

Prevalence of *Mycoplasma wenyonii* Infection on Seven Dairy Farms in Shanghai, China

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Abstract

Mycoplasma wenyonii (*M. Wenyonii*) was studied by PCR and blood smear staining in 197 cows. Clinical signs and hematological parameters of all tested cows were collected. Results confirmed that 31% populations were positive of *M. wenyonii* infection. Statistical analysis revealed that the cows of 2-4 years old were most susceptible. No clinical signs were found to be significantly associated with the infection. The infection also did not result in anemia based on hematological investigation that only showed elevation of WBC counts in infected cows. Sequence analysis of the partial 16S rRNA gene showed that three different representative strains were confirmed in the present study.

Keywords: cattle, hematological parameters, *Mycoplasma wenyonii*

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บทคัดย่อ

ความชุกของเชื้อ *Mycoplasma wenyonii* ในฟาร์มโคนมจำนวน 7 ฟาร์มในเขตเมืองเซี่ยงไฮ้ ประเทศจีน

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ทำการศึกษาการติดเชื้อ *Mycoplasma wenyonii* โดยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรสและการย้อมสีแผ่นเลือดบางในโคนมจำนวน 197 ตัวอย่าง และทำการเก็บรวบรวมอาการทางคลินิกและค่าทางโลหิตวิทยา ผลการศึกษาสามารถยืนยันการติดเชื้อ *Mycoplasma wenyonii* ในกลุ่มประชากรจำนวนร้อยละ 31 ผลการวิเคราะห์ทางสถิติ พบว่าโคนมที่มีอายุระหว่าง 2-4 ปี มีความไวต่อการติดเชื้อสูงสุด และไม่พบความสัมพันธ์ของอาการทางคลินิกกับการติดเชื้อ ซึ่งไม่ส่งผลต่อภาวะเลือดจาง แต่พบการเพิ่มขึ้นของเซลล์เม็ดเลือดขาวในกลุ่มโคที่ติดเชื้อ การวิเคราะห์หัตถ์พันธุกรรมบางส่วนของยีน 16SrRNA พบเชื้อ *Mycoplasma wenyonii* จำนวน 3 สายพันธุ์ ในการศึกษาครั้งนี้

คำสำคัญ: โคนม ค่าทางโลหิตวิทยา *Mycoplasma wenyonii*

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Introduction

Hemotropic *mycoplasma* (hemoplasma) is a widespread vertebrate pathogen that results in anemia, icterus, and fever in infected animals. Hemoplasma infection was recognized in many vertebrate hosts including humans (dos Santos et al., 2008). Commonly, such organisms are found individually or in chains on the surface of erythrocytes, but they do not penetrate. However, a recent study has confirmed that *Mycoplasma suis* can in fact invade erythrocytes, which may protect them from host immune responses and antimicrobial treatment (Groebel et al., 2009). Hemoplasma infection has gained increasing public health significance since its potential character of cross transmission from animals to humans was found (Yang et al., 2000; dos Santos et al., 2008; Yuan et al., 2009). However, the life cycle of hemoplasma has not hitherto been described. Blood-sucking insects (*Aedes aegypti*) are thought to play an important role in the transmission (Berkenkamp and Wescott, 1988; Prullage et al., 1993). Other reports demonstrated that it might be spread by close contact, vertical transmission and contaminated food (Messick, 2003). These routes of transmission need further investigation.

In cattle, *Mycoplasma wenyonii* (*M. wenyonii*) infection results in anemia, fever, lymphadenopathy, anorexia, weight loss and decreased milk production

(Smith et al., 1990). However, the majority of the infections remain subclinical (Montes et al., 1994). In China, *M. wenyonii* was firstly reported in 1983 (Zhang et al., 1983). Prevalence of *M. wenyonii* infection was confirmed in some studies in China. But the detection methods used in these studies were mainly conducted by blood smears, as a result the infection ratio varied significantly between different studies. Moreover, no study was performed to analyze the disease burden induced by *M. wenyonii* infection in China. The aim of the present study was to determine the prevalence of bovine hemoplasma infection and to evaluate its disease burden in Shanghai, China.

Materials and Methods

Sample collection: Seven farms located in the rural area of Shanghai were randomly selected for investigation. EDTA-anticoagulated blood samples were collected from all animals (n=29) showing obvious icterus and emaciation. The healthy cows (n=168) were selected from all cowhouses of these seven farms (6 animals were randomly selected in every cowhouse). Age, clinical signs and medical records were also obtained. The protocol for sample collection from the cattle was approved by the Shanghai Animal Management Committee.

Hematological detection: Parameters, including red blood cell count (RBC), hemoglobin level (Hb), hematocrit (Hct), mean corpuscular volume (MCV),

mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), platelet (PLT) and white blood cell count (WBC), were measured using an animal blood autoanalyzer (Drew Scientific, Oxford, UK) according to the manufacturer's protocol.

Blood staining: Blood staining was performed based on the previous study (Yuan et al. 2009). Blood films on microscopy slides were prepared and air dried soon after the blood collection. Wright-Giemsa mixture liquid (0.8 ml) covered the whole smear for 1 min, and then mixed with 2x vol PBS buffer (pH 7.8) with gentle blow for 5 min, followed by washing and drying. The stained smears were then examined under a compound light microscope. Positive result was defined if one infected erythrocyte was found in 200 observed RBC.

Transmission electron microscope: Positive samples confirmed by blood smear detection were selected for transmission electron microscope examination. Blood was washed with PBS buffer (pH 7.4) three times and diluted with 2x vol PBS buffer (pH 7.4). Blood cells were fixed with 2.5% glutaraldehyde for 1 hour. After being fixed, washed, post-fixed with 1% osmium tetroxide in the same buffer, samples were then embedded in epoxy resin. Thin sections were prepared, stained with lead acetate and examined by using a Hitachi 7100 transmission electron microscope, Japan.

PCR detection: DNA extraction was performed using a commercial Kit (QIAamp DNA Blood Mini Kit, QIAGEN, Hilden, Germany) according to the manufacturer's protocol. After sequence alignment of the 16S rRNA gene, a set of *M. wenyonii* specific primer was designed; Upstream 5'-gCgCCTTgATggTACTAATT-3' and Downstream 5'-gTCTTACTgATTgCCTCCACT-3'. The specificity of primers was initially evaluated by using Primer-BLAST software in Genbank. DNA materials extracted from laboratory archives included *Mycoplasma suis*, *Mycoplasma felis*, *Bartonella henselae*, *Streptococcus*, *Toxoplasma gondii*, and *Salmonella choleraesuis*. *Escherichia coli* was also selected to further determine the specificity of the primers. The 563-bp fragment was amplified with Pfu polymerase (34 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, with a 10-min extension). After electrophoresis, the PCR product was purified with a spin column and dissolved in double-distilled water. Extracted DNA was sequenced in both directions by using an automatic sequencer (Model 373A, Applied Biosystems Inc, Foster, California, USA). Since *Mycoplasma haemobos* has recently been identified as another virulent hemoplasma species in cattle, infection of *Mycoplasma haemobos* was also detected in the present study by using published primers (Tagawa et al., 2010).

Phylogenetic analysis: The 16S rRNA gene sequences of various hemotrophic *Mycoplasma* species were downloaded from public databases, and target fragments, together with the partial 16S rRNA gene sequence obtained in the present study, were aligned using Clustal X. *Mycoplasma penetrans* was chosen as the out group. A phylogenetic tree was generated by

means of the neighbor-joining method corrected for nucleotide substitutions by the use of the Kimura 2-parameter correction with the transition-to-transversion ratio set at 2. Data were resampled 1,000 times, and bootstrap analysis was used for statistical assessment of the resulting node.

Statistic analysis: Statistical analysis of the association between infection of *M. wenyonii* with age (1-10 years) and farms was performed by the use of χ^2 test. Association of infection with clinical signs (icterus and emaciation) and hematological parameters was analyzed by t test. A value of $p < 0.05$ was considered significant. The partial 16S rRNA sequences of three representative isolates obtained in the study were deposited with GenBank under accession numbers of HM048984-86.

Results

The age of the tested cows ranged from 1 to 10 years (< 1 year, 15; 1-2 years, 20; 2-3 years, 27; 3-4 years, 27; 4-5 years, 28; 5-6 years, 29; 6-7 years, 12; 7-8 years, 13; 8-9 years, 16; 9-10 years, 10). The results of blood smear and electron microscope showed *M. wenyonii* attached onto erythrocytes (Fig 1). The attachment of *M. wenyonii* led to the depression of infected erythrocytes, while uninfected cells showed a normal shape (indicated by a white arrow). Most of the *M. wenyonii* were approximately 1 μ m in diameter. No organisms were detected penetrating into erythrocytes. BLAST analysis and PCR assay confirmed the good specificity of the primers in the present study. Sixty-two cows (31.5%) were found to be infected with *M. wenyonii* by PCR. Only 48 positive samples (24.4%) were confirmed by blood staining detection. These false negative samples were re-detected by compound microscope examination. Seven additional samples were confirmed to be positive when the number of investigated erythrocytes increased to five hundred. Therefore, the final positive ratio was determined based on the results of PCR assay because of the low sensitivity of blood smear in detection of some mild infection samples. The infection rate of the seven farms was 32.3% (95% CI of 14.2-50.3%), 45% (95% CI of 20.7-69.3%), 36.4% (95% CI of 14.0-58.7%), 30.8% (95% CI of 11.1-50.4%), 40% (95% CI of 18.8-61.2%), 21.7% (95% CI of 2.7-40.8%) and 24% (95% CI of 11.2-36.8%), respectively. But no statistically significant difference was found ($\chi^2 = 3.4974$, $p > 0.05$). *M. wenyonii* was frequently found in cows of 2-4 years of age ($\chi^2 = 25.5366$, $p < 0.05$). Based on the visible clinical signs, 10 and 19 cows (14.7% of total subjects) showed icterus and emaciation, respectively. Among these, 4 and 7 animals (37.9%) were confirmed to be infected with *M. wenyonii*. Unexpectedly, the association of these signs with *M. wenyonii* infection was not supported by the T test ($z = 0.5960$ in icterus animals, $z = 0.5302$ in emaciated animals, $p > 0.05$). For a further evaluation of the pathogenicity of *M. wenyonii*, hematological parameters of all tested animals were collected. No anemia associated indexes (RBC, Hb, MCV) showed statistically significant differences between *M. wenyonii* positive and negative animals. However, WBC value in infected cows was

significantly elevated (Table 1). Sequence alignment confirmed three different genotypes (termed as MW1-3: A-G substitution at 160nt of MW 2 and 3; A-G substitution at 267nt of MW3; G-A substitution at 273nt of MW 2 and 3) in 62 positive samples. Phylogenetic analyses revealed that these three sequences branched into the clade of *M. wenyonii* and were closely related with the isolates from China (Fig 2).

Table 1 Hematologic examination of *M. wenyonii*-infected cattle

Parameters (Mean±SD)	<i>M. wenyonii</i> infection (n=62)	Negative (n=135)	Reference Values
WBC ($\times 10^3/\mu\text{l}$)	54.3±24.9*	24.9±17.1	5-13
RBC ($\times 10^6/\mu\text{l}$)	6.3±0.68	6.5±0.77	5-10.1
Hb (g/dl)	11.6±1.2	11.2±0.8	9-14
MCV (fl)	43.9±4.1	45.9±3.9	38-53
Hct (%)	36.8±6.1	37.1±9.2	28-46

*Indicate significant difference $p < 0.05$

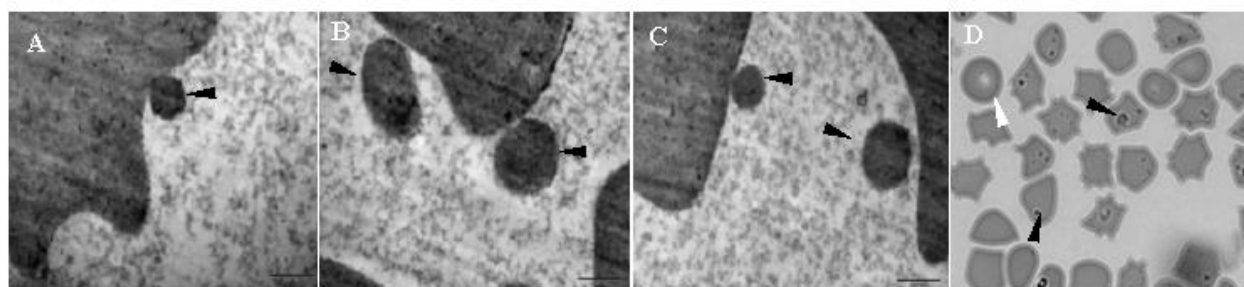


Figure 1 Morphological observation of blood samples via transmission electron microscopy (A-C) and light microscopy (D). Panel A-C: *M. wenyonii* (arrow) organisms attached to the surface of erythrocytes. Panel D: attached erythrocytes are deformed (black arrows) while uninfected ones appear normal (white arrows) shape. Bar indicates 1 μm .

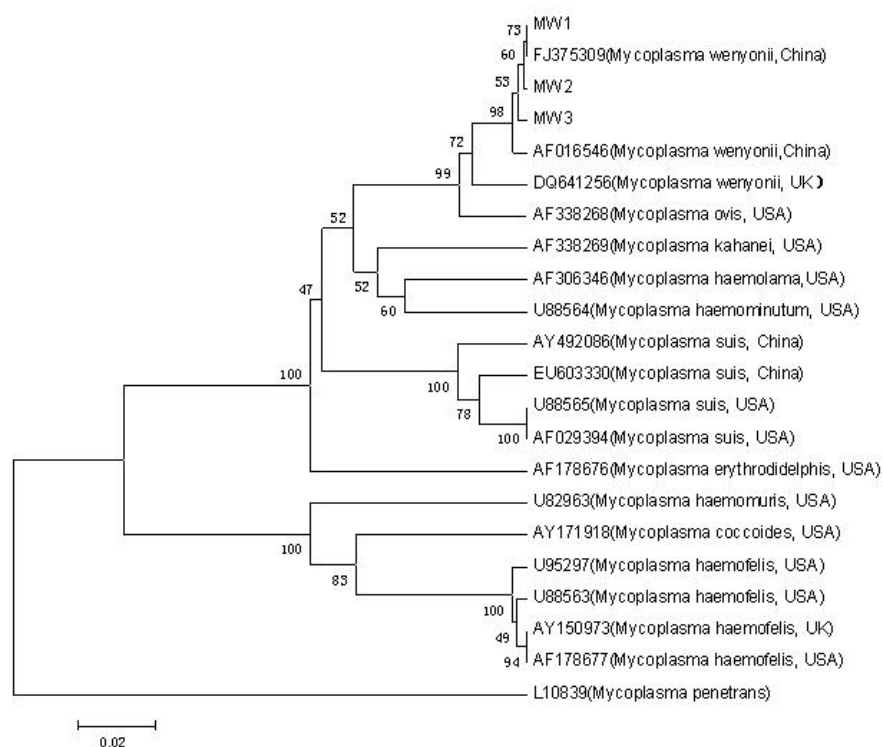


Figure 2 Phylogenetic tree generated by use of partial 16S rRNA gene sequences. Phylogenetic analysis indicated the relationship of three *M. wenyonii* strains identified in the present study with other hemoplasma species. *Mycoplasma penetrans* was selected as the out (reference) group. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is indicated at the nodes of the tree. The evolutionary distances were calculated by use of the neighbor-joining method and were corrected for nucleotide substitutions by use of the Kimura 2-parameter correction. Distance indicated by the scale bar is equivalent to base substitution/10 nucleotides.

Discussion

M. wenyonii was first described in a splenectomized calf in 1934 (Adler and Ellenbogen, 1934). Commonly, affected cattle may show signs of depression, fever, and anemia. This pathogen was also considered to be the potential cause of hindlimb edema and udder swelling (Smith et al., 1990; McAuliffe et al., 2006). However, some reports demonstrated that *M. wenyonii* infection will not produce obvious signs of anemia and icterus (Tagawa et al., 2010). In all likelihood, the occurrence of clinical signs is associated with the pathogen load. To date, three morphologically and immunologically different hemoplasma species have been identified in cattle: *E. wenyonii* (Adler and Ellenbogen, 1934), *E. teganodes* (Hoyte, 1962), and *E. tuomii* (Uilenberg, 2009). Recently, cattle infected with a novel virulent hemoplasma species, named as *Mycoplasma haemobos*, were reported in Japan and northern Germany (Hoelzlea et al., 2010; Tagawa et al., 2010). In the present study, no infection with *Mycoplasma haemobos* was determined using published primers (Tagawa et al., 2010). Most of the *M. wenyonii* infections in the present study also did not show obvious signs. However, most of the ill cows had received extensive antibiotic treatment (doxycycline) before the sample collection, which might lead to the underestimation of *M. wenyonii* prevalent in ill cows. It also hampered the evaluation of the disease burden of *M. wenyonii* infection in the present study. These studies showed that cows aged of 2-4 years were most vulnerable to *M. wenyonii* infection, but the reason for susceptibility in young animals was unclear. Here, WBC level in *M. wenyonii* infected animals showed significant elevation. Similar observations were recorded in previous studies (Welles et al., 1995; Tagawa et al., 2010). However, *M. Haemobos* infection, a more pathogenic hemoplasma species in cattle, would not induce the increasing of WBC counts (Tagawa et al., 2010). Certainly, elevation of WBCs might also induce by the coinfection other pathogens. Therefore, we can not give any implication of WBC elevation with *M. wenyonii* infection at this stage. In the present study, *M. wenyonii* infection is prevalent in dairy farms in China. Most of the infections remain subclinical. Whether the infection of *M. wenyonii* is detrimental to the host's RBC immunity (complement receptor activation and immune complex formation) which results in the possibility of coinfection with other pathogens, it is suggestive to determine the clinical significance of this subclinical pathogen.

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