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Original article

Genetic diversity of *Theileria equi* and *Babesia caballi* infecting horses of Central-Southern Italy and preliminary results of its correlation with clinical and serological status

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ABSTRACT

Babesia caballi and *Theileria equi* are tick-borne pathogens causing equine piroplasmosis infecting the Equidae family in which they cause significant sanitary and economic losses. Furthermore, equine piroplasmosis is included in the World Animal Health Organization (OIE) notifiable diseases list with possible movement restrictions for positive horses.

Thirty-nine EDTA and whole-blood samples collected during 2013 and 2014 from symptomatic and asymptomatic horses of Central-Southern Italy were included in the present study either because of their strongly positive results in Real Time (RT) PCRs targeting the 18S rRNA gene specific for each piroplasm and/or due to their serological ELISA/18S rRNA RT-PCR discordant results. A nested PCR amplifying the hypervariable V4 region of the 18S rRNA gene of both piroplasms was performed on all samples. *T. equi* positive samples were also analysed with a PCR targeting the equi merozoite antigen 1-gene (EMA-1). The sequences obtained were thirty for *T. equi*, 25 of which were for the hypervariable V4 region of the 18S rRNA gene and 13 for the EMA-1 gene, with eight samples positive for both targets, while only six 18S rRNA gene sequences were retrieved for *B. caballi*.

The phylogenetic analysis results are as follows: *T. equi* sequences of the 18S rRNA gene clustered in three different phylogenetic groups, respectively in the A (15), B (9) and C (1) while those of *B. caballi* in the A (1), B1 (3) and B2 (2) groups. *T. equi* sequences for EMA-1 gene clustered in A (11) and in B (2).

This analysis confirms that both *T. equi* and *B. caballi* sequences present a genetic heterogeneity independently of their geographical location, similar to that reported by other authors.

Statistical associations were evaluated between phylogenetic groups of *T. equi* 18S rRNA gene and each of the following variables, using Fisher's exact test: clinical signs, serological ELISA/18S rRNA RT-PCR discordant results and *T. equi* EMA-1 negativity. The different groups were found to be statistically related to the presence of signs (less present in group B samples), to ELISA negativity/18S rRNA RT-PCR positivity (more seronegative samples in group B). No statistical analysis was performed for the *B. caballi* as the number of sequences available was insufficient and for the EMA −1 sequences which almost all grouped in the same cluster.

The results here obtained provide additional information about *T. equi* and *B. caballi* sequences, which could also be used to verify the performance of serological and molecular diagnostic methods.

1. Introduction

Equine piroplasmosis (EP) is a disease caused by tick-borne pathogens *Babesia caballi* and *Theileria equi,* that infect horses, mules, donkeys and zebras and is capable of causing significant sanitary and economic losses in horses (Preston, 2001; Uilenberg, 2001). It also represents an important constraint to the international movement of horses. EP is endemic in tropical and temperate areas and can occur with the

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following clinical forms: acute, sub-acute, chronic and unapparent, the latter being the most frequent. Animals surviving the acute phase may remain seropositive carriers with low levels of parasitaemia, a condition that occurs more frequently in *T. equi* infections (de Waal, 1992). While disease due to *B. caballi* is reported as less severe than that induced by *T. equi*, clinical signs during the acute phase are common to both protozoal infections, represented by fever, depression, anaemia, jaundice, oedema, anorexia and, occasionally, mucosal petechiae and ecchymoses (de Waal, 1992). As these signs are indistinguishable for the two parasite infections, a differential diagnosis is laboratory-based, employing the following methods: stained blood smear observation, serological tests, such as complement fixation test, indirect fluorescent antibody test (IFAT), ELISA, and genome detection using piroplasm-specific PCR protocols, as described by World Animal Health Organization (OIE) Manual.[1]

The use of highly sensitive molecular tests, such as PCRs, not only overcome the difficulties in the detection of cases of low parasitaemia such as those that occur in chronic and unapparent infections but also permit genetic characterization. Different targets have been used in piroplasm detection such as equi merozoite antigen 1-gene (EMA-1) (Battsetseg et al., 2002), β-tubulin gene (Cacciò et al., 2000) and 18S rRNA gene (Nagore et al., 2004).

The small subunit ribosomal RNA (18S rRNA) gene possesses several characteristics that have led to its wide use for phylogenetic studies (Chae et al., 1999; Eickbush and Eickbush, 2007; Salim et al., 2010). In fact, variable sub-regions are also present across this gene allowing unequivocal sequence alignment allowing phylogenetic discrimination (Allsopp and Allsopp, 2006). Several 18S rRNA gene-based phylogenetic analyses reported by different authors reveal a remarkable degree of variation among and within *B. caballi* and *T. equi.* Such studies were carried out in Spain (Nagore et al., 2004, Criado-Fornelio et al., 2003), in Greece (Kouam et al., 2010) in Italy (Veronesi et al., 2014), in South Africa (Bhoora et al., 2009), in Sudan (Salim et al., 2010) in Jordan (Qablan et al., 2013) in Korea (Seo et al., 2013), and in USA (Hall et al., 2013).

Further to this gene, Bhoora et al. (2010) conducted a phylogenetic study using sequences of a region of the EMA-1 gene obtained from field isolates collected from horses of South Africa.

EMA-1 is a 34 kDa immunodominant protein of *T. equi*, coded by a family of 10 genes distributed across the genome with an amino acid identity ranging from 17 to 55% (Kappmeyer et al., 2012). It is among the merozoite surface proteins that play an essential role in the recognition, attachment and penetration of the parasites in erythrocytes and is employed as antigen in a c-ELISA diagnostic test (Knowles et al., 1991).

The aim of the present study was to assess the genetic heterogeneity of *T. equi* and *B. caballi* detected in horses of Central-Southern Italy, employing EMA-1 and 18S rRNA genes for the former parasite and just the 18S rRNA gene for the latter. Moreover, a preliminary evaluation was conducted on the correlation between the different *T. equi* genotypes relative to the 18S rRNA gene and the clinical form, the serological/PCR discordant result and the EMA-1 PCR result and implications on management of the disease are presented and discussed.

2. Material and methods

2.1. Samples

Thirty-nine *T. equi* and/or *B. caballi* PCR-positive horse blood samples of 24 symptomatic and 15 asymptomatic horses collected between 2013 and 2014 from Tuscany, Latium and Campania regions were included in the study. The inclusion criteria were based on either their strong reactivity (Ct < 24) in the piroplasm specific RT-PCRs targeting the 18S rRNA (Kim et al., 2008; Bhoora et al., 2010, Bartolomé Del Pino et al., 2016) or because the sequences were detected in animals presenting discordant ELISA/18S rRNA RT-PCR results.

2.2. Serological tests

Two commercial competitive ELISA (c-ELISA), *Babesia equi* Antibody test kit VMRD® and *Babesia caballi* Antibody test kit VMRD® were performed according to manufacturer's instructions. The first assay employs EMA-1 antigen while the second uses the Roptry Associated Protein 1 antigen.

2.3. DNA extraction from blood

DNA extraction from blood samples was performed using the QIAamp DNA Blood Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

2.4. RT-PCRs for T. equi and for B. caballi

The *T. equi* RT-PCR employed was that reported by Kim et al. (2008) amplifying an 81-bp fragment outside the V4 hypervariable region of the 18S rRNA gene; while that for *B. caballi* was performed according to Bhoora et al. (2010) that amplifies a 95-bp fragment within the V4 hypervariable region of the 18S rRNA gene. TaqMan ® Universal PCR Master Mix kit (A. Biosystems, Foster City, CA, USA) was used for both RT-PCRs.

PCR internal positive and negative controls were obtained as described by Bartolomé Del Pino et al. (2016).

2.5. End-point PCRs for regions of the EMA-1 and 18S rRNA genes and sequencing

Hall et al. (2013) reported that samples positive with other PCRs resulted negative in the EMA-1 PCR for *T. equi*. To verify if this also occurred in the present set of samples, the protocol described by Battsetseg et al. (2002) that targets a 268-bp fragment of the *T. equi* EMA-1 gene was employed on RT-PCR positive samples.

The nested 18S rRNA PCR used to obtain amplicons for sequencing was carried out as described by Nicolaiewsky et al. (2001), employing AmpliTaq Gold® DNA Polymerase (A. Biosystems, Life Technologies, Austin, TX, USA) and GeneAmp® PCR System 9700HT (A. Biosystems, Foster City, CA, USA).

PCR products were visualised in 1.5% agarose gel electrophoresis by staining with GelRed dye (Biotium, Hayward, CA, USA) and once recovered they were purified using the QIAquick® PCR Purification kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions and sequenced using reagents (BigDye Terminators) and instrumentation (3500 Genetic Analyzer) from Applied Biosystems, (Foster City, CA, USA).

2.6. Phylogenetic analysis

The evolutionary history of the sequences was inferred by the Neighbour-Joining method (Saitou and Nei, 1987; Tamura et al., 2004), using the software MEGA6[2]; the best substitution model for the nucleotides or amino acids was chosen by Model Selection (ML) analysis (Tamura, 1992).

Thirty-seven nucleotide sequences were used to construct the phylogenetic tree of a region of the hypervariable V4 region of 18S rRNA gene of *T. equi*: 3 from Bhoora et al. (2009), one for each of the groups

[1] OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2017 http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/ Chapter 2.5.8. Equine piroplasmosis (NB: Version adopted in May 2014).

[2] MEGA – MolecularEvolutionary Genetics Analysis http://www.megasoftware.net.