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Short communication

Molecular identification of *Anaplasma marginale* and rickettsial endosymbionts in blood-sucking flies (Diptera: Tabanidae, Muscidae) and hard ticks (Acari: Ixodidae)

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Abstract

In an attempt to identify the main vector and possible transmission routes of *Anaplasma* spp. in a region of Hungary with high prevalence of ovine and bovine anaplasmosis, DNA was extracted from 316 haematophagous arthropods (individually or in pools), including 4 species of ixodid ticks, 6 species of tabanid flies and hornflies. *Midichloria*-like organisms were identified with PCR (amplifying a portion of the 16S rRNA gene) and sequencing from *Dermacentor marginatus* and *Ixodes ricinus*. Significantly higher 16S positive *D. marginatus* individuals were collected in March than in April, suggesting earlier questing of ticks that contain rickettsial agents (thus endosymbionts). *Midichloria*- and *Wolbachia*-like organisms were also found in randomly caught horse flies (*Tabanus bovinus* and *T. tergestinus*) as well as hornflies (*Haematobia irritans*), respectively, with 97–99% similarity to sequences deposited in the GenBank. Although all ticks were negative in the *Anaplasma* spp.-specific *msp4* PCR, four individuals of *T. bovinus* collected near to grazing cattle were positive for *Anaplasma marginale*. The results of the present study provide the first molecular evidence for the potential mechanical vector role of *T. bovinus* in the transmission of *A. marginale*, and broaden the range of haematophagous arthropods harbouring *Midichloria*-like bacteria, for the first time in any *Dermacentor* or *Tabanus* species.

Keywords: *Anaplasma marginale*; *Tabanus*; *Dermacentor*; Horse fly; *Midichloria*; Horn fly; *Wolbachia*

1. Introduction

Representatives of the order Rickettsiales are Gram-negative, intracellular bacteria classified into the families Rickettsiaceae and Anaplasmataceae (Dumler et al., 2001). They are frequently associated with arthropods, most notably as pathogens transmitted by blood-sucking vectors into their vertebrate hosts (providing their veterinary significance). While most of the formerly known species have been molecularly characterized, the existence of a third lineage (with family rank) was postulated (Sassera et al., 2006), including symbionts (*Midichloria mitochondrii*) of ticks passed on to the next generation transovarially. Recently, *Midichloria*-related bacteria were identified in five ixodid genera and several newly examined species (Epis et al., 2008).

Hard ticks are potential biological vectors of *Anaplasma marginale* in the temperate zone (Dikmans,

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1950; de la Fuente et al., 2005a). It was also shown that certain haematophagous dipterans, such as tabanid horse flies are competent for its mechanical transmission (Wilson and Meyer, 1966; de la Fuente et al., 2005b), whereas others like the buffalo fly (*Haematobia irritans exigua*) are not (Allingham et al., 1994). Since ovine and bovine anaplasmosis have been recently recognized with high prevalence in Northeastern Hungary (Hornok et al., 2007), and the potential vectors of the causative agents are unknown in Central Europe, ixodid ticks and blood-sucking flies were collected in the endemic area for screening representatives of the family Anaplasmataceae and related microorganisms.

2. Materials and methods

2.1. Sample collection

Hard ticks were collected on several occasions at 2–3 weeks intervals with the cloth-dragging method on two pastures in Northeast Hungary, where ovine and bovine anaplasmosis is endemic. The sampling period was chosen in order to encompass the whole spring season of *Dermacentor* spp. as it corresponded well to the appearance of clinical signs of anaplasmosis in previous years. Thus, specimens evaluated in the present study included 61 adults of *Dermacentor marginatus* and 8 adults of *D. reticulatus* (collected in March and April), 48 adults and 40 nymphs of *Ixodes ricinus* and 1 adult and 8 nymphs of *Haemaphysalis concinna* (collected in April and May). All ticks were identified according to Babos (1965). Fifty tabanid flies (belonging to 6 different species) and 100 hornflies (*H. irritans*) were caught with a fine mesh in June in sites within 10 m of cattle herds. Tabanids were also collected at longer distance (more than 1 km) from sheep flocks or cattle herds. The species of tabanids were determined as described by Aradi (1958). All samples were stored in 70% ethanol until evaluation.

2.2. Pooling and DNA extraction

Dermacentor and tabanid species were processed one by one, whereas the number of individuals per pool (*n*) were 3 for adult and 20 for nymphs of *I. ricinus*, 4 for nymphs of *H. concinna* and 10 for hornflies. All specimens were washed three times: for 30 s in detergent containing water, followed by tapwater and finally in distilled water. Air-dried arthropods were minced with pointed scissors at the bottom of Eppendorf-tubes, in a small volume of phosphate-buffered saline (PBS) kept to the minimum according to the size of the specimen. DNA was isolated using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions.

2.3. 16S PCR and sequencing

For preliminary assessment of the samples primers EHR16SD (5′-GGT ACC YAC AGA AGA AGT CC-3′) and EHR16SR (5′-TAG CAC TCA TCG TTT ACA GC-3′) were used, which amplify an approximately 345 bp fragment from the 5′ region of the 16S rRNA gene from various members of the family Anaplasmataceae and closely related rickettsial agents (Brown et al., 2001). One microliter of extracted DNA was included in a 20 μl reaction mixture comprised of 1.0 unit of HotStartTaq DNA polymerase, 200 μM of each dNTP, 10 pmol of each primer and 1.5 mM $MgCl_2$ (HotStartTaq Master Mix, QIAGEN, Hilden, Germany). Amplification was performed using a T-personal 48 thermal cycler (Biometra GmbH, Göttingen, Germany). An initial denaturation step at 94 °C for 15 min was followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s. Final extension was done at 72 °C for 5 min. PCR products were subjected to electrophoresis in a 1.5% agarose gel (100 V, 40 min), pre-stained with ethidium bromide and viewed under ultra-violet light.

After purification with Wizard® SV gel and PCR clean-up system (Promega, Madison, WI, USA), ABI Prism® Big Dye Terminator v3.1 Cycle Sequencing Kit (PerkinElmer, Applied Biosystems Division, Foster City, CA, USA) was used for DNA sequencing reactions. Samples were then examined using an ABI Prism 3100 Genetic Analyser at the Agricultural Biotechnology Center (Gödöllő, Hungary). Obtained sequences were checked and edited with Chromas Lite and compared to sequence data available from GenBank®, using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4. Anaplasma-specific PCR and sequencing

All 16S positive samples were further analysed for the presence of the major surface protein 4 (*msp4*) gene of *Anaplasma* spp. The PCR was performed from 1 μl DNA with 10 pmol of each primer. For *A. marginale* and *Anaplasma ovis* MSP45 (5′-GGG AGC TCC TAT GAA TTA CAG AGA ATT GTT TAC-3′) and MSP43 (5′-CCG GAT CCT TAG CTG AAC AGG AAT CTT GC-3′); for *A. phagocytophilum* MSP4AP5 (5′-ATG AAT TAC AGA GAA TTG CTT GTA GG-3′) and MSP4AP3

(5′-TTA ATT GAA AGC AAA TCT TGC TCC TAT G-3′) were used in a 50 μl PCR volume containing 1.5 mM $MgSO_4$, 0.2 mM dNTP, 1× AMV/*Tfl* reaction buffer, 5 μm *Tfl* DNA polymerase. Cycling conditions, cloning and sequencing were reported previously (de la Fuente et al., 2004, 2005a).

2.5. Sequence accession numbers

GenBank accession numbers for partial 16S sequences are EU315771-EU315781, and those for *msp4* sequences EU315782, EU315783.

2.6. Statistical analysis

Data were compared by using Fisher's exact test, and differences were regarded significant when $P \leq 0.05$.

3. Results

Samples of *D. marginatus* (46% positive) and *D. reticulatus* (12.5% positive) as well as pools of adult *I. ricinus* (56% positive) yielded amplicons in the 16S PCR (Table 1). Regarding their temporal distribution, significantly ($P < 0.003$) more 16S-positive *D. marginatus* ticks were collected in March (24 of 25) than in April (4 of 36). In addition, PCR positivity was associated with only females of *I. ricinus*, but was higher in males than females of *D. marginatus*. Both pools of *I. ricinus* nymphs showed 16S positivity, as contrasted to the negative results with the two pools of *H. concinna* nymphs.

Unknown rickettsial agents occurred in male and female individuals of *Dermacentor* spp., whereas *Midichloria* sp.-like endosymbionts were identified in females of two species from among both hard ticks and randomly caught horse flies (Table 1), with 97–99% similarity to sequences deposited in the GenBank. On the other hand, *Wolbachia* sp.-like organisms could be demonstrated from pools of hornflies.

In the *msp4* PCR performed with 16S-positive samples four individuals of *T. bovinus* collected near to grazing cattle were positive, with 99.5% sequence similarity to *A. marginale* strains according to data available in the GenBank. One isolate differed from the others in two positions (guanine instead of adenine at positions 390 and 506; adenine in the translation initiation codon = position 1). All ticks were negative in the *msp4* PCR, and no amplicons were gained with primers specific for *A. phagocytophilum*.

4. Discussion

This is the first study providing molecular identification of *A. marginale* in *T. bovinus*. Besides clarifying important aspects of the epidemiology of bovine anaplasmosis in Central-Eastern Europe, in a broader context (and to the best of our knowledge) such evidence also compensates for previously unavailable data on this horse fly species, as it was not among those

Table 1
PCR results from adult ticks and flies

Collection site	Species name	Proportion of 16S positive samples		Proportion of *msp4* positive samples	Samples for sequencing	Results of sequencing (alignment similarity)
		Males	Females			
On the pasture	*Dermacentor marginatus*	18/30	10/31	0/28	2 ♂ 1 ♀	oR. *Midichloria*-like (97%)
	D. reticulatus	0/2	1/6	0/1	1 ♀	oR.
	Ixodes ricinus	0/7[a]	9/9[a]	0/9[a]	2 ♀[a]	*Midichloria*-like (99%)
	Haemaphysalis concinna	–	0/1	0/0	–	–
Far from sheep and cattle (<1 km)	*Tabanus bovinus*	–	4/6	0/4	2 ♀	*Midichloria*-like (99%)
	T. bromius	–	0/9	0/0	–	–
	T. quatuornotatus	–	0/6	0/0	–	–
	T. tergestinus	–	1/7	0/1	1 ♀	*Midichloria*-like (97%)
	Chrysops relictus	–	0/5	0/0	–	–
	Haematopota pluvialis	–	0/7	0/0	–	–
Near to cattle (<10 m)	*T. bovinus*	–	5/8	4/5	4 ♀	*Anaplasma marginale* (99%)
	T. tergestinus	–	0/1	0/0	–	–
	H. pluvialis	–	0/1	0/0	–	–
	Haematobia irritans (unsexed)	10/10[a]		0/10[a]	2[a]	*Wolbachia*-like (99%)

Molecular identification was attempted from some 16S and all *msp4* positive samples. *Abbreviation*: oR., other rickettsial agent.
[a] Pooled samples.

members of the genus *Tabanus* for which transmission experiments verified a mechanical vector role (reviewed by Dikmans, 1950). According to their *msp4* PCR positivity other tabanid species were also suggested to be involved in spreading *A. marginale* in Southern Europe (de la Fuente et al., 2005b).

As substantiated by former results (de la Fuente et al., 2005a,b) the methods applied here to amplify the *msp4* gene can be regarded as sensitive enough to detect the presence of *Anaplasma* spp. in ixodid ticks. Nevertheless, in previous studies only engorged ticks were used. Therefore, assuming that tick sampling was representative, the absence of *msp4* PCR positivity reflects an undetectable level or low prevalence of ruminant-infecting *Anaplasma* spp. (if present) in unfed stages of the local tick population. Since the whole spring activity period of *Dermacentor* spp. (the most important biological vectors of *A. marginale* and *A. ovis* in the temperate zone; Kocan et al., 2004) was sampled, and infected ticks were not found, results of the present study support the view that formerly emphasized transstadial transmission of *A. marginale* is less important than the intrastadial route (Eriks et al., 1993). This is further justified by the fact that nymphs of *Dermacentor* spp. tend to feed on smaller mammals (Babos, 1965), thus lowering the chance for becoming infected with *A. marginale* or *A. ovis* at this stage and being able to transmit them as adults. On the other hand, local sheep flocks reach the pastures on narrow paths and also rub their fleece coat frequently (being next to each other) while grazing, thus intermittantly feeding male ticks can easily transfer between animals. This is in favour of intrastadial transmission which may alone explain the high prevalence of ovine anaplasmosis and can account for infection of naive animals.

However, cattle are more dispersed between small farms in the relevant region and when grazing the pastures in small herds, they do not tend to get into frequent close contact with each other, in this way preventing intrastadial transmission. Therefore, according to the present results, we concluded that mechanical carry-over by tabanid horse flies may be more important than the biological vector role of hard ticks in spreading *A. marginale* infection to cattle. Tabanid females suck blood only every 3–4 days (a period too long for the survival of any mechanically transmitted infectious agents), but their painful feeding is usually interrupted by host animals several times, allowing mechanical transfer of pathogens. Although remaining able to inoculate *A. marginale* for at least 2 h (Hawkins et al., 1982) during which *T. bovinus* can cover several km with its flight speed around 5 m/s (Nachtigall, 2004), they tend to feed in the same herd until completing their blood meal (Barros and Foil, 2007). This is further supported by our findings, as no tabanids were found infected with *A. marginale* at distances longer than 1 km from cattle herds. The lack of *msp4* positivity among hornflies is also consistent with data in the literature (Allingham et al., 1994), suggesting that *Haematobia* spp. are not important mechanical vectors of *Anaplasma* spp.

Tick-associated rickettsial endosymbionts were first demonstrated from the genus *Ixodes* (Noda et al., 1997) and *Haemaphysalis* (Parola et al., 2003), in case of the latter with the same primers as used in this study. Afterwards it was clarified that these bacteria reside in the cytoplasm and mitochondria of cells in the ovaries of female ticks (Beninati et al., 2004). This may explain why 16S PCR positivity in the present results was exclusive to females of *I. ricinus*. Recently the new genus name *Midichloria* was proposed for these microorganisms (Sassera et al., 2006), and related bacteria were successfully identified in further genera (*Rhipicephalus*, *Hyalomma* and *Amblyomma*), but not in any *Dermacentor* spp. (Epis et al., 2008). Although *Dermacentor* ticks may also have symbionts, only *Francisella tularensis*-like agents were confirmed in them so far (Niebylski et al., 1997). Therefore, detecting a 16S sequence in *D. marginatus* that is highly similar to that of *Midichloria* sp. constitutes a new finding in this genus. Furthermore, the results reported herein demonstrate the DNA of *Midichloria* symbionts (apparently associated with particular species of Tabanidae) in horse flies for the first time. The fact that not only diverse ixodid tick genera, but also taxonomically distant groups may contain *Midichloria*-like bacteria (although their location and biological role in horse flies is yet to be clarified) support the view that they originally may have been acquired through blood feeding of relevant vectors on their host (Noda et al., 1997), as ruminants are important targets for the haematophagous activity of both *Dermacentor* spp. and members of the family Tabanidae. *Wolbachia* sequences could also be amplified from various arthropods (Jeyaprakash and Hoy, 2000; Floate et al., 2006) including arachnids and insects, the latter exemplified by *H. irritans* as confirmed by the present results.

Based on significantly higher 16S PCR positivity in *D. marginatus* collected in March vs. April, the presence of rickettsial agents (including *Midichloria* spp.) may be associated with earlier questing of relevant ticks. Extension of the behavioural–metabolical diapause was demonstrated for ticks if conditions are not in favour of their feeding, development and thus repro-

duction (Belozerov, 1982). These endosymbionts reside in the mitochondria of cells in the sexual organs and – interfering with their function – may play a role in the biology of relevant ticks (Sassera et al., 2006), perhaps through influencing metabolic activity and hormonal levels. Such a phenomenon as observed here (earlier tick activity, whether or not induced by their bacteria) would be a mutual interest, since it may serve to ensure host-finding and thus vertical transmission of endosymbionts to the next generation (Bandi et al., 2007), similarly to other mechanisms attributable to *Wolbachia* spp. which induce reproductive alterations of their arthropod hosts (Stouthamer et al., 1999).

Besides establishing a significant role for *T. bovinus* as possible mechanical vector of *A. marginale* in Central-Eastern Europe, the results provided herein highlight the importance to study the genus *Midichloria* in a wider range of insects and arachnids, especially due to the growing concern on the evolutionary and genomics studies of arthropod endosymbionts. These bacteria play a pivotal role in developing new strategies against vector-borne diseases as they represent possible agents for paratransgenesis (Rasgon et al., 2003), i.e. to prevent the spread of biologically or mechanically transmitted pathogens through genetic manipulation of symbiotic microorganisms in their vectors.

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