Journal of Animal and Veterinary Advances 12 (16): 1338-1342, 2013
ISSN: 1680-5593

Phylogenetic Relationships of the *18SrRNA* Gene Sequence of *Histomonas meleagridis* from Chickens in East China

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Abstract: *Histomonas meleagridis* (*H. meleagridis*) is a protozoan parasite that may cause histomoniasis, a disease in poultry of the order Galliformes. The phylogenetic relationships between *H. meleagridis* in different geographical regions of China are not well understood. By PCR, the partial *18SrRNA* gene was amplified from 30 liver samples collected from chickens infected with *H. meleagridis* in East China. The obtained sequences were aligned with other known *18SrRNA* gene sequences of *H. meleagridis* strains and two taxonomically related protozoan species. The alignment results showed that the *18SrRNA* gene sequence of *H. meleagridis* was highly conserved in East China with nucleotide sequence identity of 98.4-100.0%. Further phylogenetic analysis revealed these sequences were clustered into five groups, suggesting the presence of different genotypes. These results throw a light on molecular diagnosis, epidemiological investigation and population genetics of *H. meleagridis* in China.

Key words: *Histomonas meleagridis*, 18SrRNA, homology, phylogenetic relationships, China

INTRODUCTION

Histomoniasis, caused by *Histomonas meleagridis* (*H. meleagridis*) is a protozoal disease in poultry of the order Galliformes. It is characterized by liver necrosis, cecum swelling and sulphur-yellow diarrhoea (McDougald, 2005). The head may become cyanotic (bluish in colour) and hence the common name of the disease is "blackhead". In recent years, the disease occurred in many regions of China and caused mortality rate of 20-30% in some farms due to serious infection (Chen *et al*., 2010). For food safety, the developed countries in Europe and the Americas have prohibited the use of most effective drugs for treatment and prevention of the disease which makes its outbreaks and prevalence more frequent (McDougald, 2005).

Clear phylogenetic relationships and exact taxonomy are of significance for better understanding of the infectivity, transmission and virulence of pathogens and thus also serve the disease control. Molecular biological methods have been widely used to investigate phylogenetic relationships in parasites because they can provide comparable quantitative evolutionary information which is not based on morphology and biogeography and not affected by environmental factors and subjective judgment of researchers (Dlugosz and Wisniewski, 2006). Ribosomal RNA (rRNA) is present and rich in all cells of eucaryotes. It also has highly conserved sequences and important functions in life activities. Therefore, it is an ideal molecular marker for taxonomic and phylogenetic studies in parasites (Dlugosz and Wisniewski, 2006). Currently, some researches have been conducted to study taxonomic and phylogenetic features of *H. meleagridis* by using 5.8SrRNA, 18SrRNA and Internal Transcribed Spacer (ITS) sequences (Van der Heijden *et al*., 2006; Mantini *et al*., 2009; Lollis *et al*., 2011). Liu *et al*. (2011) analyzed the phylogenetic relationships of the 18SrRNA sequences of 10 *H. meleagridis* isolates from Hunan Province of China and they speculated the existence of different genotypes. However, the evolution and taxonomy of *H. meleagridis* in other regions of China are still unclear.

East China has the largest number of poultry raised and also has the highest incidence of histomoniasis (Chen *et al*., 2010). It is necessary to determine whether there are different genotypes of *H. meleagridis* in this region and to find out the genetic and evolutionary relationships among them which will serve the diagnosis, molecular epidemiology and population genetics of histomoniasis. Therefore, researchers analyzed partial *18SrRNA* gene sequences of *H. meleagridis* collected from different areas of East China.

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MATERIALS AND METHODS

Tissue samples: Thirty liver samples (HM1-HM30) were collected from naturally infected chickens with typical histomoniasis lesions in East China in 2011. The liver samples collected from SPF chickens were used as control. All samples were frozen at -20°C before use.

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). The DNA concentration was diluted to 50-100 ng μL^{-1} and stored at -20°C until further use.

PCR amplification: A pair of primers was designed according to the report of Grabensteiner and Hess (2006) to amplify the *18SrRNA* gene and the primer sequences are as follows: 5'-GAA AGC ATC TAT CAA GTG GAA-3' (P1) and 5'-GAT CTT TTC AAA TTA GCT TTA AA-3' (P2). The primers were synthesized by Sangon Biotech (Shanghai, China). PCR amplification was performed in a reaction system containing 100 ng DNA template, 2.00 μL of 10× PCR buffer, 2.52 μL of 25 mmol L^{-1} $MgCl_2$, 0.40 μL of 10 mmol L^{-1} dNTPs, 0.20 μL of 50 μmol L^{-1} each primer and 7.5 units of Taq DNA polymerase (Promega, Madison, USA) made up to a final volume of 20.00 μL using deionized water. The temperature profile are as follows: denaturation at 95°C for 2 min, followed by 40 amplification cycles (95°C for 35 sec, 57°C for 35 sec, 72°C for 45 sec) and a final extension cycle (72°C for 5 min). The PCR products were separated by electrophoresis on 1.5% agarose gel, stained by ehidium bromide and photographed using a gel imaging system.

Cloning and sequencing: The target band was carefully excised under an UV light and purified using agarose gel DNA purification kit (TaKaRa, Dalian, China). 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. Then, the positive plasmids identified by blue-white screening and enzyme digestion were sequenced by Invitrogen Trading (Shanghai, China). Finally, the obtained sequences were submitted to the GenBank.

Table 1: *18SrRNA* gene sequences indexed in this research

Parasites	Geographic origin	GenBank accession No.
Histomonas meleagridis	Austria	AJ920323
Histomonas meleagridis	Australia	AY730405
Histomonas meleagridis	France	AF293056
Histomonas meleagridis	France	EU647884
Histomonas meleagridis	France	EU647885
Histomonas meleagridis	France	EU647886
Histomonas meleagridis	France	EU647887
Histomonas meleagridis	Italy	JQ677147
Histomonas meleagridis	Italy	JQ677148
Tetratrichomonas gallinarum	Austria	AJ920324
Tritrichomonas foetus	America	AY754332

Sequencing and phylogenetic analysis: Using the DNAStar Software, the obtained sequences were aligned with other *18SrRNA* gene sequences from *H. meleagridis*, *Tetratrichomonas gallinarum* (*T. gallinarum*) and *Trichomonas foetus* (*T. foetus*) which had been published in the GenBank (Table 1). The phylogenetic tree of the *18SrRNA* gene was constructed using the maximum parsimony method included in the MEGA Software. The reliability of the branching orders was evaluated by the bootstrap test.

RESULTS

***18SrRNA* gene cloning:** By PCR about 550 bp fragments of *18SrRNA* gene were specifically amplified from all liver samples except the control with the expected length (Fig. 1).

Sequencing analysis of *18SrRNA*: Thirty obtained *18SrRNA* gene sequences were deposited in the GenBank with accession numbers of JX963642-JX963670 and JQ277354. The sequencing analysis showed the partial *18SrRNA* gene sequence was highly conserved among the *H. meleagridis* in East China with nucleotide sequence identity of 98.4-100.0%. The partial *18SrRNA*

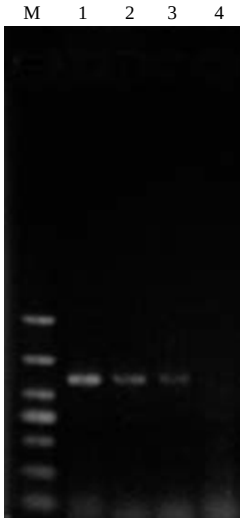

Fig. 1: Electrophoresis of *18SrRNA* gene PCR products of *Histomonas meleagridis*. M: DL1000 DNA ladder; 1-3: Positive liver sample named HM1-HM3; 4: Control liver sample from SPF chicken

Table 2: Identity and divergence of *18SrRNA* gene of partial *Histomonas meleagridis* and taxonomically related protozoan species from Eastern China and other regions

	Perecent identity																		
Divergence	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1		99.3	99.0	99.0	99.8	99.1	99.7	99.5	99.8	99.3	99.1	99.8	98.8	98.8	99.0	99.0	99.7	81.8	67.1
2	0.5		99.7	99.5	99.5	99.8	99.1	99.0	99.5	100.0	99.8	99.5	99.5	99.5	99.7	99.7	99.3	82.1	67.6
3	0.9	0.3		99.3	99.1	99.7	99.0	98.8	99.1	99.7	99.7	99.3	99.3	99.3	99.5	99.5	99.1	81.9	67.5
4	0.9	0.5	0.7		99.0	99.7	99.3	99.1	99.0	99.5	99.7	99.3	99.3	99.3	99.5	99.5	99.1	82.1	67.3
5	0.2	0.3	0.7	0.9		99.3	99.7	99.5	100.0	99.5	99.3	99.0	99.0	99.0	99.1	99.1	99.8	81.8	67.3
6	0.7	0.2	0.3	0.3	0.5		99.3	99.1	99.3	99.8	100.0	99.7	99.7	99.7	99.8	99.8	99.5	82.1	67.6
7	0.3	0.7	0.9	0.5	0.3	0.5		99.8	99.7	99.1	99.3	99.0	99.0	99.0	99.1	99.1	99.8	81.9	67.1
8	0.3	0.7	0.9	0.5	0.3	0.5	0.0		99.5	99.0	99.1	98.8	98.8	98.8	99.0	99.0	99.7	81.9	67.3
9	0.2	0.3	0.7	0.9	0.0	0.5	0.3	0.3		99.5	99.3	99.0	99.0	99.0	99.1	99.1	99.8	81.8	67.3
10	0.5	0.0	0.3	0.5	0.3	0.2	0.7	0.7	0.3		99.8	99.5	99.5	99.5	99.7	99.7	99.3	82.1	67.6
11	0.7	0.2	0.3	0.3	0.5	0.0	0.5	0.5	0.5	0.2		99.7	99.7	99.7	99.8	99.8	99.5	82.1	67.6
12	0.9	0.3	0.5	0.5	0.7	0.2	0.7	0.7	0.7	0.3	0.2		99.3	99.3	99.5	99.5	99.1	81.9	67.5
13	1.0	0.5	0.7	0.7	0.9	0.3	0.9	0.9	0.9	0.5	0.3	0.5		100.0	99.8	99.8	99.1	82.1	67.6
14	1.0	0.5	0.7	0.7	0.9	0.3	0.9	0.9	0.9	0.5	0.3	0.5	0.0		99.8	99.8	99.1	82.1	67.6
15	0.9	0.3	0.5	0.5	0.7	0.2	0.7	0.7	0.7	0.3	0.2	0.3	0.2	0.2		100.0	99.3	82.1	67.6
16	0.9	0.3	0.5	0.5	0.7	0.2	0.7	0.7	0.7	0.3	0.2	0.3	0.2	0.2	0.0		99.3	82.1	67.6
17	0.3	0.5	0.7	0.7	0.2	0.3	0.2	0.2	0.2	0.5	0.3	0.5	0.7	0.7	0.5	0.5		81.8	67.3
18	19.5	18.8	19.1	18.8	19.5	18.8	19.2	19.1	19.5	18.8	18.8	18.9	18.8	18.8	18.8	18.8	19.5		69.5
19	12.7	11.7	12.0	12.2	12.4	11.7	12.7	12.7	12.4	11.7	11.7	11.7	11.7	11.7	11.7	11.7	12.4	9.0	

1: JQ277354; 2: JX963642; 3: JX963643; 4: JX963644; 5: JX963645; 6: JX963646; 7: JX963647; 8: JX963648; 9: JX963649; 10: JX963650; 11: JX963651; 12 : AF293058; 13: EU647884; 14: EU647885; 15: EU647886; 16: EU647887; 17: AJ920323; 18: AJ920324; 19: AY754332

Fig. 2: Phylogenic tree of *Histomonas meleagridis* (HM) based on the *18SrRNA* gene sequences. HM1-HM30 are the sequences from East China. The GenBank accession numbers are given in parentheses

gene sequence had identity higher than 98.4% to that of the France strain and Austria strain and also had identity of 88.5-90.0% to that of the Italy strain and Australia strain. In addition, these sequences had higher identity to *T. gallinarum* than that to *T. foetus* (81.6-82.3% vs. 66.7-67.6%). Table 2 represents the identity of *18SrRNA* gene sequence among 11 isolates from East China.

Phylogenetic relationship of *18SrRNA*: The phylogenetic tree of the *18SrRNA* gene was constructed successfully using the maximum parsimony method. The thirty isolates from East China were grouped into five lineages (Fig. 2), suggesting the presence of different genotypes. In detail, HM5, HM10, HM14, HM20, HM22, HM23 and HM26 were clustered with the France strain while the HM4, HM6,

HM7, HM8, HM13, HM17, HM28 and HM30 with the Austria strain. All sequences from East China had relatively distant genetic distance with the Australia and Italy strains and more distant genetic distance with *T. gallinarum* and *T. foetus* which are the members of other related genera.

DISCUSSION

The *18SrRNA* gene is used as a reliable scientific indicator in taxology because its nucleotide composition and sequence are conservative in the evolutionary process and the rate of nucleotide substitution is low. In recent years, it has become a trend to explore intra or inter-species genetic relationships and phylogenetic evolution by analyzing variations of *18SrRNA* gene sequence (Dlugosz and Wisniewski, 2006). In this study, researchers analyzed the nucleotide sequences of *18SrRNA* genes of *H. meleagridis* from East China. The results showed that these sequences had high identity (≥88.5%) of *18SrRNA* gene sequence to other foreign strains which is in line with the investigation results in Hunan Province of China (Liu *et al*., 2011). As the 18SrRNA can be used to distinguish between genera or between species, it has been widely used in the molecular diagnosis of histomoniasis (Hafez *et al*., 2005; Huber *et al*., 2005; Grabensteiner and Hess, 2006; Liebhart *et al*., 2006; Hauck *et al*., 2010; Mostegl *et al*., 2010). The 5.8SrRNA can also be applied to distinguish between genuses or between species because its sequence identity was found to be about 89.7% between different isolates (Lollis *et al*., 2011).

Besides the *18SrRNA* gene, the 5.8SrRNA and ITS regions have also been used to analyze phylogenetic evolution and genovariation of *H. meleagridis* (Van der Heijden *et al*., 2006; Mantini *et al*., 2009; Liu *et al*., 2011; Lollis *et al*., 2011). Among them, the ITS, the non-coding region of *rRNA* genes is the most valuable. Based on the *ITS-1* sequence, Van der Heijden *et al*. (2006) divided *H. meleagridis* into three genotypes (I, II and III) using the C-Profiling Method. By analyzing the *5.8SrRNA* gene sequence and ITS flanking sequence, Hauck *et al*. (2010) showed the presence of four genotypes (A, B, C and D) of *H. meleagridis*. In the study, the phylogenetic tree based on the *18SrRNA* gene sequence showed the *H. meleagridis* isolates from East China were located at five different branches suggesting the possible presence of different genotypes. Considering the vast territory, a large number of poultry and serious prevalence of *H. meleagridis* in China, researchers believe that to obtain more geographical isolates is essential for systematic identification, classification and evaluation using molecular biological methods. To determine the genotypes and variation of *H. meleagridis* will also help the prevention and treatment of histomoniasis. In the future, researchers will analyze other related genes to determine the genotypes of *H. meleagridis* using the C-Profiling Method.

CONCLUSION

The partial *18SrRNA* gene sequence is highly identical among the *H. meleagridis* from different areas of East China and *18SrRNA* gene thus can be used as a target gene for molecular diagnosis. Moreover, its genetic diversity suggests the presence of different genotypes. Further genotyping researchs are required in the future studies.

ACKNOWLEDGEMENTS

This research was supported by the Natural Science Foundation of Jiangsu Province (Grant No. BK20131229), the Priority Academic Program Development of Jiangsu Higher Education Institutions, the New Century Talent Project of Yangzhou University (Grant No. 2012). The researchers thank Dr. Zhongbin Deng at University of Louisville and Dr. Huigang Shen at Kansas State University for their helps in the preparation of the manuscript.

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