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Short communication

Molecular investigation of transplacental and vector-borne transmission of bovine haemoplasmas

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ABSTRACT

The present study was carried out in a herd with concurrent infections of *Mycoplasma wenyonii* and 'Candidatus *M. haemobos*', to investigate if transplacental and/or vector-borne transmission is possible for one or both bovine haemoplasma species. For this purpose blood samples were collected from 38 mother animals and their newborn calves; as well as from 17 uniseminated cows twice three months apart. In addition, 311 mosquitoes and blood-sucking flies (Diptera: Culicidae, Tabanidae, Muscidae) were caught near the animals. DNA was extracted from all samples, followed by real-time PCR analysis. In 10.5% of neonate calves, that were born to cows harbouring both haemoplasmas, *M. wenyonii* and/or 'Candidatus *M. haemobos*' positivity was detected. Copy numbers in positive samples from cows and their calves indicated that – in comparison with *M. wenyonii* – 'Candidatus *M. haemobos*'-bacteraemia had usually lower levels. In samples of uniseminated cows the rate of infection with the latter species decreased. These findings may explain why *M. wenyonii* was significantly more frequently detected in blood-sucking flies, than 'Candidatus *M. haemobos*'. In conclusion, molecular evidence is provided for the first time on the transplacental transmission of bovine haemoplasmas. Regarding their spread by blood-sucking arthropods, new potential vectors were identified, i.e. the horn fly (*Haematobia irritans*), the stable fly (*Stomoxys calcitrans*) and two species of horse flies (*Tabanus bovinus*, *T. bromius*).

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1. Introduction

Haemotropic mycoplasmas or haemoplasmas (formerly *Haemobartonella* and *Eperythrozoon* spp.) are Gram-negative, wall-less epierythrocytic bacteria (Neimark et al., 2001). They can cause acute or chronic disease, most notably haematological disorders in various mammals (Messick, 2004). In cattle formerly only one species, *Mycoplasma (E.) wenyonii* was regarded as valid, since data in the literature on the existence of other species – including *H. bovis* – are controversial (Hoelzle et al., 2010). However, based on its 16S rRNA genotype, a novel

microorganism, 'Candidatus *M. haemobos*' was also discovered (Hofmann-Lehmann et al., 2004; Tagawa et al., 2008; Hoelzle et al., 2011). These two bovine haemoplasmas have a worldwide occurrence, and were reported to elicit anaemia, oedema, reproductive problems and various other clinical signs (Smith et al., 1990; Messick, 2004; Tagawa et al., 2008; Hoelzle et al., 2011). According to a recent study 'Candidatus *M. haemobos*' appears to be more pathogenic (Tagawa et al., 2010).

Regarding the epidemiology of haemoplasmoses in general, routes of infection are incompletely characterised. Transplacental transmission was suggested and verified for only a few species (Berrier and Gouge, 1954; Almy et al., 2006). Although no biological vector was ever identified for haemotropic *Mycoplasma* spp., blood-sucking arthropods were shown to contribute to the spread of infection – most

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likely mechanically – in case of certain species (Daddow, 1980; Neimark et al., 2001; Messick, 2004). For bovine haemoplasmas in particular, vector competency in transmitting *M. wenyonii* was proven only for *Dermacentor andersoni* (Neimark and Kocan, 1997). However, *D. andersoni* is not indigenous outside North America, and European *Dermacentor* spp. collected from haemoplasma-infected cattle were found negative for these disease agents (Hornok et al., in press). Similarly, vector potential in spreading *M. wenyonii* was suggested for *Haematopinus* lice (Hofmann-Lehmann et al., 2004), but *H. eurysternus* removed from cattle in several herds of a haemoplasma-endemic region were found all negative for this species (Hornok et al., 2010). At the same time there is no report on the analysis of vector-borne transmission in case of 'Candidatus *M. haemobos*'.

In order to compensate for such lack of data or inconsistency in the literature, the present study was undertaken to clarify if transplacental and/or vector-borne transmission is possible for one or both bovine haemoplasma species.

2. Materials and methods

2.1. Sample collection

Sampling was performed in a privately owned herd of 100 limousine beef cattle, in the first half of 2010. These animals were kept extensively in northern Hungary, where high prevalence of bovine haemoplasmosis was diagnosed (Hornok et al., in press). From 38 pregnant cows housed separately EDTA-anticoagulated blood samples were collected at around delivery. Precolostral blood sample from their calves was taken shortly after birth. From a group of further 17 unseminated cows (recently imported from another part of the country) two blood samples were obtained three months apart. In this way altogether 110 blood samples were included in the study. Blood sucking insects (Diptera: Culicidae, Tabanidae, Muscidae) were collected near the animals by fine mesh. The species or genera for vector-candidates were identified according to standard keys, and all specimens were stored in 70% ethanol until evaluation.

2.2. DNA extraction and molecular methods

DNA was extracted with the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. From blood samples 200 µl was used for the

extractions. Regarding potential vectors, smaller species were pooled, whereas larger ones were processed individually (Table 2). All insects were washed sequentially in detergent containing water, in tapwater and in distilled water. Air-dried specimens were minced with pointed scissors at the bottom of Eppendorf-tubes in 100 µl of phosphate-buffered saline (PBS). For DNA extraction from these samples an overnight digestion step (incubation at 56 °C for at least 8 h) with tissue lysis buffer and Proteinase-K (QIAGEN, Hilden, Germany) was also included.

The presence of amplifiable DNA was tested by using an 18S rRNA gene TaqMan real-time PCR (Applied Biosystems, Rotkreuz, Switzerland) as described previously (Boretti et al., 2009). In case of high CT values (inhibition) another 1:10 dilution was prepared and analysed further. Real-time PCR evaluation of samples for bovine haemoplasmas was done in two species-specific assays based on the amplification of the 16S rRNA gene (Meli et al., 2010), with different sets of primers and probes for *M. wenyonii* and 'Candidatus *M. haemobos*'. Based on the lack of false positives during their preliminary evaluation, both assays had 100% specificity, and they were sensitive enough to detect one copy of gene per reaction (Meli et al., 2010). For quantitation by standards, tenfold serial dilutions of cloned plasmid DNA with known copy number were used in each test of the present study.

2.3. Statistical analysis

For rates of PCR-positivity exact confidence intervals (CI) at the level of 95% were calculated according to Sterne's method (Reiczigel, 2003). Prevalences were compared by using Fisher's exact test, and mean values by Student's *t*-test. Differences were regarded significant when $P < 0.05$.

3. Results

3.1. Transplacental transmission

All 38 mother cows were PCR positive to *M. wenyonii* and/or 'Candidatus *M. haemobos*'. Neonatal PCR positivity was detected in 10.5% of calves (4 out of 38). Despite their mother's dual infections, two of the calves were positive only for 'Candidatus *M. haemobos*', one only for *M. wenyonii*, and another for both haemoplasmas. In samples of newborn calves the mean copy number (i.e. bacterial load) of *M. wenyonii* was higher, than of 'Candidatus *M.*

Table 1

Results of bovine haemoplasma-specific real-time PCRs with samples of cows and calves.

Sample source	<i>M. wenyonii</i>		'Candidatus <i>M. haemobos</i> '	
	Positives/all tested	Mean copy number in positive samples	Positives/all tested	Mean copy number in positive samples
Mother cows	36/38	6.6×10^3	37/38	3.6×10^2
Calves	2/38	1.2×10^6	3/38	6.3
Unseminated cows (A) ^a	16/17	3.3×10^3	12/17	5.1×10^2
Unseminated cows (B) ^a	17/17	1.5×10^2	8/17	2.0

^a A and B refer to consequent samplings three months apart.

Table 2

Results of bovine haemoplasma-specific real-time PCRs with blood-sucking insects analysed in pools or individually.

Dipteran species	No. of specimens in one sample	<i>M. wenyonii</i>		' <i>Candidatus M. haemobos</i> '	
		Positives/all tested	Mean copy number in positive samples	Positives/all tested	Mean copy number in positive samples
<i>Haematobia irritans</i>	8	1/20	1.9×10^2	2/20	1.0
<i>Stomoxys calcitrans</i>	4	6/20	1.0×10^2	1/20	1.0
<i>Aedes/Culex</i> spp. ^a	8	0/5	0	0/5	0
<i>Haematopota pluvialis</i> ^a	1	0/7	0	0/7	0
<i>Tabanus bromius</i> ^a	1	2/8	6.1×10^1	0/8	0
<i>T. bovinus</i> ^a	1	5/16	3.2×10^1	1/16	1.3×10^1

^a Only females suck blood and were included.

haemobos' (Table 1). Three of the four PCR-positive calves were males.

The mean age of cows delivering infected calf was lower (5 ± 0.8 years) than that of others (6.6 ± 2.1 years), but this was a non-significant association ($P = 0.16$). There was also no significant difference between the mean copy number in samples of mother animals with a PCR-negative or with PCR-positive calf, neither for '*Candidatus M. haemobos*' nor for *M. wenyonii* (data not shown). However, taking together all infected and inseminated cows, the mean copy number was significantly ($P = 0.015$) higher for *M. wenyonii*-positive samples, than in case of '*Candidatus M. haemobos*'-positive ones (Table 1).

3.2. Persistence of bacteraemia

Among uninseminated cows sampled three months apart, the rate of PCR positivity for *M. wenyonii* increased from 94% (CI: 71.3–99.9%) to 100% (Table 1). On the contrary, the prevalence of '*Candidatus M. haemobos*'-infection decreased from 71% (CI: 44–89.7%) to 47% (CI: 23–72.2%), since four previously PCR-negative cows became PCR-positive, but detectable level of infection disappeared from eight formerly PCR-positive cows. Clearance of the latter haemoplasma from the peripheral blood was not related to the age of relevant animals (data not shown). The mean copy number (reflecting bacterial load) was higher for *M. wenyonii*, than for '*Candidatus M. haemobos*' on both sampling occasions (Table 1).

3.3. Occurrence in potential vectors

Muscid flies (*Haematobia irritans* and *Stomoxys calcitrans*), mosquitoes (*Aedes/Culex* spp.) and horse flies (*Haematopota pluvialis* and two *Tabanus* spp.) were caught near the animals. Results of vector analysis are shown in Table 2. Accordingly, at least four species of dipterans may be potentially involved in the transmission of bovine haemoplasmas. Altogether, *M. wenyonii* was significantly more frequently found in blood-sucking insects, than '*Candidatus M. haemobos*' ($P = 0.022$).

4. Discussion

This is the first report of transplacental infection in case of bovine haemoplasmas and the first molecular con-

firmation of their presence in blood-sucking dipterans (for '*Candidatus M. haemobos*' in any potential vector).

In the present study neonatal PCR-positivity of calves to bovine haemoplasmas should have been a consequence of transplacental infection of the fetus, since other post-parturient routes (including colostral/galactogenic, vector-borne infection) can be discounted. Vertical (between-generation) transmission was suggested and proven for only very few other haemotropic *Mycoplasma* spp., with some controversy in the literature. The present results also raise the possibility that intrauterine infection may be influenced by the genotype, since two of the species (*M. haemosuis* and *M. haemolamae*) for which this route was verified (Berrier and Gouge, 1954; Almy et al., 2006) cluster together with *M. wenyonii* based on 16S rRNA phylogeny (Messick, 2004). In this study the manifestation of transplacental infection apparently did not depend on bacterial load – as reflected by copy numbers – and age of mother cows. Moreover, the sex of the fetus (three males, one female) seemed not to have played a role; however, these numbers were too small to draw any final conclusions.

In a previous work applying the same quantitative PCR the bacterial loads of the two bovine haemoplasma species were not compared (Meli et al., 2010). In the present study – relative to '*Candidatus M. haemobos*' – *M. wenyonii*-infection was accompanied by higher levels (loads) of bacteraemia in calves, as well as in mother- and uninseminated cows. Among the latter a decreasing prevalence of '*Candidatus M. haemobos*'-positivity was also noted, as contrasted to that of *M. wenyonii*. However, data on the persistence of bacteraemia in this case are limited to only two samplings, therefore they should not be interpreted as if obtained from permanent monitoring.

Vector species evaluated in the present study were shown to be capable mechanical transmitters of other intra- or epierythrocytic bacteria (Foil, 1989; Prullage et al., 1993) except for *Haematobia irritans*. Consequently, to the best of our knowledge this is the first molecular detection of any haemotropic bacteria in the latter species. PCR-positivity for *M. wenyonii* and '*Candidatus M. haemobos*' was demonstrated in small size muscid flies as well as large horse flies. This may indicate that the amount of ingested blood may have been enough in both cases for mechanical transmission, taking into account that one infected red blood cell is sufficient to inoculate *M. ovis* (Mason and Statham, 1991). By contrast, in another study

(Hornok et al., in press), all evaluated hard ticks that recently sucked blood on haemoplasma-infected cattle turned out to be PCR-negative and therefore are regarded as unlikely vectors.

On the other hand, negative PCR results of the horse fly *Haematopota pluvialis*, and of mosquitoes in the present study does not exclude a vector role for the relevant species in the epidemiology of bovine haemoplasmoses, and may be solely attributable to the low number of analysed pools. For instance, recently in another study one pool of mosquitoes was found (with similarly sensitive methods) to carry *M. wenyonii* (Lin et al., 2009).

Regarding the comparative occurrence of bovine haemoplasmas in blood-sucking dipterans, *M. wenyonii* predominated in the PCR-positive samples, suggesting that it may be more effectively transferred (at least mechanically) to new susceptible hosts, than '*Candidatus M. haemobos*'. This may also be related to a more persistent and/or higher level bacteraemia in case of the former species, as concluded from the results above, rendering it more available for potential blood-sucking vectors.

In summary, transplacental infection and vector-borne transmission may contribute to the spread of both bovine haemoplasmas as demonstrated for the first time. However, detection of bacterial DNA in blood-sucking insects may only indicate a potential to carry over the relevant disease agents mechanically. Since the actual vector competency of flies evaluated in the present study is unknown, and in general, the biological vector(s) of any haemotropic *Mycoplasma* spp. remain to be identified (if they exist at all), further studies are encouraged to clarify these aspects in the epidemiology of haemoplasmoses.

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