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Original Article

Molecular detection and characterization of *Histomonas meleagridis* in fighting cocks Thailand

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Abstract

Histomoniasis caused by the protozoa *Histomonas meleagridis* is a disease of gallinaceous birds which is of special importance to the poultry industry. The turkey is highly susceptible, which results in high mortality. However, the disease is less severe in chickens but sometimes leads to mortality, especially in those reared in the free-range system. During the present study, four dead fighting cocks were presented to the Veterinary Diagnostic Laboratory, Livestock Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University for post-mortem examination. Based on history and pathological changes, it was preliminarily diagnosed as histomoniasis. The affected livers and caeca samples were collected from which the genomic DNA was extracted. Using species-specific primers, the 18S rRNA gene was amplified by PCR and sequenced. The sequences thus obtained were analyzed using the BLAST search algorithm demonstrating that they are specific for *H. meleagridis*. The sequences were aligned and compared with the other related sequences published in the GenBank database. The four Thailand isolates showed 98-100% nucleotide sequence identity. In comparison, it shows 93-100%, 93-99%, and 88-99% nucleotide sequence identity to China, France, and Austria genotypes, respectively. Phylogenetic analysis of 18S rRNA gene fragments with pair-wise deletion of all gaps and insertion showed that three of the 4 Thailand isolates were closely related to China and France isolates and formed a distinct cluster. While the other Thailand isolates formed a separate cluster along with Austria isolates. Therefore, the PCR method sequences and phylogenetic tree analysis based on the 18S rRNA gene carried out during the present study revealed that the PCR method is highly sensitive for the detection of *H. meleagridis* and there is the genetic diversity of *H. meleagridis* which indicates the presence of different genotypes of this parasite in Thailand.

Keywords: *Histomonas meleagridis*, molecular detection, 18S rRNA gene, phylogenetic relationship, Thailand

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Introduction

Histomoniasis, also known as 'blackhead disease' or 'enterohepatitis,' is a disease of gallinaceous birds caused by an anaerobic protozoan, *Histomonas meleagridis*, belonging to the family Monocercomonadidae in the class Trichomonadea (Tyzzer, 1934). The disease is characterized by liver necrosis, caecal enlargement, and sulfur-yellow diarrhea. Hepatic lesions are variably present and consist of portal or multifocal-to-coalescing heterophilic granulomas and/or necrosis. The parasite is distributed worldwide and transmission occurs primarily via ingestion of embryonated eggs of caecal nematode *Heterakis gallinarum* containing *H. meleagridis* trophozoites or by ingestion of earthworms that have ingested nematode eggs (Hauck & Hafezm, 2013; McDougald 2005). The disease is mainly associated with turkeys due to their susceptibility to *H. meleagridis* resulting in high mortality. It may also occur in chickens, peafowl, guinea-fowl, pheasant, partridge, and quail. However, the disease is comparatively less severe in chickens, resulting in a decrease in production accompanied by some mortality, especially in free-range birds (Esquenet *et al*., 2003). Histomoniasis in chickens has been noted in some European countries i.e. Belgium (Esquenet *et al*., 2003), Austria (Grafl *et al*., 2011), Netherlands (Van der Heijden and Landman, 2011), Denmark (Stokholm *et al*., 2010), Germany (Hauck *et al*., 2010), USA (Homer and Butcher, 1991), and in Asia: Malaysia (Ganapathy *et al*., 2000), India (Banerjee *et al*., 2006; Patra *et al*., 2013).

Nowadays, there is very limited information about histomoniasis in Thailand apart from only one case diagnosed by histopathological examination (Somkid *et al*., 2006). It has been observed that direct demonstration of the parasite in tissue based on histopathological examination is difficult when a small number of parasites is present. In this study, we used molecular techniques to detect and characterize *H. meleagridis* to determine if there is any genetic variability of this parasite which will be very important information for the diagnosis, molecular epidemiology, treatment, and assessment of differences in virulence between the strains.

Materials and Methods

Sample collection: Four dead chickens were presented to the Veterinary Diagnostic Laboratory, Livestock Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, Thailand for post-mortem examination in May 2022. The chickens were kept as fighting cocks. The chickens were reared in the open-farm system with commercial feed and untreated underground water. The chickens showed signs of depression, weakness, and greenish and whitish diarrhea. Despite the antibiotic treatment, the chickens did not respond and died later. A complete necropsy was performed. The affected tissues were collected and fixed in 10% buffered formalin for histopathological investigation. Portions of the affected liver and caeca samples were collected and kept at -20 °C until use.

Genomic DNA extraction: About 25 mg of both liver and caeca tissue as recommended by the manufacturer covering the evident lesions were cut with the help of a small pair of scissors and genomic DNA was extracted using NucleoSpin® Genomic DNA from tissue (Duren, Germany) according to the manufacturer's protocol. In the final step, DNA was eluted with 20 µl of elution buffer and was quantified using NanoDrop™ Spectrophotometer and stored at -20°C until further use.

PCR amplification: A pair of *H. meleagridis* specific primers (Forward primer 5′-GAAA GCATCTATCAAGTGGAA-3′ and reverse primer 5′-GATCTTTTCAAATTAGCTTTAAA-3′) designed by Grabensteiner & Hess (2006) were used to amplify the 18S rRNA gene. The PCR amplification was carried out in a 25 µl reaction mixture using the "Go Taq Green Master Mix 2X (Promega, Madison, USA). PCR amplification was performed in a reaction system containing 12.50 µl of Go Taq Green Master Mix 2X, 1.00 µl of 10 pmol L-1 of each primer, 100 ng of DNA template, and made up to a final volume of 25 µl using Nuclease Free Water (NFW). Negative control (without the template DNA) was used throughout the specimen preparation and PCR progress. The PCR cyclic condition described by Grabensteiner & Hess (2006) was used with slight modification. The cycling conditions were as follows: following the initial denaturation at 95°C for 15 min, the reaction mixtures were subjected to 40 cycles of heat denaturation at 94 °C for 30 sec, primer annealing at 56 °C for 1 min, and DNA extension at 72 °C for 30 sec. Then, the samples were maintained at 72 °C for 10 min for the final extension step and stored at 4 °C until electrophoresis was carried out.

Phylogenetic analyses: A multiple sequence alignment was generated using the sequences obtained from this study and other related sequences published in the GenBank database using the multisequence alignment Clustal W program within the MEGA11. The phylogenetic tree was constructed using the neighbor-joining tree using the Kimura 2-parameter model with pair-wise deletion of all the gaps and insertions using the MEGA 11 software. The bootstrap values were determined for 100 replicates of the data sets. *Trichomonas gallinae* homolog was used as an outgroup to root the tree.

Results

The affected chickens were in poor body condition characterized by atrophy of the pectoral and leg muscles with low subcutaneous and coelomic adipose tissue. A striking lesion was observed in the liver and cecum. The liver lesion revealed irregular yellowish-white to dark green necrotic areas with raised borders (Fig. 1A). The caecum was greatly distended and thickened with a caseous core in the lumen (Fig.1C). The irregular fibrin flags were attached to the caecal serosa leading to adhesiveness with the adjacent organs. Catarrhal content with nematodes identified as *Ascaridia galli* which were collected and preserved in 70% alcohol for further study, was also seen in one chicken. The remaining organs were grossly normal. Microscopically, the liver demonstrated multiple

necrotic foci with infiltration of mixed inflammatory cells, predominately heterophil and macrophages. Myriad histomonads were noticed in the parasitophorous vacuoles (Fig. 1B). Pyogranulomatous transmural typhlitis with massive histomonads was observed in the caeca (Fig. 1D).

H. meleagridis specific primers were used and amplified the 18S rRNA of *H. meleagridis* in the affected liver and caeca of both chickens. The PCR amplicon of 574 bp was generated for 18S rRNA (Fig. 2). These amplicons were purified using NucleoSpin® DNA RNA and Protein purification kit (Duren, Germany) and sequenced. The sequence analysis applying the BLAST search algorithm demonstrated that all four Thailand isolates showed sequence specific for *H. meleagridis* (Fig.3). Alignment of partial nucleotide sequences of these four isolates showed sequence variability through addition, deletion, and substitution of nucleotides at various positions and had 98-100 % nucleotide sequence identity.

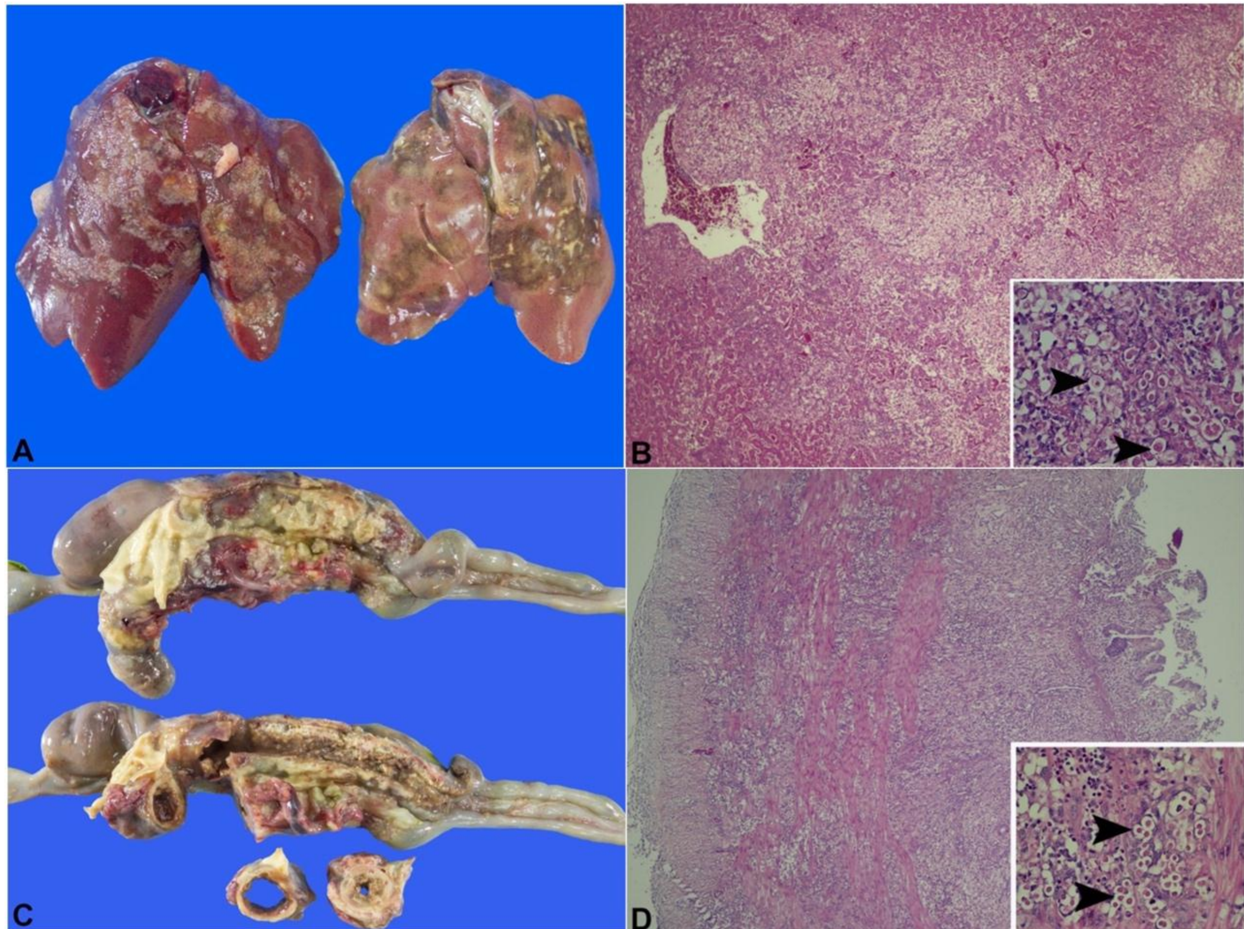

Figure 1 The pathological changes in histomoniasis affected chickens. The irregular necrosis of the liver (A). Massive necrosis of the liver with infiltration of mixed inflammatory cells (B) and histomonads in the parasitophorous vacuole (arrowhead, inset, B). The thick caecal wall with caseous core and fibrin flag on the serosa (C). Pyogranulomatous transmural typhlitis (D) with histomonads (arrowhead, inset, D).

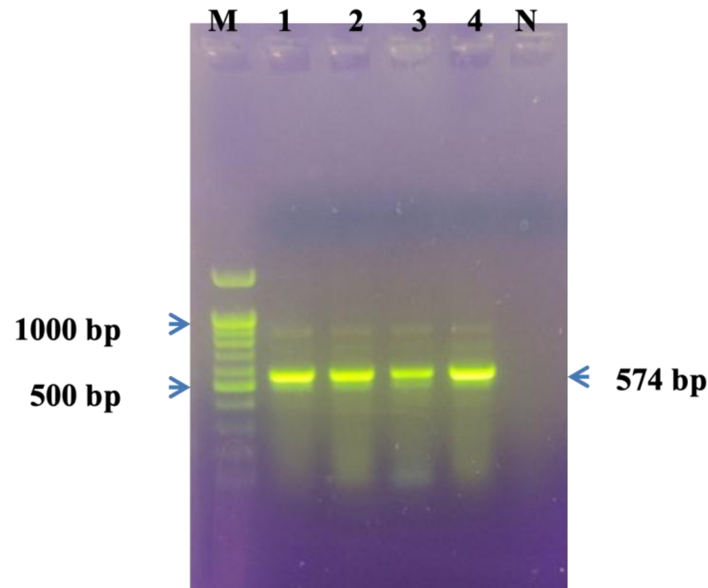

Figure 2 PCR amplification of 18S rRNA of *H. meleagridis*. Lane M: 1.5 kb DNA Ladder. Lane 1 to 4: PCR amplification of 574 bp products of 18S rRNA from genomic DNA of *H. meleagridis*. Lane N: Negative control.

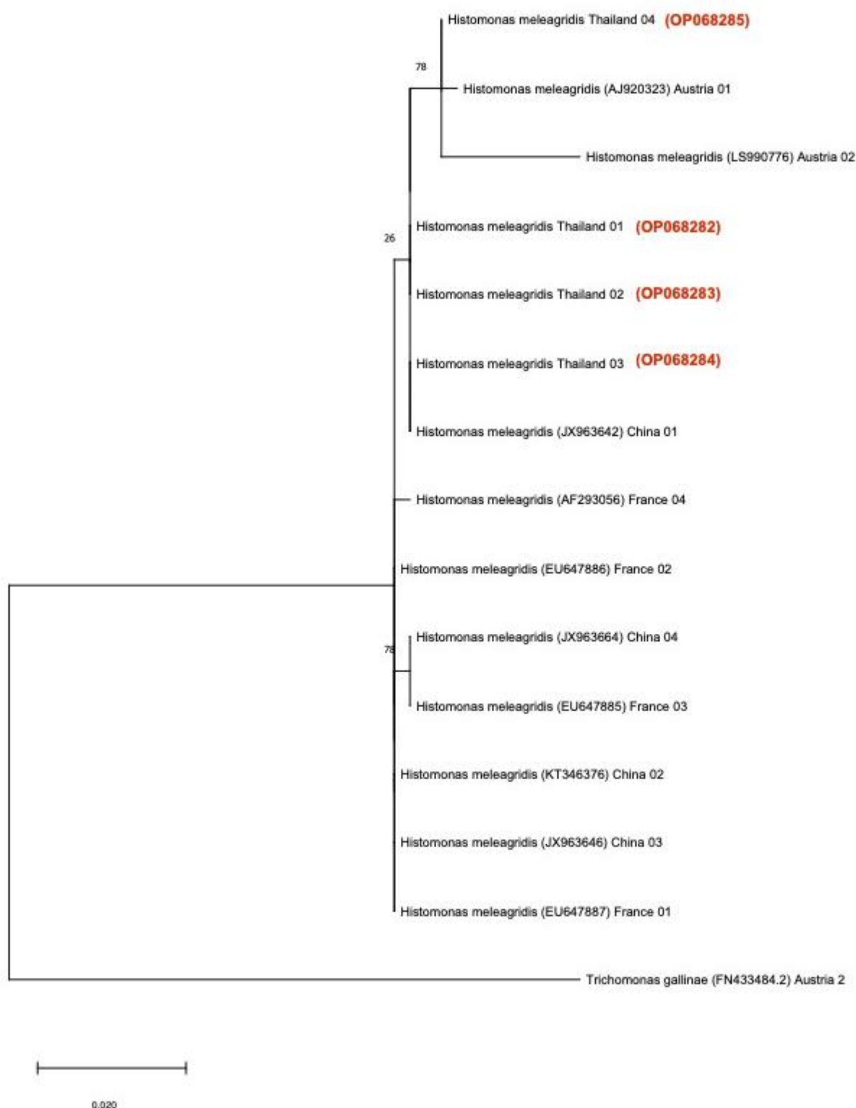

Figure 3 The maximum-likelihood phylogenetic tree showed the genetic relation between *Histomonas meleagridis* from Austria, China, France, and Thailand using the 18s rRNA gene. *Trichomonas gallinae* (FN433484) was used as an outgroup.

Discussion

H. meleagridis was first described under the name *Amoeba meleagridis*, but the discovery of flagellate characteristics led Tyzzer to rename the protozoan *H. meleagridis* (Tyzzer and Collier, 1925). *H. meleagridis* infected chickens showed severe liver and caecal lesions and morbidity. Generally, the mortality rate of the infected chicken is low but sometimes high virulence strains of Histomonas may be found in chicken resulting in high mortality exceeding more than 30%, especially in naturally occurring infections (McDougald, 2003). The pathological changes were seen in both liver and caeca as previously documented (Dandan *et al*., 2018; Jinjun *et al*., 2016). The caseous core of caeca and enlargement, necrosis, and parasites in the liver have also been reported (Kemp and Springer, 1984). As the invading histomonads go deeper through the caecal mucosa, they take the hepatic portal route and are carried to the liver, where they cause multi-focal necrosis (Bon Durant and Wakenell, 1994).

In this study, both *Heterakis gallinarum* and *Ascaridia galli* worms were detected in the caeca and small intestines of the birds, respectively. The birds might have died due to concurrent histomoniasis and *A. galli* infections which synergistically aggravate the infection. *H. gallinarum* is a common intestinal nematode of domestic chicken and turkey. The presence of *H. gallinarum* indicates its vector potentiality for *H. meleagridis* as the main route of histomonas infection through ingestion of embryonated eggs of the caecal worm, *H. gallinarum*. The trophozoites of *H. meleagridis* possess only a low tenacity (Lotfi *et al*., 2012). Thus, eggs of *H. gallinarum* are regarded as a very important vector for disease transmission (Mc Dougald, 2005).

Diagnosis of histomoniasis can be done by several methods including microscopic examination of caecal content, pathognomonic lesions of liver and caeca, histopathological examination of the affected liver and caeca, culture and isolation of parasite, serological techniques like indirect and sandwich ELISA. However, the most specific and sensitive method of diagnosis is molecular techniques like PCR, Nested-PCR, Real-time PCR, etc. PCR has proven successful in detecting *H. meleagridis* (Hafez *et al*., 2005; Huber *et al*., 2005). Huber *et al*. (2005) reported a PCR technique for simultaneous detection of *H. meleagridis* and *T. gallinarum* in caecal droppings of turkeys using only one primer pair. Hafez *et al*. (2005) also developed a PCR, nested-PCR, and real-time PCR to diagnose histomonosis. PCR has also been used to detect *H. meleagridis* in samples and infected birds, as well as to differentiate from *T. gallinarum* and *Blastocystis* spp. (Landman *et al*., 2015; Liu *et al*., 2011). Grabensteiner and Hess (2006) developed a PCR method to amplify the 18S rRNA of *H. meleagridis* and it was found to be a useful diagnostic tool for the rapid and sensitive detection of *H. meleagridis*. During the present study, using the *H. meleagridis* specific primers as described earlier, we could amplify the 18S rRNA of *H. meleagridis* in the affected liver and caeca of both chickens.

Sequence analysis is often used to study phylogenetic relationships to infer diversity or similarity between a set of organisms. Phylogenetic analysis has shed some light on the taxonomic placement of *H. meleagridis*. The Cavalier-Smith system placed *H. meleagridis* in the phylum Parabasalea, Class Trichomonadea, and Family Monocercomonadidae (Cavalier-Smith, 1998). The taxonomic placement of *H. meleagridis* was revised placing it in Class Tritrichomonadea and Family Dientamoebidae (Cepicka *et al*., 2010). In this study, alignment of partial nucleotide sequences of these four isolates showed sequence variability through addition, deletion and substitution of nucleotides at various positions and had 98-100 % nucleotide sequence identity.

Further, this sequence analysis also showed that the Thailand isolates of *H. meleagridis* have 93-100 %, 93-99%, and 88-99% nucleotide sequence identity to the China, France, and Austria genotypes respectively, when compared with already published data in the GenBank database. The 18S rRNA PCR carried out during the present study is specific for the detection of *H. meleagridis* and to differentiate it from the other closely related parasites like *Tetratrichomonas gallinarum* and Blastocystis spp. Therefore, the result of sequence analysis of the 18S rRNA gene carried out during the present study confirmed that the species which is infecting the chickens and leading to their mortality is *H. meleagridis*. This is the first report on the molecular detection and characterization of *H. meleagridis* of chicken from Thailand. The nucleotide sequences of the 18S rRNA gene generated during the present study were registered at GenBank with accession numbers OP068282-OP068285 (Fig. 3). Similar findings were also observed when 18S rRNA and internal transcribed spacer (ITS)-1 sequence analysis demonstrated a clear distinction between *H. meleagridis* isolates and other trichomonads such *as D. fragilis* (Munsch *et al*., 2009). Further, analysis of 18S rRNA, a-actinin1, and rpb1 genetic loci revealed two different phylogenetic clusters of *H. meleagridis* isolates in Europe and further identified two genotypes; in contrast, a probed sequence and partial 18S rRNA have displayed genetic similarity of six purportedly different isolates (Lollis *et al*., 2011; Bilic *et al*., 2014).

Phylogenetic analysis of 18S rRNA gene fragments with pair-wise deletion of all the gaps and insertions revealed that *H. meleagridis* parasite of three Thailand isolates formed a distinct cluster with the isolates already reported from China and France with bootstrap support of 78. While the other Thailand isolate is found to be more closely related to Austria isolates and formed a separate clade away from the China and France isolates. In the phylogenic tree, the outgroup *Tetratrichomonas gallinarum* isolate is clustered away from all the *H. meleagridis* isolates. In contrast, a tree constructed only on the 5.8S rRNA grouped all but one *H. meleagridis* sample into one clade, including GenBank accessions submitted from Europe. There was no correlation between genotypes and host species or geographic location, suggesting that *H. meleagridis* moves freely between multiple avian species in the sampled regions (Lollis *et al*., 2011).

In this study, it showed that the PCR method for the detection of *H. meleagridis* is very useful for identifying genetic diversity and further study is essential to find out the various genetic variants of this

parasite that are prevailing in Thailand affecting the poultry population. Furthermore, molecular detection can assist veterinarians and farmers in checking the histomonad status in Thai poultry farms.

Conflicts of interest: There were no conflicts of interest that may have biased the work reported in this study.

Ethical statement: No animals/ poultry were specifically harmed for the purpose of this study. All the samples were obtained from the deceased birds and presented to the Veterinary Diagnostic Laboratory, Livestock Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, Thailand for post-mortem examination as per standard protocol. And all the data were acquired from diagnostic laboratories or from contributing veterinarians.

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