VECTOR IDENTIFICATION AND THEIR ROLE IN EPIDEMIOLOGY OF CANINE BABESIOSIS

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Canine babesiosis is increasing in incidence and prevalence and is now a major problem for dogs. During this study, a total of 6204 dogs were examined for babesiosis over a 12 month period round the year in Lahore and 2.62 % were found positive. The dogs were grouped on the basis of age, sex, season and breeds. The male dogs were-more prone to disease than female dogs (3.39 vs. 1.32%) whereas, the incidence of disease was higher in younger dogs (6.9%) than older age groups. Crossbreds were more prone to the infection (10.9%) than purebreds. However, none of them were completely resistant. Warm and humid season played a key role in the spread of disease. Predominant vector of the disease was Rhipicephalus spp. Canine babesiosis is caused by two distinct species of genus Babesia, the large Babesia canis and the small Babesia gibsoni. The former, is subdivided into three subspecies, that is, Babesia canis canis, transmitted by Dermacentor reticulates in Europe, Babesia vogeli transmitted by Rhipicephalus sanguineus in tropical and subtropical regions and Babesia canis rossi transmitted by Haemaphysalis leachi in South Africa (Uilberg et al., 1989). Babesia gibsoni has been reported from Asia, North America, Northern and Eastern Africa, Australia and Europe (Birkenheuer et al., 1999; Muhnickel et al., 2002; Criado-Fonnelio et al., 2003). The present study was undertaken to identify the vector species and the species of genus Babesia harbored by the ticks removed from the bodies of dog, by PCR-RFLP in and around Lahore. The most frequent tick genera found was Rhipicephalus from 491 (97.2%) dogs out of 507. Genus Dermacentor was found on 14 dogs (0.08%), and Hemaphsalis was found on 2 dogs (0.004 %). Out of the total ticks (507) studied 401 (82%) Rhipicephalis sanguineus ticks harbored Babesia gibsoni, 10 (71 %) Demacentor reticulates contained Babesia canis vogeli, while Haemaphysalis leachi contained no babesia in their bodies. This indicates that the babesial species harbored by the ticks seems to be species specific.

Introduction

The incidence of canine babesiosis is on increase causing severe disease throughout the World due to global warming (Muller et al., 2010). It has got zoonotic potential as well (Spolidorio et al., 2010). Until now there is no evidence in Pakistan about the zoonosis causes by any of species of geninus Babesia. However, Babesia divergens in Europe and Babesia microti have been reported from many countries to be zoonotic (Gray, 2004).

The disease is characterized by haemolytic anaemia, icterus, and haemoglobinuria. Canine babersiosis is caused two distinct species of genus Babesia, the large Babesia canis and the small Babesia gibsoni. The former, is subdivided into three subspecies, that is, Babesia canis canis, transmitted by Dermacentor reticulates in Europe, Babesia vogeli transmitted by Rhipicephalus sanguineus in tropical and subtropical regions and Babesia cants rossi transmitted by Haemaphysalis leachi in South Africa (Uilberg et al., 1989). Babesia gibsoni has been reported from Asia, North America, Northern and Eastern Africa, Australia and Europe (Birkenheuer et al., 1999; Muhnickel et al., 2002; Criado-Fonnelio et al., 2003). The present study was undertaken to identify the vector species and the species of babesia harbored by the ticks removed from the bodies of dog, by PCR-RFLP in and around Lahore.

Materials and Methods

A total of 100 dogs infested by ticks were included in this study and 507 ticks were removed from various parts of the body of dogs, and collected in the specimen bottles. The ticks were identified using the method described by Soulsby (1982). For identification of various species of genus Babesia, the ticks were chopped finely, separately and about 1 ul material was applied on Flinders Technology Associates (FTA) Classic Cards (Whatman' International Ltd, UK), which were cut into one cm wide strips (vertically). Each time a sterile blade was used to avoid DNA contamination of card. The impregnated cards were stored at room temperature in sealed plastic bag containing silica

desiccant, until analyzed for the presence babesia species.

Nested set of primers (Table-1) was used to amplify a partial region of the 18S rRNA gene of Babesia species (Thompson et al., 1994) alignment was performed using complete 18S rRNA gene sequences of c, available from the Gene Bank database. An external and internal set of primers (Table-1) were designed on the basis of conserved regions of DNA between the aligned sequences using Amplify 2.1 (Eagles, W., University of Wisconsin, Madison). The external primer set amplified an approximately 90bp product, while the internal set amplified an approximately 800bp product (Jefferies, et al., 2003).

One µl of 50 g/µ1 Proteinase K (Master Pure, Madison, Wisconsin) was diluted into 150 µlof 2X T and C lyses solution (Master Pure, Madison, Wisconsin) for each sample. The sample was transferred to a micro-centrifuge tube and 151 µl of 2X T and C lyses solution containing the Proteinase K was added and mixed thoroughly. Then 150µ1 of 1 xPBS was added to the sample and mixed on vortex. The mixture was incubated at 55^0C for overnight; and then vortexed 5 times. Next day the sample was incubated on ice for 5 minutes. One hundred and seventy five microlitre of MPC Protein Precipitation Reagent (Master Pure, Madison, Wisconsin) was added to 300 µl of lysed sample and mixture was mixed vigorously on the vortex for 10 seconds. The debris was pelleted by centrifugation for 10 minutes at 10,000 rpm in a micro-centrifuge. If the resultant pellet was clear, or small, or loose, an additional 25 µl of MPC Protein Precipitation Reagent, was added and mixed, the debris was pelleted again. The supernatant was transferred to a clean labeled 1.5 ml micro-centrifuge tube and pellet was discarded. Five hundred microlitre of isopropanol was added to the recovered supernatant. All tubes were inverted 40 times. The DNA pelleted by centrifugation for 10 minutes at 10,000 rpm. Isopropanol was carefully pipetted off without dislodging the DNA pellet. Pellet was rinsed twice with 75% ethanol, carefully without dislodging the pellet. All of the residual enathol was removed with a pipette tip or tissue. The DNA was re-suspended in a 20 µl of TE Buffer. The sample was mixed on vortex and left at room temperature approximately for an hour to rehydrate the DNA pellet. The protocol recommended by the Whatman is available at http://www.whatman.com/ FTANecleicAcidCollectionStorageandPurification.aspx.

The DNA was amplified and subjected to RFLP as mentioned latter. The sensitivity of the extraction
method and the limit of detection were assessed by cutting the sample applied to the card according to their weight, which were 0.5g, 1 g, 1.5g, 2g, 2.5g.

The tick samples were subjected to PCR-RFLP to detect Babesia gibsoni and Babesia canis vogeli, as single infection or co-infections of both the Babesia species. The primer sequences were BLAST, to find out whether they do or do not amplify any other DNA.One µl of extracted DNA was added to a 24 µl reaction mixture comprising 0.6875 units of the taq Plus DNA polymerase (Fisher Biotech, Australia), 200 µM of each dNTP, 12.5 pico moles of the forward and reverse primers (Invitrogen, Australia), 2.5µ1 10x PCR buffer (Fisher Biotech, Australia), and 1.5µ1 MgCI2 (Fisher Biotech, Australia), Positive (1 µl of B.gibsoni or B. canis DNA) and negative (1 µl of dH2 0) control samples were included with each set of PCR reactions.

Amplification was performed on a GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, USA). For the primary round of amplification, an initial activation step at 94°C for 3 minutes, 58°C for 1 minute, 72°C for 2 minutes, was followed by 45 cycles of amplification (94°C for 30 seconds, 58°C for 20 seconds, 72°C for 30 seconds) and a final extension step of 72°C for 7 minutes for 25µ1 reaction. The same conditions were followed for secondary round of amplification, except that the annealing temperature was increased to 62°C, using 1 µl of DNA template from the primary reaction (Jefferies et al., 2003).

Gel electrophoresis of PCR products was performed using 1 % agarose (Promega, Madison, USA) gels in TAE buffer (40mM Tris-HCL, 20mM acetate, 2mM EDTA). Gels were restained with ethidium bromide (Amresco, USA). A 1 kb bp molecular weight marker (Promega, Madison, USA) was run for all gels to

determine the size of PCR product. Electrophoresis was performed using a Minisub electrophoresis cell (Biorad) at 90 V for 40 minutes and DNA was visualized by UV transillumination.

Purification was carried out using the freeze squeeze method. Amplified DNA was electrophoresed on a 1.5% agarose gel (90 V, 40 minutes). The gel was viewed under ultra-violet light and the appropriate sized band was cut out using a scalpel blade. Each band was cut out using a separate scalpel blade to avoid contamination of DNA. The bands were each placed in separate 1.5ml eppendorf tubes and allowed to freeze overnight. The gel was squeezed between 2 folded halves of parafilm to extract liquid. This liquid was placed in fresh eppendorf tube. To a tenth of the extract volume, 3M sodium acetate was added and to double the extract volume, 70% ethanol was added. The mixture was vortexed and then centrifuged at a 14000 rpm for 5 minutes. The supernatant was discarded and the pellet was vacuum dried. Finally, 10µl of water were added to the dried pellet.DNA was sequenced using an ABI PrismTM Dye Terminator Cycle Sequencing Kit (Applied Biosystems, (ABI). Foster city, California) according to the manufacturer's instructions, with the following modifications: Amplification was carried out in 10 µ1 reaction mixture containing the following: 1 µl of the forward or reverse primer diluted to 3.25pmol/ µl, 1 µ1 of dye terminator solution, 1.5 µ1 of sequencing buffer (Genpak Inc., Stony Brook, New York), 5.5 µ1 of H20 and 1 µ1 of purified template. 38 cycles of amplification (96 ^{o}C for 2minutes, 96 ^{o}C for 10 seconds, 60 ^{o}C for 5 seconds and 60 ^{o}C for 4 minutes) was preceded by an initial denaturation of 60 ^{o}C for 1 minute and followed by a holding temperature of 15 ^{o}C.

Transferred the amplified DNA to 0.6ml tube. To 10 µl amplified DNA, Twentyfive microlitres of 100% ethanol, 1 µl of 125mM EDTA and 1 µl of 3M sodium acetate were added. This solution was mixed gently using a pipette, then incubated at room temperature for 20 minutes and then centrifuged for 30 minutes at 14000 rpm. The supernatant was carefully removed with a pipette and 125 µ1 of 70% ethanol was added to the remaining precipitate. The tube was spun for 5 minutes at maximum speed. The supernatant was carefully removed and the insides of the tubes were dried with kimwipes (Kimberly-Clark, Australia Pty. Limited). The sample was vacuumed dried in a vacuum desiccator for 1 hour.

The sequenced products were analyzed using the program seqEd v.1.0.3 (ABI) and were compared to sequence data available from GenBankTM, using the BLAST 2.1 program (http:// www.ncbi.nlm.nih.gov/BLAST/).

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP).

A restriction fragment length polymorphism (RFLP) technique was designed to permit discrimination between each of the canine piroplasm species and, in particular, Babesia canis and Babesia gibsoni in ticks picked from the dogs in Lahore. Complete sequences of the 18S rRNA gene each of the canine species and subspecies available on GenBank database were imported into the program Amplify 2.1 (Engels, W., University of Wisconsin, Madison) and the target region of DNA was determined using the internal primer set (BTF2 and BTR2). The sequence of the amplified internal PCR product was then used in DNA Strider^{TM}1.0 (Marck, 1988) to determine the most suitable restriction enzymes for discriminating between the canine Babesia species. Six µl of amplified DNA from the secondary PCR reaction was subjected to restriction enzyme digestion in a reaction mixture of 16.3 µ1 of dH20, 2.0 µl of Buffer B (Promega, Madison, USA), 0.2 µl of Bovine serum albumin acetylated (Promega, USA). The reaction mixture was gently mixed 0.5 µ1 of the appropriate restriction enzyme, (Hinf I, Hinc II or Ava II) (Promega, USA) was added and then incubated at 37^{0} C for 2 hours. Restriction Products were then subjected to electrophoresis at 80 volts for 1 hour on a 3% agarose gel (Promega, USA) stained with Ethidium bromide and visualized using UV illumination.

Table 1: External and Internal primer sets for the amplification of a partial region of the 18S rRNA gene of most Piroplasmida species.

Primer name	Sequence

BTF1 (external)	5’ GGCTCATTACAACAGTTATAG 3’
BTR1 (external)	5’ CCCAAAGACTTTGATTTCTCTC 3’
BTF2 (internal)	5’ CCGTGCTAATTGTAGGGCTAATAC 3’
BTR2 (internal)	5’ GGACTACGACGGTATCTGATCG 3’

Results and Discussion

The data regarding the ticks removed from the dogs in and around Lahore. The most frequent tick genera found was Rhipicephalus from 491 (97.2%) dogs out of 507. Genus Dermacentor was found on 14 dogs (0.08%), and Hemaphsalis was found on 2 dogs (0.004 %). Out of total 507 ticks 274 were males and 234 were females.

Table 2: Showing Various Genera of Ticks on Dogs

Tick Genus	Number of Ticks
Amblyomma	-
Boophilus	-
Hyalomma	-
Rhipicephalus	491
Dermacentor	14
Ixodes	-
Haemaphysalis	2
Total	507

Table 3: Various species of Genus Babesia recovered from Ticks

Tick Genus	B. canis canis	B. canis vogeli	B. canis rossi	B. gibsoni
Rhipicephalus sanguineus		10(8 females and 2 males)		401(395 females and 6 males)
Dermacentor reticulatus				
Haemaphysalis leachi				

The diagnostic tests for babesiosis can be divided into three categories; the conventional tests including microscopy and culture, serological techniques, and molecular based tests. A combination of detection techniques is desirable. In the present study morphological and molecular techniques were used to identify the Babesia species.

Out of the total ticks (507) studied 401 (82%) Rhipicephalis sanguineus ticks harbored Babesia gibsoni, 10 (71 %) Demacentor reticulates contained Babesia canis vogeli, while Haemaphysalis leachi contained no babesia in their bodies. This indicates that the babesial species harbored by the ticks seems to be species specific. The results obtained by nested PCR-RFLP were confirmed by sequencing of amplified DNA sequences.

Canine babesiosis is caused two distinct species of genus Babesia, the large Babesia canis and the small Babesia gibsoni. The former, is subdivided into three subspecies, that is, Babesia canis canis, transmitted by Dermacentor reticulates in Europe, Babesia vogeli transmitted by Rhipicephalus sanguineus in tropical and subtropical regions and Babesia canis rossi transmitted by Haemaphysalis leachi in South Africa (Uilberg et al., 1989). Babesia gibsoni has been reported from Asia, North America, Northern and Eastern Africa, Australia and Europe and Eastern Africa, Australia and Europe (Birkenheuer et al., 1999, Muhlnickel et al., 2002 and Criado-Fornelio et al., 2003). Gray et al. (2004) has reported that babesiosis is most prevalent arthropod disease of humans in the temperate northern hemisphere. The main parasites involved are Babesia divergens in Europe and Babesia microti in USA diagnosed by new molecular tools.

Growing incidence of canine babesiosis was recorded in Slovakia during the last decade. The highest prevalence of B. canis canis was observed in D. reticulates, from eastern Slovakia, (14.7%; n=1250). Notably, all the 874 D. reticulates collected from low lands were negative for any Babesia canis species (Kubelona et al., 2011). The findings of present study are not in agreement with the findings of latter

workers. This might be due to geographical factor.

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