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A simple, effective and inexpensive method to isolate the nucleic acid (DNA/RNA) from a single tick for molecular detection of various pathogens

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ABSTRACT

Objectives: Ticks are vectors of a number of pathogens, which cause diseases with fatal consequences, therefore it is essential to detect such pathogens from single tick. Molecular methods like polymerase chain reaction (PCR) are offering such possibilities. At present, cumbersome methods involving liquid nitrogen, cutting ticks with scalpels as well as pooled ticks are being used worldwide. Our goal is to develop a reliable and fast method to obtain nucleic acid (DNA/RNA) from the single tick shipped at room temperature to detect various pathogens.

Methods: We developed a mechanical crushing method with mini column nucleic acid isolation from a single tick shipped at room temperature with postal or currier service in a letter. PCR detection was done for *Borrelia burgdorferi* and tick-borne encephalitis virus as examples.

Results: This method was used successfully for the isolation of nucleic acid from single tick and later used for PCR detection of *B. burgdorferi* and tick-borne encephalitis virus on 17 single tick samples as examples, but for last 18 years, this method was used on more than 250 ticks from Germany. Spectrometric values indicate the presence of sufficient yield of DNA and RNA (up to 900 µg/mL per tick) during the isolation.

Conclusions: This may be the first report about a number of one single tick cases, which were sent at room temperature in letters with postal services for isolation of the nucleic acid with mini column kit and used later on for PCR detection of various pathogens. This inexpensive and simple method may be used in any laboratory worldwide for monitoring the presence of tick-borne pathogens.

Keywords: Tick, tick borne pathogens, nucleic acid isolation, *Borrelia burgdorferi*, polymerase chain reaction

Tick borne pathogens are one of the major threats to human beings and animals because the ticks are vectors of many different pathogens with fatal consequences in many incidences around the world. These pathogens are *Borrelia burgdorferi, Babesia microti, Babesia bovis, Anaplasma genus*, tick borne encephalitis virus etc. Polymerase chain reaction (PCR) is one of the important detection methods to detect various pathogens like SARS CoV-2. Influenza virus, Hepatitis *C virus, Coxiella* burnetii and many other pathogens [1-9]. Moreover there are large number of RNA viruses in ticks, which are needed to be addressed [10].

To conduct the PCR, there is a need of isolated nucleic acid from the source. In the case of ticks, user needs to isolate this from tick itself, but there are many

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methods described in the literature.

Various groups have developed various DNA isolations for conducting molecular analysis. The ticks were preserved and cumbersome nucleic acid isolation method was developed [11]. The ticks were collected in liquid nitrogen and isolated with mini column methods to detect the various RNA viruses [10] In another work, researchers digested the ticks in lysis buffer with Proteinase K overnight at 56 °C, vortexed and centrifuged the tick to get the tick exoskeleton to isolate the nucleic acid from them in order to detect Rickettsia in PCR test [12]. Another group has developed a different method to isolate DNA and RNA from single tick as the tick was washed with PBS and dried. After that it was cut with sterile scalpel and the isolation was performed with chloroform and commercial solution [13]. This method is very cumbersome as there are many ticks, which are very small, hence this method is not suitable. In another method: beads were applied to beat the ticks in a microtube in lysis buffer containing proteinase K [14]. Mortar and Pestle method was being used for a pool of 4-10 ticks, which were washed in ethanol and washed with sterile water. After that they were dried and PBS was added to crush them physically. The DNA from the homogenized extract were isolated with chloroform method [15]. There was a comparison done with different homogenizers to isolate nucleic acid from medical relevant ticks [16]. There are research works, where no mechanical crushing is used to isolate the ticks, but ticks were cut with scalpels in two halves. One part is stored in 80% ethanol and other half was used for isolation and subsequently used molecular detection of Anaplasma and *Ehrlichia gena* [17].

In literature, there are a number of publications about the detection of pathogens from pooled ticks, but there is a need of simple nucleic acid isolation method, which can be applied under field conditions from a single tick, where the tick can be transported under field conditions without any storage chemicals as some research workers are using liquid nitrogen, which is not easily available under fields. Therefore, our laboratory has decided to develop a simple, inexpensive and effective isolation method to detect the various pathogens from single tick. Hence such research about detection of the tick-borne pathogens from single ticks with various molecular methods can be very accurate to have the reliable data about the prevalence of pathogens in a particular area. This type data can be used to develop the preventive and therapeutic measurements in a particularly area.

METHODS

Ticks were sent to Genekam laboratory from different areas at room temperature without any storage chemicals through German postal service in plastic microtubes or glass vials. In many cases, they were fixed with adhesive film on a piece of paper in such a way that the tick was trapped between the folded piece of paper. (Ticks were not pasted on adhesive tape as they were put in the folded paper. If one puts the tick on the adhesive tape, it can cause problem during isolation process) so that they cannot move or go out the containers. On the arrival of these ticks, they were kept at room temperature in restricted area before their processing. Most of the shipments or letters were containing one tick, but sometimes, there were two ticks. In this work, each tick was processed as single tick, not pooled samples of two ticks.

Isolation of nucleic acid from single tick: Mortar and pestle (size and dimensions are mentioned elsewhere) was prepared as followings: they were rinsed in soaped water for 10 minutes, washed with tap water and at the end rinsed with single distilled water twice. After that they were air dried. Before the use, mortar and pestle were sterilized with flame of gas burner while exposing for 1 to 2 minutes that the flame should destroy any remaining DNA from previous isolation, if any and flame should not harm the mortar and pestle. The whole area (particularly bottom) of mortar and head of pestle were exposed to the flame. This is very important step during isolation of nucleic isolation to avoid the contamination from previous samples. It was repeated 3 to 4 times. After that mortar and pestle allowed to cool.

Pincettes were also cleaned with soaped water and washed with tap water, but finally washed with single distilled water. The ends of pincettes were exposed to flame in order to destroy any contamination on them. They were allowed to cool. Alternatively, these were washed thoroughly with soap solution and later with distilled water twice.

Three hundreds µL of lysis buffer from Genekam DNA/RNA kit was also added to mortar. Now single

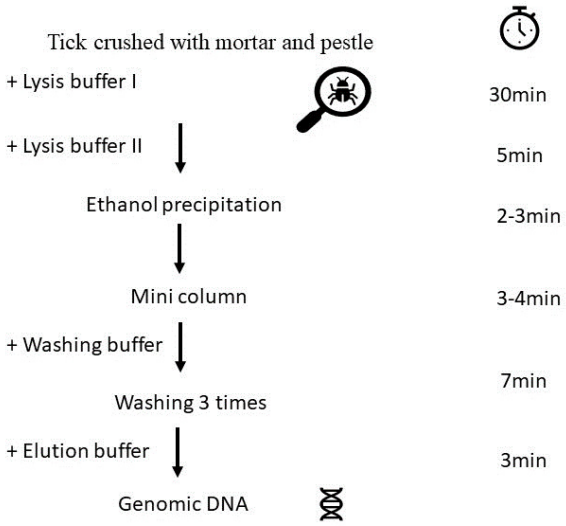

Fig. 1. The sheme of isolation method of almost 50-55 minutes.

tick was observed with a hand lens and with help of pincettes put in lysis buffer (dead ticks) or the single tick was just thrown into the lysis buffer from container (This was used with live tick, donot use pincettes to catch live ticks as they may fall or escape!). The tick was crushed with pestle to get the extract solution. The extract solution was put in microtube. After that 30 µL Proteinase K was added. This was put at 56 °C for 30 minutes or overnight in the heating block. After that DNA/RNA was isolated with standard method from Genekam nucleic acid kit. The protocol is available with manufacturer. The eluted volume was 50 to 100 µl for conducting the PCR testing for different pathogens. The diagram of steps of DNA isolation is shown with the time line in Fig. 1.

Detection of Borrelia. Burgdorferi

Two µL of isolated nucleic acid was used to perform the real time or conventional PCR with Genekam PCR kits. The conventional PCR was a nested PCR. It was performed with thermoycler (Biometra) and results were observed in 2% gel agarose. Positive and negative controls were also included. Real time PCR was also performed with 2 µL of isolated nucleic acid on ABI 7500 (Thermofisher, USA). Positive and negative controls were used.

Detection of Tick-Borne Encephalitis Virus

It was done with conventional PCR kit (Genekam). 2 µl RNA was used here to perform the PCR kit on conventional thermocycler (Biometra) and results were seen on gel agarose. Positive and negative controls were also included. Real time PCR was performed on ABI7500 with 2µl of isolated nucleic acid with real time PCR kit (Genekam, Germany). Negative and positive controls were used.

Protocols for conducting the conventional and real time PCR for both pathogens are available with manufacturer.

Spectrometric measurements of isolated DNA and RNA were measurement with Nanodrop (Thermofischer, USA). 2 µL of elution buffer of isolation kit was used to calibrate the instruments and 2 µL each sample was used to measure the amount of DNA and RNA in each sample. After the measurement, each amount of isolated DNA and RNA was calculated in µg per mL.

RESULTS

We are working with this method since 2005, during these 18 years more than 250 ticks have been used to isolate their nucleic acid and a number of different assays are conducted on them. In this research work, the results of 17 ticks are presented, where isolated nucleic acid was analyzed with real time and conventional PCR for Borrelia burgdorferi and Tick-borne encephalitis virus.

The flame to sterilize the mortar and pestle was used. It is shown as Fig. 2. The size of mortar and pestle is an important factor; therefore, a smallest mortar

Fig. 2. White colored mortar and pestle.

and pestle has the size of 8 cm in diameter with a wall thickness of 0.7 cm and depth of 4 cm. The pestle has a length of 11.5 cm and thickness of 2.5 cm. A white mortar is advantageous because the tick is then easily visible. The ticks (also removed from body as well as live) were shipped to laboratory in a glass vial, small box, carton as well as microtube or taped between a folded paper.

The results showed that nucleic acid was isolated successfully from all ticks with mortar and pestle method using mini column isolation method. Out of 17 ticks, there were only 8 ticks were positive for *B.*

Table 1. Molecular and spectrometric values of isolated nucleic acid of different *Borrelia burgdorferi* positive and negative ticks

Sample	*Borrelia burgdorferi* PCR	DNA (µg/mL)	RNA (µg/mL)
1	positive	17.2	12.7
2	positive	24.6	16.1
3	positive	83.3	61.95
4	positive	41.1	25.15
5	positive	20.2	12,1
6	positive	8.75	4.85
7	positive	41.65	37.65
8	positive	66.45	52.1
9	positive	3.65	1.15
10	positive	10.65	8.6
11	negative	3.3	0.75
12	negative	8.15	5.3
13	negative	2.5	0.8
14	negative	33.8	20.65
15	negative	5.05	5.05
16	negative	7.15	4.5
17	negative	16.15	11.85

burgdorferi in conventional and real time PCR test. There was no signal in negative controls and positive control gave the signal. These are indicators of successful real time and conventional PCR detection (Table 1). Out of 17 ticks, there was only 1 tick positive for tick borne encephalitis virus.

These all results of spectrometric measurements for DNA and RNA are shown in a table 1, where the values show that amount of DNA per tick varies between 3.65 µg/mL to 83.6 µg/mL in ticks with PCR positive results for *B. burgdorferi*, but in *B. burgdorferi* negative ticks DNA yield varies between 3.3 and 33.8 µg/mL.

It is found from results of Table 1 that there is a tend to have less amount DNA in PCR negative ticks. Similarly, RNA yield varies between 1.15 and 61.95 µg/mL for *B. burgdorferi* positive ticks and it varies between 0.75 µg/mL and 20.65 µg/mL in *Borrelia burgdorferi* negative ticks. Isolated RNA output was most of the time less than that of isolated DNA in *B. burgdorferi* positive and negative ticks. These measurements are also indicators of successful isolation of nucleic acid.

DISCUSSION

In this research work, we have shown successful isolation of nucleic acid from a single tick. In literature, there are reports of using different methods for collection of the ticks e.g., liquid nitrogen during the transportation, which is not possible under field conditions [10]. During the isolation of nucleic acid, the pooled ticks are washed with ethanol, dried and cut with scalpel. [13].) These all steps are not feasible and cumbersome under field conditions. Our method is very simple and easy to use. User needs a small mortar and pestle, which can be washed and sterilized with a flame of gas burner. Moreover, these articles can be purchased in any store or online store in any country around the world. The tick can be collected in glass vial or taped between a folded piece of paper. It can be shipped to laboratory with normal post or currier service.

This method may be used with other mini column isolation kit available on the market as we have used Genekam isolation kit in this work as well as other 2 manufacturers from Germany. (data not shown) The spectrometric measurements gave us a surprise as they show that ticks contain most of the times huge amount of DNA and RNA particularly when they are infected with *B. burgdorferi* against PCR negative ticks. We are going to investigate this aspect whether there is some kind of correlation of amount of isolated nucleic acid and the presence of a pathogen in the tick. The presence of nucleic acid in each sample shows that our isolation method was successful for single tick. There are many publications, where the users are pooling the ticks between 3-10 ticks for PCR analysis, but there are no spectrometric values. Our values per tick are indicating that there is huge amount of DNA or RNA presence in samples particularly pathogen positive ticks. It means that users with pooled ticks might have been gotten huge amount nucleic acid with our method.

In another study, the comparison between different DNA isolation methods were done, but it was found that up to 2000 µg/mL DNA can be isolated with chloroform phenol method, it is not mentioned whether they used pooled samples, but it seems that this study used pooled ticks and used a cumbersome method to remove the parts of ticks, in spite of this, our mentioned method here is also in position to provide high amount of isolated nucleic acid from single tick [7].

Comparison between our results and another study done in 2015 shows that our method is going provide higher yield of DNA as this study compared different isolation commercial methods and the yield of isolated was 0.6 to 7 µg/mL, which is many folds lower against the method mentioned in this research work (up to 900 µg/mL per tick). It may indicate our method is going to achieve better yield of isolated DNA, but a comparison between the studies may be needed [18].

Our results show that isolated DNA and RNA were sufficient to conduct PCR analysis for different pathogens like *B. burgdorferi* and tick-borne encephalitis virus. We are conducting other assays on isolated nucleic acid also to be published later.

The method presented here offers to conduct PCR analysis on a single tick, so that one can conduct the prevalence of different pathogens in particular area to develop the preventive measurements. Such methods should be part and parcel of medical studies in the medical institutes to teach the students new possibili-

ties of molecular medicine. This method should be called Genekam Single Tick nucleic acid isolation method as we developed over the past 18 years to make it prefect to be used under field conditions.

CONCLUSION

In this work, it may be the first report about a new simple and inexpensive method to isolate nucleic acid from SINGLE tick in molecular biology laboratory for detection of various pathogens, which cause a number of fatal diseases. The tick is being transported at room temperature with normal post in currier or postal letter. This method may lead to better preventive measurements as well as quicker therapeutic interventions.

Authors' Contribution

Study Conception: SB, GB; Study Design: SB, GB; Supervision: SB, GB; Funding: Institute; Materials: Institute; Data Collection and/or Processing: SB, GB; Statistical Analysis and/or Data Interpretation: SB, GB; Literature Review: SB, GB; Manuscript Preparation: SB, GB and Critical Review: HS.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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