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EVALUATION OF DNA EXTRACTION PROTOCOLS FROM IXODID TICKS

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Abstract: Ticks are important vectors of disease in man and animals and transmit an extensive array of viral, bacterial and protozoan pathogens. Molecular detection of pathogens in ticks could be accomplished only by efficient nucleic acid extraction. The aim of this study was to compare the salting out procedure, commercial kit method and phenol-chloroform procedures for DNA extraction from ticks. The results indicated that salting out method is an alternative for the extraction of the genomic DNA from the ticks as it allowed eliminating all the blood residuals, debris, and other possible inhibitory materials from the ticks and permitted detection of DNA from tick-borne pathogens.
Keywords: Tick, DNA Extraction, Salting out method.

Introduction

The distribution of ixodid ticks of the genera *Rhipicephalus* and *Haemaphysalis* spp. in the tropical climate of Kerala state contributes to many serious tick borne parasitic and rickettsial infections such as babesiosis, ehrlichiosis and hepatozoonosis in domestic and wild livestock. Identification of ticks is typically based on morphological examination which demands considerable technical expertise (Barker and Walker, 2014). Screening of ticks for pathogens by using conventional techniques is also quite demanding in terms of time and expertise. Molecular tools such as PCR provide a better alternative to species identification, pathogen detection and may serve as reliable indicators of tick-borne pathogens in particular geographic environment. Nucleic acid extraction is key to such molecular approaches. Several commercial and non-commercial approaches have been reported for DNA extraction from ixodid ticks. Commercial kits were utilised for DNA extraction from ticks for detection of various pathogens *viz.*, *Anaplasma platys* (Inokuma *et al*., 2000), *Babesia gibsoni* (Trapp *et al*., 2006), *Babesia* spp. (Blaschitz *et al*., 2008), *Ehrlichia canis* (Masala *et al*., 2012), *Rickettsia* spp, *Borrelia* spp and *Babesia* spp (Schreiber *et al*., 2014) and spotted fever group

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rickettsiae (Kumsa *et al*., 2015). Alternate method of DNA extraction from ixodid ticks including mechanical crushing and enzymatic digestion followed by phenol chloroform protocol was compared with commercial kit method by Halos *et al*. (2004). Rodriguez *et al.* (2014) had compared four variants of salting out procedure for DNA extraction from ixodid ticks, at different stages of their life cycle, with phenol chloroform and ammonium hydroxide methods. The present communication places on record the results of a study designed to compare and evaluate the different DNA extraction protocols from adult ticks recovered from dogs in terms of efficiency based on quality and quantity of DNA for downstream processes.

Materials and methods

Extraction of DNA is a preliminary step for testing the vector potentiality of ticks by molecular screening. *Rhipicephalus sanguineus* (n=126) and *Haemaphysalis spp* (n=36) ticks were collected from dogs presented to the University Veterinary hospitals of Mannuthy and Kokkalai as well from local kennels of Thrissur. They were morphologically identified as per the standard keys (Sen and Fletcher, 1962) and subjected to DNA extraction. Three to four ticks of the same species obtained from the same animal formed a tick pool. In this study, we compared three different extraction protocols based on an enzymatic digestion by proteinase K.

Protocol 1: Kit Method

Genomic DNA extraction was performed using the Qiamp DNA extraction kit for tissue protocol (Qiagen, Hilden, Germany). The ticks were first roughly torn to pieces in 180 µL ATL buffer (provided in the kit) and treated with proteinase K (20 mg/mL) for 16 h (56 °C). The subsequent steps were carried out according to the manufacturer's instructions.

Protocol 2: Phenol-chloroform Method

The ticks were either dissected and homogenized or inserted into liquid nitrogen and then ground into a fine powder in a mortar pre-chilled with liquid nitrogen. The homogenised tissue/powder was transferred into a microfuge tube containing 600 µl of ice cold lysis solution and homogenised quickly with a microfuge pestle. Then, added 25µl of proteinase-K into the lysate and incubated at 55°C for at least 3 h and not more than 16 h. DNA extraction was then followed by phenol-chloroform method(Sambrook and Russell., 2001; Aravindakshan, *et al*., 1998). The DNA was then precipitated with absolute ethanol (two volumes) and resuspended in TE buffer.

Protocol 3: Salting-out Method

The protocol of Rodriguez *et al*. (2014) was adopted with modifications. The ticks were either dissected and homogenized or inserted into liquid nitrogen and then ground into a fine powder in a mortar pre-chilled with liquid nitrogen. The homogenised tissue/powder was transferred into a microfuge tube containing 600 µl of ice cold lysis solution and homogenised quickly with a microfuge pestle. Then, added 25µl of proteinase-K into the lysate and incubated at 55°C for at least 3 h and not more than 16 h. The digest was allowed to cool to room temperature and added 20 µl of 4 mg/ml DNase free RNase enzyme (Sigma Aldrich, USA) and incubated at 37°C for 15- 60 min. Allowed the contents to cool to room temperature and then 200 µl of potassium acetate solution was added. Mixed contents vigorously for 20 sec in a vortex. The protein/SDS complex was pelletted by centrifugation at maximum speed for 3 min at 4°C in a microfuge. When the pellet was not visible at the bottom of the tube, the lysate was incubated on ice for 5 min and repeated the centrifugation step. Transferred the supernatant into a fresh tube containing 600 µl of isopropanol. Mixed the solution and recovered the precipitate of DNA by centrifugation at maximum speed for one minute at room temperature. The supernatant was then removed by aspiration and added 600 µl of 70 % ethanol. Mixed the contents by inversion several times and centrifuged at maximum speed for one minute. Then the supernatant was removed by aspiration and dried the pellet in room temperature for 20 min. The pellet of DNA was re-dissolved in 100 µl TE (pH 7.6) by keeping at 65°C for 1 h or at room temperature for 16 h.

The efficiency of each nucleic acid extraction was evaluated by electrophoresis in 0.8% (w/v) agarose gels containing ethidium bromide and visualized under ultraviolet light, nanodrop spectrophotometry (Nanodrop 2000 UV-Vis, Thermo Scientific USA) for quality and quantity of DNA and amplification of 18S rRNA fragment for *Babesia canis vogeli* using the specific primers as per Duarte *et al*. (2008) was done by PCR. PCR amplicons were electrophoresed in 2% (w/v) agarose gels.

Results and Discussion

Genomic DNA could be successfully extracted from 67% tick pools with protocol 1, 87% tick pools with protocol 2, and 100 % tick pools with protocol 3. The yield of DNA was lowest for the first protocol. The extraction efficiency using salting out was significantly higher than other two methods based on the yield of DNA and quality. The absorbance ratio at 260 nm and 280 nm, obtained for all the DNA samples by protocol 3 were in between 1.79 and 1.99, indicating good quality. Hard ticks possess a chitinous exoskeleton which must be

broken prior to DNA extraction. Moreover, genomic DNA appeared to be highly susceptible to degradation (Rodriguez *et al*., 2014). Fine crushing prior to enzymatic protein digestion ensures mechanical destruction of polysaccharide chains of chitin (Halos *et al*., 2004).

The engorged ticks may contain PCR inhibitors, the removal of which is imperative for successful pathogen detection by molecular techniques. To ensure the absence of PCR inhibitors in the DNA extracted from the ticks by salting out method, PCR for amplifying tick borne parasites was attempted. The DNA from *Rhipicephalus sanguineus* collected from known parasite positive dogs were tested by PCR for the screening of *Babesia canis.* Specific amplification products of 590bp was obtained from template DNA obtained by salting out method (Fig.1). The tick template DNA samples extracted by other two method yielded positive amplicons upon PCR in very few cases due to the lack of purity of the DNA sample or due to the presence of PCR inhibitors. Several studies suggested that the genomic DNA obtained by the salting out procedure from mosquitoes and triatomines has an excellent quality as target in molecular techniques based on amplification by the PCR such as the random amplified polymorphic DNA analysis (Fraga *et al*., 2004; De Armas *et al*., 2005; Fraga *et al*., 2011). Taking in to account the advantages of salting out procedure over the phenol-chloroform and commercial kit method the former would be a rapid and reliable method of tick DNA extraction useful for poorly resourced laboratories. It would serve as an useful adjunct to assess the epidemiological status of tick borne pathogens of man and animals and to map endemic areas.

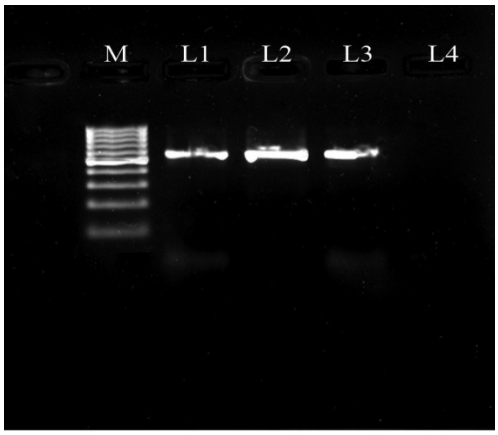

Figure 1 –Amplicons of *B.canis vogeli* (590bp) Lane: M, 100bp-ladder (Promega); Lane 1-3 tick DNA extracts using salting out method, Lane 4-NTC.

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