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Assessing effects of different processing procedures on the yield of *Treponema pallidum* DNA from blood☆

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ABSTRACT

In this work, we employed real-time PCR analysis targeting *tp0574* to investigate the effects of different processing procedures on the yield of *T. pallidum* DNA from blood to improve assay sensitivity. The *T. pallidum* DNA yields following red blood cell lysis pretreatment were 40.4 times greater from whole blood and 32.4 times greater from residual hematocytes than yields without pretreatment. For the simulated whole-blood experiments, the *T. pallidum* DNA yields from the lower layer were 2.8, 4.6, 7.3, 12.6, 15.24, 16.7, 65.1 and 73.1 times those from the upper layer following centrifugation at 500×, 1000×, 2000×, 4000×, 5000×, 7000×, 10,000× and 20,000 × *g*, respectively. However, the *T. pallidum* DNA yields from blood clots were only 1.0% at different centrifugal forces. The experiment with infected rabbit blood showed results similar to those mentioned above. In addition, sample processing time (within 48 h) and storage temperature (4 °C and 25 °C) did not affect *T. pallidum* DNA extraction efficiency. The *T. pallidum* DNA yield can be significantly improved by red blood cell lysis pretreatment and appropriate centrifugation. Furthermore, the *T. pallidum* DNA extraction yield is greater from whole blood or residual hematocytes from anti-coagulated blood than from plasma, serum or blood clots.

Introduction

Syphilis, which is caused by the noncultivatable spirochetal pathogen *Treponema pallidum* subsp. *pallidum (T. pallidum)*, has been recognized by clinicians and the general public for hundreds of years [1]. The diagnosis of syphilis remains a challenge. Serological testing has emerged as the mainstay of syphilis diagnosis and is divided into treponemal and nontreponemal tests. The interpretation of syphilis-related serological results can be problematic in primary syphilis because serological responses might be delayed, often requiring the patient to return for re-testing. Dark-field microscopy of secretions from chancres can provide a rapid point-of care test result, but the sensitivity is limited, and personnel with the skill and meticulous care required for specimen collection and microscopy are rarely available. HIV infection is more likely to be associated with exaggerated and delayed serological responses to syphilis infection. This problem can result in a syphilis diagnosis being missed or the treatment being delayed [2]. Polymerase chain reaction (PCR) tests have been developed to aid in the diagnosis of early, primary syphilis. These tests have proven to be both sensitive and specific for primary syphilis tests based on swab samples but are less sensitive for blood tests [3]. In the past, to improve the sensitivity of PCR tests to detect *T. pallidum* DNA in the blood, almost all studies have focused on the clinical stages of syphilis and the targets and types of PCR tests but have made little progress [4–6]. To the best of our knowledge, there have been few studies considering *T. pallidum* density when performing PCRs to detect *T. pallidum* DNA in blood. Because the density of *T. pallidum* (1.051 k/cm^3) [7] is greater than the densities of serum (1.020 k/cm^3) and plasma (1.025 k/cm^3), *T. pallidum* may sink during centrifugation and become redistributed in the blood. In addition, different processing procedures and the analysis of blood fractions may also influence the yields of *T. pallidum* DNA from blood.

We used real-time PCR tests targeting the *tp0574* (*tp47*) gene to measure *T. pallidum* DNA in a series of *in vitro* simulated experiments and in an experiment with infected rabbit blood. In these experiments,

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we investigated differences in *T. pallidum* DNA yield achieved using different processing methods (including methods that vary centrifugal force, processing time and storage temperature) with different sample types (whole blood, plasma, serum, hematocytes, and blood clots) and with red blood cell lysis pretreatment.

Materials and methods

Preparation of T. pallidum

The Nichols strain of *T. pallidum* (provided by Lorenzo Giacani, University of Washington, Seattle, USA) was serially passaged by intratesticular inoculation of a male New Zealand white rabbit as previously reported [8]. Treponemes were extracted by mincing the testes and then gently rotating the tissue in a saline solution. The extract was centrifuged at 400 × *g* for 10 min at room temperature to pellet gross debris. Treponemes in the supernatant were counted by darkfield microscopy, and the *T. pallidum* concentration was adjusted to 1.0×10^7 treponemes/mL. The research involving animal use complied with all the relevant national regulations and institutional policies for the care and use of animals. All animal experiments followed the protocols approved by the experimental animal ethics committee of the Medical College of Xiamen University.

Red blood cell lysis pretreatment

Anti-coagulated or coagulated blood (0.4 mL) was spiked *in vitro* with *T. pallidum* at 0.1 mL of 1.0×10^7 treponemes/mL. All tubes were inverted 10 times and then centrifuged at 2000 × *g* for 10 min. The lower layers were mixed with red blood cell lysis buffer (Solarbio Science & Technology Co., Ltd, Beijing, China) according to the manufacturer's instructions and then centrifuged at 20,000 × *g* for 15 min. The supernatant was discarded, and the pellet was re-suspended using molecular-grade water to a volume of 0.5 mL for DNA extraction. Blood clots were fully ground using a plastic pestle before adding the red blood cell lysis buffer.

DNA extraction

All of the samples in this study were mixed with 2× lysis buffer (20 mM Tris HCl (pH 8.0), 0.2 M EDTA, 1% SDS) at same volume as the original sample and were frozen at −80 °C if DNA extraction was not performed immediately. DNA was extracted using the QIAamp DNA Blood Midi Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Finally, eluted DNA was precipitated using Dr.GenTLE® Precipitation Carrier (Takara Inc., Dalian, China) and re-suspended in 100 μL of buffer AE (10 mM Tris HCl, 0.5 mM EDTA (pH 9.0)).

Analyzing T. pallidum DNA yield with different processing times and different storage temperatures

An anti-coagulated blood sample obtained from a healthy volunteer was divided into 0.4-mL aliquots in 12 1.0-mL tubes and spiked with 0.1 mL of *T. pallidum* at 1.0×10^7 treponemes/mL. All tubes were inverted 10 times; six tubes were stored at 4 °C and six at 25 °C. After the red blood cell lysis pretreatment, the DNA was extracted from whole blood for 0, 2, 6, 12, 24 or 48 h,.

Analyzing the T. pallidum DNA yield with different centrifugal forces in normal saline, serum, and plasma

Pure liquid medium samples, including serum and plasma from a healthy volunteer and normal saline, were divided into 0.4-mL aliquots in 1.0-mL tubes, and each tube was spiked with 0.1 mL of *T. pallidum* at 1.0×10^7 treponemes/mL. All tubes were inverted 10 times and then centrifuged for 15 min at 500×, 1000×, 2000×, 4000×, 5000×, 7000×, 10,000× or 20,000 × *g*. The centrifuged samples were divided into two layers (a 0.4-mL upper layer and a 0.1-mL lower layer) for DNA extraction.

Analyzing the T. pallidum DNA yield with different centrifugal forces in anti-coagulated and coagulated blood

For the simulated *in vitro* experiment, anti-coagulated and coagulated blood from a healthy volunteer was equally divided into 0.4-mL aliquots and immediately spiked with 0.1 mL of *T. pallidum* at 1.0×10^7 treponemes/mL. All blood tubes were inverted 10 times, and 8 tubes were then centrifuged for 15 min at 500×, 1000×, 2000×, 4000×, 5000×, 7000×, 10,000× or 20,000 × *g*. The centrifuged sample was divided into two layers, including the upper layer (serum or plasma) and the lower layer (blood clot or residual hematocytes from anti-coagulated blood) based on visual assessment. Then, DNA was extracted from all of the blood fractions described above. The samples with red blood cells were pretreated before DNA extraction.

For the experiment with infected rabbit blood, anti-coagulated or coagulated blood from a rabbit infected with *T. pallidum* was divided into 0.5-mL aliquots in 1.0-mL tubes, and samples were centrifuged for 15 min at 2000 × *g* (a common centrifugal force in clinical use) and 20,000 × *g* (the maximum centrifugal force for concentrating *T. pallidum*). The centrifuged samples were divided into an upper layer (serum or plasma) and a lower layer (blood clot or residual hematocytes from anti-coagulated blood). Then, DNA was extracted from all of the blood fractions described above. The samples with red blood cells were pretreated before DNA extraction, and anti-coagulated blood that was not centrifuged (0.5 mL) was employed as a control.

Real-time PCR testing for the tp47 gene

T. pallidum 47-kDa protein gene (*tp47*) plasmids were constructed following the protocol reported in a previous study [9]. A standard curve was constructed using 10-fold serial dilutions of linearized plasmids, ranging from 10^6 to 10^1 copies/μL. Five microliters of each DNA sample was amplified in a 20-μL reaction mixture containing 10 μL of Maxima Probe qPCR Master Mix (Thermo Scientific, Waltham, USA), the sense primer (TCCCAAGTACGAGGGGAACA) and antisense primer (AAGCTTAGGCTGTCCATCGG) at 0.5 μM each, 0.2 μM TaqMan probe (FAM-CGATATTGGGTTGAAGGGGAAGGTG-TAMRA) and 1× ROX (Takara Inc., Dalian, China) in a 96-well reaction plate with a ViiA 7 Real-Time PCR System (Applied Biosystems, USA). The precise conditions for PCRs were as follows: 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 32 s. Positive DNA and control reactions without template (distilled water and negative extraction control) were included in each assay. All samples were tested in triplicate.

Statistical analysis

The analyses were performed using SPSS version 19 for Windows (IBM SPSS, Chicago, IL, USA). A variance analysis with a completely random design was performed to determine the differences in means among three or more different groups. Student's t-tests were used to compare the means between two groups. All statistical analyses were 2-sided, and a *P* value less than 0.05 was considered to indicate statistical significance.

Results

Performance characteristics of the tp47 PCR assay

Fig. 1 shows the standard curve for the *tp47* PCR assay, which has a slope of −3.33, an efficiency of 98.6% and a correlation coefficient (R