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Research Article

Keywords: Tick, Phi29, Rickettsia, Genotyping

Posted Date: November 30th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1027057/v1>

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Pathogen Detection and Genotyping With Trace Sample by A Phi29 Based Unbiased Exponential Amplification

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Abstract

Ticks are vectors for many infectious diseases such as spotted fever group (SFG) rickettsioses and borrelioses. Ticks are valuable material for pathogen ecology study. Ticks have several growth stages with significant varying size, and therefore, in most cases, the collected ticks cannot provide sufficient DNA for subsequent studies, particularly for multiple pathogen screening and genotyping. Unbiased pretreatment of the tick samples for subsequent analysis is an urgent need for subsequent ecological survey and other studies. Phi29 DNA polymerase, an enzyme with strand displacement activity, could exponentially amplify DNA randomly and non-biasedly, generating large quantities of DNA. In the present study, we developed a Phi29 based unbiased exponential amplification (PEA) assay for unbiased treatment of sample nucleic acid to provide sufficient DNA for genetic analysis. By using tick borne pathogen detection and genotype as a model, we tested and evaluated the feasibility of the assay. Nucleic acid were extracted from single ticks and subjected to PEA. The results showed that tick DNA could be amplified up to 10^5 folds. The amplified products were successfully used for pathogen screening and genotyping. With the amplified DNA from single tick, *Rickettsia* was successfully detected and genotyped. A new genotype of *Rickettsia* was identified from ticks collected from Dandong city, Liaoning province, Northeast China. This PEA assay is universal and can also be extended to other applications where samples are greatly limited.

Keywords: Tick, Phi29, *Rickettsia*, Genotyping

Introduction

The recent outbreaks of Ebola, Zika and Dengue virus worldwide remind us infectious diseases are still a challenge for human being. Of the emerging infectious diseases, a significant proportion are vector borne diseases. That is, vector borne diseases are important source of emerging infectious diseases. Ticks, one of the most important vectors, are hosts for many pathogens of zoonoses¹⁻³. Therefore, many investigations on pathogen ecology focus on ticks to survey and evaluate transmission risk of infectious diseases, especially emerging ones.

Ticks have several four growth stages, including eggs, larvae, nymphs and adult worms⁴. Their size can vary for over 300 times before and after bleeding. Free ticks, which do not suck blood, are usually very small in size⁵. This make it difficult to use them for many subsequent analysis, for only limited DNA could be extracted from them. Large amounts of DNA are frequently required for use multiple pathogen screening and genotyping⁶. Actually, in our practice, the limited amount DNA extracted from a single tick is always a critical obstacle to meet requirement of subsequent applications. How to make enough DNA for subsequent use is of great value for tick borne disease survey.

Nucleic acids could be amplified with many polymerases, most of which have biased amplification with short sequences. Phi29 DNA polymerase, an enzyme with strand displacement activity, could exponentially amplify DNA randomly and non-biasedly, generating large quantities of DNA⁷. Particularly, extremely long DNA products and the high DNA yields could be generated from exceedingly low amounts of starting material^{8,9}. This make the Phi29 an ideal tool for trace DNA treatment. In the present study, we firstly tested the amplification efficacy of Phi29 for tick DNA, and then a new Phi29 based unbiased exponential amplification (PEA) assay was developed and evaluated its applications in etiology detection and genotyping. The results showed that the method could amplify trace DNA to thousands of times and increase the detection rate without influence the genotyping.

Results

Principle of the PEA based single tick detection and genotyping

To realize single tick based detection and genotyping, a Phi29 based strategy was designed as shown in Figure 1. Briefly, free or parasitic ticks were collected from natural environment or removed from their hosts, such as goat, cattle. The collected ticks were washed with 70% alcohol to remove any contaminated bacteria or microorganisms. DNA were extracted with standard procedures. A small proportion of extracted DNA was subjected to Phi29 amplification at 37°C. After amplification, both genomic DNA of ticks and pathogens will be amplified by orders of magnitude, generating large quantity of template DNA. The amplification products were diluted and used for subsequent detection, genotyping or other applications.

Optimized Phi29 amplification efficacy of tick DNA

To test the feasibility of the PEA assay for amplification of tick DNA, one Rickettsia positive sample was used as a test model. A total of 20 randomly selected ticks were subjected to DNA extraction. These DNA were amplified with primers of ompB. As shown in Table 1, 12 of the samples were positive for Rickettsia. One of the positive samples, EDG241, was selected for PEA. Firstly, 10 ng/μl of tick DNA was used for amplification. The incubation time was set to 8, 16 and 24 hours. As shown in Figure 1, after amplification, the amplification products for 8,16, and 24 hour incubation was 220, 680 and 700ng/μl. That is, 16 hours is enough for the amplification. Then, original tick DNA was diluted to concentration of 100ng/μl,10 ng/μl,1 ng/μl, and 0.1 ng/μl, and then amplified with PEA. The concentrations of amplification products for the four different starting concentration of 100ng/μl,10 ng/μl,1 ng/μl, and 0.1 ng/μl were 579.1, 550.5, 576.8 and 544.4 μl. That is, the final concentration did not differ significant, even though the original tick concentration differed 1000 times. The amplification folds for the four templates were calculated by dividing the final amplification concentration with starting concentration. The four tick DNA were amplified for 5444, 576, 55 and 5.79

folds respectively, about 1000 fold differences.

Detection of *Rickettsia* DNA from the amplified products

We then test whether PEA also improved pathogen DNA quantity in the products. Both the original DNA and PEA amplified DNA were diluted to 100ng/μl, 10 ng/μl, 1 ng/μl, and then with PCR for *ompB* gene. As shown in Figure 2, for original tick DNA, only concentration of 100 and 10 ng/μl generated positive amplification, while for the PEA amplified DNA, in addition of 100 and 10, the concentration of 1 ng/μl also generated positive amplification. This result indicated that pathogen DNA in the tick DNA were also significantly amplified by PEA. According to the dilution factors for tick DNA and PEA products, the pathogen DNA in original tick DNA was amplified for at least 2000 folds. This high amplification efficacy *Rickettsia* DNA indicated that both tick DNA and pathogen DNA are unbiasedly amplified.

Improved pathogen detection rate by PEA

After proving that pathogen DNA are unbiasedly amplified, we test whether the PEA could improve the positive rate of pathogen detection. A total of 20 ticks with various size were extracted by genomic DNA kits. As shown in Figure 3, the DNA concentration varied significantly among the ticks, as little as 6ng/μl to 552 ng/μl. The mean concentration for all 20 ticks was 154 ng/μl. 10 ng of original tick DNA was subjected to PEA. The DNA concentrations were improved to over 700ng/μl, with an average of 200 folds of amplification. The amplified DNA were diluted and amplified for *ompB* gene. As shown in Table 1, 11 of the 20 samples were positive for *Rickettsia* before amplifications. For the PEA products, 17 out of the 20 samples were positive. That is, 6 samples were successfully detected after PEA. The positive rate was increased 30% by PEA.

***Rickettsia* genotyping with PEA amplified DNA**

The PCR products of *ompB* amplified from PEA amplified products and original tick DNA were sequenced and compared. The sequences generated from PEA products were 100% identical to that from original tick DNA. This indicated that PEA did not incorporate any mutations into the products during the amplification. This is one of the most important requirement for genotyping, for any introduction of error bases will generate incorrect genotypes. The high fidelity of PEA make it possible for use of the products for pathogen genotyping. The generated sequence of *ompB* was compared with those in Genbank database and phylogenetic tree was obtained with NG modules implemented in MEGA software. As shown in Figure 4, the *Rickettsia* located separately from other genotypes and adjacent to *R. heilongjiangensis* and *R. japonica*, implying that *R. lldd* represents a novel genotype.

Discussion

In this study, a new Phi29 based amplification assay, which we term PEA, was developed and evaluated. With the highly active strand displacement activity of Phi29 enzyme, the PEA is a powerful technique for DNA amplification. The most important advantages of PEA include long product, the high efficiency and fidelity of the amplification. All these advantages make PEA very powerful and find its use in many application fields.

With PEA, it is possible to amplify sample DNA for up to 5 orders of magnitude. In our present study, tick DNA was amplified for up to 5000 times. This produced sufficient DNA for our subsequent analysis. In our study, we also found that the final concentrations of PEA products were correlated with the incubation time, but not with the original template DNA concentration. As shown in Figure 2, the starting DNA concentration ranged from 0.1 to 100 ng/μl, with a 1000-fold difference, however, after 16 hours of amplification, all final PEA products concentrations reached about 550ng/μl. This provides a very efficient means for generating template DNA for future use.

PEA is unbiased, a great advantage over many other amplification technologies. As shown in this study, *Rickettsia* DNA could be detected in the PEA products, but not in the original tick DNA. However, even with this high efficiency of PEA, the starting DNA concentration could not be reduced unlimitedly. Extremely low starting template DNA might

bring two important problems, representativeness and amplification bias. We have tested this by serial dilution of tick DNA. Although amplification products remains comparable, excessive dilution resulted negative detection of pathogen DNA (data not shown). The reason for this might be that low quantity of starting DNA does not contain pathogen DNA due to its original low concentration in tick¹⁰.

Feasibility of PEA for tick DNA amplification for pathogen detection and genotyping was then validated with ticks collected from Dandong, Liaoning province, China. As shown in Table 1, with PEA amplification, the detection rate of *Rickettsia* DNA in these samples were increased 30%. This validated that PEA is very efficient not only for amplification of tick DNA, but also for pathogen DNA. To further test the PEA efficacy, tick DNA was diluted to as low as 0.1 ng/μl, under which concentration, *Rickettsia* DNA could be detected after MDA amplified, but not be detected without amplification (data not shown). This confirmed that PEA amplify both tick and pathogen DNA. Pathogen genotyping is very important for genetic analysis and outbreak source tracing. High fidelity of amplification are key for the genotyping¹¹. PEA results in DNA amplification up to 5 orders of magnitude, any errors incorporated in early stage will generate great changes in final products. We compared the *ompB* sequences of original and PEA amplified, no sequence differences were observed between them, indicating that in present study, the amplification is of high fidelity. Considering the high amplification efficiency and orders of magnitude, we recommend use of higher starting template DNA concentration to decrease the amplification index, accordingly reducing the error rate.

In the PEA amplification and pathogen detection, we also found a difficult choice of starting quantity of tick DNA. Because the presence or quantity of pathogen DNA in tick DNA remains unknown, it is difficult to define the optimal starting DNA¹⁰. Total DNA isolated from a single tick may vary greatly. As shown in Figure 3, the concentration of original tick DNA varied from 6 to 552 ng/μl, about 100 folds difference. In our practice, we found that for blood sucked ticks produced greatly higher concentration of DNA than those did not suck blood. The DNA quantity of ticks correlated with their sizes. Therefore, for PEA is more appropriate for non-blood sucked ticks. On the other hand, the presence and quantity of pathogen DNA in tick also vary greatly. For the 20 ticks, 11 were positive by PCR before PEA amplification, and another 6 were positive when PEA applied. With these results, we could not conclude that the other 3 samples were negative for *Rickettsia*. Two reasons are possible, one is that concentration of *Rickettsia* is too low or definitely absence, the other is that the starting DNA for PEA is too low to produce detectable concentration of *Rickettsia* DNA. With these situations, some modifications of the PEA procedures might be a possible resolution. In our ongoing study, we are trying to find solutions to improve concentration of pathogen DNA in final PEA products.

Conclusion

In the present study, in face of trace sample for tick related ecological survey, we developed a PEA assay by using unbiased strand displacement amplification activities of Phi29 DNA polymerase. With PEA, both tick and tick borne pathogen DNA could be unbiasedly amplified, making sufficient sample DNA for subsequent detection and genotyping. The PEA is a universal assay for genetic analysis with trace samples.

Materials and Methods

Ticks collection and DNA extraction

Ticks were collected from goats raised in village of Dandong city, Liaoning province, North-east China. Ticks were collected from animal skin manually without damage and then carefully placed into 70% ethanol¹². Informed consent was obtained from the animal owners for the collection of tick sample. All samples were sent to the laboratory of Shenyang Agricultural University and stored for further examination. DNA was extracted using a DNA extraction kit

(Tiangen Biochemical Technology (Beijing) Co., Ltd.) according to the manufacturer's protocols and stored at -20°C.

Amplification of single tick DNA

To determine the optimal amplification of single tick DNA, the amplification time was compared and optimized. The reaction time was set for 8h, 16h and 24h. The amplification was performed mainly using phi29 DNA Polymerase according to the instructions. Primarily, master mix was prepared by adding 4.3µl ddH₂O, 2.5µl random primer, 1µl DNA sample and 10* Phi29 DNA into a microcentrifuge tube, mixed by vortex and centrifuge briefly. Place the master mix in metal bath at 95 ° C for 3 minutes, and then put it on ice for 15min. The final reaction mixture was set up by adding 0.5µl Deoxynucleotide (dNTP) Solution Mix, 0.2µl 100*BSA and 0.5µl phi29 DNA Polymerase to 10µl. The reaction was performed at 30°C for 16h. REPLI-g Mini DNA Polymerase was inactivated by heating the mixture at 65°C for 3 minutes. Measure the concentration of the amplified DNA, and dilute with appropriate multiples and used as templates for subsequent analysis.

Rickettsia detection and genotyping from single tick

The diluted amplification products were subjected to PCR amplification of ompB gene for Rickettsia detection (Amann et al., 1995)¹³. The ompB gene was amplified with primer ompB-F (5'-GGG TGC TGC TAC ACA GCA GAA-3') and ompB-R (5'-CCG TCA CCG ATA TTA ATT GCC-3') as reported previously. The PCR reactions are as follows: 94°C for 5min denaturation, then 37cycles of 94°C for 30s, 58°C for 40s and 72°C for 40s¹⁴. PCR products of *ompB* was sequenced and the generated sequences compared with those from database for phylogenetic analysis. Phylogenetic tree was constructed based on the sequence distance method using the neighborhood-joining (NJ) algorithms implemented in the Molecular Evolutionary Genetics Analysis (MEGA) 7 software.

All animal sampling operations were performed properly according to the protocols proposed by NEON and AfVIP: TOS Protocol and Procedure: Tick and Tick-borne Pathogen Sampling and Ticks: Tick surveillance. Informed consent was obtained from the animal owners for the collection of tick sample. Molecular identification was performed in the Biosafe Laboratories of Shenyang Agricultural University using the relevant equipment with formal approval.

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Acknowledgments

This work was supported by the State Key Program of National Natural Science of China (U1808202), Project funded by China Postdoctoral Science Foundation (2021M692233), NSFC International (regional) cooperation and exchange program (31961143024), The National Key Program for Infectious Disease of China (2018ZX10101002-002), Key Program of Inner Mongolia (2019ZD006).

Author contributions

Z. Chen, H. Zhang and X. Han conceived and designed the study, X. Zhang, J. Chen, H. Xu and Q. Zhang and performed the experiments and analyzed the data, X. Zhang and Z. Chen draft and revised the manuscript.

Figures

Figure 1. Principle and procedures of single tick based detection and genotyping

Free or parasitic ticks were collected from natural environments or their hosts. After washing with 70% alcohol, the ticks as subjected to DNA extraction with genomic kits. A small part of the tick DNA were amplified with MDA to generate MDA products, which was then diluted for subsequent use. The amplified DNA could be used for PCR detection, genotyping, or genomic analysis.

Figure 2. MDA amplification efficacy of single tick DNA

(A) Effects of incubation time on final MDA product concentration; Tick DNA was amplified for 8, 16 and 24 hours, the final products concentrations were determined. (B) Amplification index for different amount of starting DNA. (C) Effects of starting tick DNA concentration on final MDA products concentrations. (D) Amplification of *ompB* gene from original tick DNA with different template concentrations; M, marker, 1-4, represent original tick concentration of 100 ng/μl, 10 ng/μl, 1 ng/μl and 0.1 ng/μl, 5 negative control. (E) Amplification of *ompB* gene from MDA products. M, marker, 1-4, represent diluted MDA product concentration of 100 ng/μl, 10 ng/μl, 1 ng/μl and 0.1 ng/μl, 5 negative control.

Figure 3. DNA concentrations and MDA amplification index for singular ticks

(A) Concentration distribution of original tick DNA and MDA amplified DNA; (B) MDA amplification index for single ticks.

Figure 4. Phylogenetic tree of *Rickettsia* with *ompB* amplified from MDA products

The trees were constructed with sequence of *ompB* using maximum-likelihood (ML) algorithms implemented in the Molecular Evolutionary Genetics Analysis (MEGA) 7 software. Black dot represents sequence acquired from this study.

Sample ID	DNA Concentration (ng/ul)		Amplification Index		Rickettsia Detection	
	Tick DNA	MDA Products	Tick DNA	Starting DNA	Tick DNA	MDA Products
EDG011	54.6	727.5	13.3	72.8	+	+
EDG015	43.7	1006.9	23.0	100.7	+	+
EDG039	27.9	962.8	34.5	96.3	+	+
EDG044	31.2	890.2	28.5	89.0	+	+
EDG047	46.7	885.4	19.0	88.5	+	+
EDG149	19.9	785	39.4	78.5	+	+
EDG124	461.5	887.5	1.9	88.8	+	+
EDG135	382.8	825.6	2.2	82.6	+	+
SD002	31.3	692.3	22.1	69.2	+	+
PH008	438.7	875.5	2.0	87.6	+	+
LZG109	87.7	885.5	10.1	88.6	+	+
PH014	278.2	994.8	3.6	99.5	-	+
PH172	552.2	1079.4	2.0	107.9	-	+
LZG102	148.3	941.2	6.3	94.1	-	+
EDG071	6.2	945.9	152.6	94.6	-	+
EDG103	6	889.8	148.3	89.0	-	+
SD010	50.3	1021.9	20.3	102.2	-	-
KD219	121.7	410	3.4	41.0	-	-
KD213	23.3	1351.5	58.0	135.2	-	-
EDG241	272.5	619	2.3	61.9	+	+

Table 1. MDA amplification of tick DNA and Rickettsia detection