Fast Diagnosis of Bovine Theileriosis by Whole Blood PCR and Microchip Electrophoresis

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Theileria buffeli (*T. buffeli*/*orientalis*/*sergenti*) is a tick-transmitted, intracellular apicomplexan parasite that causes theileriosis in mammals throughout the world.[1,2] The typical signs of theileriosis include anemia, weight lose followed by death in grazing calves, stressed animals, and/or immuno-supressed adult cattle due to aggressive tick-sucking, especially during the spring to late summer seasons. There is no available effective drug for treatment or vaccine for prevention of *T. buffeli*-infection in cattle. However, when infected cattle were treated with diminazene diaceturate (Berenil®), buparvaquone and chlortetracycline during the early parasitemic stage, the growth of parasites was significantly inhibited.[3,4] However, screening and detection methods for whole herds prior to treatment are time-consuming. Therefore, prevention and management of this disease using insecticides and preimmunization with infected ticks on the grazing ranch is very important.

The demonstration of hemo-parasites in blood film and lymph tissues using microscopy, complement fixation test (CFT), indirect immunofluorescent assay (IFA), and enzyme linked immunosorbent assay (ELISA) are generally used for the diagnosis of theileriosis.[4,5] Recently, diagnostic methods like PCR have been developed for the rapid and accurate detection of Theileria spp.[6,7] Many PCR diagnostic tests use blood as the biological material. Typical DNA used in the PCR assay is usually extracted from the blood according to the phenol-chloroform,[8] by a "salting-out" rapid purification[9] and/or a base method.[10] However, these methods are time consuming and also have a risk of introducing contamination during DNA preparation.[11] Sample transfers increase the risk of contaminating the operator by infectious materials that may be in the sample. Formamide low temperature (FoLT) PCR is a rapid PCR protocol that was designed to allow the direct PCR amplification from whole blood.[12-17] FoLT PCR allows all the manipulations to occur in a single tube and the whole process can be automated. Unfortunately, the FoLT PCR method may not be sensitive enough for some PCR studies such as vial DNA detection or chimerism detection after grafts involving the amplification of "marginal" sequences.[18] The major problem for PCR in the blood is inability of the DNA polymerase to access the target DNA.[13] Because of the potential Taq polymerase inhibitors (such as hemoglobin), whole blood used in too large an amount may also totally inhibit the amplification reaction. Therefore, identifying the conditions that reduce the amount of protein coagulation and whole blood may be a superior way of amplifying directly from blood.

The application of microfabrication technology to microchip electrophoresis (ME) has been increasing in the interdisciplinary field in analytical chemistry. ME separation is significantly faster than conventional gel electrophoresis, and is usually completed in seconds to minutes.[19] In this report, the investigation focused on the first successful demonstration for the diagnosis of bovine theileriosis in the electrophoretic microchip after amplifying the target 816-bp DNA using only 200 nL of whole blood. A combination method using whole blood PCR and ME for the diagnosis of bovine theileriosis would be a very simple and ultrafast methodology for use in a clinical diagnostic laboratory.

Experimental Section

Chemicals. For direct PCR, 10 × PCR buffer, 25 mM $MgCl_2$, formamide, ethidium bromide and 2.5 mM dNTP mixes were purchased from Promega (Madison, WI, USA). The *Taq* DNA polymerase (5 U/μL) was obtained from Super-Bio (Suwon, Korea). An 816-bp DNA fragment from the 18S rRNA of *T. buffeli* (buffeli/orientalis/sergenti) was amplified with the forward primer (5'-AAA CTG CGA ATG GCT CAT-3') and the reverse primer (5'-ACA TCC TTG GCA AAT GCT-3') synthesized by GenoTech (Daejeon, Korea). A 100-bp DNA ladder (100 μg/mL) was purchased from Genepia (Seoul, Korea). The standard 816-bp DNA for calibration curve was obtained from the theileria 18S rRNA cloning by the transform of *E. coli* (Waters Lab Protocols, Department of Molecular Biology, Princeton University, NJ, USA).

Whole blood PCR. Blood samples were provided by the Bio-Safety Research Institute at Chonbuk National University. DNA purification from whole blood was achieved on the QIAamp DNA blood mini kit (QIAGEN Inc., Valencia, CA, USA) according to the "Blood and Body Fluid Spin Protocol" of QIAGEN.[20] Whole blood PCR amplification

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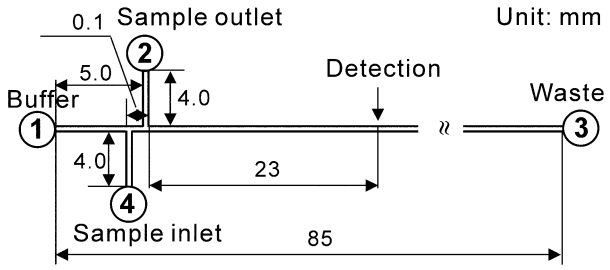
(A) Schematic diagram of microfluidic chip



(B) Applied voltage condition

Reservoir	1	2	3	4
Introduction voltage (V)	240	480	240	0
Separation voltage (V)	0	250	1650	250

Figure 1. (A) Schematic diagram of a microfluidic chip with (B) introduction and separation voltages in each reservoir.

was performed directly from a 200-nL whole blood sample. The reaction was performed in a thermal cycler (Perkin-Elmer model 2400, USA) using the same procedure described by Kang *et al.*[16] at the following temperatures: incubation at 80 °C for 10 min; 40 cycles of denaturing at 80 °C for 30 s, annealing at 40 °C for 60 s, and extension at 60 °C for 60 s; followed by 1 cycle of holding at 60 °C for 7 min. The 10-μL reaction mixture had the following final composition: 3 mM $MgCl_2$, 1 μL of 10 × PCR buffer, 0.25 mM of dNTP, 0.4 μM of each forward and reverse primer, 16% formamide, 2 U of *Taq* DNA polymerase and 200 nL of whole blood (or purified DNA). Finally, 4.5 μL of each amplified product was run on a 1% agarose gel stained with ethidium bromide.

Microchip electrophoresis. ME was performed on a DBCE-100 Microchip CE system (Digital Bio Technology Co., Korea) equipped with a diode-pumped solid-state laser (exciting at 532 nm and collecting fluorescence at 605 nm) and a high-voltage device (DBHV-100, Digital Bio Technology Co., Korea). The electrophoretic microchip was a standard microfluidics chip (MC-BF4-TT100, Micralyne Inc, USA). The chip channel was 50 μm wide and 20 μm deep. The reservoirs were 2.0 mm in diameter and 1 mm deep. A double-T injector with a 100 μm offset was selected to provide the precise injection volume. The separation channel was 85 mm long and detection was performed at 23 mm from the injection-T (Figure 1A). The sample introduction was accomplished into the injection-T region by applying 480 V at the sample outlet reservoir (2) followed by grounding the sample inlet reservoir (4) for 60 s (Figure 1B). Subsequently, separation was achieved by applying 0, 250, 1650, and 250 V at the buffer (1), sample outlet (2), waste (3) and sample (4), respectively. The reservoir positions are shown in Figure 1A. The 1 × TBE buffer was prepared by dissolving 8.5 g of premixed TBE buffer powder (Amerosco, Solon, OH, USA) in 500 mL of deionized water. Ethidium bromide was incorporated into the TBE buffer with a final concentration of 0.5 μg/mL. The sieving matrix was made by dissolving 1% (w/v) of 1,300,000 M_r polyvinylpyrrolidone (PVP) from Sigma into the 1 × TBE buffer with a dye. The mixture was shaken for 2 min and left standing for 1 h to remove any bubbles. The sieving matrix was hydrodynamically filled using a vacuum aspirator (EYELA A-3S, TOKYO RIKAKIKAI Co., Japan). Peak areas were calculated by OriginPro7.5 software (OriginLab Co., Northampton, MA, USA). The PCR product was quantitatively analyzed using the calibration curve of a standard 816-bp DNA fragment obtained by theileria 18S rRNA cloning. The amount of target DNA fragment (816-bp DNA) in the PCR product was calculated from the regression equation of the calibration curve.

Results and Discussion

In order to optimize the PCR amplification conditions, conventional slab-gel analyses of PCR products at various PCR conditions were carried out using 100-200 nL of whole blood and formamide. Figure 2 shows that an 816-bp DNA fragment from the 18S rRNA of *T. buffeli* was successfully amplified in both case using the fresh whole blood of an infected bovine (lane 1) and using the purified DNA from the whole blood of an infected bovine (lane 4). In the whole blood PCR, the formamide dissolved blood cells and the low incubation temperatures reduced protein coagulation. FoLT PCR allowed the direct PCR amplification from whole

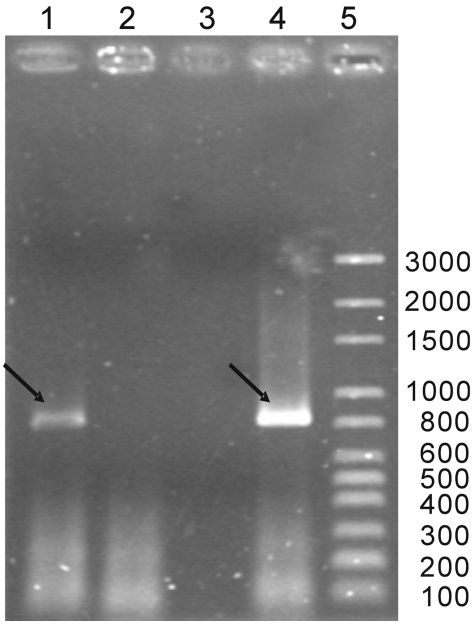


Figure 2. PCR amplified fragments from the 18S rRNA of *T. buffeli* electrophoresed on 1% agarose gel by ethidium bromide staining. Lane 1: whole blood of an infected cow. Lane 2: whole blood of a non-infected cow. Lane 3: negative control. Lane 4: purified DNA from the whole blood of an infected cow. Lane 5: 100-bp DNA ladder. The ratio of the PCR mixtures (total volume: 10 μL): blood, 2%; $MgCl_2$, 3 mM; 10 × PCR buffer, 10%; dNTP, 0.25 mM; primer (up/down), 0.4 μM; formamide, 16%; Taq DNA polymerase, 0.4 μL; whole blood (or purified DNA), 0.2 μL. *Arrow indicates the amplified 816-bp DNA.

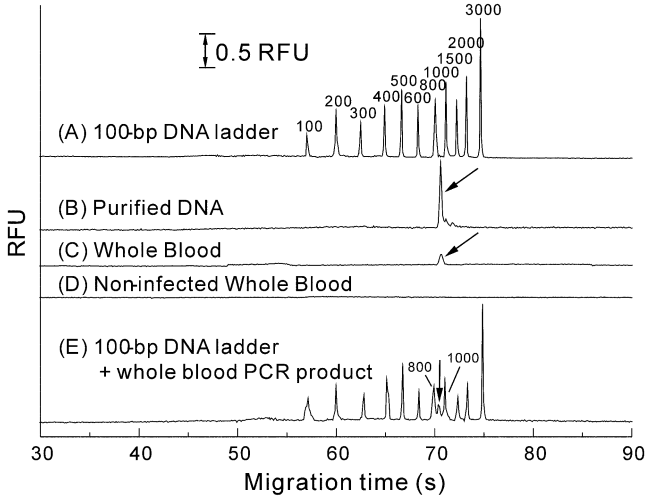


Figure 3. ME gene analysis of an 816-bp *Theileria buffeli* fragment. A: 100-bp DNA ladder (diluted 10-fold with run buffer). B: purified DNA from the whole blood of infected cattle. C: whole blood of infected cattle. D: whole blood of non-infected cattle. E. 100-bp DNA ladder plus the PCR product of whole blood of infected cattle. ME conditions: glass chip (50 μm width × 20 μm depth × 85 mm total length, 23 mm to the detection); run buffer, 0.5 ppm ethidium bromide in 1 × TBE; sieving matrix, 1% PVP (M_r 1,300,000); applied voltage, 194 V/cm; injection, 50 μm double-T (injection volume, 0.166 pL). *Arrow indicates the amplified 816-bp DNA. RFU: Relative fluorescence unit.

blood. It is always desirable to amplify the DNA directly from the clinical sample material because of the volume error involved in genetic analysis. The target DNA in the *T. buffeli* was contained in the red blood cells, which decreased the PCR efficiency as an inhibitor. According to a previous study,[16] it is very important to carefully choose the whole blood sample volume because the inaccurate quantification caused the failure of amplification of the target DNA at PCR. However, at our PCR condition, there was no notable difference between the purified DNA and the whole blood samples. These data suggest that using whole blood is as sensitive and specific as using purified DNA as starting material for PCR assays from clinical samples, provided a temperature step modification and an addition of formamide are used. Generally, the efficiency of whole blood PCR relies on alternating the heating-cooling steps since a single, longer heating incubation results in very poor amplification. This study successfully amplified the fragment up to 816-bp DNA from the 18S rRNA of *T. buffeli* using fresh whole blood samples.

The fast separation of the amplified 816-bp DNA fragment from the 18S rRNA of *T. buffeli* on a microfabricated chip (Figure 3) was accomplished using various PVP concentrations ranging from 0.5 to 5.0%. Higher PVP concentrations showed a better resolution of the 100-bp DNA ladder, but a longer migration time. One percent (w/v) of the PVP at the other optimum conditions could separate the PCR product (816-bp DNA) of *T. buffeli* within 71 s (Figure 3), which was approximately 200-fold faster than by conventional gel electrophoresis. The results are consistent with those obtained using purified DNA (Figure 3B), whole blood (Figure 3C) and slab gel electrophoresis (Figure 2). Addition of whole blood PCR product (Figure 3C) to the 100-bp DNA ladder (Figure 3A) gave the results as shown in Figure 3E. The correspondence peak further confirms the identify of the amplified 816-bp DNA fragment as a whole blood PCR product. The calibration curve for the standard 816-bp DNA fragment was linear over the concentration range 17.51-52.54 μg/mL. The regression equation of the curve was calculated as $y = 0.0051x + 0.0762$ (correlation coefficient, $r = 0.9988$), where y is the peak area and x is the concentration of the standard 816-bp DNA. The contents of the amplified 816-bp DNA fragments in the PCR products of purified DNA and whole blood were 353.35 μg/mL and 94.65 μg/mL, respectively. The relative standard deviations ($n = 5$) for the peak area and the migration time of the whole blood PCR product were 2.592% and 0.267%, respectively.

In conclusion, this study is the first to demonstrate the application of ME and direct PCR to the diagnosis of bovine theileriosis using whole blood without DNA purification. A rapid ME was used to separate and quantify the amplified 816-bp DNA fragment produced by FoLT PCR of the whole blood samples. This rapid and convenient procedure provides an alternative to the tedious DNA purification method and prevents possible errors inherent to the DNA extraction procedure such as sample inversion or contamination. This method is very straightforward and should be suitable for a point-of-care diagnosis or on a lab-on-a-chip where an immediate test of the collected sample is required. The sample can be analyzed immediately and also has the advantage of reducing the consumption of expensive reagents compared to normal PCR and general slab gel electrophoresis. These simple, yet powerful, demonstrations of whole blood PCR and ME might be applied in other clinical diagnoses.

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