Persian J. Acarol., 2021, Vol. 10, No. 2, pp. 223–230.
https://doi.org/10.22073/pja.v10i2.64420
Journal homepage: http://www.biotaxa.org/pja

Determination of an efficient and reliable method for PCR detection of borrelial DNA from engorged ticks

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ABSTRACT
Since ticks have been recognized as one of the most important vectors of pathogens causing serious diseases in humans, a number of studies have focused on identifying the pathogen composition as well as transmission and infection mechanisms. Although a plethora of detection methods is available today, PCR-based approach is regarded as the most sensitive and rapid. However, common challenges in molecular analyses conducted on ticks are weak amplification results because of present inhibitors, either from a mammalian bloodmeal or a male DNA in female reproductive organs. Present study aimed to evaluate which body part of an engorged tick is the most preferable as a starting material in DNA extraction for molecular detection of *Borrelia burgdorferi sensu lato*, causative agent of Lyme borreliosis. We analyzed 58 *Ixodes ricinus* ticks removed from patients in The Center for Emergency Medical Assistance of the Sarajevo Canton, Bosnia and Herzegovina. Our findings suggest using the anterior half of semi-engorged and fully-engorged ticks for DNA extraction with the purpose of *Borrelia* detection.

KEY WORDS: anterior half of body; *Borrelia burgdorferi sensu lato*; engorged ticks; *Ixodes ricinus*; molecular detection.

PAPER INFO.: Received: 20 January 2021, Accepted: 17 February 2020, Published: 15 April 2021

INTRODUCTION

Ixodes ricinus (Linnaeus, 1758) is the main vector of spirochete from *Borrelia burgdorferi sensu lato* complex in Europe that is causing Lyme borreliosis (LB) (Steere *et al*. 2017). Available detection methods of *Borrelia* sp. in ticks are *in vitro* cultivation, serological assays, microscopy (Direct Fluorescent Antibody and Indirect Fluorescent Antibody) and PCR-based assays. The advantage of the molecular approach is its sensitivity, simplicity and rapidity. Pal and Buyuktanir (2018) summed up protocols for molecular detection of *B. burgdorferi s.l.* in ticks. In all tested protocols the whole individuals were used for DNA isolation. This is a common practice when small organisms, such as ticks, are used for molecular analyses (Williams *et al.* 1992). However,

How to cite: Ušanović, L., Lasić, L., Hanjalić, J., Ćakić, S., Destanović, D. & Kalamujić Stroil, B. (2021) Determination of an efficient and reliable method for PCR detection of borrelial DNA from engorged ticks. *Persian Journal of Acarology*, 10(2): 223–230.

subsequent molecular analyses are often impaired when DNA extracted from the entire tick is used as a template due to presence of various inhibitors from blood such as haemoglobin, lactoferrin and immunoglobulin G (Lempereur *et al.* 2011). As a result of poor PCR efficiency, studies regarding the genetic diversity of ticks have shown the presence of size homoplasy, allelic dropout, false alleles, and mistaken alleles (Pompanon *et al.* 2005). In the study where whole *Dermacentor variabilis* ticks were used for DNA extraction, fragment analysis of 12 microsatellite loci resulted in allelic dropout and amplification of non-specific alleles (Dharmarajan and Rhodes 2011). Consequently, inhibition of efficient PCR amplification has been reported in *B. burgdorferi* detection studies (Schwartz *et al.* 1997; Lempereur *et al.* 2011; Noda *et al.* 2013; Okeyo *et al.* 2019) due to the remnants of blood in a sample or the engorgement of a tick. The probable sources of inhibitors causing a poor amplification are bloodmeal in engorged ticks and male DNA in reproductive organs of fertilized females (Dharmarajan and Rhodes 2011). In addressing the problems with PCR inhibition and poor amplification, two studies suggested different approaches. According to Schwartz *et al.* (1997), sufficiently rigorous extraction step should be used for removing any potential PCR inhibitors. On the other hand, part of the tick body excluding bloodmeal and reproductive organs should be used as a starting material for DNA extraction according to Dharmarajan and Rhodes (2011). Lejal *et al.* (2019) tested for 32 tick-borne pathogens in dissected midgut and salivary glands of field-collected, unfed *I. ricinus* males and females. A surprising finding of their study was the presence of *B. burgdorferi s.l.* genospecies in both inspected body parts of females. This was opposed to previous belief that a bloodmeal is needed to start multiplication and migration of these pathogens from the gut to salivary glands of a tick. Similar results were seen in study by Sertour *et al.* (2018) in which *B. burgdorferi s.l.* was detected in the salivary gland of females prior to a bloodmeal. They also proved the ability of these bacteria to infect mice during 24 hours after a tick bite. However, neither study could conclude if a salivary gland is a genuine reservoir or the pathogen remained there after moulting because not all bacteria were transmitted to the host.

The aim of this study was (a) to compare the success of molecular-based detection of *B. burgdorferi s. l.* in engorged *I. ricinus* ticks removed from human patients and (b) to identify which part of tick body should be preferably used for DNA extraction in order to minimize the presence of PCR inhibitors.

MATERIALS AND METHODS

Total of 58 ticks were removed from patients in The Center for Emergency Medical Assistance of the Sarajevo Canton (Bosnia and Herzegovina). None of the patients, treated for tick bite, complained or showed any signs of Lyme disease at the time of medical assistance. The sampling was performed with patients' consent in March 2019. All samples were stored in separate 1.5 ml tubes with 96% ethanol until the species identification was done according to Estrada-Peña *et al.* (2004).

We defined three degrees of engorgement of collected ticks according to the change in body volume and color after a blood meal: fully-engorged (N = 19), in which the body volume is greatly increased and body color starts to change to most often silver, silver-gray or white, semi-engorged (19), in which volume changes are evident, but with no change in body color, and partially engorged (20), in which diverticula are filled with blood but the changes in body volume are not evident. There were no collected ticks removed from patients that could be classified as non-engorged.

We carried out three groups of extractions depending on the amount of starting material (Fig. 1). The first extraction group included the whole individual of partially engorged ticks as starting material (N = 12). The second extraction group included anterior half of semi-engorged (N = 9) and fully-engorged ticks (N = 11) as starting material (Fig. 1B). Lastly, the third group comprised

paired DNA extractions carried out on each tick from semi-engorged (N = 5) and fully-engorged (N = 4) category. The first of paired DNA extractions utilized capitulum and basis capituli (further referred to as mouthparts), without bloodmeal and reproductive organs as a starting material, while the second used body part covered by scutum, without capitulum and basis capituli (bellow referred to as a part of scutum) that comprises midgut diverticula, but without reproductive organs. Paired DNA extraction was conducted on five specimens from each semi- and fully-engorged group, totaling to 68 extraction reactions. Ticks were washed in distilled water before the extraction step. DNA extraction was done using the commercially available GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's recommendation. Extraction success was confirmed via visualization on 1.5% agarose gels stained with Midori Green (Nippon Genetics EUROPE GmbH).

Figure 1. Different amount of starting material – **A.** The whole individual of partially engorged tick; **B.** Anterior half of fully-engorged tick (above red line); **C.** Paired DNA extraction – mouthparts (1) and a part of scutum (2).

We tested ticks on the presence of *B. burgdorferi s. l.* using optimized protocol for nested PCR, targeting intergenic *rrf* (5S) – *rrl* (23S) region, as in our previous study (Lasić *et al.* 2020). A positive AMPLIRUN *Borrelia burgdorferi* DNA control (Vircell, Spain), a negative control and a 50 bp ladder (BioLabs, New England) were run in parallel with samples. A single-factor ANOVA (Excel, Microsoft) was performed to evaluate whether there was a significant difference in PCR efficiency between three tick categories and between different groups of DNA extractions.

RESULTS AND DISCUSSION

DNA was successfully obtained from 58 collected ticks, in all of 68 isolation reactions, regardless of the extraction group.

All specimens that showed the presence of 250 bp band that corresponds to the size of expected targeted intergenic *rrf* (5S) – *rrl* (23S) region of *B. burgdorferi s.l.* complex were considered as *Borrelia*-positive samples, regardless of the gel electrophoresis band quality. In 70.7% of tested tick samples such band was evident, but in only 14.63% of those cases a clear, single 250 bp band (Fig. 2, A1) was observed. Based on the quality of detected amplicons, three other PCR outcomes were identified in the rest of the samples: non-specific alleles, poor amplification, and a combination of poor amplification and non-specific alleles (Fig. 2, A2, A3, A4, respectively). Also, an unsuccessful amplification was detected in 66.66% ticks treated by paired DNA extractions (Fig. 2, B1).

Regarding the amount of starting material, the highest success rate (41%) of a positive unambiguous amplification was seen in the first extraction group in which the whole, partially-engorged ticks were used (Fig. 3). There were no failed PCRs in this group, but poor amplification

alone or in combination with non-specific alleles was detected (42% and 17%, respectively). Comparing the PCR results on DNA isolated from an anterior half of semi- and fully-engorged specimens, an unambiguous amplification was seen in the latter only. In almost half of the cases, PCRs failed when performed on an anterior half of semi-engorged ticks. Different PCR outcomes were seen in the samples from the paired DNA extractions group, for both semi- and fully-engorged animals (Fig. 3). Consistent positive amplification was evident only for the fully-engorged ticks for which a body section with midgut was used to extract DNA. However, regardless of the starting material in this extraction group, there were no cases in which a single, clear 250 bp band was present.

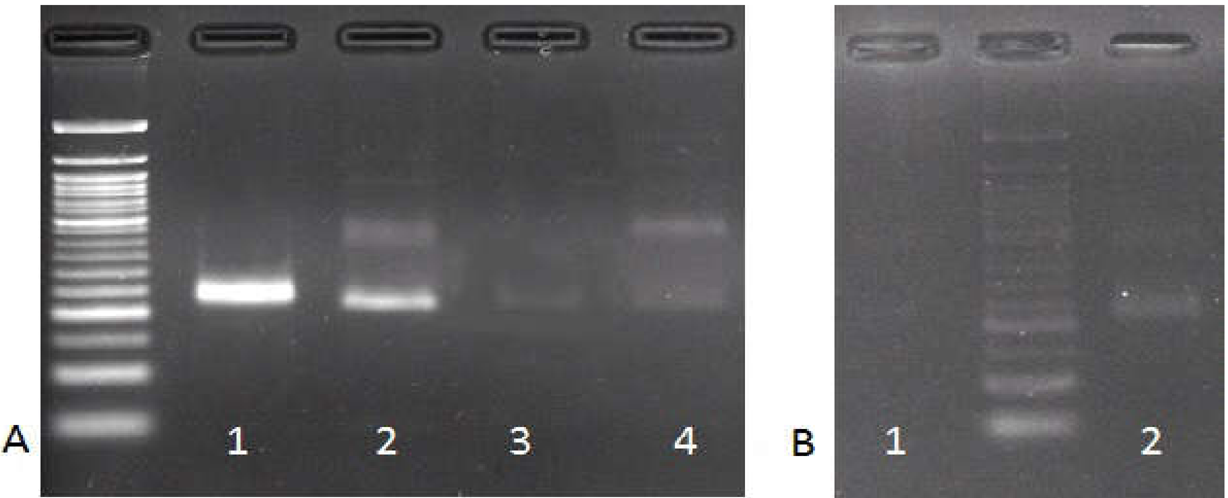

Figure 2. Results of PCR amplification – **A1.** Efficient PCR with clear band of 250bp presented as successful amplification, determined by using a positive control; All amplified bands with sizes differing from the positive control were defined as non-specific alleles; Four types of different PCR results: **A2.** Non-specific alleles; **A3.** Poor amplification; **A4.** A combination of poor amplification and non-specific alleles; **B.** Unsuccessful amplification detected in paired DNA extraction, after using DNA obtained from mouthparts **(B1)**, whereas target region was amplified for the same sample but using DNA obtained from a part of scutum **(B2)**; a 50 bp DNA ladder (BioLabs, New England) (A, B).

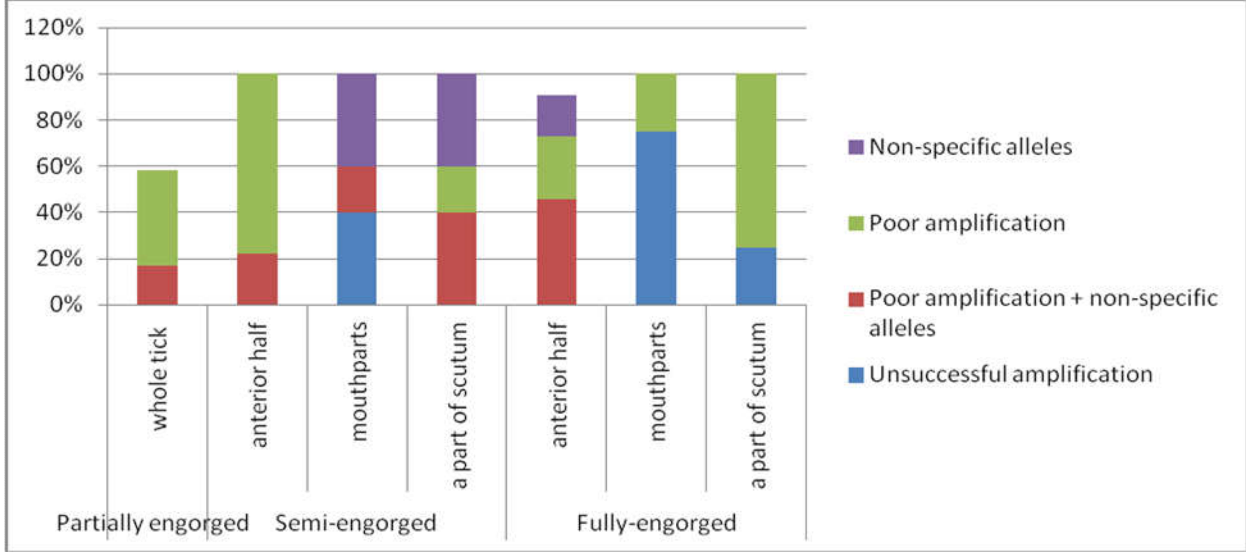

Figure 3. Ratio of four different PCR types in presented ticks categories and isolation groups.

The ambiguous amplification results in all positive semi- and fully-engorged ticks were not unexpected, because PCR inhibition rises with an increase in tick engorgement level. Tick engorgement status is directly correlated with feeding duration and amount of blood fed. Components that are found in mammalian blood, such as haemoglobin, urea and heparin, are common PCR inhibitors (Schwartz *et al.* 1997; Wilson 1997). Additionally, we were not provided with information if patients treated ticks before removal with any agent that could inhibit PCR. Although ticks were washed before the extraction step, there is a possibility that inhibitor traces still remained. This implies that washing with distilled water alone was not enough and other liquids such as 70% ethanol need to be used.

Unsuccessful amplification was seen in paired DNA extractions, for both semi- and fully-engorged ticks. An unsuccessful amplification as a non-efficient PCR result was also found in paired DNA extractions in the population genetic study conducted on *Dermacentor variabilis* (Dharmarajan and Rhodes 2011). Lejal *et al.* (2019) also did not detect any *Borrelia* in salivary glands alone of *I. ricinus*. In some of the cases, they found the presence of *B. spielmanii* (Schwartz *et al.* 1997) and *B. afzelii* (Pal and Buyuktanir 2018) only in the midgut. However, in most of the positive detections, the pathogen (*B. lusitaniae*, *B. spielmanii* and *B. garinii*) was seen in both organs. Similarly, in our study, for fully-engorged ticks, amplification on DNA extracted from mouthparts failed, while poor amplification was obtained for the same individuals when PCR was performed on DNA extracted from a part of scutum. Only in one case a poor amplification was seen in a sample from mouthparts, with no amplicon observable in that sample from a part of scutum. When a semi-engorged group is considered, 40% amplifications failed on mouthparts samples, while in 60% of cases amplification was evident in both types of samples (mouthparts and a part of scutum).

Results of a single-factor ANOVA showed that there was no statistically significant difference regarding the type of PCR results in this study between the three engorgement categories ($p = 0.18$), neither between three groups of starting material for DNA isolation ($p = 0.42$).

Assessing the level of tick's engorgement prior to extraction could be a helpful step in deciding which part of the body would be best suited as a DNA source for optimal detection of *Borrelia*. Using a whole body for DNA extraction might be justified in case of a partially engorged specimen. Results of our study showed the highest success rate (41%) of a positive unambiguous amplification in this group. In such individuals the quantity of a bloodmeal is not that large to cause a significant change in their body volume, therefore indicating that a concentration of possible inhibitors should be low. Thus, macerating the whole individual should maximize the chance of a successful detection of *Borrelia* presence. However, based on our findings, we suggest performing DNA extraction from the anterior half of semi-engorged and fully-engorged ticks for the purpose of *Borrelia* detection. We obtained a single, clear 250 bp band in 9% of the cases in a fully-engorged group, while the rest of samples in both groups (semi- and fully-engorged) revealed a positive amplification with lower quality. By using this part of the tick's body, both of the potential reservoirs (salivary gland and midgut) are included in the sample. As most of the gut containing a bloodmeal is avoided, the interference of the inhibitors from the mammalian blood is reduced, therefore increasing the chance of *Borrelia* detection. This could especially be important for the laboratories which do not have more sensitive methods such as real-time PCR at their disposal, but use end-point PCR method in their routine testing for *Borrelia*.

Funding

This work was supported by the Ministry of Education, Science and Youth of Sarajevo Canton (grant number 11-05-14-26465-1/18).

ACKNOWLEDGEMENTS

We are grateful to The Center for Emergency Medical Assistance of the Sarajevo Canton for their

help in collecting ticks and also to the patients who agreed to participate in the study.

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یافت روشی موثر و قابل اعتماد برای آشکار کردن پی سی آر دی ان ای بورلیایی از کنه‌های تغذیه‌کرده

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چکیده

چون کنه‌ها به عنوان یکی از مهم‌ترین ناقلان عوامل بیماری‌های سخت در انسان‌ها هستند بررسی‌ها در زمینه شناسایی ترکیب عوامل بیماریزا و سازوکار انتقال و آلودگی متمرکز شده است. اگرچه امروزه انبوهی از روش‌های شناسایی در دسترس است، اما روش مبتنی بر PCR حساس‌ترین و سریع‌ترین روش در نظر گرفته می‌شود. با این حال، چالش‌های رایج در تجزیه و تحلیل مولکولی انجام شده در مورد کنه‌ها، به دلیل نتایج تکثیر ضعیف مهارکننده‌های موجود گرفته شده از خون پستانداران یا دی ان ای نر در اندام‌های تولیدمثلی ماده است. هدف بررسی حاضر این است که کدام قسمت از بدن کنه تغذیه شده به عنوان ماده اولیه در استخراج DNA برای تشخیص مولکولی *Borrelia burgdorferi sensu lato*، عامل ایجاد بیماری لایم، بیشترین ترجیح را دارد. ۵۸ نمونهٔ کنهٔ *Ixodes ricinus* که از بیماران در مرکز کمک‌های فوری پزشکی کانتون سارایوو ، بوسنی و هرزگوین برداشته شده بود، مورد تجزیه و تحلیل قرار گرفت. یافته‌های ما نشان می‌دهد که نیمهٔ جلویی بدن کنه‌های نیمه تغذیه شده و کامل تغذیه شده برای استخراج DNA با هدف تشخیص بورلیا می‌تواند استفاده شود.

واژگان کلیدی: نیمهٔ جلویی بدن؛ *Borrelia burgdorferi sensu lato*؛ کنهٔ تغذیه کرده؛ *Ixodes ricinus*؛ تشخیص مولکولی.

اطلاعات مقاله: تاریخ دریافت: ۱۳۹۹/۱۱/۱، تاریخ پذیرش: ۱۳۹۹/۱۱/۲۹، تاریخ چاپ: ۱۴۰۰/۱/۲۶

AN EFFICIENT AND RELIABLE METHOD FOR PCR DETECTION OF BORRELIAL DNA