Prevalence of *Mycoplasma suis* (*Eperythrozoon suis*) infection in swine and swine-farm workers in Shanghai, China

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Objective—To determine the prevalence of *Mycoplasma suis* infection in swine, swine-farm workers, and swine veterinarians in Shanghai, China.

Sample Population—172 swine and 65 workers and veterinarians from 19 commercial swine farms.

Procedures—Blood samples were collected from all study subjects. Blood samples were examined for the presence of *M suis* by means of compound and scanning electron microscopy. A species-specific PCR assay was developed for detection of *M suis* DNA extracted from blood samples. Relationships between infection status of swine and sex, age, geographic location, and clinical signs of disease were evaluated by use of a χ^2 test. The phylogenetic relationship between partial 16S ribosomal RNA (rRNA) sequences from swine and human isolates of *M suis* was determined.

Results—86% (148/172) of swine and 49% (32/65) of humans had positive PCR assay results for *M suis* infection. Swine infection status was not associated with any variable, with the exception of pyrexia and subcutaneous bleeding. The partial 16S rRNA sequences from human and swine isolates of *M suis* were 98% homologous and in the same phylogenetic cluster as a previously identified swine isolate of *M suis*.

Conclusions and Clinical Relevance—A large proportion of swine and humans in close contact with those swine were infected with *M suis* in Shanghai, China. The close phylogenetic relationship between swine and human isolates of *M suis* suggested possible interspecies transmission; however, additional research is required to better assess that possibility. (*Am J Vet Res* 2009;70:890–894)

Microorganisms of the genus *Eperythrozoon*, formerly considered to be *Rickettsiae*, are uncultivatable, obligate parasites that live on the surface of erythrocytes in vertebrate hosts. Results of a molecular assay based on 16S rRNA gene sequence alignment indicate that *Eperythrozoon* should be reclassified as *Mycoplasma*, specifically hemotrophic *Mycoplasma*.[1,2] However, the suggested reclassification has not yet been well accepted.[3]

Infected animals generally become latent nonclinical carriers of hemotrophic *Mycoplasma* organisms.[4] However, malnutrition or other diseases can result in substantial bacteremia and clinical manifestations of *Mycoplasma* infection.[5,6] Deformation of affected erythrocytes and exposure of erythrocytes to autoantigen results in the clearance of these cells through the spleen.[7] Moreover, attachment of hemotrophic *Mycoplasma* organisms to erythrocytes can increase cell permeability and fragility, causing the cells to rupture.[8]

The acute phase of *Mycoplasma suis* infection in swine reportedly involves febrile anemia and icterus and is associated with low morbidity but high mortality rates.[4] Chronic *M suis* infection in swine results in low reproductive efficiency, growth retardation, and an increased incidence of concurrent disease, compared with the incidence in uninfected swine.[4,9] The first case of human infection with *M suis* was reported in 1986,[10] but the source of human infection with this organism has not yet been determined. Infected humans can develop mild pyrexia, hemolytic anemia, and icterus. Moreover, congenital infection via transplacental transmission has been reported.[11]

Abbreviation

rRNA	Ribosomal RNA

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China is one of the highest producers and consumers of pork in the world. Nonetheless, *M suis* is not recognized as a common pathogen in swine in this country. The purpose of the study reported here was to determine the prevalence of *M suis* infection in swine, swine-farm workers, and swine veterinarians in Shanghai, China.

Materials and Methods

Participants and sample collection—Blood samples from swine and feeders and veterinarians who had close contact with the swine were collected from commercial pig farms in rural Shanghai. Information was collected on age, sex, and geographic region for each swine sample. For humans and swine, clinical manifestations of disease at the time of sample collection were recorded, and medical records were also obtained. The protocol for sample collection from swine was approved by the Shanghai Animal Management Committee. Blood samples were obtained from human participants after acquiring their consent.

General procedures—Blood samples were evaluated to determine the infection status of swine and humans by means of 3 techniques: microscopy, PCR assay, and scanning electron microscopy. A blood sample from a pig known to be infected with *M suis* was maintained in the laboratory as a positive control sample. This sample had been obtained from an experimentally infected specific-pathogen–free pig, and infection was confirmed via microscopy and PCR assay. For a negative control sample, a blood sample from an uninfected specific-pathogen–free pig was used.

Compound microscopy—Blood films on microscopy slides were prepared and allowed to dry. Wright-Giemsa stain (0.8 mL) was applied to cover each blood film for 1 minute, then a 2× volume of PBS solution (pH, 7.8) was applied and mixed with the stain by means of gentle blowing for 5 minutes. Slides were subsequently rinsed with water and dried, then examined with a compound microscope.

PCR assay—A standard phenol-chloroform–isoamyl alcohol protocol[12] was used to extract DNA from each blood sample (500 µL). Attempts to amplify the whole 16S rRNA gene by use of universal primers failed. Therefore, a 16S rRNA gene fragment was amplified by use of the following primers: upper primer, 5′–CACGCCGTAACGATGGGTAT–3′ and lower primer, 5′–CAGCCCAAGGCATAAGGGG–3′.

The DNA materials extracted from laboratory archived and confirmed *Escherichia coli*, group E *Streptococcus* (serotype II), *Toxoplasma gondii*, and *Salmonella choleraesuis*, and samples of blood from healthy humans and pigs were selected as control samples to evaluate the specificity of the PCR assay. Rigorous precautions were taken to avoid bacterial contamination. The 396-bp fragments were amplified with *Pfu* polymerase (36 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, with a 10-minute extension). After electrophoresis, the PCR product was purified with a spin column[a] and dissolved with double-distilled water. Extracted DNA was sequenced in both directions by use of an automatic sequencer.[b]

Scanning electron microscopy—The procedure for examination of blood samples via scanning electron microscopy is described elsewhere.[2] Three blood samples identified as positive for *M suis* DNA via the PCR assay and 2 blood samples identified as negative were randomly selected from among all swine blood samples. Fresh samples of anticoagulated blood were washed with PBS solution (pH, 7.4) 3 times and diluted with a 2× volume of PBS solution (pH, 7.4). A drop from each sample was then placed on a polylysine-coated slide that had been moistened with distilled water, and the slide was placed in a covered Petri dish for 30 minutes. The material was then fixed on the slide by application of 2.5% (vol/vol) glutaraldehyde (pH, 6.8) for 30 minutes. Afterward, 2% osmium tetroxide (wt/vol) in 0.1M HEPES buffer (pH, 6.8) was applied for 30 minutes. The slide was subsequently rinsed again with HEPES buffer and dehydrated by application of ethanol at an increasing concentration (70% to 95%). At the critical point, the slide was dried by use of liquid CO_2 in a critical point drier, sputter coated with gold-palladium (60:40), and examined with a field-emission scanning electron microscope.[c]

Phylogenetic analysis—The 16S rRNA gene sequences of various hemotrophic *Mycoplasma* spp were downloaded from the database of the National Center for Biotechnology Information, and their corresponding fragments together with the partial 16S rRNA gene sequence obtained in the present study were aligned by use of a multiple-sequence alignment program. *Mycoplasma penetrans* was chosen as the out (reference) group. A phylogenetic tree was generated by means of the neighbor-joining method, which was corrected for nucleotide substitutions by 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.[2]

Statistical analysis—Statistical analysis of the association between seropositivity for *M suis* infection in swine and sex, age, geographic region (ChongMin, BaoShan, JinShan, PuDong, JiaDing, SonJiang, FenXian, QingPu, NanHui, or MinHang), and clinical signs of infection was performed by use of a χ^2 test.[d] A value of $P < 0.05$ was considered significant.

Nucleotide sequence accession number—The partial 16S rRNA sequences of pig and human genetic isolates obtained in the study were deposited with GenBank under accession Nos. EU371555 and EU371556, respectively.

Results

Blood samples were obtained from 172 swine and 65 swine feeders and veterinarians from 19 pig farms distributed throughout rural Shanghai. Seventy-nine (46%) of the swine were male, and 93 (54%) were female. The distribution of swine by age group was as follows: < 30 days, 18; 31 to 60 days, 18; 61 to 100 days, 63; 101 to 150 days, 28; 151 to 190 days, 19; 191 days to 1 year, 17; and

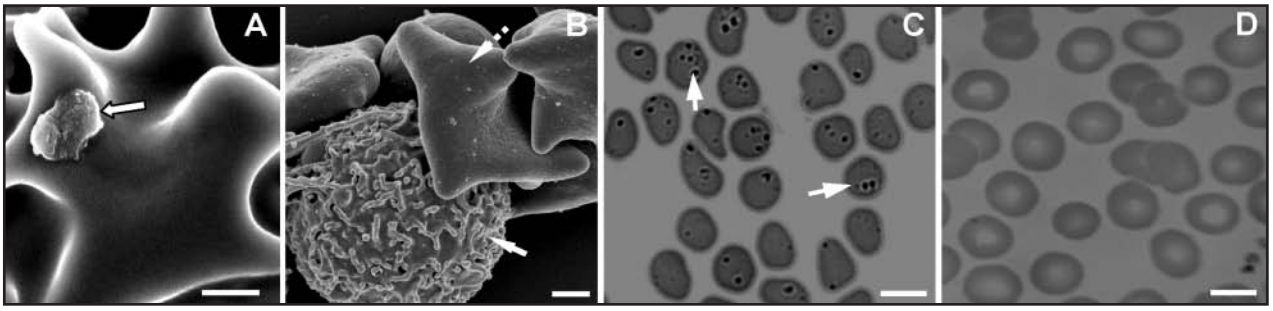

Figure 1—Photomicrographs of blood samples from swine evaluated for evidence of *Mycoplasma suis* infection via scanning electron microscopy (A and B) and compound microscopy (C and D). In panel A, an *M suis* (arrow) organism is attached to the surface of an erythrocyte in a blood sample from an infected pig. In panel B, erythrocytes (dashed arrow) surround a lymphocyte (solid arrow) in a blood sample from an uninfected pig. In panel C, several *M suis* organisms (arrows) are evident on the surface of erythrocytes in a blood film from an infected pig. In panel D, no *M suis* organisms are evident in a blood film from an uninfected pig. A and B—Bar = 1 µm. C and D—Wright-Giemsa stain; bar = 5 µm.

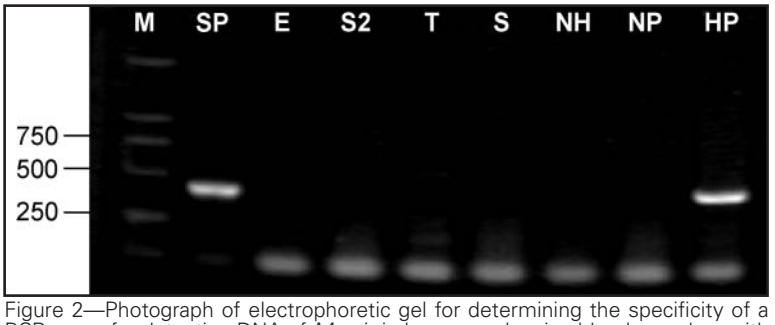

Figure 2—Photograph of electrophoretic gel for determining the specificity of a PCR assay for detecting DNA of *M suis* in human and swine blood samples, with DNA from other microorganisms used for reference. Values to the left of the gel represent molecular weight (kDa) of bands. M = Molecular weight marker. SP = *M suis*–positive swine sample. E = *Escherichia coli*. S2 = Group E *Streptococcus* (serotype II). T = *Toxoplasma gondii*. S = *Salmonella choleraesuis*. NH = *M suis*–negative human sample. NP = *M suis*–negative swine sample. HP = *M suis*–positive human sample.

> 1 year, 9. The distribution of swine by geographic regions was as follows: ChongMin, 17; BaoShan, 16; JinShan, 21; PuDong, 11; JiaDing, 16; SongJiang, 16; FenXian, 23; QingPu, 17; NanHui, 20; and MinHang, 15.

Compound and scanning electron microscopy revealed *M suis* attached to erythrocytes (**Figure 1**) in 32 of 65 (49%) human blood samples and 132 of 172 (77%) swine blood samples. When *M suis* was detected, the number of organisms was typically small. Most of the organisms were round, measured approximately 1 µm in diameter, and were arranged singly, in pairs, and in clusters on erythrocytes. Scanning electron microscopy revealed that *M suis* had attached to the surface of porcine erythrocytes but had not penetrated them.

The PCR assay revealed that 32 (49%) blood samples from humans and 148 (86%) blood samples from swine contained *M suis* DNA. In total, 15 swine blood samples in which *M suis* organisms were not detected via the compound microscopic method were identified as containing *M suis* DNA via the PCR assay. On the other hand, there was no microscopic evidence of *M suis* in any of the human and swine blood samples in which the PCR assay failed to detect *M suis* DNA.

Results of evaluation of the specificity of the PCR assay indicated that the 16S rRNA gene fragment could be amplified only in positive control samples but not in the negative control samples (*E coli*, group E *Streptococcus* [serotype II], *T gondii*, and *S choleraesuis*; **Figure 2**). Sequence alignment analysis confirmed that the microorganisms for which DNA was recovered from the blood samples were hemotrophic *Mycoplasma* spp.

Statistical analysis revealed there was no association between *M suis* infection status in swine and sex ($\chi^2 = 1.73$; $P > 0.05$), age ($\chi^2 = 2.80$; $P > 0.05$), or geographic location ($\chi^2 = 1.26$; $P > 0.05$). During the study, 3 of 65 (5%) humans with icterus and 4 (6%) with a history of anemia were identified. Among these, the blood sample from only 1 person with icterus contained *M suis* DNA. The PCR assay did not detect *M suis* DNA in the blood samples of 7 of 73 (10%) swine with mild pyrexia and 6 of 60 (10%) swine with subcutaneous bleeding, but it did detect *M suis* DNA in the blood samples of 66 (90%) other swine with mild pyrexia and 54 (90%) other swine with subcutaneous bleeding. Statistical analysis confirmed these 2 clinical signs of disease were associated with *M suis* infection in swine ($\chi^2 = 7.04$ and $\chi^2 = 6.63$, respectively; $P < 0.01$). Moreover, 46 of the 54 (85%) infected swine with subcutaneous bleeding were also mildly pyrexic. There was no association between clinical signs of disease in swine and geographic location. Although icterus was evident in the visible mucosa of some swine, this factor was not associated with *M suis* infection status. There were no associations between clinical signs of disease or medical history and infection status in humans.

Phylogenetic analysis yielded an evolutionary tree that indicated the relationship among the 16S rRNA of human and swine isolates in this study with the 16S rRNA of other hemotrophic *Mycoplasma* spp. The 16S rRNA genes of human and swine isolates were highly similar (98% homology) and in the same phylogenetic cluster as a previously identified swine isolate (**Figure 3**).

Discussion

Many species of vertebrates are susceptible to hemotrophic *Mycoplasma* infections. In Europe and the United Kingdom, hemotrophic *Mycoplasma* DNA was

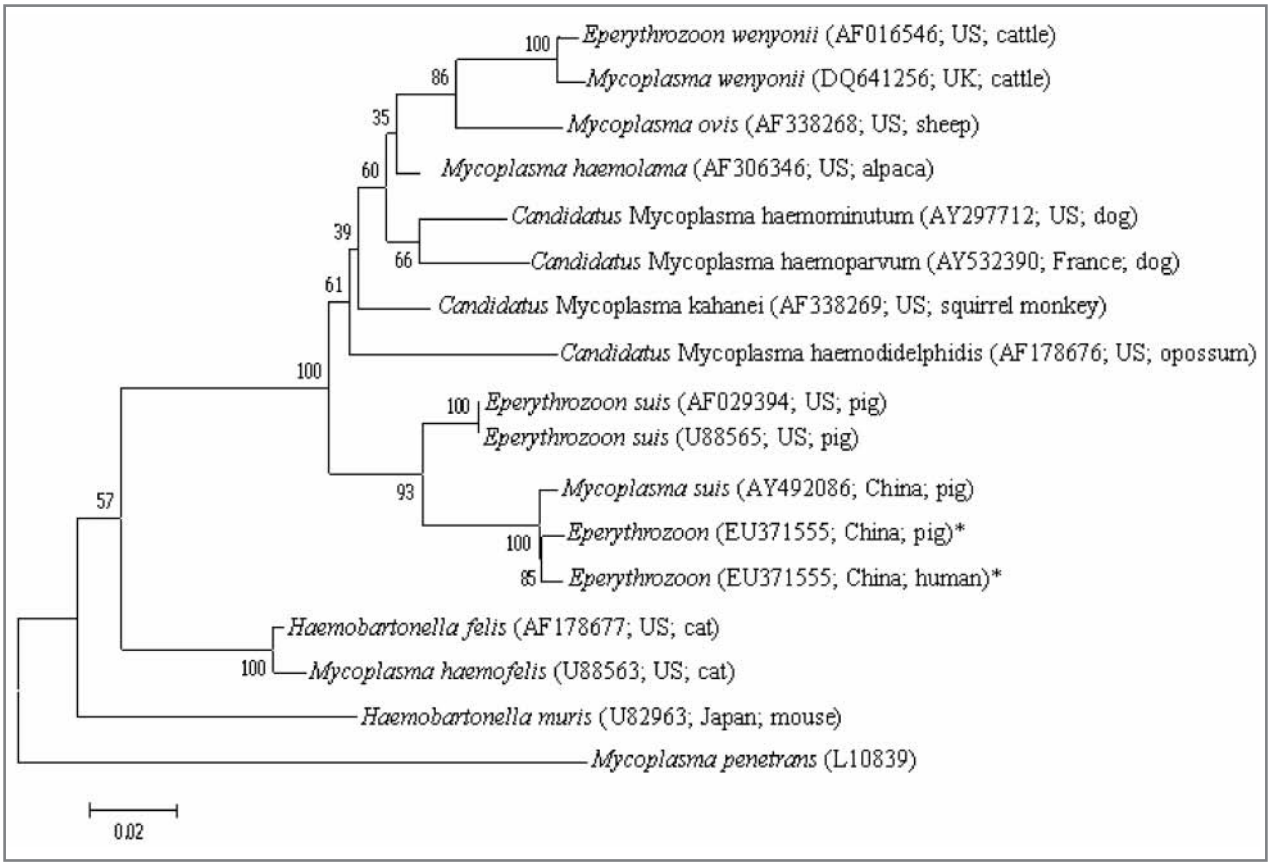

Figure 3—Phylogenetic tree generated by use of partial 16S rRNA gene sequences, indicating the relationship of 2 *Eperythrozoon* spp identified in swine and humans in the present study (asterisk) with other *Eperythrozoon* spp and *Haemobartonella* spp. The accession number and origin of each species are provided in parentheses when available. Nomenclature used is that which was assigned when the species sequences were originally identified and reported. *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 (1,000 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. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Distance indicated by the scale bar is equivalent to 1 base substitution/10 nucleotides. UK = United Kingdom. US = United States.

identified via PCR assay in blood samples of between 10% and 23% of dogs and cats tested between 2003 and 2006.[13–15] A large-scale study[11] of the prevalence of hemotrophic *Mycoplasma* in humans in Inner Mongolia, China, revealed that 35.3% of the population was infected, and infection status appeared to be associated with occupation and season. People that had close contact with livestock were significantly more likely to be infected than those that did not have close contact. In 2007, 4 swine farms in Brazil were evaluated, and 33.1% of swine in that study[16] were identified as infected with *M suis* via Southern blot and PCR techniques. The PCR assay used in the study reported here revealed a higher prevalence of infection in humans with close contact with swine (49%) and the swine with which they had contact (86%), compared with the prevalences reported for other studies.[11,13–16] Poor sanitary conditions in the commercial swine farms in the present study provided a good environment for breeding of mosquitoes (*Aedes* spp), which are believed to play an important role in transmission of *M suis* among swine.[17] Other situations conducive to the transmission of *M suis* among swine, such as ingestion of food contaminated with blood from infected swine or reuse of syringes by farm workers between swine, were common in these farms as well. These factors might have contributed to the high prevalence of infection in the swine in our study.

Infection with *M suis* is typically subclinical in humans and swine, and symptoms or clinical signs can vary among those infected. In the study reported here, data on clinical manifestations of disease and medical histories of all tested humans were collected, but no association of these data with *M suis* infection was detected (data not shown). Therefore, it was concluded that the low numbers of *M suis* in human blood samples and, hence, the low degree of infection in humans, failed to result in symptoms. The lack of symptoms could also have been attributable to a loss in the degree of pathogenicity after the pathogen was transmitted from swine to humans, assuming swine were the source of human infection. Common clinical signs in *M suis*–infected swine were mild pyrexia and subcutaneous bleeding. We speculated that precipitation and stacking of erythrocytes in blood of *M suis*–infected swine would lead to formation of immune complexes that would become trapped in capillaries, reducing blood flow, causing injury to those vessels, and leading to subcutaneous bleeding. However, whether the subcutaneous bleeding

was actually attributable to infection with *M suis* would require additional research.

To the authors' knowledge, there is no molecular evidence to suggest that *M suis* can be transmitted from swine to humans. In the study reported here, results of phylogenetic analysis revealed a high degree of homology between the 16S rRNA from humans and swine and among that 16S rRNA and the 16S rRNA isolated from a different strain of *M suis*. The similarity of the human and swine strains suggested that transmission of *M suis* to humans may take place. To investigate this possibility further, it would be beneficial to determine whether the hemotrophic *Mycoplasma* spp from humans from the same geographic regions who had no contact with swine differ from the species detected in the present study.

a. QIAqucik Gel Extraction kit, QIAGEN, Valencia, Calif.
b. Model 373A, Applied Biosystems Inc, Foster City, Calif.
c. SIRION 200 field-emission scanning electron microscope, FEI, Oxford, England.
d. Clustalx, University College Dublin, Dublin, Ireland. Available at: www.clustal.org. Accessed January 3, 2008.

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