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SHORT COMMUNICATION

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Discrimination between *Theileria lestoquardi* and *Theileria annulata* in their vectors and hosts by RFLP based on the 18S rRNA gene

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Abstract *Theileria lestoquardi* and *T. annulata* can occur in similar vectors, and current available probes based on the 18S rRNA gene showed cross-reaction between the two species. However, we developed a species-specific RFLP test based on the *MspI* restriction enzyme, able to cut amplified products from *T. lestoquardi* only and to discriminate the two species in both tick and blood samples.

Introduction

Theileria lestoquardi and *T. annulata* are haemoprotozoan parasites, morphologically and biologically similar, transmitted by ixodid ticks of the genus *Hyalomma*. Both are the causative agents of serious disease—theileriosis of small and large ruminants in tropical and subtropical countries—but they are different in the capacity to infect their hosts. *T. lestoquardi* readily infects sheep but is unable to infect cattle. On the other hand, *T. annulata* infects both cattle and sheep (Brown et al. 1998; Leemans et al. 1999a, 1999b).

The aim of the study was the differentiation of *T. lestoquardi* and *T. annulata*, using the 18S rRNA gene, as they could be present in the same tick or the same host, and previous discriminating molecular probes have failed.

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Materials and methods

Isolates of *T. lestoquardi* (from Iran) and *T. annulata* (from Italy) were used in this study.

Primers R2 (5′-CTA AGA ATT TCA CCT CTG ACA GT-3′) and F2 (5′-GAC ACA GGG AGG TAG TGA CAA G-3′), covering the hypervariable region V4 adopted from Gubbels et al. (1999), were used for PCR amplification of the 18S rRNA gene. PCR products were hybridized to a reverse line blot (RLB) membrane with the specific oligonucleotide probes shown in Fig. 1, and containing an N-terminal (TFA)–C6 amino linker (Isogen). The preparation and hybridization of the RLB membrane was done as described by Gubbels et al. (1999) and Bekker et al. (2002).

The amplified products were digested with *MspI* restriction enzyme (Sigma) as described by the supplier, and analyzed by agarose gel electrophoresis on a 1.2% gel.

Results and discussion

Alignments of sequences of *T. lestoquardi* and *T. annulata* are different only in a few sites; one of them is at the oligonucleotide probe location for *T. lestoquardi* (Schnittger et al. 2004) and *T. annulata* (Gubbels et al. 1999) on the 18S rRNA (Fig. 1). By RLB hybridization, these two pathogens can not be distinguished (Fig. 2). The probe for *T. annulata* (Gubbels et al. 1999) is cross-reacting with *T. lestoquardi* DNA, and the *T. lestoquardi* probe (Schnittger et al. 2004) cross-reacts with *T. annulata* DNA. The necessity to differentiate between *T. lestoquardi* and *T. annulata* is implicit mainly from their occurrence in tick vectors, because the vectors could be the same for both pathogens, and *T. lestoquardi* and *T. annulata* can both be found in small ruminants. Therefore, to distinguish them, we developed a restric-

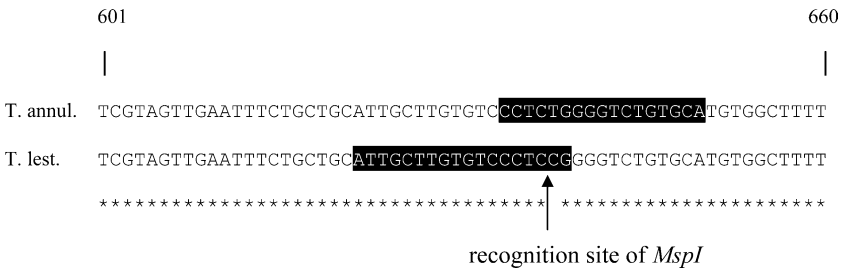

Fig. 1 Alignment showing the locations of the species-specific oligonucleotide probes in the 18S rRNA V4 hypervariable region (*T. annulata* probe from Gubbels et al. 1999; *T. lestoquardi* probe from Schnittger et al. 2004)

tion fragment length polymorphism (RFLP) method by the restriction endonuclease enzyme *MspI*, which recognizes the following sequence: CCGG. The *MspI* enzyme could digest PCR products of the 18S rRNA gene of *T. lestoquardi* but not *T. annulata* (Fig. 3).

Furthermore, we checked whether mixed infections could interfere with this test. We amplified different DNA ratios of *T. lestoquardi*/*T. annulata* (R = 1, 10, 100), and results showed (Fig. 3) that unless the DNA ratio is about 100 and above, we can not see anymore the mixed infection. However, in this case we could consider that *T. lestoquardi* is the predominant pathogen when the DNA ratio is above 100.

Pathogens can not be distinguished in the salivary glands of infected ticks by traditional staining methods such as Feulgen or methyl green-pyronin because of their morphological similarity. Serological methods are not very effective either, as antibodies are cross-reacting. Kirvar et al. (1998, 2000) developed a PCR using specific primers to amplify only *T. lestoquardi* or *T. annulata* fragments of the gene coding a 30-kDa merozoite surface protein. The 18S rRNA gene is commonly used, compared to the gene coding the 30-kDa merozoite surface protein. This paper shows for the first time a test able to differentiate between *T. lestoquardi* and *T. annulata* occurring in identical vectors and hosts. Seeing that RLB is able to discriminate *Theileria* species from *Babesia* species, mixed infections other than *T. lestoquardi*/*T. annulata* should not be a problem.

In Mediterranean countries, for instance, where these species are often endemic, it will be of great importance to use such a method to identify the prevalence of each pathogen in tick populations, to check if small ruminants or cattle are more at risk.

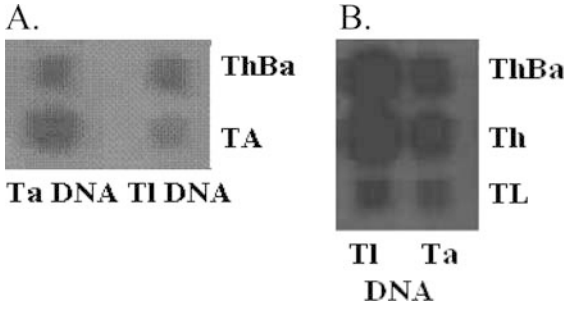

Fig. 2A, B Reverse line blot (RLB) hybridization of PCR products of *Theileria* species. *ThBa* Oligonucleotide probe catches all *Theileria* and *Babesia* species; *Th* oligonucleotide probe catches all *Theileria* species; *TL* oligonucleotide probe, *Theileria lestoquardi* (*Tl*; Schnittger et al. 2004); *TA* oligonucleotide probe, *T. annulata* (*Ta*; Gubbels et al. 1999)

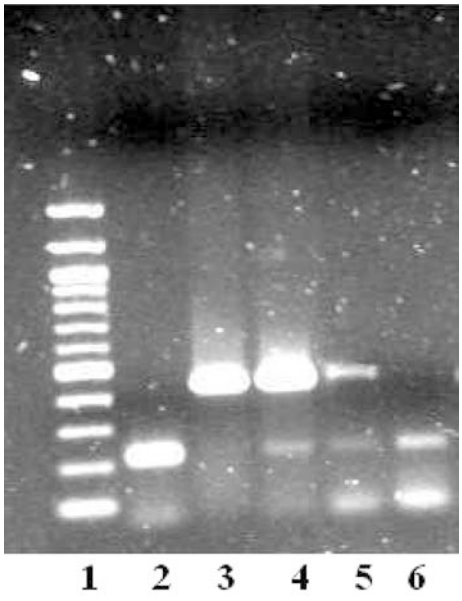

Fig. 3 Restriction fragment length polymorphism (RFLP) of PCR products of the 18S rRNA gene of *Theileria lestoquardi* and *T. annulata*. *Lane 1* 100 bp molecular marker, *lane 2 T. lestoquardi*, *lane 3 T. annulata*, *lane 4 T. lestoquardi*/*T. annulata* ratio 1:1, *lane 5 T. lestoquardi*/*T. annulata ratio* 10:1, *lane 6 T. lestoquardi*/*T. annulata* ratio 100:1

We presented here a single PCR method based on the 18S ribosomal RNA gene, followed by RFLP able to give a rapid discrimination between *T. lestoquardi* and *T. annulata*. Knowing if ticks or livestock are carrying one or the other would give valuable epidemiological data, allowing to understand the prevalence and the distribution for each pathogen and the appropriate treatment method.

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