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Original article

Protozoan and bacterial pathogens in tick salivary glands in wild and domestic animal environments in South Africa

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ABSTRACT

A total of 7364 ticks belonging to 13 species was collected from 64 game animals (belonging to 11 species) and from 64 livestock animals (cattle and sheep) living in close vicinity at 6 localities in 3 South African Provinces (Free State, Mpumalanga, and Limpopo). The geographic distribution of all tick species was congruent with the literature except for *Haemaphysalis silacea*. From each infested host, a maximum of 10 males and 10 females of each tick species were dissected to isolate the salivary glands. Salivary glands were screened for tick-borne pathogens using polymerase chain reaction followed by reverse line blotting and sequencing. This approach allowed us to evaluate the exposure of wild and domestic hosts to tick-borne pathogens in their respective environments. Among the 2117 examined ticks, 329 (15.5%), belonging to 8 species, were infected and harboured 397 infections. Among those, 57.7% were identified to species level and were assigned to 23 pathogen species of the genera *Babesia*, *Theileria*, *Anaplasma*, and *Ehrlichia*. In 3 out of 6 localities, salivary glands from ticks infesting wild ruminants displayed significantly higher infection prevalence and pathogen mean density than salivary glands from ticks infesting livestock animals. Four piroplasm species [*Theileria bicornis*, *Babesia* sp. (sable), *Theileria* sp. (giraffe), and *Theileria* sp. (kudu)] were detected for the first time in ticks. The tick species *Rhipicephalus evertsi evertsi*, *Rhipicephalus (Boophilus) decoloratus*, *Hyalomma rufipes*, *Rhipicephalus appendiculatus*, and *Amblyomma hebraeum* were associated with a broader pathogen range than previously known, and thus new vector–pathogen combinations are described. In addition, previously unknown coinfection patterns in tick salivary glands are reported.

Introduction

Ticks are vectors of a great variety of tick-borne pathogens. In the recent decades, the development of molecular tools increased their known number and their known variety. The majority of tick-borne pathogen species that circulate between ticks and both game and livestock animals have been reported in the vertebrate hosts they infect and less in the ticks. Indeed, in early studies that aimed to detect tick-borne pathogens in wild African ungulates, animal sera were screened (Neitz and Du Toit, 1932; Neitz, 1935; Löhr and Meyer, 1973; Löhr et al., 1974; Carmichael and Hobday, 1975). More recently, in studies reporting tick-borne pathogens affecting game animals, host blood was examined, but not the associated tick species (Nijhof et al., 2003, 2005; Spitalska et al., 2005; Brothers et al., 2011; Oosthuizen et al., 2008, 2009; Pfitzer et al., 2011). In addition, several tick-borne pathogens with known tick vectors display a broader host range than formerly thought. An example is *Theileria* sp. (sable) that infects the sable (*Hippotragus niger*) and roan (*H. equinus*) antelope and that has been newly described in red hartebeest (*Alcelaphus buselaphus*) (Spitalska et al., 2005), cattle (Yusufmia et al., 2010), and nyala (*Tragelaphus angassii*) (Pfitzer et al., 2011). For these pathogens, a broader host range could mean that additional, so far unknown vectors, especially when associated with a wide host variety, might be involved in their transmission. From this point of view, the two-host tick *R. e. evertsi*, the three-host ticks *Amblyomma hebraeum* and *R. appendiculatus* as well as the one-host tick *R. (Boophilus) decoloratus* are good candidates. These tick species were recorded from a great variety of host species and are geographically widely distributed in southern Africa (Walker et al., 2000, 2003).

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Therefore, in this study, we focused on the presence of *Babesia*, *Theileria*, *Anaplasma*, and *Ehrlichia* in the salivary glands of different tick species collected from wild and domestic ruminants in South Africa. Detection of pathogens in salivary glands of ticks allowed us to distinguish pathogens that infected the ticks before they attached to the hosts from those taken up by ticks during their blood meal. Thus, this approach allows to evaluate the exposure of wild and domestic ruminants to tick-borne pathogens in their respective environments. The presence of a pathogen in tick salivary glands strongly suggests that it may be transmitted to the host, but this is not sufficient to prove its vector role, which would require transmission experiments. The purpose here was (i) to evaluate the possible role of ticks in the transmission of recently described pathogen species, (ii) to verify whether pathogens with a broad host range have a broader vector range than currently known, (iii) to investigate the exposure of game and livestock to tick-borne pathogens in their respective environments, and (iv) to obtain information on coinfections in ticks.

Materials and methods

Study areas

Ticks were collected in 2009 (May to July), 2010 (January to May), and 2011 (April to June) at 6 localities in 3 South African Provinces. In the Free State, 3 provincial nature reserves, Tüssen-Die-Riviere, Willem Pretorius, and Sandveld, as well as several livestock farms at their surroundings were investigated (highveld). In the Mpumalanga Province, one game farm and one livestock farm were investigated near Bethal (highveld). Finally, 5 game farms and 5 livestock farms were investigated in the Limpopo Province in the Thabazimbi and Lephalale areas (lowveld).

Tick sampling

At each locality, tick samplings were performed on domestic and wild animals within the same week. Ticks were sampled from an equal number of livestock animals living on farms sharing a common border with the reserves or game farms or located in the close surroundings (within the range of 40 km). Ticks were collected from game animals during game capture, culling operations, and hunts. Cattle were maintained in holding facilities during sampling, and sheep were immobilised by hand in small camps. Hosts were visually examined for ticks; palpation helped to localise specimens attached on flanks, back, belly, neck, and legs. All the found ticks were removed with tweezers. Tick identification was performed according to Matthysse and Colbo (1987) and Walker et al. (2000, 2003). Ticks were pooled per species, host, and developmental stage and stocked in alcohol in 50-ml labelled tubes.

Detection and identification of pathogen species in tick salivary glands

From each individual host, a maximum of 10 males and 10 females from each tick species was analysed. To distinguish pathogens that had infected ticks before they attached to the host from those taken up by ticks during their current blood meal, each tick was dissected and the salivary glands of dissected ticks were analysed for pathogens. Salivary glands were carefully removed with tweezers – special attention was paid to avoid contamination with the midgut, and washed twice in PBS in 96-well plates (Milian®, Geneva, Switzerland). Instruments were sterilised for a few seconds in 5 M HCl and 5 M NaOH (Aktas et al., 2009) and dried with sterile wipes between each dissection.

DNA from tick salivary glands was extracted using QIAamp® DNA Micro kit (Qiagen, Hombrechtikon, Switzerland) following the manufacturer's instructions with modifications. Tissue lysis buffer and proteinase K were added in the 1.5-ml tubes before the salivary glands. DNA was stored at −20 °C.

An approximately 500-base pair (bp) fragment of the 16S ribosomal RNA (rRNA) gene spanning the hypervariable V1 region of the genera *Anaplasma* and *Ehrlichia* and an approximately 400-bp fragment of the 18S rRNA gene spanning the V4 hypervariable region of the genera *Babesia* and *Theileria* were amplified by PCR (Tonetti et al., 2009). Positive control, included in each run, consisted of DNA of *A. phagocytophilum* (provided by Ana Sofia Santos, Instituto Nacional de Saude, Lisboa, Portugal) and *B. divergens* (provided by Simona Casati, Institut Cantonal de Microbiologie, Bellinzone, Switzerland). PCR products were analysed using reverse line blotting (RLB) (Tonetti et al., 2009). In addition to the original 15 oligonucleotide probes, which are listed in Tonetti et al. (2009) (2 genus-specific Babesia/Theileria and Anaplasma/Ehrlichia, and 13 species-specific), 26 probes were added: one genus-specific Theileria spp. (Nagore et al., 2004) and 25 species-specific probes, B. ovis, B. crassa (Schnittger et al., 2004), B. major (Georges et al., 2001), Babesia sp. (sable) (Oosthuizen et al., 2008), B. caballi (Butler et al., 2008), B. occultans (Ros-Garcia et al., 2011), B. orientalis (Hea et al., 2011), B. gibsoni (from Pfitzer, 2009), B. rossi (Matjila et al., 2004), B. bicornis (Nijhof et al., 2003), B. motasi, Theileria sp. (greater kudu), Theileria sp. (sable) (Nijhof et al., 2005), T. separata, T. lestoquardi, T. ovis (Schnittger et al., 2004), T. buffeli (Gubbels et al., 1999), T. bicornis (Nijhof et al., 2003), Theileria sp. (buffalo) (Oura et al., 2004), T. equi (Butler et al., 2008), T. annulata (Georges et al., 2001), Ehrlichia sp. (Omatjenne) (Bekker et al., 2002), and 4 A. phagocytophilum (from Pfitzer, 2009) that replaced the original degenerated probe. Samples reacting only with the Babesia/Theileria probe were considered *Babesia* spp. since a genus-specific probe was included for the genus *Theileria*. To test for theoretical specificity, oligonucleotide probes were aligned with various sequences of targeted species available from the National Centre for Biotechnology Information (NCBI) using a software package: CLC Sequence Viewer 6 (CLC bio, Aarhus, Denmark).

Sequencing

PCR products that reacted only with genus-specific probes Babesia/Theileria, Theileria spp., or Anaplasma/Ehrlichia and did not hybridise with the panel of species-specific probes were sequenced. Prior sequencing, PCR products were purified using Wizard® SV and PCR Clean-Up System (Promega, Madison, USA) following the manufacturer's instructions except that we eluted with 35 μl rather than 70 μl Nuclease-Free Water. Sequencing was performed by Microsynth AG (Balgach, Switzerland). Sequences were compared and corrected with CLC Sequence Viewer 6 (CLC bio, Aarhus, Denmark) and Bioedit (Tom Hall Ibis Biosciences, Carlsbad). Corrected sequences were compared with available sequences retrieved from GenBank using NCBI Basic Local Alignment Search Tool (BLAST).

Data analysis

Data were analysed with "R" 2.14 for Windows (R Development Core Team, 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL: http://www.R-project.org/), using software packages (Skaug et al., 2010; Husson et al., 2012). A generalised linear mixed model (GLMM) with negative binominal errors was used to evaluate tick salivary gland infection (taking into account the 4 main infected tick species) collected from wild and domestic ruminants originating from 6 localities. In each locality, the significance of the factors LOCATION and HOST TYPE (i.e. ticks infesting wild vs. domestic ruminants) as well as the