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Original article

A comparison of DNA extraction protocols from blood spotted on FTA cards for the detection of tick-borne pathogens by Reverse Line Blot hybridization

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ABSTRACT

An essential step in the molecular detection of tick-borne pathogens (TBPs) in blood is the extraction of DNA. When cooled storage of blood under field conditions prior to DNA extraction in a dedicated laboratory is not possible, the storage of blood on filter paper forms a promising alternative. We evaluated six DNA extraction methods from blood spotted on FTA Classic® cards (FTA cards), to determine the optimal protocol for the subsequent molecular detection of TBPs by PCR and the Reverse Line Blot hybridization assay (RLB). Ten-fold serial dilutions of bovine blood infected with *Babesia bovis*, *Theileria mutans*, *Anaplasma marginale* or *Ehrlichia ruminantium* were made by dilution with uninfected blood and spotted on FTA cards. Subsequently, DNA was extracted from FTA cards using six different DNA extraction protocols. DNA was also isolated from whole blood dilutions using a commercial kit. PCR/RLB results showed that washing of 3 mm discs punched from FTA cards with FTA purification reagent followed by DNA extraction using Chelex® resin was the most sensitive procedure. The detection limit could be improved when more discs were used as starting material for the DNA extraction, whereby the use of sixteen 3 mm discs proved to be most practical. The presented best practice method for the extraction of DNA from blood spotted on FTA cards will facilitate epidemiological studies on TBPs. It may be particularly useful for field studies where a cold chain is absent.

1. Introduction

Over the years, a number of different molecular diagnostic assays have been developed to detect tick-borne pathogens (TBPs) with high sensitivity and specificity (Criado-Fornelio, 2007). One assay which has found widespread adaptation in the detection and differentiation of tick-borne pathogens is the Reverse Line Blot (RLB) hybridization assay. This macroarray is capable of simultaneously detecting multiple TBPs in a single sample (Bekker et al., 2002; Gubbels et al., 1999).

An essential step in the molecular detection of TBPs in blood is the extraction of DNA. For most DNA extraction methods, the use of a functional molecular biology laboratory is essential, but the cold or frozen storage of blood samples prior to analysis in a dedicated laboratory may be difficult in field situations where a cold chain is not readily available.

In the absence of a functional cold chain, the storage of blood spotted on filter paper forms a promising alternative. Flinders Technology Associates (FTA®) technology (Whatman) has improved filter paper based systems, as it protects the sample from spoiling and degradation, allowing for long-term storage and archiving of nucleic acids at room temperature (RT) (Ahmed et al., 2011). In addition, potential pathogens are lysed and become inactivated on FTA cards, making the samples safe to handle and transport (Abdelwhab et al., 2011).

Several methods to extract DNA from blood spotted on filter paper for the molecular detection of TBPs have been described. These include phenol–chloroform-isoamyl alcohol based extraction (PCI) (Cardoso et al., 2010), the use of FTA purification reagent (Simuunza et al., 2011), Saponin followed by Chelex® (Devos and Geysen, 2004), Saponin combined with PCI (Tani et al., 2008) and commercial solid-phase extraction kits (Salih et al., 2007). Here we report on the evaluation of six different DNA extraction methods from blood spotted on FTA cards, to determine the optimal protocol in terms of sensitivity and cost-effectiveness for subsequent

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molecular detection and epidemiological investigation of TBPs by PCR and RLB.

2. Materials and methods

2.1. Parasite stocks

Four blood samples, stored in liquid nitrogen and infected with *Babesia bovis* (6% parasitemia), *Theileria mutans* (unknown parasitemia), *Anaplasma marginale* (20% parasitemia) and *Ehrlichia ruminantium* (unknown parasitemia) were used in this study. The samples were thawed at RT and subsequently diluted ten-fold with freshly collected whole blood from a non-infected calf to a final dilution of 10^{-10}.

2.2. Preparation of samples for DNA extraction

For each TBP, 125 μl of infected blood was spotted on FTA cards, in quadruplicate. After overnight air drying at RT, 3 mm diameter discs were punched out using a Harris Micro-Punch (Whatman) and placed together into Eppendorf tubes for DNA extraction. In order to avoid carryover contamination between samples, discs from FTA cards containing the highest dilution were punched out first and five discs were cut from a blank filter paper after each sample. As negative extraction controls, discs were punched from blank FTA cards and processed together with the samples to be analysed.

2.3. DNA extraction

DNA was extracted from FTA cards using the following six DNA extraction protocols:

2.3.1. Whatman FTA protocol

FTA discs were prepared for PCR using FTA purification reagent following Whatman Protocol BD08 with some modifications. Briefly, the discs were washed twice for 15 min with 500 μl FTA purification reagent (Whatman) followed by two rinses of 15 min with 1.0 ml TE^{-1} buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). The discs were left to dry at RT and then transferred to PCR tubes as template for the PCR reaction.

2.3.2. Standard phenol-chloroform-isoamyl alcohol (PCI) protocol

The standard PCI method was carried out according to the protocol adapted from Ausubel et al. (1995). Briefly, discs were placed in a 1.5 ml Eppendorf tube. Then 400 μl extraction buffer (10 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA) and 10 μl proteinase K (20 mg/ml) were added and the mixture was vortexed and incubated at 56 °C for a minimum of 2 h with agitation. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, Sigma-Aldrich) was subsequently added, after which the samples were vortexed briefly and centrifuged for 10 min at 13,000 × *g*. The upper aqueous layer containing the target DNA was preserved and mixed with 50 μl of 3 M sodium acetate (pH 5.2) by vortexing. Then, 800 μl of ice-cold 100% ethanol was added to the mixture and precipitated at −20 °C for at least 90 min. Next, the precipitate was pelleted by centrifugation at 13,000 × *g* for 10 min. Following removal of the supernatant, 1 ml of 70% ethanol was added to the tube and it was centrifuged at maximum speed for 4 min. The supernatant was then completely discarded followed by air drying until no visible liquid remained. Pellets were finally rehydrated with 100 μl of distilled water.

2.3.3. Saponin – PCI method

A Chelex-based method in combination with saponin washing and PCI extraction (Tani et al., 2008) was adapted follows: the FTA discs were eluted with 1 ml of 0.5% saponin in PBS, briefly shaken, incubated at 4 °C for 2 h and centrifuged at 8,200 × *g* for 5 min. The supernatant was removed and replaced with 1 ml PBS and centrifuge once again at 8200 x g for 5 min. The supernatant was again removed and replaced with 300 μl of a 10% Chelex® 100 (Bio-Rad Laboratories, Hercules, California, USA) solution. The tubes were then incubated in a heating block at 95 °C for 10 min and centrifuged at 8200 × *g* for 5 min. The supernatant was transferred to a new tube and centrifuged at 13,000 × *g* for 5 min. The supernatant was used, mixed with an equal volume of PCI after which DNA was extracted as described in the PCI protocol above.

2.3.4. NucleoSpin® tissue kit

A NucleoSpin® Tissue Kit support protocol for dried blood spots was followed according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany). The concentrated DNA was eluted with 100 μl of elution buffer prewarmed to 70 °C.

2.3.5. FTA purification reagent and extraction using Chelex® 100 resin

For this method, a protocol previously used for the detection of *Trypanosoma* species (Ahmed et al., 2011) was adapted as follows: the FTA discs were washed and prepared using FTA purification reagent following Whatman Protocol BD08 as described above under I. After drying at 45 °C for at least 60 min, discs were incubated for 30 min at 90 °C in 100 μl of 5% (w/v) aqueous suspension of Chelex® 100 resin. This was followed by centrifugation of the sample for 3 min at 20,000 × *g*. The supernatant was subsequently transferred to a new sterile pre-labeled microcentrifuge tube, without disturbing the Chelex® 100 resin pellet.

2.3.6. Microwave irradiation

Microwave irradiation based DNA extraction was modified after a recently published method (Port et al., 2014). Briefly, 50 μl of direct whole blood sample dilutions or 8 dried FTA discs were assessed as starting materials. The dried discs from FTA cards were moisturized in 30 μl sterile PBS. Both types of samples were transferred into 0.5 ml tubes and treated at 900 W for 2 min in a microwave (Model R-939IN-A, Sharp, Thailand) until precipitated and condensed droplets were visible on the tube walls. After spinning down the condensed droplets, 2.5 μl of the clear precipitated watery solution containing DNA was used as a template for the PCR.

2.3.7. DNA extraction from whole blood samples

For comparison purpose, DNA was also extracted directly from 50 μl of infected whole blood sample in a final elution volume of 100 μl using the NucleoSpin® Blood kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions.

2.4. Quantification of DNA

The extracted DNAs were quantified by using Epoch™ Multi-Volume Spectrophotometer System (BioTek, Winooski, VT, USA). A volume of 2 μl was placed in duplicate on a Take 3™ plate and absorbance was measure at 260 nm. The data was collected and analysed using the Gen5 software. Normalization of DNA amounts was conducted for the dilution series of *B. bovis*, to rule out an effect of varying DNA amounts associated with the efficiency of the different extraction methods on the assay's sensitivity.

2.5. Comparison of different number of 3 mm FTA discs

From serially diluted blood infected with *B. bovis*, DNA was extracted from different numbers of 3 mm FTA card discs (1, 2, 4, 8 and 16 discs) using protocol V.

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