Original Article

Molecular characterization of *Histomonas meleagridis* in clinical samples of chickens from Eastern China

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Abstract

Histomonas meleagridis (*H. meleagridis*) is a protozoan parasite that may cause histomoniasis, a disease of special importance to the poultry industry and public health. The molecular characterization of *H. meleagridis* in China has not been established. The 5.8S and flanking ITS regions were amplified by polymerase chain reaction from 15 liver samples of chickens which were preliminarily diagnosed with *H. meleagridis* infection by observing clinical symptoms and macroscopic changes in the organs in Eastern China between 2012 and 2013. The obtained sequences were aligned and compared with other known sequences of *H. meleagridis* and related protozoan species based on ITS1-5.8S rRNA-ITS2 or 5.8S rRNA region alone. Out of the 15 obtained sequences, 8 sequences were identified as *H. meleagridis* and were grouped into five clades, suggesting the possibility of multiple genotypes within the samples. Among the remaining 7 sequences, 4 sequences were more related to *Trichomonas* and 3 sequences were more related to *Tetratrichomonas*, which suggests the possibility of misdiagnosis or coinfection with other protozoans. Therefore, there is obvious genetic diversity of *H. meleagridis* based on the 5.8S and flanking ITS regions, which suggests the presence of different genotypes in chickens from Eastern China.

Keywords: *Histomonas meleagridis*, internal transcribed spacer sequence, 5.8S rRNA, homology, phylogenetic relationship

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Introduction

Histomoniasis (also known as infectious enterohepatitis and blackhead disease) is a poultry disease caused by *Histomonas meleagridis* (*H. meleagridis*), a protozoan parasite of the order Galliformes (Tyzzer, 1934). It is characterized by liver necrosis, cecum swelling and sulphur-yellow diarrhoea (Tyzzer, 1934). For food safety, developed countries in Europe and the United States have prohibited the use of most effective drugs for treatment and prevention of the disease (McDougald, 2005). However, this has led to the re-emergence of histomoniasis with considerable economic losses, especially in free-range birds of these regions (Hauck et al., 2010; van der Heijden and Landman, 2011; Popp et al., 2010). In recent years, this disease has also occurred in many regions of China and caused mortality rate of 20-30% in some free-range farms due to serious infection (Chen et al., 2010; Xu et al., 2014).

Genetic variability and phylogenetic relationship between different isolates or regions are of great importance for the understanding of infectivity, transmission and virulence of *H. meleagridis* and also for better disease control (Hess et al., 2015). The 5.8S rRNA, 18S rRNA and flanking internal transcribed spacer (ITS) regions have been widely used as molecular markers for comparative sequence analysis of parasites (Długosz and Wiśniewski, 2006; Sansano-Maestre et al., 2009; Grabensteiner et al., 2010). Genetic variation of *H. meleagridis* in Dutch layer chickens was found using C-profiling of the ITS-1 region, which yielded 3 significant genetic variants (van der Heijden et al., 2006). Four types of *H. meleagridis* in German poultry flocks were found using C-profiling of the 5.8S and flanking ITS regions (Hauck et al., 2010). Significant genetic variation within *H. meleagridis* sequences of the 5.8S and flanking ITS regions was also demonstrated in the United States (Lollis et al., 2011). Recently, two different genotypes have been demonstrated in France and other European countries by multi-locus typing (Bilic et al., 2014). In China, the possibility of different genotypes existing in this region is unclear.

Eastern China has the largest number of poultry raised and thus the highest incidence of histomoniasis in China. Clinical pattern of the disease showed variation in severity and duration in some cases (Chen et al., 2010; Xu et al., 2014). It is necessary to determine whether there is genetic variability of *H. meleagridis* and to find out whether there are unknown species in this region, which will be important for the diagnosis, molecular epidemiology and assessment of differences in virulence between strains. In this study, the 5.8S and internal transcribed spacer sequences of *H. meleagridis* in clinical samples collected from this region were analyzed.

Materials and Methods

Sample collection: Fifteen liver samples (YZ1–YZ15) of chickens originating from different free-range flocks (Table 1) were collected in Eastern China from 2012 to 2013. The chickens had been preliminarily diagnosed with histomoniasis at the animal hospital of Yangzhou University based on symptoms and macroscopic changes in the organs. The liver samples showed gross lesions appearing as multifocal or diffuse nodular necrosis (Figure 1A and 1B), with which caseous core formed in the caeca of the same bird. Portions of the liver samples were collected and frozen at -20°C before use. Liver samples collected from normal SPF chickens were used as negative control.

Table 1 Information about sources of liver samples collected in this study

Year	Age of birds (days)	Type of birds	Initial mortality (%)	Locality	Chart ID	GenBank accession no.
2012	67	Broiler	6.8	Jiangsu	YZ1	KJ863540
2012	72	Layer	6.5	Jiangsu	YZ2	KJ863541
2012	42	Layer	7.4	jiangsu	YZ3	KJ863542
2012	53	Broiler	8.5	Anhui	YZ4	KJ863543
2012	55	Broiler	9.0	Jiangsu	YZ5	KJ863544
2012	95	Layer	4.5	Shangdong	YZ6	KJ863545
2012	60	Broiler	8.5	jiangsu	YZ7	KJ863546
2013	58	Layer	7.3	jiangsu	YZ8	KJ863547
2013	65	Broiler	6.8	jiangsu	YZ9	KJ863548
2013	73	Layer	5.5	Zhejiang	YZ10	KJ863549
2013	64	Broiler	6.2	Jiangsu	YZ11	KJ863550
2013	112	Layer	2.3	Jiangsu	YZ12	KJ863551
2013	77	Layer	4.7	Anhui	YZ13	KJ863552
2013	43	Broiler	8.1	Jiangsu	YZ14	KJ863553
2013	48	Broiler	7.4	Jiangsu	YZ15	KJ863554

Genomic DNA extraction: About 30 mg liver tissue covering evident lesions was cut up with a small pair of scissors, and genomic DNA was extracted using miniprep DNA purification kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. DNA concentration was diluted to 50-100 ng μL^{-1} and stored at -20°C until further use.

PCR amplification: A pair of Trichomonadidae wide primers was designed according to previous reports (Sansano-Maestre et al., 2009; Lollis et al., 2011; Bilic et al., 2014) to amplify the 5.8S and flanking ITS1 and IST2 regions. PCR amplification was performed in a reaction system containing 100 ng DNA template, 12.50 µL of 2×Easy Taq PCR SuperMix (TransGen Biotech, Beijing, China) and 1.00 µL of 10 µpmol L^{-1} of

each primer, and made up to a final volume of 25 µL using deionized water. The cycling conditions were as follows: denaturation at 95°C for 2 min, followed by 40 amplification cycles (94°C for 30 sec, 52°C for 35 sec, 72°C for 45 sec) and a final extension cycle at 72°C for 5 min. PCR products were separated by electrophoresis on 2.0% agarose gel, stained by ethidium bromide, and photographed using a gel imaging system (Biorad Gel Doc XR+, Bio-Rad Laboratories, California, USA).

Cloning and sequencing: The target band was carefully excised under UV light and purified using agarose gel DNA purification kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The purified gene segment was ligated with the pGEM-T-Easy vector (Promega, Madison, USA), and the ligation products were transformed into *E. coli* competent cells DH5α. To confirm the validation of the sequences, three random selected positive plasmids from each sample were identified by blue-white screening and enzyme digestion, then were sequenced in both directions by Invitrogen Trading (Shanghai, China). Finally, the obtained sequences of each sample were submitted to GenBank.

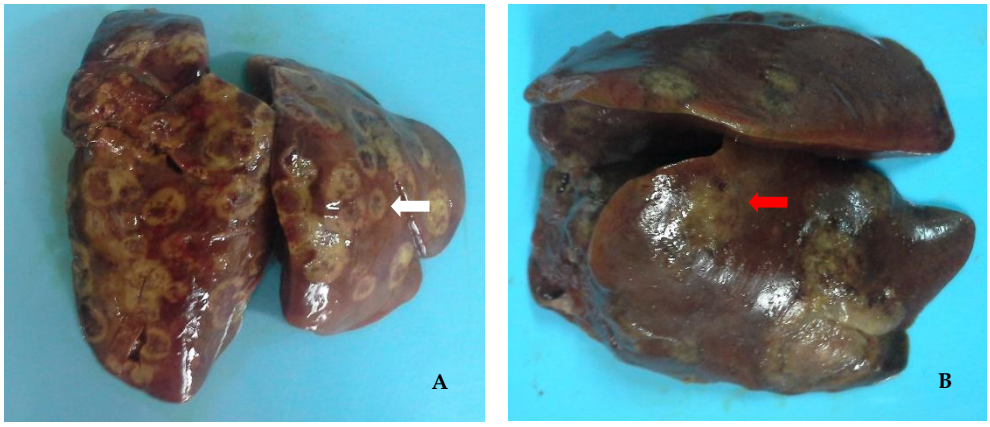

Figure 1 Macroscopic changes in liver from clinical samples preliminarily diagnosed with histomoniasis. (A) Multifocal necrosis (white arrow) in liver (B) Diffuse nodular necrosis (red arrow) in liver

Phylogenetic analysis: Using the DNAStar software (DNASTAR, Madison, Wisconsin, USA), the obtained sequences were aligned with other sequences of ITS1-5.8S rRNA-ITS2 region from related species including *H. meleagridis*, *Tetratrichomonas gallinarum* (*T. gallinarum*), *Trichomonas foetus* (*T. foetus*), and *Dientamoeba fragilis* (*D. fragilis*), which were selected from the GenBank database. A phylogenetic tree was constructed using the maximum parsimony method included in the MEGA 5.0 software with default setting. Reliability of the branching orders was evaluated by the bootstrap test using 1000 replicates.

Results

Cloning and sequencing analysis of 5.8S and flanking ITS regions: Using PCR, about 350 bp fragments were specifically amplified from all fifteen liver samples except for the control of SPF chickens, with the expected length. The amplified fragments were successfully cloned into pGEM-T-Easy vector, which were demonstrated by enzyme digestion and sequencing. Fifteen obtained sequences of YZ1 to YZ15 were deposited in GenBank with accession numbers of KJ863540 to KJ863554, respectively. Table 2 represents the identity among the obtained sequences and other related sequences. As shown in Table 2, significant genetic variation of *H. meleagridis* in Eastern China and other locations abroad could be observed, and the nucleotide sequence identity varied widely with the locations of samples and the species of parasites.

Phylogenetic relationship of 5.8S and flanking ITS regions: The phylogenetic tree of the 5.8S and flanking ITS regions was constructed successfully using the maximum parsimony method (Figure 2). The figure revealed significant genetic variation within *H. meleagridis* and related organisms, with maximum parsimony values supporting this significant variation. Among the fifteen sequences, YZ2, YZ3, YZ4, YZ6, YZ11, YZ12, YZ14, and YZ15 were sequences of *Histomonas*. These sequences were grouped into five clades, displaying polytomies, which suggests the presence of different genotypes. In detail, YZ4, YZ6, YZ12, and YZ15 formed one clade with 87% bootstrap support; YZ2, YZ3, YZ14, USA-1, USA-2, USA-3, and USA-4 formed their own clade; while YZ11 was clustered with the German strain with 69% bootstrap support. Although the sequences of YZ1, YZ5, YZ7, and YZ8 became a sister taxon of *H. meleagridis* with 99% bootstrap support, they had relatively distant genetic distance with the known strains of *Histomonas*, and were unresolved sequences, and could belong to other species whose sequences are currently unavailable in the database. The remaining three sequences (YZ9, YZ10, and YZ13) were not clustered with *H. meleagridis*, and more related to *T. gallinarum.*

Phylogenetic relationship of 5.8S region: The phylogenetic tree of the 5.8S region was also constructed successfully using the maximum parsimony method (Figure 3). The results were broadly similar to the analysis of the 5.8S and flanking ITS regions. Among the fifteen sequences, YZ2, YZ3, YZ4, YZ6, YZ11, YZ12, YZ14, and YZ15 were sequences of *Histomonas*. In detail, YZ4, YZ6, YZ12 and YZ15 were clustered in one clade with 99% bootstrap support; YZ2, YZ3, YZ11 and YZ14 formed their own clade

separately; while YZ2 and YZ3 became sister taxa of USA-2. The sequences of YZ1, YZ5, YZ7, and YZ8 were also unresolved sequences with 99% bootstrap support; while YZ9, YZ10, and YZ13 were not clustered with *H. meleagridis*, and were related to *T. gallinarum*.

Table 2 Percent identity of 5.8S rRNA and flanking ITS region genes of *Histomonas meleagridis* and related parasites

	Percent identity of sequences from China (%)														
	YZ1	YZ2	YZ3	YZ4	YZ5	YZ6	YZ7	YZ8	YZ9	YZ10	YZ11	YZ12	YZ13	YZ14	YZ15
A	30.5	51.2	51.2	51.5	30.2	51.5	30.8	30.5	78.5	77.8	50.5	51.5	77.5	50.7	51.5
B	28.0	54.5	54.5	53.8	28.7	53.8	28.7	29.0	57.0	57.8	53.2	53.8	57.8	53.5	53.8
C	39.0	76.8	76.8	75.0	39.5	75.0	39.5	39.8	61.8	61.0	75.2	75.0	61.0	75.2	75.0
D	36.8	91.0	90.8	91.0	37.0	91.0	37.5	37.2	55.5	56.8	92.5	91.0	56.5	91.5	91.0
E	37.8	84.8	84.5	83.5	38.0	83.5	38.5	38.2	53.2	55.5	84.2	83.5	55.5	85.5	83.5
F	39.0	91.0	91.2	90.5	39.5	90.5	40.0	40.0	59.0	59.2	90.2	90.5	58.8	91.2	90.5
G	39.2	79.5	79.2	78.0	39.0	78.0	39.5	39.5	69.2	70.8	77.8	78.0	70.5	78.8	78.0
H	20.5	49.5	49.5	48.0	20.2	48.0	20.5	20.5	26.0	27.0	48.2	48.0	26.8	49.5	48.0

Note: A: *Tetratrichomonas gallinarum* (JN619424); B: *Tritrichomonas foetus* (HM046255); C: *Dientamoeba fragilis* (DQ233461); D: HM-Germany (HM229784); E: HM-USA-1 (HQ334180); F: HM-USA-2 (HQ334181); G: HM-USA-3 (HQ334185); H: HM-USA-4 (HQ334191)

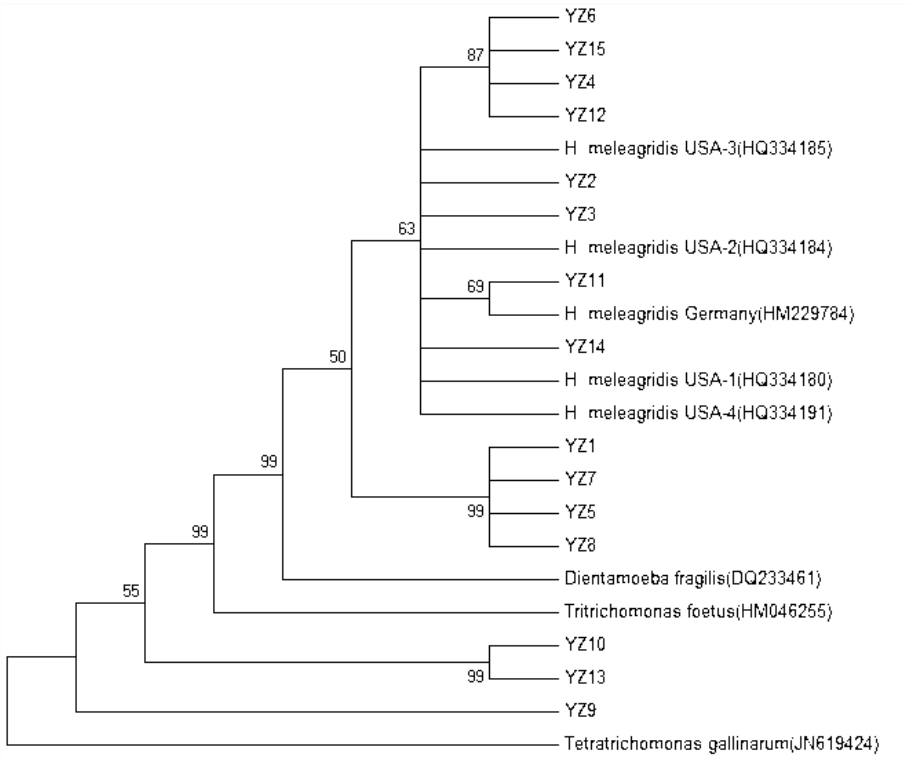

Figure 2 Phylogenetic analysis of *Histomonas meleagridis* sequences and other related parasites based on 5.8S rRNA and flanking ITS regions. The tree was constructed using the maximum parsimony method. *Tetratrichomonas gallinarum* was used as out-group. Bootstrap values are located at nodes.

Discussion

In this study, 15 sequences were successfully obtained from a total of 15 samples of possible histomoniasis cases collected in Eastern China. The freshly collected tissues contributed to the high recovery rate, while other formalin-fixed tissues could result in low recovery rates (Hauck et al., 2010; Lollis et al., 2011). The significant genetic variation among samples collected from different locations of this region was observed by the sequences and phylogenetic analysis of 5.8S rRNA and flanking ITS regions. The present results strongly suggest the possibility of different genotypes of *H. meleagridis* in Eastern China.

Genetic variations of *H. meleagridis* have been reported in several countries or regions by using various molecular markers and methods, and various results have been acquired. Of these markers, the ITS1-5.8S-ITS2 region has been mostly used to elucidate the molecular phylogeny (Lollis et al., 2011; Bilic et al., 2014). As demonstrated in our results, it is still not a perfect marker for genotyping with traditional molecular methods. C-profiling of the ITS-1 region, a novel genotyping method, has been developed to describe genetic variation of *H. meleagridis* in Denmark, and three genotypes (type I, type II, and type III) were found (van der Heijden et al., 2006). Moreover, four types of *H. meleagridis* (type A, type B, type C, and type D) in German poultry flocks were found using C-profiling of the 5.8S and flanking ITS regions (Hauck et

al., 2010). However, this method is still less suitable for genotyping due to the presence of heterogeneous ITS sequences in a single clone or a mixed infection (Hauck et al., 2010; Lollis et al., 2011; Bilic et al., 2014), even though it has not been observed in this study (confirmed by sequencing of three different positive plasmids of the same sample, data not shown). Recently, about 3,000 genes have been identified in *H. meleagridis* (Klodnicki et al., 2013), and further knowledge of the gene and genome will make it possible to design new perfect markers or methods for the genotyping of *H. meleagridis*.

YZ1, YZ5, YZ7, and YZ8, which shared 98.8-99.8% identify within the ITS1-5.8S-ITS2 region, formed a sister taxon of *H. meleagridis* with 99% bootstrap support. This cluster had relatively distant genetic distance with the known strains of *Histomonas*, and was unresolved. As for the 5.8S region alone, which is better for the separation of genera and species in the parabasalids (Lollis et al., 2011), the phylogenetic tree further constructed, however, failed to place these sequences into the *Histomonas* cluster, and these sequences were more related to *Trichomonas.* Besides, YZ9, YZ10, and YZ13 obviously were not clustered in *Histomonas* by comparing with the ITS1-5.8S-ITS2 region or 5.8S alone, and were more related to *Tetratrichomonas.*

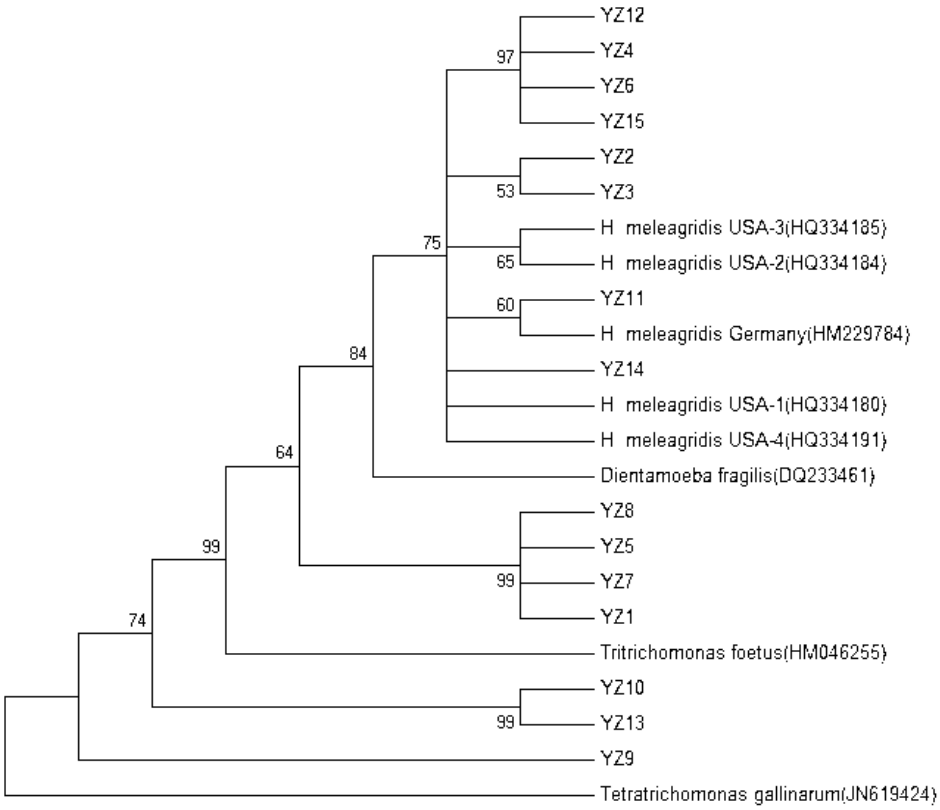

Figure 3 Phylogenetic analysis of *Histomonas meleagridis* sequences and other related parasites based on 5.8S rRNA region. The tree was constructed using the maximum parsimony method. *Tetratrichomonas gallinarum* was used as out-group. Bootstrap values are located at nodes.

A total of 46.7% (7/15) of the sequences were away from *H. meleagridis* in this study, and the negative rate was very high. Similar results also have been reported (Hauck et al., 2010; Lollis et al., 2011; Bilic et al., 2014). For a long time, some unknown species or coinfection with other protozoans such as *Trichomonas* or *Pentatrichomonas* were considered as the cause of blackhead disease (Allen, 1941). However, these unknown species which cause tricomoniasis-like diseases in chickens or turkeys have not been pursued in recent years, and further work should be urgently required to identify the species represented by these samples.

It is generally accepted that there is no clear correlation between subtypes and hosts or geographic locations of *H. meleagridis* (Hauck et al., 2010; Lollis et al., 2011). Conversely, recent reports have described some correlations between subtypes and locations (Bilic et al., 2014). In this study, although the nucleotide sequence identity varied widely with the locations of samples and the selected species of parasites, no significant correlations with the locations could be determined. This is due to the limited number of samples and hosts in this study. It is, therefore, recommended that other locations and host species in China should be further investigated.

In conclusion, there is obvious genetic diversity of *H. meleagridis* based on the 5.8S and flanking ITS regions in chickens from Eastern China. Considering the large number of poultry and the high prevalence of *H. meleagridis* in China, it is necessary to obtain more geographical isolates or samples for systematic identification, classification, and evaluation using molecular biological methods. Moreover, its genetic diversity suggests the presence of different

genotypes, and further genotyping is required in future studies.

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บทคัดย่อ

การอธิบายลักษณะระดับโมเลกุลของเชื้อ *Histomonasmeleagridis* จากตัวอย่างไก่ในเขตตะวันออกของประเทศจีน

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เชื้อ *Histomonas meleagridis (H. meleagridis)* เป็นเชื้อโปรโตซัวที่ทำให้เกิดโรค histomoniasis ซึ่งเป็นโรคที่มีความสำคัญต่ออุตสาหกรรมการเลี้ยงสัตว์ปีกและการสาธารณสุข เนื่องจากในประเทศจีนยังไม่มีรายงานการศึกษาลักษณะระดับโมเลกุลของเชื้อ *H. meleagridis* ในการศึกษาครั้งนี้ได้ตรวจพิสูจน์หา 5.8S และ flanking ITS โดยวิธี polymerase chain reaction จากตัวอย่างชิ้นเนื้อตับไก่ จำนวน 15 ตัวอย่าง จากตัวอย่างไก่ที่ตรวจยืนยันว่าติดเชื้อ *H. meleagridis* ระหว่างปี ค.ศ. 2012 ถึง 2013 ผลจากการเปรียบเทียบรหัสพันธุกรรมที่บริเวณ ITS1-5.8S rRNA-ITS2 หรือ 5.8S rRNA ของเชื้อ *H. meleagridis* กับเชื้อโปรโตซัวอื่นๆพบว่าใน 15 ตัวอย่างมี 8 สายพันธุกรรมที่มีลักษณะใกล้เคียงกับเชื้อ H. meleagridis และยังสามารถจัดแบ่งออกเป็น 5 กลุ่ม ซึ่งแสดงถึงความเป็นไปได้ที่มีเชื้อหลาย genotype ในตัวอย่างที่ศึกษา ส่วนตัวอย่างอีก 7 สายพันธุกรรม มีลักษณะใกล้เคียงกับเชื้อ *Trichomonas* 4 ตัวอย่าง และเชื้อ *Tetratrichomonas* 3 ตัวอย่าง ซึ่งแสดงให้เห็นถึงการตรวจพิสูจน์เชื้อที่ผิดพลาด หรือการติดเชื้อร่วมกับโปรโตซัวชนิดอื่น โดยสรุปการศึกษาครั้งนี้แสดงให้เห็นความหลากหลายทางพันธุกรรมของเชื้อ *H. meleagridis* ด้วยการศึกษาบริเวณ 5.8S และ flanking ITS โดยพบเชื้อหลากหลายจีโนไทป์ในไก่ในเขตตะวันออกของประเทศจีน

คำสำคัญ: *Histomonasmeleagridis* internal transcribed spacer sequence 5.8S rRNA ความคล้าย ความสัมพันธ์ทางพันธุกรรม

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