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Haemotrophic *Mycoplasma* Infection in Horses

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Abstract
Haemotrophic mycoplasmas (HM) are parasites on the surface of red blood cells and known to infect a wide range of animals. However, there are no previous evidences of HM infections in horses. In this study HM were detected for the first time in the blood of two horses suffering from poor performance, apathy, weight loss, and anaemia. Using a HM specific PCR assay and subsequent sequencing the infective agents isolated from the blood of said horses were confirmed as closely related to the HM species *Mycoplasma haemofelis* and ‘*Candidatus Mycoplasma haemobos*’.

Keywords
Haemotrophic *Mycoplasma*, horse, anaemia, 16S rDNA PCR, phylogenetic classification

1. Introduction
Haemotrophic mycoplasmas (HM; formerly classified as *Eperythrozoon* and *Haemobartonella*) are uncultivable bacteria, which parasitize erythrocytes of a variety of mammals, such as pigs, cattle, cats and dogs (Hoelzle, 2008; Groebel et al., 2009).

Significance of HM as pathogens of several animal species was previously reported. Animal infections with HM are clinically marked either by an overt live-threatening haemolytic anaemia or a subtle chronic anaemia, by illthrift, infertility, and immune suppression (Messick et al., 2004; Hoelzle, 2008).

To date almost nothing is known about HM infections in horses. One indication of a possible incidence of equine HM infections is provided by a 30-year-old report on a case of equine “haemobartonellosis” in Nigeria. Affected horses exhibited clinical signs including fever,
apathy, lymphadenitis, circulatory disorders, and pale mucosa. Blood smears from these animals revealed bacteria of approx. 0.3 µm on the surface of erythrocytes (Gretillat, 1978). In this report we provide the first molecular proof that HM infections do occur in horses.

2. Materials and methods

2.1. Animals and samples

Two horses (Hanoverian mares aged 11 and 18 years) from Northern Germany, Luneburg Heath, were presented for clinical examination. EDTA-anticoagulated blood from these horses was subjected to haematological and bacteriological investigation. Blood smears were stained with Giemsa and acridine orange and examined microscopically.

2.2. Molecular analysis: PCR and sequencing

Genomic DNA was extracted as described elsewhere (Hoelzle et al., 2003). To screen specimens for HM a real time SYBR Green PCR assay being able to detect the entire group of known HM was performed according to Willi et al. (2009). For sequencing purposes PCR amplification of the 16S rDNA was performed using the HotStarTaq Polymerase (Qiagen, Hombrechtikon, Switzerland) and oligonucleotides targeting 16S rDNA regions specific for HM (16S_HAEMOforw: GGCCCATATTCCT(AG)CGGGAAG; 16S_HAEMOrev 5AC(AG)GGATTACTAGTGATTCCA; Hoelzle et al., 2010). Sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany). The 16S rDNA sequences obtained were compared with each other and to GenBank entries using the BLAST tool provided by NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and the FASTA algorithm (Biocomputing service, University Zurich, www.bio.unizh.ch/).

2.3. Phylogenetic analyses

To classify the sequences derived from equine blood phylogenetic analyses were carried out applying the ARB software package (Ludwig et al., 2004). For tree calculation the sequences
of the two novel equine isolates as well as sequences of selected *Mycoplasma* species and *Bacillus subtilis* were integrated into an ARB database and aligned using the ClustalW tool. Minimum-similarity filters were calculated retaining only positions conserved in at least 0 %, 25 % or 50 % of the selected sequences. Phylogenetic analyses were performed applying each filter in combination with each of the following treeing methods: distance matrix methods (Phylip NEIGHBOR), maximum parsimony (Phylip DNAPARS), and maximum likelihood (RAxML). In case of maximum parsimony and likelihood analyses trees were re-sampled 1000 times by bootstrapping.

2.4. Nucleotide sequence accession numbers

The 16S rDNA sequences obtained from both horses were deposited at GenBank database with the following accession numbers: FN421445 and FN421443.

3. Results

3.1. Clinical, haematological, bacteriological and microscopic findings

The horses were reported by their owners to show unthriftiness, loss of weight and condition, segregation of flock, and tucked up belly. Clinical examination revealed horses suffering from apathy and rough hair. All other physical examination findings e.g. temperature, pulse and respiratory rate were within normal limits. Haematological analysis revealed a decreased haematocrit of 28 % and 30 % (normal range 31-45 %). Other haematological parameters were within normal ranges. Bacteriological cultures yielded no results. In acridine orange stained blood smears distinct roundish particles of approximately 0.4 µm in diameter were observed on the surface of erythrocytes rendering strong evidence of an infection with HM (Figure 1).

3.2. PCR results and sequence analysis
Both equine DNA samples reacted positive in the universal HM SYBR Green PCR assay (Willi et al., 2009). By using conventional PCR targeting 16S rDNA, fragments of approx. 900 bp were amplified from the blood of both horses. Both fragments were sequenced. The sequences (isolate designations 30/7 and 32/3; GenBank accession numbers FN421445 and FN421443) showed homologies of 97.8 % with each other. Furthermore, the isolates demonstrated the highest homology to Candidatus Mycoplasma haemobos (98.1 % and 98.3 %; Acc. no. EF460765), and to Mycoplasma haemofelis (94.0 % and 94.4 %; Acc. no. U95297).

3.3. Phylogenetic analyses

Construction of a phylogenetic tree confirmed the relationship to Candidatus M. haemobos and M. haemofelis. All trees calculated showed stable topologies among each other and were in good agreement with previously published phylogenies of HM (e.g. Neimark et al., 2001; Tagawa et al., 2008). A representative tree is shown in Figure 2. The novel equine HM isolates clustered within the “haemofelis”-group, which includes amongst others the well established species M. haemofelis, M. haemocanis (formerly known as Haemobartonella felis and H. canis) and ‘Candidatus M. haemobos’.

4. Discussion

This study provides the first molecular proof of HM infections in horses using a specific SYBR Green PCR assay and 16S rDNA sequencing. Indications of HM infections in horses based on microscopical findings were reported in 1978 in Nigeria (Gretillat, 1978). By using molecular biological methods the novel equine HM isolates were demonstrated to be closely related to Candidatus M. haemobos. Our phylogenetic analyses classified the new equine HM isolates within the so-called “haemofelis”-cluster (Peters et al., 2008), which comprises the representatives of HM formerly classified as Haemobartonella. Thus, the novel HM isolates...
from the horses clearly belong to the cluster comprising those HM formerly classified within
the genus *Haemobartonella*. It is noteworthy that already in the first description of equine
“haemobartonellosis” in Nigeria in 1978, Gretillat classified the bacteria on the surface of the
equine erythrocytes by means of morphological criteria as *Haemobartonella*. It is of particular
interest to note that clinical signs observed in the Nigerian horses affected by
*Haemobartonella*-like bacteria closely match with the clinical signs reported in the German
horses investigated here. This argues for a pathogenic role of HM in horses. The two horses
presented here were affected by a syndrome characterised by unspecific signs including
anaemia. No evidence for infections with other anaemia-associated agents e.g. *Theileria equi*
or *Babesia caballi* was found in Giemsa stained blood smears (data not shown). Other
bacteria than haemotrophic mycoplasmas could be excluded as aetiological cause since PCRs
targeting universal 16S rDNA regions revealed no other results. Infections with the equine
anaemia virus could be excluded, too, due to clinical and laboratory findings. In our case the
horses were not specifically treated since an effective anti-mycoplasmal therapy using
tetracycline is discussed controversially in horses because of concerns over potentially fatal
adverse gastrointestinal effects (Dowling and Russel, 2000). The horses merely received a
diet rich in vitamins and minerals and recovered completely 3-6 months later. In future, the
etiologic and pathogenic significance of HM infections in horses needs to be elucidated as the
symptomatology is rather unspecific.

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References


Figure Captions

Figure 1
Blood smear of affected horse stained with acridine orange. Bacterial structures similar to those known from infections with haemotrophic mycoplasmas in other animal species were detected on the surface of erythrocytes (1000x magnification).

Figure 2
Comparative sequence analysis using 16S rDNA sequences showing the position of the two equine haemotrophic isolates among the haemotrophic *Mycoplasma* (HM) group. They clearly belong to the so-called “haemofelis”-cluster (Peters et al., 2008), which comprises HM representatives formerly classified as *Haemobartonella*. All trees calculated showed stable topologies. The tree shown was produced applying the maximum likelihood RAxML method (25 % SAI, ARB software package; Ludwig et al., 2004). For reconstruction of trees sequences of selected *Mycoplasma* species and *Bacillus subtilis* were included (GenBank acc. no. are given behind the strain designation). The numbers at the nodes indicate bootstrap values in percent (1000 bootstraps). The bar represents the estimated evolutionary distance.