Ticks and Tick-borne Diseases 10 (2019) 1041–1045

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Comparison of methods for economic and efficient tick and *Borrelia* DNA purification

Mercy Okeyo[a,*], Christina Hartberger[a], Gabriele Margos[a], Reinhard K. Straubinger[b], Andreas Sing[a], Volker Fingerle[a]

[a] Bavarian Health and Food Safety Authority, German National Reference Centre for Borrelia, Veterinärstr. 2, 85764 Oberschleißheim, Germany
[b] Bacteriology and Mycology, Institute for Infectious Diseases and Zoonoses, Ludwig-Maximilians-Universität München, Veterinärstraße 13, 80539 München, Germany

ARTICLE INFO

Keywords:
Borrelia burgdorferi sensu lato
DNA extraction
Ammonium hydroxide
Tick lysis

ABSTRACT

DNA purification is a critical step in the processing of samples for molecular diagnosis and/ or identification of pathogens via polymerase chain reaction (PCR). Especially when handling vectors like ticks, purifying the DNA always poses a challenge.

In this study, we compared factors that may have an influence on DNA extraction namely commercially available DNA extraction kits vs alkaline hydrolysis for DNA extraction. The methods were applied to questing *Ixodes* (*I.*) *ricinus* ticks and *Borrelia* cultures of defined cell concentrations. A total of 69 questing *I. ricinus* ticks were collected. From 34 ticks, total DNA was extracted using a commercial DNA extraction kit. Thirty-five ticks were treated with 1.25% ammonium hydroxide (NH_4OH). Six ticks from each batch were placed in 70% ethanol (EtOH) for one week prior to DNA extraction to see the effect of EtOH preservation on total DNA yield. DNA yield was estimated in field-collected ticks using conventional PCR targeting the *Ixodes* Cytochrome C oxidase (*coi*) gene and in cultured *Borrelia* isolates using quantitative real-time PCR (qPCR) targeting the FlaB encoding gene of *Borrelia*.

Column DNA extraction yielded slightly better results than NH_4OH treatment when tested in a PCR targeting a tick-specific *coi* gene (96% PCR-positive vs 86% PCR-positive results, respectively). EtOH preservation had a slightly negative effect on DNA yield and – again – slightly stronger PCR products were observed by commercial kit extraction. A Shapiro-Wilk test conducted revealed a significance-level of 90% for both the methods, indicating a normal distribution of the values generated by BioNumerics quantification. A two-sided *t*-test conducted revealed a significant ($p < 0.01$) mean difference between the methods. Similarly, qPCR on cultured specimen DNA of *Borrelia burgdorferi* sensu stricto (*B. burgdorferi* s.s.) (B31) with different concentrations revealed a better yield for kit extraction in comparison to NH_4OH treatment; a difference of approximately 3 Ct-values was ascertained between extraction methods. A one-sided *t*-test showed a significant difference between the methods at lower concentration of *Borrelia* i.e. better extraction with a commercial kit at lower borrelial DNA concentration, while at higher concentration (10^6 cells per ml) the difference was not significant.

1. Introduction

As molecular tools are increasingly being employed for diagnostics, DNA purification is a critical step in analysis/ diagnosis of infectious agents and is indispensable in daily laboratory life. It is therefore essential to balance affordable DNA purification methods with maximum yield when handling field samples, as only low quantities of target DNA might be present.

In general, regarding ticks, there are a wide number of methods, which have been described to be efficient for DNA extraction. Such methods include mechanical beating of samples, which may involve the use of beads, freezing in liquid nitrogen followed by manual crushing with mortar and pestle, or manual crushing with spatula and subsequent DNA extraction using proteinase K and diverse buffers such as sodium dodecyl sulfate (SDS), phenol extraction, EtOH precipitation amongst others (Hill and Gutierrez, 2003). Some laboratories opted to use a commercial kit after manual tick crushing (Maggi et al., 2010). Halos and colleagues compared three different DNA extraction protocols on *Dermacentor* sp., *Rhipicephalus* sp. and *Ixodes* (*I.*) *ricinus*; in their study fine crushing with beads, proteinase K digestion and subsequent

* Corresponding author.
E-mail address: mercyakinyi2012@mail.com (M. Okeyo).

https://doi.org/10.1016/j.ttbdis.2019.05.002
Received 14 November 2018; Received in revised form 7 May 2019; Accepted 7 May 2019
Available online 08 May 2019
1877-959X/

Table 1
Number of ticks tested (A), results on the two-sided *t*-test conducted (B) and culture dilutions (C) processed using different DNA purification methods.

Methods	Commercial Kit (Qiagen)		NH_4OH	
A: Number of ticks processed using different purification methods				
EtOH-Preservation	without	with	without	with
Total Ticks	34		35	
Ticks	28	6	29	6
Male, n / positive	2/2	1/1	9/7	4/4
Female, n / positive	3/3	3/3	11/9	1/1
Nymph, n / positive	23/22	2/2	9/9	1/1
B: Two-sided t-test on the different methods used assuming equal variance				
Average	65615		49808	
Variance	227195838		151761455	
t-statistic	4.40			
P(T < =t) two-sided	4.77E-05			
Critical t-value for two-sided t-test	2.00			

C: Ct-values of real-time PCR on serial dilutions of *B. burgdorferi* sensu stricto DNA

	10^4 Roche*	10^4 NH_4OH	10^5 Roche	10^5 NH_4OH	10^6 Roche	10^6 NH_4OH
First Run	34.09	38.28	31.11	33.4	26.7	28.72
Second Run	33.13	36.81	31.03	32.15	26.93	29.16
Third Run	35.25	36.93	30.22	34.58	25.87	26.12
Mean Ct-Value	34.16	37.34	30.79	33.38	26.5	28
Standard Deviation	0.867	0.666	0.402	0.992	0.455	1.341
T-test p-value	0.0267		0.0559		0.0697	

n = number of ticks/ positive = number of positive.
DNA was purified from cultured *B. burgdorferi* s.s. with known number of cells: 10^4, 10^5 and 10^6 per ml and DNA eluted in 60 µl RNA and DNA-free water. 5 µl were used as template DNA for PCR, corresponding to 833, 8.333 and 83.333 cells/ PCR, respectively. Three experiments were run (first run, second run, and third run) to show the reproducibility of the results.
* DNA from cultured specimen purified with different extraction methods (commercial DNA extraction kit vs alkaline hydrolysis).

DNA extraction by a commercial kit was the most effective protocol (in 30/30 ticks DNA extraction was successful) (Halos et al., 2004).

Since ticks do not just harbor bacterial but also viral disease-causing agents, some laboratories have modified commercially available kits creating protocols, which are also suitable for extracting both tick DNA and RNA simultaneously (Crowder et al., 2010).

Commercial kits for DNA extraction differ in prices and may add up to prohibitively costs when large numbers of ticks are being investigated. However, methods that are more economical are available. DNA extraction methods using guanidinium thiocyanate have been used successfully (Hubbard et al., 1995). Another method used by a number of laboratories working on tick-borne pathogens uses NH_4OH which allows efficient handling of large numbers of samples (Guy and Stanek, 1991).

In this study, we have tested several parameters that are known to play a role in DNA purification from complex samples including commercial DNA purification kits vs alkaline hydrolysis (NH_4OH) and have quantified extraction success. Our data show that commercial kits are slightly better than alkaline hydrolysis regarding DNA yield. Nevertheless, the latter method is very well suited and more economical for a large sample scale. Inhibitory factors that may be present in some samples following DNA extraction using alkaline hydrolysis can be removed by additional use of a commercial kit.

2. Materials and methods

2.1. Tick collection

Ticks were collected by drag-sampling using a white cotton cloth measuring 1 m^2 (Rulison et al., 2013). A total of 69 *Ixodes* spp. ticks were collected, 18 of which were female, 16 were male and 35 nymphs. These ticks were identified morphologically as *I. ricinus*. EtOH storage is widely used as a very convenient method for tick storage. To obtain information on EtOH storage on DNA yield using the different methods, 12 ticks were kept in 70% EtOH for one week.

2.2. Tick homogenization and DNA extraction

As preliminary results comparing different homogenization methods (manual vs mechanical disruption of tick cuticle), and different lysis buffers had given similar results (data not shown), samples in this study were manually crushed (as this was the more economical alternative) with the buffers recommended in the DNA extraction kit. To make results comparable, the same elution volume for DNA extraction was used for both methods (approximately 60 µl), whether NH_4OH hydrolysis method was used or a commercial kit. EtOH-preserved ticks were washed shortly (10 s) with aqua destillata (aq. dest.) prior to DNA extraction. From 34 ticks DNA was extracted using a commercial kit (Qiagen, one for all vet kit; Hilden Germany) according to the manufacturer's manual. Thirty-five ticks were treated with 1.25% NH_4OH (Sigma Aldrich, Munich, Germany) as described (Guy and Stanek, 1991). Briefly, for NH_4OH treatment single ticks were placed in 1.5-ml Eppendorf safe lock tubes and manually crushed using an individual sterile spatula for each tick. For adult ticks 200 µl of freshly prepared 1.25% NH_4OH solution was added and for nymphs 120 µl. Closed tubes were put in a heat block at 100 °C for 20 min followed by a short centrifugation step (10–30 s). The tubes were placed back to the heat block with open lids at 100 °C until the desired extraction volume (approximately 60 µl) was reached (between 15–40 min). They were again shortly centrifuged (10–30 s) and stored at 4 °C until further use. Several Tubes (8%) that were handled in the same way but without ticks present and placed between the sample containing tubes were used as negative DNA extraction controls. The whole procedure was performed in a fume hood.

In the case of four samples, for which the initial NH_4OH extraction and *coi* gene amplification was not successful, DNA was re-extracted from tick-NH_4OH homogenate using High Pure PCR Template Preparation Kit (Roche, Mannheim Germany) according to the manufactures' protocol.

2.3. Borrelia culture DNA

Serial DNA dilutions (10^6, 10^5 and 10^4 cells) obtained from a cultivated *B. burgdorferi* s.s. B31 isolate were also included in this study.

This experiment was carried out to test the influence of the different extraction methods on the sensitivity of a real-time PCR targeting the *Borrelia* FlaB encoding gene. For both methods, cell pellets from 1 ml of culture after 20 min centrifugation at 12,000 rpm (Eppendorf Centrifuge 5424, Hamburg, Germany) were used and the same conditions and elution volumes were used as above.

Taken together 69 ticks that were available for this study were handled as follows (Table 1A); in 34 ticks, DNA was extracted using a commercial kit, in 35 ticks DNA was extracted using a NH_4OH hydrolysis method. Six ticks from each batch were preserved in 70% EtOH prior to DNA extraction.

2.4. Confirmation of tick DNA extraction by PCR

Since it is not possible to determine directly the DNA concentration in samples that were subjected to alkaline hydrolysis, samples to be compared were subjected to PCR after NH_4OH treatment/ DNA extraction. DNA extraction controls containing nuclease-free water instead of a sample DNA (8% of the total number of samples) were randomly distributed amongst the tubes containing the samples during DNA extraction and in the subsequent PCR reactions. To determine the success of DNA extraction in tick samples, a PCR amplifying the tick mitochondrial Cytochrome C oxidase (*coi*) gene was used as described (Dinnis et al., 2014). The primer set had the following forward and reverse sequences, respectively 5`-ATTTTACCGCGATGAYTWTW-CTC-3` and 5`-ATTTTACCGCGATGAYTWTWCTC-3`. My Taq Mix, 2x from Bioline was used (Bioline, Luckenwalde-Brandenburg, Germany). The thermal profile for the *coi* gene PCR was as follows: 94 °C for 3 min, five cycles of 94 °C for 15 s, 51 °C for 30 s, 68 °C for 30 s, 35 cycles of 94 °C for 15 s, 53 °C for 30 s, 70 °C for 30 s, 70 °C for 5 min. This was done in a total volume of 20 µl; 10 µl master mix, 2 µl of 10 pmol/ µl forward primer, 2 µl of 10 pmol/ µl revers primer, 0.5 µl of 25 mM/ ml $MgCl_2$, 3.5 µl nuclease free water and 2 µl template DNA solution. PCR products were visualized on a 1.5% agarose gel (Biozym Biotech Vienna, Austria) containing 0.08% gel red (Biotium, Darmstadt Germany).

For statistical analysis BioNumerics version 7.6 was used to quantify the PCR product bands in agarose gels. The values were subsequently analyzed employing the Shapiro-Wilk test (Wilk and Shapiro, 1965) and a two-sided *t*-test (Ned, 2015; Pillemer, 1991).

A real-time PCR (qPCR) targeting the *flaB* gene encoding the *Borrelia* flagellin protein (Schwaiger et al., 2001) was carried out on DNA extracted from *Borrelia* cultures (QuantiTect Multiplex No Rox Master- MixQiagen) as described. Primers and probe were used as described (Schwaiger et al., 2001). The thermal profile was as follows: the ta-q polymerase was activated at 95 °C for 15 min, followed by 45 cycles of 95 °C 15 s and 60 °C 1 for min. Total reaction volume was 25 µl; 12.5 µl master mix, 0.75 µl forward primer, 2.25 µl revers primer, 0.5 µl $MgCl_2$, 3.5 µl nuclease-free water and 5 µl template DNA. All primers had a concentration of 10 µM/ ml.

3. Results and discussion

A total of 75 samples were included in this study; 69 ticks and two sets of three serial DNA dilutions obtained from a *B. burgdorferi* s.s. B31 culture.

In all 12 samples, which were stored in 70% EtOH for one-week period prior to DNA extraction, the *coi* gene locus was amplified by PCR regardless of the extraction method. In 33 of the 34 ticks (96%) that were used fresh (i.e. without EtOH preservation) for DNA extraction via a commercial kit, the *coi* gene fragment was successfully amplified by conventional PCR. In samples in which DNA was extracted using NH_4OH, only 31 of the 35 (86%) were positive in a conventional PCR targeting the *coi* gene (Fig. 1 Panel A and Panel B showing a subset of these results). Thus, for five ticks (two females, two males and one nymph; one included in commercial DNA extraction kit, four in alkaline hydrolysis) the amplification was not successful, likely due to inhibitory factors present in the samples (Table 1A). Moreover, it was apparent that PCR amplification on DNA purified using a commercial kit produced slightly stronger bands compared to NH_4OH extracted DNA (Fig. 1 Panel A and B). In general, no considerable difference was found whether DNA was extracted from adults or nymphs.

Tick DNA PCR product bands in gels were quantified using BioNumerics version 7.6 software. Afterwards, a Shapiro-Wilk test was conducted to determine normal distribution of the values. For both NH_4OH treatment and commercial DNA extraction kit the values revealed a significance level of 90% normal distribution. Thereafter a two-sided *t*-test was conducted. A significant ($p < 0.01$) mean difference was determined to be 4.40, which is greater than the critical t-value for the two-sided *t*-test in this study, which was determined to be 2.00 (Table 1B).

To quantify the PCR results and to exclude the possibility of inhibitory factors being present in NH_4OH ticks, a serial dilution of eight samples was conducted from 10^{-1} to 10^{-6}; two samples from each group (Fig. 1 Panel C to F). These data indicate that *coi* gene amplification was successful in freshly prepared samples in dilutions up to 10^{-2} in NH_4OH DNA (Panel D) and up to 10^{-3} in kit extracted DNA (tick 14, Panel C). Following EtOH preservation and DNA purification by NH_4OH (Panel F), only in the dilution of 10^{-1} a PCR product was obtained.

Since inhibitory factors possibly present in the four samples extracted by alkaline hydrolysis might have resulted in failure of PCR amplification of the *coi* gene fragment, DNA was re-extracted using a commercial DNA purification kit (Fig. 1 Panel G).

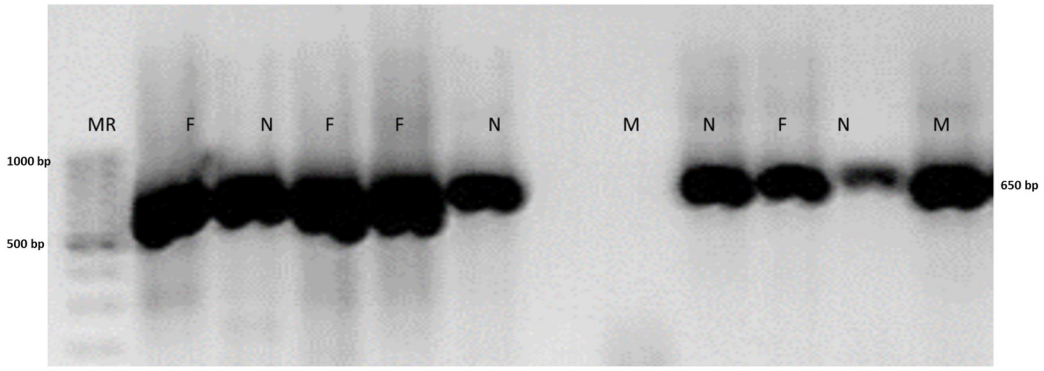
In all four re-purified samples, a PCR product was obtained with three samples producing very prominent bands after DNA re-extraction using a commercial kit. These results support the notion that inhibitory factors were removed by column-mediated DNA extraction. It is known that for ticks that have taken a blood meal alkaline hydrolysis is not the method of choice due to inhibition of PCR amplification (Rodríguez et al., 2014). Thus, one possibility is that these ticks, although collected by drag sampling and apparently questing, had taken blood from a host (Richter et al., 2012) (see below).

Table 1C shows Ct-values of column-based DNA vs NH_4OH extraction methods performed with three different dilutions of cultured *Borrelia* in three different runs. A difference of 3–5 Ct-values was observed between dilution steps (i.e. from 10^6 to 10^5 to 10^4). A difference of 2 to 3 Ct-values was observed between the different DNA extraction methods, i.e. commercial kit vs alkaline hydrolysis, with higher discrepancies in lower cell concentrations. Based on our experience 3 Ct values correspond to approximately 10 fold dilution. This agrees with what was observed in our tick DNA dilution experiment (Fig. 1 Panel C–D). Thus, our results indicate that the detection limit is reduced in samples when alkaline hydrolysis is used for DNA extraction. Purified DNA of cell dilutions was eluted in 60 µl of buffer and 5 µl of the eluate was used for PCR amplification. By extrapolation, this would mean that, we were able to detect at least 800 to 1000 bacterial cells in samples where DNA was extracted by alkaline hydrolysis.

NH_4OH DNA treatment has been described and used previously (Guy and Stanek, 1991; Rijpkema and Bruinink, 1996; Szekeres et al., 2017; Vollmer et al., 2011). For the control of possible contaminations during DNA extraction, tubes containing only nuclease free water were included (8% of the total number of samples). These DNA extraction controls were also included in the PCR reaction and were all negative each time. Our results indicated that for 4/35 samples in the group of alkaline hydrolysis the PCR was unsuccessful. Amplification of the target locus, the tick *coi* gene, ought to have been possible in all samples but this was not the case in our study. Although we observed some fluctuation in the amount of DNA depending on the extraction method used, we suggest that the lack of PCR amplification might be due to some inhibitory factors present in the sample. PCR inhibition in samples treated with NH_4OH is not surprising, as this method does not include protein digestion and/or filtration. Moreover, we cannot exclude that

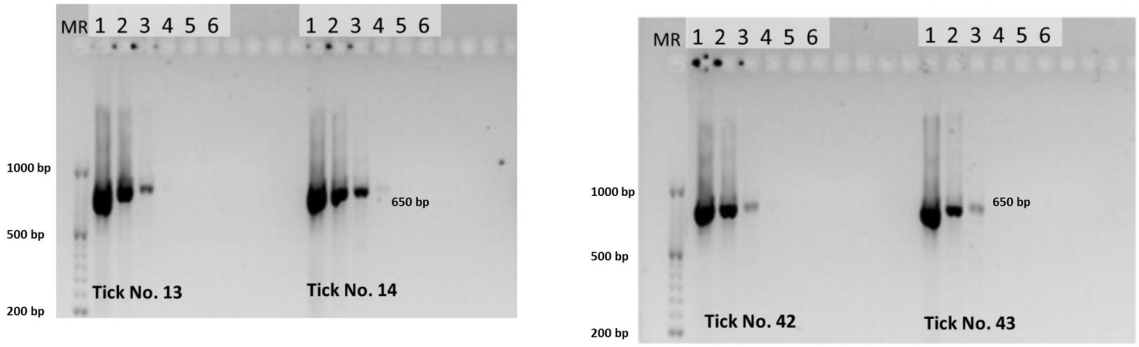
Panel A: Extracted DNA with DNA extraction kit

Panel B: NH_4OH-DNA extraction



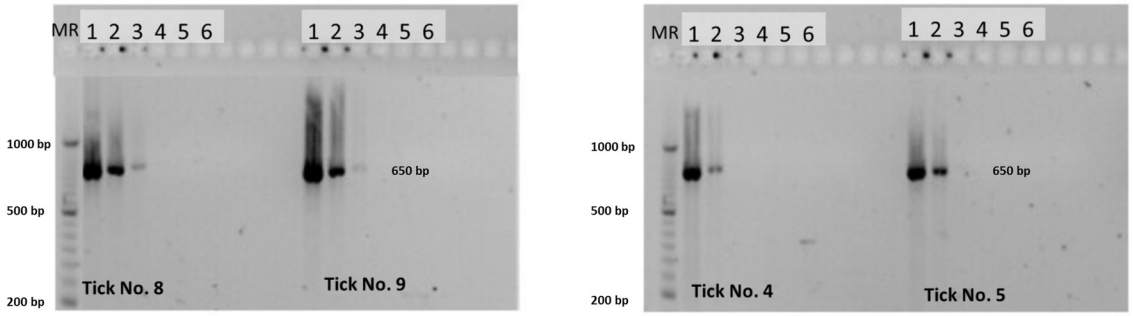
Panel C: Direct DNA extraction with DNA extraction kit

Panel D: Direct NH_4OH-DNA extraction

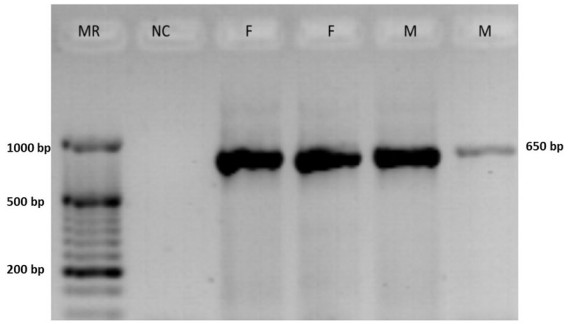


Panel E: EtOH preserved ticks prior to column DNA extraction

Panel F: EtOH preserved ticks prior to NH_4OH-DNA extraction



Panel G: Re-extracted DNA using commercial kit



(caption on next page)

Fig. 1. Agarose gels of *coi* gene PCR products from samples after DNA extraction by different methods. Panels A and B show a subset of samples from freshly handled ticks (i.e. without preservation in EtOH). Panel A: PCR products (*coi* gene) obtained from tick samples purified with commercial DNA extraction kits, Panel B: DNA extracted with alkaline hydrolysis.
Panels C–F show the PCR amplification in samples of eight nymphs after a serial dilution from 10^{-1} to 10^{-6}. Panel C and D are ticks which were handled freshly (i.e. without preservation in EtOH). Panel E and F are ticks, which were preserved in 70% EtOH for one week prior to DNA extraction. Panel G: PCR results of tick samples after the initial alkaline hydrolysis negative results; DNA was re-extracted using a commercial kit. MR = DNA low molecular marker (Orange Ruler 50-bp DNA marker, Thermo scientific, Karlsruhe, Germany); NC = negative control; N = nymph; F = female; M = male, 1 = undiluted, 2 = 10^{-1}, 3 = 10^{-2}, 4 = 10^{-3}, 5 = 10^{-4}, 6 = 10^{-5}

the ticks that we collected, although questing and flat, had perhaps taken a small interrupted blood meal that caused PCR inhibition in some samples (Apanaskevich and Oliver, 2014; Schwartz et al., 1997). Indeed, when DNA was re-extracted using a commercial kit in these PCR negative samples, a *coi* gene PCR product became visible. Other inhibiting components present, for example proteins interacting with the DNA or chelating agents in the biological samples may have contributed to the failure of PCR. In conclusion, the NH_4OH DNA extraction method may lead to false negative results.

In contrast to previous reports suggesting that alkaline hydrolysis does not work on nymphal ticks (Ammazzalorso et al., 2015), we were able to extract DNA using this method also from nymphs as shown by positive PCR results. Despite the lower efficiency of alkaline hydrolysis for DNA extraction from ticks and from cultured *Borrelia* organisms, which was ascertained in this study, we propose that NH_4OH DNA hydrolysis in conjunction with manual/ fine crushing may still be the method of choice especially in low resourced laboratories. Considering the fact that DNA purification with a commercial kit is approximately 90 times more expensive per sample than with NH_4OH, the latter method is an inexpensive alternative to commercially available DNA extraction kits.

Samples containing low numbers of *Borrelia* could, however, be missed when DNA is extracted using NH_4OH and this should be put into consideration while evaluating results. Although bead beating as a DNA preparation method could be expensive, it generated the most reliable results (Halos et al., 2004). It is also worth mentioning that NH_4OH extraction is only suited for (questing) ticks that have not taken (even a small) blood meal; for engorged ticks one should use either commercially available DNA extraction kits or potassium acetate (Rodríguez et al., 2014).

In conclusion, we showed that alkaline hydrolysis is an economical and inexpensive method for DNA extraction from vectors of pathogens. In our study at least 800 bacteria cells from cultured *Borrelia* were detected. Thus, using it on field samples it may include the bulk of infected ticks as it has been shown that the average infection burden of *I. scapularis* was around 800 *Borrelia* per tick (Barbour et al., 2009). Inhibition of PCR did occur in a number of samples. As it is known that alkaline hydrolysis is not suitable for blood fed vectors, we consider it likely that the observed inhibition was related to uptake of small amounts of blood and re-questing of ticks.

Funding

This work was supported by the Robert Koch Institute through funding the German National Reference Centre for *Borrelia* and a scholarship through German Academic Exchange Service (DAAD) to Mercy Okeyo.

Acknowledgments

We are grateful to Cecilia Hizo-Teufel and Silvia Stockmeier in the NRZ-*Borrelia* laboratory for their assistance during the course of this research.

Also, a big thank you to Dr. Christian Tuschak from the Hygiene Laboratory, Bavarian Health and Food Safety Authority, for help with BioNumerics quantification.

References

Ammazzalorso, A.D., Zolnik, C.P., Daniels, T.J., Kolokotronis, S.O., 2015. To beat or not to beat a tick: comparison of DNA extraction methods for ticks (*Ixodes scapularis*). PeerJ 3, e1147.
Apanaskevich, D., Oliver, J., 2014. Life cycles and natural history of ticks. In: Sonenshine, D.E., Roe, R.M. (Eds.), Biology of Ticks. Oxford University Press.
Barbour, A.G., Bunikis, J., Travinsky, B., Hoen, A.G., Diuk-Wasser, M.A., Fish, D., Tsao, J.I., 2009. Niche partitioning of *Borrelia burgdorferi* and *Borrelia miyamotoi* in the same tick vector and mammalian reservoir species. Am. J. Trop. Med. Hyg. 81, 1120–1131.
Crowder, C.D., Rounds, M.A., Phillipson, C.A., Picuri, J.M., Matthews, H.E., Halverson, J., Schutzer, S.E., Ecker, D.J., Eshoo, M.W., 2010. Extraction of total nucleic acids from ticks for the detection of bacterial and viral pathogens. J. Med. Entomol. 47, 89–94.
Dinnis, R.E., Seelig, F., Bormane, A., Donaghy, M., Vollmer, S.A., Feil, E.J., Kurtenbach, K., Margos, G., 2014. Multilocus sequence typing using mitochondrial genes (mtMLST) reveals geographic population structure of *Ixodes ricinus* ticks. Ticks Tick Borne Dis. 5, 152–160.
Guy, E.C., Stanek, G., 1991. Detection of *Borrelia burgdorferi* in patients with Lyme disease by the polymerase chain reaction. J. Clin. Pathol. 44, 610–611.
Halos, L., Jamal, T., Vial, L., Maillard, R., Suau, A., Le Menach, A., Boulouis, H.J., Vayssier-Taussat, M., 2004. Determination of an efficient and reliable method for DNA extraction from ticks. Vet. Res. 35, 709–713.
Hill, C.A., Gutierrez, J.A., 2003. A method for extraction and analysis of high quality genomic DNA from Ixodid ticks. Med. Vet. Entomol. 17, 224–227.
Hubbard, M.J., Cann, K.J., Wright, D.J., 1995. Validation and rapid extraction of nucleic acids from alcohol-preserved ticks. Exp. Appl. Acarol. 19, 473–478.
Maggi, R.G., Reichelt, S., Toliver, M., Engber, B., 2010. *Borrelia* species in *Ixodes affinis* and *Ixodes scapularis* ticks collected from the coastal plain of North Carolina. Ticks Tick Borne Dis. 1, 168–171.
Ned, K., 2015. One-tailed or two-tailed P values in PLS-SEM? Int. J. e-Collab. 11, 1–7.
Pillemer, D.B., 1991. One- versus two-tailed hypothesis tests in contemporary educational research. Educ. Res. 20, 13–17.
Richter, D., Debski, A., Hubalek, Z., Matuschka, F.R., 2012. Absence of Lyme disease spirochetes in larval *Ixodes ricinus* ticks. Vector Borne Zoonotic Dis. 12, 21–27.
Rijpkema, S., Bruinink, H., 1996. Detection of *Borrelia burgdorferi* sensu lato by PCR in questing *Ixodes ricinus* larvae from the Dutch North Sea island of Ameland. Exp. Appl. Acarol. 20, 381–385.
Rodríguez, I., Fraga, J., Noda, A.A., Mayet, M., Duarte, Y., Echevarria, E., Fernández, C., 2014. An alternative and rapid method for the extraction of nucleic acids from ixodid ticks by potassium acetate procedure. Braz. Arch. Biol. Technol. 57, 542–547.
Rulison, E.L., Kuczaj, I., Pang, G., Hickling, G.J., Tsao, J.I., Ginsberg, H.S., 2013. Flagging versus dragging as sampling methods for nymphal Ixodes scapularis (Acari: Ixodidae). J. Vector Ecol. 38, 163–167.
Schwaiger, M., Peter, O., Cassinotti, P., 2001. Routine diagnosis of *Borrelia burgdorferi* (sensu lato) infections using a real-time PCR assay. Clin. Microbiol. Infect. 7, 461–469.
Schwartz, I., Varde, S., Nadelman, R.B., Wormser, G.P., Fish, D., 1997. Inhibition of efficient polymerase chain reaction amplification of *Borrelia burgdorferi* DNA in blood-fed ticks. Am. J. Trop. Med. Hyg. 56, 339–342.
Szekeres, S., Lugner, J., Fingerle, V., Margos, G., Foldvari, G., 2017. Prevalence of *Borrelia miyamotoi* and *Borrelia burgdorferi* sensu lato in questing ticks from a recreational coniferous forest of East Saxony, Germany. Ticks Tick Borne Dis. 8, 922–927.
Vollmer, S.A., Bormane, A., Dinnis, R.E., Seelig, F., Dobson, A.D.M., Aanensen, D.M., James, M.C., Donaghy, M., Randolph, S.E., Feil, E.J., Kurtenbach, K., Margos, G., 2011. Host migration impacts on the phylogeography of Lyme Borreliosis spirochaete species in Europe. Environ. Microbiol. 13, 184–192.
Wilk, M.B., Shapiro, S.S., 1965. An analysis of variance test for normality (complete samples). Biometrika 52, 591–611.