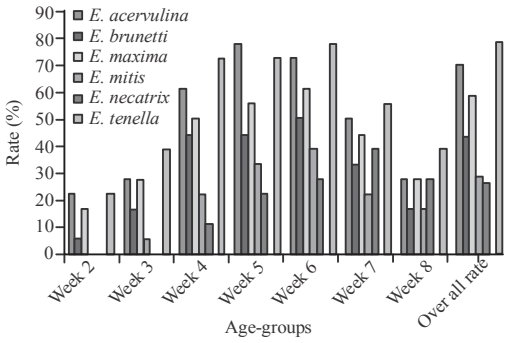


Fig. 3: The rate of *Eimeria* sp. infection on chickens in different age-groups

In case of among scavenging native chickens in household, commercial farm and semi-commercial farm in Bacgiang of Vietnam was also different visibly (Fig. 4). This indicated that different breeding conditions were influence on the infection of *Eimeria* sp. in chickens visibly.

The preponderant species were *E. tenella*, *E. maxima*, *E. acervulina* and *E. brunetti*. These results were different with previous report of some researchers relying only on prevalence estimates or individual species identification about the preponderance of *Eimeria* sp. (*E. tenella*, *E. necatrix*, *E. acervulina*, *E. maxima* and *E. mitis*) in Vietnam.

Concurrently, the difference of infection rate of *Eimeria* sp. between colour broiler and broiler was not significant. However, there were differences between two above candidates and layer chickens. These differences showed that different old day of chicken the rate of

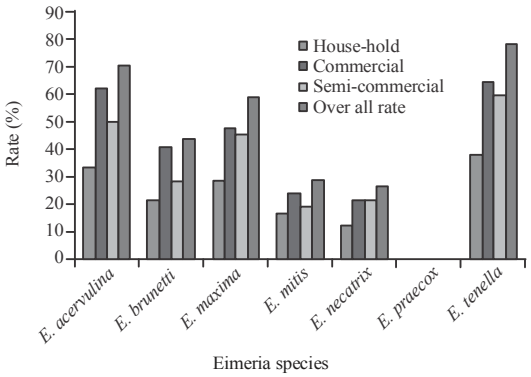


Fig. 4: The rate of *Eimeria* sp. infection on chickens in different breeding conditions

infection of coccidiosis in general and the infection of *Eimeria* species in particular was not similar. In general, *Eimeria tenella* and *Eimeria acervulina* were high prevalent and preponderance in chickens. Reported prevalence of *Eimeria* sp. in China, the infection rate of identified *Eimeria* sp. in the farms was 90, 88, 72, 68, 60, 26 and 8% for *E. tenella*, *E. praecox*, *E. acervulina*, *E. maxima*, *E. mitis*, *E. necatrix* and *E. brunetti*, respectively (Sun *et al*., 2009). On the other hand, the nearest report in Nigeria showed that the prevalence of 71 and 57.7% for the mixed infection of 29 and 42.3% for the single infections in layer and broiler, respectively (Jatau *et al*., 2012).

## CONCLUSION

The present study also showed that there was poor agreement between PCR and traditional identification for diagnosis of *Eimeria* species. Traditional methods are not sufficiently reliable for specific diagnosis of *Eimeria* species in chickens. Alternatively, occurrence of multiple infections in a single bird and the fact that *Eimeria* species with low oocyst frequency in the mixture may be missed indicates that PCR based amplification of DNA sequence of parasite could resolve this problem and overcame the limitation in analysis of small amounts of oocyst in mixed infections. Hence, in the future, the sufficiently reliable method for specific diagnosis of *Eimeria* species in chickens and PCR based amplification of DNA sequence of parasite would have been on behalf of traditional methods. In addition, reported more purifier, reduced both labour and time-consuming method for DNA purification for further molecular studies in general and instead of Phenol-Chloroform Isoamyl Alcohol DNA extraction method.

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