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Evaluation of preservation and extraction methods of total DNA from "hard ticks" (ixodidae family)

Abstract

This study evaluated the methods for preserving and extracting total DNA from ticks of the family Ixodidae, which are important pathogen vectors. Different preservation techniques, including isopropyl alcohol and RNAlater®, and DNA extraction methods (HotSHOT, acetate/acetic acid, salting-out, and phenol-chloroform) were used. The effect of maceration on the process of obtaining total DNA was also evaluated. Significant differences were absent between the methods performed with and without maceration. Among the extraction techniques evaluated, the HotSHOT and phenol-chloroform methods stood out, with no significant difference between the two. However, as the HotSHOT technique without maceration is inexpensive and requires fewer steps, it was considered superior to the phenol-chloroform technique. Regarding preservation, greater DNA amplification was obtained with RNAlater® than with isopropyl alcohol and was more beneficial for maintaining DNA amplification even after exposure to different conditions (storage time at room temperature [24–30°C] or freezing). These results highlight the importance of adequate preservation for successful molecular research, demonstrating the efficiency of RNAlater®. Our findings emphasize the relevance of appropriate extraction and preservation techniques for molecular research on ticks and contribute to an understanding of the most effective methods in this context.

Keywords: extraction of genetic material from ixodids, RNA later, HotSHOT, conservation, molecular analysis

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Abbreviations

PCR, polymerase chain reaction

Introduction

In the group of arthropods responsible for carrying hemoparasites, ticks (class *Arachnida*, subclass *Acari*, order *Ixodida*) are highly important agents, as they require blood at least one stage in their life cycle for their survival. Prolonged attachment to the host facilitates the transmission of viral, bacterial, and parasitic pathogens.[1] The scientific field has undergone remarkable growth with the development of molecular techniques for research linked to the detection of various pathogens transmitted by ticks. Polymerase chain reaction (PCR) is one of the most commonly used methods, owing to its high sensitivity and specificity.[2,3] Despite its advantages, PCR has limitations such as the use of inhibitors. However, major problems may arise before the technique is implemented, such as the challenges of sample preservation because of the susceptibility of tick DNA to degradation and total DNA extraction from arthropods owing to the presence of a hard chitinous exoskeleton and small amounts of microbial nucleic acids.[4–6] These challenges are even more worrisome when extracting DNA from pathogens such as rickettsiae and borreliae because of the small amounts within the initial life-cycle stages of the tick.

Different methods of isolating tick DNA have been investigated and evaluated,[4,5,7–11] corroborating the advancement of new experiments. However, a consensus on the most effective method of isolating total tick DNA, as well as the preservation method, is lacking. The aim of the present study was to evaluate preservation methods (isopropyl alcohol and RNAlater®), use of ixodid maceration, and total DNA extraction methods (HotSHOT, acetate/acetic acid, salting-out, and phenol-chloroform) in total DNA amplification of ixodids.

Materials and methods

Samples

A total of 164 ticks of different species (*Rhipicephalus sanguineus*, *Amblyomma sculptum*, and *Rhipicephalus microplus)* in various life-cycle stages (larva, nymph, and adult) were used. The analyses were performed using 100 larvae (50 individuals preserved in isopropyl alcohol and 50 preserved in RNAlater® at -20°C) of *R. sanguineus*, 20 nymphs and 20 adults (10 males and 10 females) of *A. sculptum*, and 24 engorged females of *R. microplus* collected from the floor of cattle stalls. The specimens were divided into 15 groups (G1–G15). Groups G1 to G5 comprised *R. sanguineus* larvae and were used to evaluate the extraction methods. Groups G6 to G11 consisted of *A. sculptum* nymphs and adults and were used to evaluate the HotSHOT extraction method. Engorged *R. microplus* females were distributed across groups G12 to G15 and were used to evaluate the preservation of tick DNA using RNAlater®.

Extraction methods in larvae

The extraction methods for ticks were evaluated by dividing *R. sanguineus* larvae into five groups, each group containing 10 larvae in isopropyl alcohol and 10 larvae in RNAlater® at -20 °C, all preserved for 7 d. Total DNA for each group was extracted according to the manufacturer's protocol. For all groups, larvae were dried on paper towels and individually placed in 1.5 mL polypropylene tubes. The specimens were previously washed with 300 µL of sterile distilled water to remove alcohol and RNAlater®. Total DNA from groups G1 (larvae without previous maceration) and G2 (larvae with previous maceration) was extracted using the HotSHOT method[12] adapted for tick extraction. The larvae were washed in autoclaved phosphate-buffered saline (PBS+TWEEN20), followed by addition of 150 µL

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of PBS+TWEEN20, and then left overnight at -20 °C to remove excess fixative and rehydrate the samples. The following day, all PBS+TWEEN20 was removed and 150 µL of PBS+TRITON was added, followed by centrifuging for 30s. Next, all the buffer was removed and 70 µL of alkaline lysis solution (25 mM NaOH and 0.2 mM EDTA; pH = 12) was added (after maceration in G2). The tube was then centrifuged at 6000 rpm for 15 s and placed in a Thermo-Shaker® at 99 °C for 60 min.

After this step, all tubes were removed, centrifuged for 15s, and partially covered with ice flakes for 5 min. Then, 70 µL of neutralizing solution (40 mM Tris – HCl, pH = 5) was added. This material was then centrifuged for 10 s and 100 µL was transferred to another sterile 1.5 mL Eppendorf tube and then stored at -20 °C for later evaluation of the extraction using PCR as described below. For groups G3, G4, and G5, larvae without previous maceration were placed individually in 400 µL of digestion buffer (20 mg/mL Proteinase K, 20 mM Tris – HCl, 20 mM EDTA, 400 mM NaCl, 1% sodium dodecyl sulfate, and 10 mM $CaCl_2$). Digestion was performed overnight at 56 °C. After digestion, the samples were subjected to one of the following DNA extraction methods:

Total DNA from group G3 was extracted using the method described by Sambrook et al.[13] with some modifications. To the solution containing the digested larvae, 240 µL of protein precipitation solution (3M potassium acetate, 11% acetic acid) was added. After vigorous shaking, 160 µL of chloroform was added, followed by another vigorous shaking and centrifuging at 16,000 × *g* for 10 min. The supernatant was transferred to a new tube containing 400 µL of phenol: chloroform (1:1) and shaken and centrifuged again at 16000 × *g* for 10 min. The supernatant was then subjected to DNA precipitation and washing. DNA extraction from group G4 was performed using the methodology described by Miller et al.,[14] with some modifications. Four hundred microliters of 5 M NaCl were added to the digested material and mixed vigorously, followed by centrifuging for 10 min at 16000 × *g*. The supernatant was then transferred to a new tube for DNA precipitation and washing. For DNA extraction from group 5 (G5), the protocol described by McIntosh et al.[15] was followed, with some modifications. Five hundred microliters of phenol: chloroform (1:1) were added to the digested material, followed by vigorous shaking and centrifuging at 16000 × *g* for 10 min. The supernatant was transferred to a new tube containing 400 µL of phenol/chloroform (1:1), shaken and centrifuged again at 16000 × g for 10 min. The supernatant from the second round was washed and used for DNA precipitation.

Precipitation and washing of total tick DNA

DNA precipitation and washing were performed in the same manner in groups G3, G4, and G5. Isopropanol (700 µL) was transferred to the supernatant obtained from the previous step and gently homogenized by inversion. The mixture was centrifuged at 16000 × *g* for 4 min. The supernatant was then discarded by inversion and the pellet formed was washed with 1000 µL of 100% ethanol (first wash) and 1000 µL of 70% ethanol (second wash); the solution was centrifuged at 16000 × *g* for 2 min in each wash. After drying on a stove at 56 °C, the DNA present in the pellet was dissolved in TE buffer (10 mM Tris-base pH 8, 1 mM EDTA pH 8) and kept at 56 °C for 15 min followed by storage at -20 °C.

Evaluation of the HotSHOT method for A. sculptum nymphs and adults

To evaluate the HotSHOT DNA extraction method for nymphs and adults, ticks were divided into four groups. In this test, DNA of all ticks was extracted using the HotSHOT method as described above for groups G1 and G2. The characteristics of each group are described below. In group G6, before starting the DNA extraction, the specimens (10 nymphs of *A. sculptum*) were individually macerated. In group G7, specimens were not macerated (10 nymphs of *A. sculptum*). In group G8, the adult specimens (five males and five females of *A. sculptum*) were individually macerated before starting the DNA extraction. In group G9, specimens were not macerated (10 *A. sculptum* females). Groups G10 and G11 comprised 10 males each, wherein individuals from G10 were macerated, whereas those in G11 were not.

Evaluation of tick DNA preservation using RNA later®

To evaluate the preservation of ticks collected in the field using RNA later®, 24 females were collected and individually stored in 1.7 mL polypropylene tubes and divided into four groups: G12–G15. In group G12, six specimens were immediately frozen at -20 °C. For the remaining groups, 500 µL of RNA later® was added to the tubes containing the individual specimens and treated as follows: immediately frozen (G13); kept at a temperature of 2–8 °C for 8 d and then frozen (G14); and kept at room temperature (24–30 °C) for 8 d and then frozen (G15). Groups G12, G13, G14, and G15 were kept frozen for another 6 months at a temperature between -20 °C and -15 °C until DNA extraction. After washing the ticks with 1000 µL of sterile distilled water to remove RNA later®, the specimens were macerated with a pestle and the DNA extracted using the phenol-chloroform method (used for group G5) as described above.

Polymerase chain reaction

The DNA extracted from each sample of the 15 groups was subjected to PCR using primers that amplified a fragment of approximately 460 bp of the mitochondrial *16S rDNA* of ticks using the protocol described by Mangold et al. The reaction products were subjected to electrophoresis in a 1.5% agarose gel and stained with ethidium bromide (0.5 µg/mL). The amplified products were visualized under ultraviolet light using a transilluminator.

Statistical analyses

Statistical analyses of the different groups of ticks were performed using the parametric Fisher's Exact Test, with significance set at a level of 5%. Data relating to different extraction methods (HotSHOT, acetate/acetic acid, salting-out, and phenol-chloroform), preservation methods (isopropyl alcohol and RNAlater®), and tick maceration were compared.

Results

All evaluated methods allowed sufficient extraction of total DNA from the evaluated ticks for detection of mitochondrial *16S rDNA* by PCR. However, a significant difference was observed in extraction efficiency between the extraction and preservation methods for *R. sanguineus* larvae (Table 1). The results of the salting-out and acetate/acetic acid methods were contrary to those obtained with the other evaluated methods. When analyzing the differences between the two, the acetate/acetic acid method stood out compared with salting out as the best option (Table 1). However, a significant difference was absent between the HotSHOT and phenol-chloroform methods, although the HotSHOT method achieved 100% efficiency (Table 1). When comparing the RNA later® and isopropyl alcohol preservation methods, an amplification of 92% (46/50) and 66% (33/50), respectively, was achieved and the difference between the methods was significant (p = 0.0015). The HotSHOT method for DNA extraction from *A. sculptum* nymphs and adults preserved in isopropyl alcohol yielded 81.67%

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positivity (49/60). No significant differences were present between groups with and without maceration ($p = 1.000$) (Table 2). All groups of engorged *R. microplus* females (G12, G13, G14, and G15) exhibited 100% positivity (24/24), demonstrating that RNA later® is an efficient method for DNA preservation at room temperature (24–30 °C) for up to 8 d (G15).

Table 1 Comparison between different DNA extraction methods from *Rhipicephalus sanguineus* larvae

Techniques	Positive/Total	frequency
HotSHOT	40/40	100%
Acetate/ Acetic acid	13/20	65%
Salting out	9/20	45%
Phenol-chloroform	19/20	95%

Table 2 Assessment of amplification of hard ticks in relation to different conservation methods (isopropyl alcohol and RNA later ®), absence or presence of maceration and DNA extraction protocols (HotSHOT and Phenol-chloroform)

	Maceration	Positives/Total
Stage		
Nymphs		
G6	Yes	7/10
G7	No	7/10
Adults ♀		
G8	Yes	8/10
G9	No	9/10
Adults ♂		
G10	Yes	9/10
G11	No	9/10

Discussion

Proper preservation, extraction, and purification of tick nucleic acids are necessary steps in the field of molecular research. If these steps are not performed correctly, any subsequent molecular analysis procedure, and ultimately, the results, will be compromised. Therefore, it is important to evaluate and compare the most appropriate preservation and extraction techniques for molecular research. In the present study, the collected ticks were divided into several groups to analyze the potential of specific extraction and preservation techniques. Storage of samples in RNA later® presented the most benefits, as tick mitochondrial *16S rDNA* of the samples was adequately amplified, even after exposure to different temperatures and time periods (days and months). The samples stored in isopropyl alcohol presented some disadvantages, as in some techniques, amplification by PCR was absent.

Recent studies reinforce the superiority of RNA later® for preserving tick DNA, confirming its ability to maintain the integrity of genetic material in different environmental conditions and during prolonged periods of storage. This is crucial for long-term research, as well-preserved samples are essential for obtaining reliable and consistent molecular data.[16] Jose et al.[8] compared salting-out, phenol-chloroform, and commercial Qiamp DNA extraction kits for tissue techniques (Qiagen, Hilden, Germany) for DNA extraction from ixodid ticks. The results of that study suggested that the salting-out technique is an alternative method for genomic DNA extraction from ticks because it allows the removal of blood residues, debris, and other inhibitors and enables the detection of DNA from tick-borne pathogens. In our study, the results were contradictory with the technique demonstrating a lower efficiency than the other methods. A possible reason could be that reproducibility may vary between different laboratories, as some details may have been elaborated upon according to the executor's own experience.

Rodríguez et al.[17] tested four variants of DNA extraction protocols using potassium acetate in ixodid ticks at different life-cycle stages. These protocols were compared with extraction methods using phenol-chloroform and alkaline hydrolysis with ammonium hydroxide. All variants of potassium acetate extraction were efficient in obtaining quality DNA for PCR detection based on the tick *16S rRNA* targets. The authors of that study suggest that considering its advantages over the phenol-chloroform method, as verified in the comparison, the potassium acetate extraction method is a protocol that can be useful for DNA extraction from engorged ticks. In our study, samples processed with phenol-chloroform showed more than a 90% advantage over the salting-out and acetate-acetic acid techniques, which is important because it generates protein denaturation in an extremely efficient manner. Furthermore, a 100% efficiency was achieved with the HotSHOT technique without maceration when compared with the phenol-chloroform technique. Although the results of the two techniques did not present significant differences, the HotSHOT method without maceration stands out for its simplicity of execution and does not present a carcinogenic risk, unlike the phenol-chloroform method. In addition, the extraction process is reduced by one step because it does not require maceration of the specimens to obtain total DNA. Several studies have used the phenol-chloroform and HOTSHOT techniques to extract total DNA from ticks to detect pathogenic bacteria and protozoa.[18–21] Therefore, the evaluation and adaptation of these techniques is necessary to obtain more accurate results.

The HotSHOT method is extremely effective for small and resistant organisms, such as ticks, due to its ease of use and low risk of cross-contamination. When applied to parasites, shows high efficiency, allowing the recovery of quality DNA even under adverse preservation conditions.[12,22] In addition, it could be ideal for certain meta-genomic analyses in ticks and for identifying specific organisms in the microbiome of these arthropods.[23] The demonstration of efficiency in DNA detection by PCR in non-macerated larvae can help in the identification and description of new species, as the external morphology may not be altered. Therefore, the specimen can be evaluated, both molecularly and morphologically, because of the need for preparation on embalmed slides. The HotSHOT method was used to extract DNA from *A. sculptum* nymphs and adults fixed in isopropyl alcohol with or without maceration because fixation in alcohol increases the rigidity of the carapace of these ixodids, with the possibility of failure in the extraction of total DNA. In the present evaluation, a significant difference was absent; however, the non-amplification of some specimens compromised the detection of gene targets under these conditions. It is possible that the preservation method influenced total DNA extraction; however, this was not evaluated.

In a comparative study of techniques, the HotSHOT method overcame several limitations. The method was considered as simple, single-tube, and easy to implement; increases extraction efficiency by processing multiple samples simultaneously with a minimal risk of cross-contamination; and is successful in producing DNA samples for preservation over long periods.[12] Alasaad et al.[24] described the technique as advantageous owing to its characteristics of being fast, cheap, and easy to perform. Although that study was carried out with mites, the authors suggest that this technique may be widely applicable to the extraction of gDNA from other parasites with small sizes and hard bodies, such as ticks. The same features were

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highlighted by Senne et al.[22] who compared extraction techniques involving HotSHOT, salting-out, and phenol-chloroform in sandflies. HotSHOT showed great potential as a cheap and efficient technique for studying vector-pathogen interactions. Our findings also highlight the importance of proper preservation, extraction, and purification of tick nucleic acids for successful molecular research. The results revealed that storing samples with RNAlater® offers significant benefits. We described five extraction methods with important adaptations for use with ticks. The advantage of using at least two of these methods is presented, demonstrating their effectiveness compared to methods used in previous studies, in addition to demonstrating that RNAlater® is an efficient preservation method. In addition, our work shows that a tick can remain at least 8 d at room temperature (24–30 °C) under the protection of RNAlater®.

Conclusion

This study demonstrated that RNAlater® is an effective preservation method for ixodids, presenting high amplification, even at room temperature (24–30 °C). There were no significant differences between the methods evaluated with and without maceration. Regarding the extraction methods, HotSHOT and phenol-chloroform were demonstrated to be useful and efficient, with no significant differences between them. However, the HotSHOT method without maceration stands out because of its simplicity and low execution cost, in addition to reducing the total DNA extraction process by one step.

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Conflicts of interest

The authors declared that there are no conflicts of interest.

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