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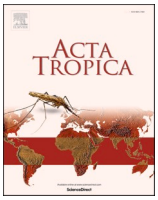

An elution independent collection device (EICD) for rapid collection of *Anaplasma marginale* DNA from blood samples

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ABSTRACT

Currently utilized molecular detection methods are based mainly on nucleic acid extraction, amplification, and detection procedures that may require costly equipment, numerous reagents, and highly trained personnel. These requirements make diagnostic tests expensive, time-consuming, and not suitable for point-of-care applications. There is an increasing demand for simple, low-cost portable technologies. To overcome these challenges, a paper-based elution independent collection device (EICD) was designed to collect microorganisms and recover nucleic acids for molecular biology applications with minimal steps. In this study, we demonstrate a simpler *Anaplasma marginale* detection that uses an EICD for nucleic acid collection combined with recombinase polymerase amplification (RPA), and a lateral flow dipstick for detection of the specified target. A pre-lysis blood treatment was optimized that uses Triton X-100 lysis buffer and bovine serum album in wash buffer. Blood samples were incubated for 5 min at room temperature and run through the EICD. Four 1-mm diameter discs excised from EICD were used as template in basic RPA and lateral flow (*nfo*) (endonuclease IV) RPA assays. Each disc of soluble central membrane (SCM) carried circa 0.249 pg/μl of *Anaplasma* DNA. The percentage of nucleic acid recoverable from the SCM ranged between 60% - 70%. Blood samples infected with *A. marginale* were treated with Triton X-100 pre-lysis protocol. All samples tested positive by PCR and RPA methods. EICD-driven collection of blood samples is a practical method successfully adapted to detect *Anaplasma* spp. or blood-borne pathogen DNA and has potential for point-of-care detection in resource-limited settings.

1. Introduction

Development of medical and veterinary diagnostics based on nucleic acids have been targeting molecular biomarkers to detect and discover disease-causing organisms from biological samples (Chin et al., 2011; Denes and Wiedmann, 2014; Kagan et al., 2011; Niemz et al., 2011). Commonly used biological samples are blood, urine, feces, and saliva (Cummins et al., 2016). Commercial kits and detergent-based methods are widely used for DNA extraction (Demeke and Jenkins, 2010); however, these methods are time-consuming, require trained personnel, reagents, equipment, and kits which are costly (McFall et al., 2015; Roberts et al., 2012). Different nucleic acid extraction protocols to address issues related to DNA or RNA extraction from blood samples have been developed. Collection/extraction protocols include paper devices such as EICD (elution independent collection device)(patent #: US9423398B2), commercially available filter-trapping paper (FTA cards), FINA (filtration isolation of nucleic acid) and origami paper-based extraction (Govindarajan et al., 2012; Jangam et al., 2009; Josue-Caasi, 2012; Lu et al., 2016; Tang et al., 2017a). Paper-based devices are low-cost, portable, easy-to-use in remote places, and allow the storage of specimens for long periods of time (Tang et al., 2017b).

There is a need to continue developing rapid and easy-use devices or methods for long term storage for blood samples taken from field settings for direct use in point-of-care diagnostics and microbial forensic applications. Such methods should be not complicated or expensive and need to provide easy access to stored nucleic acids for faster detection. One of the most utilized paper-based methods for capturing DNA and RNA from blood samples is FTA (Flinders Technology Associates) cards (Castellanos-Gonzalez et al., 2018; Malsagova et al., 2020; Nzelu et al., 2019). DNA extraction from DBS (Dried Blood Spots) or FTA cards is uncomplicated, but requires additional steps to use extracted nucleic acids in molecular techniques such as Chelex-100, TE buffer, methanol

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and/or phenol-chloroform (Ahmed et al., 2013; Baidjoe et al., 2013; Bereczky et al., 2005; Freeman et al., 2018; Golassa et al., 2013; Panda et al., 2019). Additionally, issues related with drying and storage DBS in field conditions may influence the quality and quantity of DNA extracted for diagnosis (Lim, 2018). The need for reliable yet accessible means to access DNA from blood samples is becoming more critical as detection and diagnostic methods targeting pathogen DNA are shifting toward isothermal-based assays like LAMP (Loop-mediated isothermal amplification) and RPA (Recombinase polymerase amplification).

An elution independent collection device (EICD) was developed to collect plant pathogens, rapidly recover the nucleic acids, provide room temperature storage, and still be applicable for molecular biology assays (Josue-Caasi, 2012). Containing a soluble biomaterial as central element (Josue-Caasi, 2012), the EICD provides the means to process field-collected fluidic specimens directly into reaction tubes which reduces the time needed for molecular detection (PCR, qPCR, or isothermal-LAMP, Helicase dependent amplification (HDA) or RPA) (Josue-Caasi, 2012). An advantage of the EICD is that the elution step using buffers is not required to elute the filtered pathogens before an amplification reaction since the solid matrix which traps the pathogens is soluble in the assay reaction mix (Josue-Caasi, 2012). The EICD was developed and initially tested using plant tissue and has not been tested for collection and subsequent detection of pathogens in blood.

One of the important diseases that impacts livestock production worldwide is bovine anaplasmosis, caused by *Anaplasma marginale*, an obligate intracellular parasite that develops in the erythrocytes of cattle (Kocan et al., 2010; Rymaszewska and Grenda, 2008). *Anaplasma marginale* causes significant economic losses in countries where livestock significantly features into the economy such as the United States, Latin America, and Africa (Rymaszewska and Grenda, 2008). The parasite is transmitted mechanically via the bites of flies (*Stomoxys calcitrans*) and blood-contaminated medical instruments and biologically through various species of ticks (Rar and Golovljova, 2011; Scoles et al., 2005).

This study aimed to adapt the EICD for the rapid field collection of *Anaplasma* DNA from blood samples coupled to gel-based RPA and multiplex lateral flow RPA assays. This entailed two main aims: 1) to establish a method to pre-treat *A. marginale* infected blood for efficient extraction of DNA; and 2) develop a protocol to directly use DNA from the EICD for direct testing by gel-based and multiplex lateral flow RPA assays.

2. Materials and methods

2.1. Reference positive controls and pre-lysis blood treatments

Reference positive controls consisting of ten *A. marginale*-infected bovine blood samples in DMSO were provided by Dr. Kathy Kocan (Oklahoma State University College of Veterinary Medicine, Stillwater, Oklahoma). After the removal of DMSO, the *A. marginale* blood samples were combined with fresh whole cattle blood. *Anaplasma marginale* DNA was extracted using commercial QIAamp Blood mini kit (Qiagen Inc., USA) which was used as a reference positive extracted DNA. Blood was treated separately with two pre-lysis buffer protocols using Triton X-100 and ammonium chloride as described by Butzler et al. (2017) and the fastest pre-lysis procedure was selected and optimized.

Comparative studies optimized the fastest pre-lysis procedure, testing the intra-step incubation times for each of the four main steps involved: 10% formaldehyde (10, 5, and 0 min), Triton X-100 solution (30, 20, 10, 5 min), wash buffer with bovine serum albumin, and centrifugation (10 and 0 min) (Table 1). In each experiment, the incubation time of each reagent or solution was reduced.

Each lysed blood sample was filtered through a patented EICD (patent No.: US 9423398 B2). The EICD consisted of a backing support, sample pad, soluble central membrane, and wick (Supplement Fig. 1). Essentially, the lysed blood was lateral flow-filtered through a sample pad and into the central membrane where microorganisms, virus, and nucleic acids were retained. Once the targeted pathogen of lysed sample nucleic acid was captured in the soluble central membrane (SCM), small discs for direct use in PCR or RPA reactions could be excised. In this study, the sample was collected from the EICD by excision of 1 mm disc from two sections of SCM containing the target DNA.

Table 1
Description of total numbers of tested blood samples, combinations of Triton X-100 pre-lysis optimization assays, and numbers of PCR reactions.

Optimization assay	Blood samples (n)	Triton X-100 lysis *	PCR & RPA^ (# reactions)
1	3	F(10)+T(20)+WB+C	24
2	3	F(10)+T(10)+WB+C	24
3	3	F(5)+T(10)+WB+C	24
4	3	F(5)+T(5)+WB+C	24
5	1	F(5)+T(0)+WB+C	8
6	1	F(0)+T(5)+WB+C	8
7	1	F(0)+T(0)+WB+C	8
8	1	F(0)+T(0)+WB+C	8
9	1	F(0)+T(0)+WB	8
10	1	F(0)+T(0)+WB+I(5)	8
11	1	F(0)+T(0)+WB+I(0)	8
12	1	T(0)+WB+I(5)	8
13	1	T(0)+WB+I(0)	8

* **F:** 10% formaldehyde (10, 5, and 0 min), **T:** Triton X-100 solution (20, 10, 5, 0 min), **WB:** wash buffer with bovine serum albumin, **C:** centrifugation, and **I:** incubation time (0 or 5 min).

^ Endpoint PCR with RPA and published PCR primers, Sections 1 and 2 of SCM.

2.2. End-point PCR with soluble membrane of EICD

Each of the treated samples (3 experimental *A. marginale* infected blood samples treated with 4 pre-lysis assays (Table 1 assays 1-4) and 1 experimental *A. marginale* infected blood sample treated with 4 pre-lysis assays (Table 1 assays 5-13)) were filtered through EICD and endpoint PCR reactions were performed using one SCM disc of Sections 1 and 2, separately (Supplementary Fig 1) to observe the bacterial DNA amplification and evaluate the optimal pre-lysis blood treatment. As a comparative analysis, two endpoint PCR amplifications were developed, 1) using published PCR primers for *A. marginale* (Torina et al., 2012) and 2) using *A. marginale* RPA primers (Salazar et al., 2021), which were also adapted for end-point PCR by performing a temperature gradient in order to obtain the optimal annealing temperature. Each PCR reactions were performed in duplicate.

The reaction mix for end-point PCR using published primers targeting the *A. marginale msp4* gene (Torina et al., 2012) contained 10 µl of 2X GoTaq Green Master Mix (Promega, USA), 0.5 µl of each primer (AmargMSP4Fw (5'-CTGAAGGGGGAGTAATGGG-3') and AmargMSP4Rev (5'-GGTAATAGCTGCCAGAGATTCC-3') (10 µM)), 1 disc (1mm diameter) excised from the EICD, 9 µl of nuclease-free water (Promega, USA) for a final volume of 20 µl. The test was performed in a thermal cycler (Eppendorf, Germany) with cycling parameters consisting of initial denaturation of 94°C for 4 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 10 min. The PCR reaction mix using the *A. marginale* RPA primers developed in this study consisted of 10 µl of 2X GoTaq Green Master Mix (Promega, USA), 0.5 µl of each RPA sense and antisense primers (10 µM) (Am3L_msp4: ACGAAGTGGCTTCTGAAGGGGGAGTAATGGGAG, Am3R_msp4: GACTCACGCATGTCGAAC

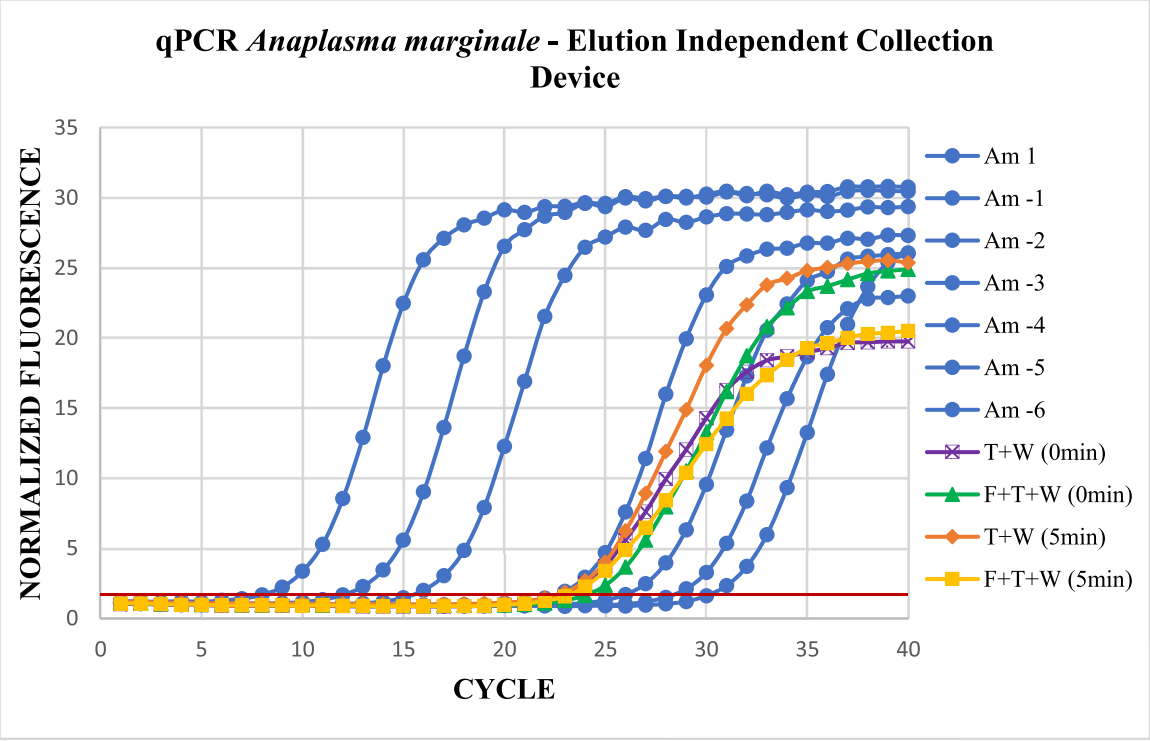

Fig. 1. Quantitative PCR assay using ten-fold serial dilution of *A. marginale* plasmid from 1ng/µl to 1fg/µl and total DNA filtrated with EICD prototype (one *A. marginale*-infected blood sample). Plasmid concentration: Am 1 = 1 ng/µl, Am -1 = 0.1 ng/µl, Am -2 = 0.01 ng/µl, Am -3 = 1 pg/µl, Am -4 = 0.1 pg/µl, Am -5 = 0.01 pg/µl, Am -6 = 1 fg/µl. Treatments: T+W (0 min) = Triton + wash buffer (no incubation), F+T+W (0 min) = formaldehyde + Triton + wash buffer (no incubation), T+W (5min) = Triton + wash buffer (5 min incubation), F+T+W (5min) = formaldehyde + Triton + wash buffer (5 min incubation). Cycle or Ct values ≤ 29 are strong positive reactions indicative of abundant target DNA in the sample (SCM) and cycle or Ct values of 30-35 are positive reactions indicative of moderate amounts of target DNA.

GAGGTAACAGAA), 1 disc (1mm diameter) excised from the EICD, 9 µl of nuclease-free water (Promega, USA) for a final PCR reaction volume of 20 µl. PCR reactions were performed in a thermal cycler (Eppendorf, Germany) with the following cycling parameters: initial denaturation of 94°C for 4 min, then 40 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis in a 2% agarose gel in 0.5X TAE (Tris-acetate-EDTA) buffer and SYBR safe (Invitrogen, USA).

2.3. LC green quantitative PCR using EICD soluble membrane

To quantify the recovered bacterial DNA from the EICD membrane and analyze the optimal pre-lysis blood protocol, samples of *A. marginale* infected blood were pre-lysed by four optimization assays (Table 1, assays 10-13) and filtered through the EICD. Additionally, two concentrations (5 and 10 ng/µl) of plasmid carrying the diagnostic *A. marginale* target were filtered through EICD prototypes and two types of EICD soluble central membrane (SCM) (ASW-50 and ASW-240), which vary in thickness, were also evaluated. One disc containing *A. marginale* DNA was excised from each of the EICD SCM membranes and tested by LC green qPCR.

LC green qPCR was performed based on ten-fold serial dilution of plasmid containing the targeted diagnostic segment of *A. marginale* from 1ng/µl to 1fg/µl. The qPCR amplification assays were performed in a Rotor Gene 6000 series (Corbett Research, Qiagen Inc., USA) and consisted of 10 µl of One Taq Hot Start DNA Polymerase (Biolabs, USA), 0.5 µl of each RPA sense and antisense primers (10 µM), 2 µl of LC green (BioChem, USA), either 1 µl of plasmid or 1 disc (1mm diameter) of the EICD, 6 µl nuclease-free water (Promega, USA) for a final volume of 20 µl. Each reaction was performed in three replicates. The cycling parameters consisted of initial start of 50°C for 3 min, initial denaturation of 94°C for 4 min, then 40 cycles of denaturation at 95°C for 20 s, annealing at 62°C for 20 s, extension at 72°C for 20 s and a final extension at 72°C for 4 min. The mean of each set of replicates was calculated.

2.4. RPA using EICD soluble central membrane

To validate the RPA assay, *A. marginale* infected blood treated with optimal pre-lysis protocol was filtered through EICD and 2-4 SCM discs were used as template in separate RPA reactions. These experiments demonstrated the optimal amount of SCM discs for accurate isothermal amplification. The RPA assay was performed with TwistAmp basic kit (TwistDx, United Kingdom) consisting of 29.5 µl of rehydration buffer, 2.4 µl of each RPA forward and reverse primer (10 µM), 10 µl betaine (5M) (Thermo Fisher Scientific, USA), two to four discs (1mm diameter) of EICD, nuclease-free water (Promega, USA) and 2.5 µl of magnesium acetate (280mM) (to activate the reaction) for a final reaction volume of 50 µl. RPA reactions were performed in a dry bath incubator (GeneMate/Bioexpress, USA) and incubated at a constant temperature (37°C) for 20 min. At the end of the reaction, the temperature was increased to 80°C for 5 min to deactivate the enzyme complex. The amplified RPA product was purified by QIAquick PCR Purification Kit (Qiagen Inc., USA) and analyzed by electrophoresis on a 2% agarose gel in 0.5X TAE buffer and SYBR safe (Invitrogen, USA). This step was necessary to visualize the RPA products in agarose gel electrophoresis.

The multiplex RPA reaction using a lateral flow device (PCRD Nucleic Acid Detector, Abingdon Health, United Kingdom) targeting the *A. marginale msp4* gene and the GAPDH gene (internal control) was performed with a TwistAmp *nfo* kit (TwistDx, United Kingdom) consisting of 29.5 µl of rehydration buffer, 10 µl betaine (5M) (Thermo

Fisher Scientific, USA), 0.2 µl of the *A. marginale nfo* probe [5'Label-FAM] TAGCTTTTACGTGGGTGCGGCCT[THF]CAGCCCAGCATTTCC [3'block-C3spacer] (10 µM), 0.2 µl of the internal control *nfo* probe [5'Label-DIG]CTGGCAAAGTGGACATCGTCGCCATCAATGACCC[THF] TTCATTGACCTTCACT[3'block-C3spacer] (LGC Biosearch Technologies, USA), 1.8 µl of each *A. marginale* and internal control RPA forward and biotin-labeled reverse primers (10 µM), two or four discs (1mm diameter) of EICD, and nuclease-free water (Promega, USA) for a final reaction volume of 50 µl. RPA reactions were incubated in a dry bath incubator (GeneMate/Bioexpress, USA) for 20 min at 37°C. At the end of the reaction, the temperature was increased to 80°C for 5 min to deactivate the enzyme complex. After amplification, 6 µl of RPA product was mixed with 84 µl of buffer (Abingdon Health, UK) and 75 µl of the diluted sample was added to the PCRD test cassette. Results were read after 10 min.

The presence of control line C confirmed the lateral flow test was working properly. The development of Test lines 1 and 2 consistently appeared in positive controls (sample and plasmid) in each assay to demonstrate the amplification of the GAPDH gene target and *A. marginale*, respectively. Artificial positive control (APC) based on tandem of forward, reverse complement and *nfo* probe sequences of *Anaplasma* spp. and internal control RPA primers was used as the positive control in *nfo* RPA reactions.

3. Results

3.1. Optimization of pre-lysis buffer and end-point PCR with EICD soluble membrane

No differences occurred as a result of the pre-treatment of 12 *A. marginale* samples using the ammonium chloride or Triton X-100 lysis protocols nor was amplification observed with non-template control (water) (data not shown). Because of the ease and fast performance time, the Triton-X-100 pre-lysis protocol was selected for further optimization.

Three *A. marginale* infected blood samples treated with different combinations of the Triton X-100 protocol were filtered through the EICD (Table 1 assays 1 – 9). All endpoint PCR reactions using an excised SCM disc (Sections 1 and 2, Supplementary Fig 1) performed in each optimization assay amplified the expected product size using RPA primers (103 bp) as well as published PCR primers (344 bp). Four assays were also developed to optimized pre-lysis treatment (Table 1 assays 10 – 13): optimization assay (OA) 10 (10% formaldehyde + Triton X-100 solution + wash buffer, incubation: 5 min), OA 11 (10% formaldehyde + Triton X-100 solution + wash buffer, no incubation), OA 12 (Triton X-100 solution + wash buffer, incubation: 5 min), OA 13 (Triton X-100 solution + wash buffer, no incubation). Thirty-two endpoint PCR assays (1 blood sample x 4 OAs (Table 1 assays 10-13) x 2 endpoint PCR reactions (published and RPA primers) x 2 sections of EICD (Supplementary Fig 1) x duplicate analysis) were run and the expected product size of 103 bp using RPA primers and 344 bp using published PCR primers were visible (Table 1 assays 10 – 13). The results were consistent in each experiment and no differences in target amplification were detected. The optimal, faster, simple and easy-to-use pre-lysis protocol consisted of mixing blood sample, Triton X-100 solution, and wash buffer, then either incubating for 5 min at room temperature or without incubation.

3.2. LC green quantitative PCR from EICD soluble central membrane

LC green quantitative PCR was used to estimate the amount of *A. marginale* DNA captured in the EICD after the lysis step (Triton X-100 protocol). The concentration of *A. marginale* DNA recovered from Treatment 1 (Triton + wash buffer (no incubation)) was 0.239 pg/µl, Treatment 2 (formaldehyde + Triton + wash buffer (no incubation)) was 0.118 pg/µl, Treatment 3 (Triton + wash buffer (5 min incubation)) was 0.249 pg/µl, and Treatment 4 (formaldehyde + Triton + wash buffer (5 min incubation)) was 0.219 pg/µl (Fig. 1). Treatments 1 and 3 did not show difference in DNA quantity, and the incubation time did not affect the amount of DNA spiked onto the SCM. However, the use of formaldehyde reduced DNA concentration (Treatment 2). Hence, the optimal pre-lysis treatment selected was Triton X-100 solution and wash buffer incubated for 5 min with or without incubation at room temperature.

Results of qPCR performed to measure two *A. marginale* plasmid concentrations (5 and 10 ng/µl) are described in Fig. 2. Quantity of DNA recovered when EICD filtered 10 ng/µl and 5 ng/µl of plasmid using ASW-50 was 6.00495 ng/µl and 3.6403 ng/µl, respectively; while when EICD filtered 10 ng/µl and 5 ng/µl of plasmid using ASW-240 was 6.0475 ng/µl and 3.5176, respectively. The amount of plasmid DNA recovered was slightly different in each type of SCM used. The percentage of recovered plasmid in both soluble central membranes was approximately between 60 – 70%, allowing the use of either membrane type of SCM.

3.3. RPA using soluble central membrane of EICD

Optimized basic and lateral flow RPA reactions were performed using a template consisting of different numbers of SCM discs (2, 3, and 4) containing DNA of the target pathogen (*A. marginale)*. The results showed that the intensity of band depended on the number of discs added to RPA reactions; thus, optimal number of SCM discs was four which produced a clear and intense band (Fig. 3A). In this assay, only *A. marginale* was targeted (Lane 2). The approximate amount of *Anaplasma* DNA in each disc was 0.249 pg/µl; therefore, RPA reaction produced a faint band with 0.498 pg/µl of DNA, and intense band with 0.996 pg/µl of DNA. Based on these results, a simple lateral flow RPA amplification was developed using SCM as a template. Detection strips showed clear and intense test line (2) where four discs were added in RPA reaction (4D-Am); however, only a faint test line appeared where two discs were added in RPA reaction (Fig. 3B). Only the control line could be observed for non-template control (water). This result confirmed that four discs of SCM were required for accurate isothermal amplification.

3.4. End-point PCR, gel-based RPA, multiplex lateral flow RPA using soluble membrane of EICD

For the validation test of the system, eight different *A. marginale*-infected blood samples were pre-treated with the optimized Triton X-100 lysis protocol and tested by endpoint PCR. The expected PCR products of RPA primers (103 bp) and published PCR primers (344 bp) were visible for each sample. Two replicates were performed from each section of SCM (Supplementary Fig 1). Additionally, gel-based and lateral flow RPA assays were developed using four discs of SCM and all samples tested positive for *A. marginale* by both RPA methods (Fig. 4). The internal control test line (1) appeared in only three samples. No amplification was observed with non-template control (water) of both of the assays. The expected RPA product size of 103 bp was visible with *A. marginale* reference positive control. Two test lines (1, 2) were observed with APC and *A. marginale* reference positive control in PCRD cassette (Fig. 4). These results demonstrated that pre-lysis treatment lysed the *A. marginale* -infected blood cells and released bacterial DNA that was collected in a soluble central membrane (SCM); therefore, DNA extraction from blood can be performed using Triton X-100 pre-lysis treatment and EICD.

4. Discussion

Elution independent collection devices (EICD) were developed for rapid sample collection to streamline sampling and subsequent PCR. However, EICDs have been only applied in plant and insect samples which have previously been physically macerated (Josue-Caasi, 2012). This is the first study to demonstrate the applicability and use of an EICD

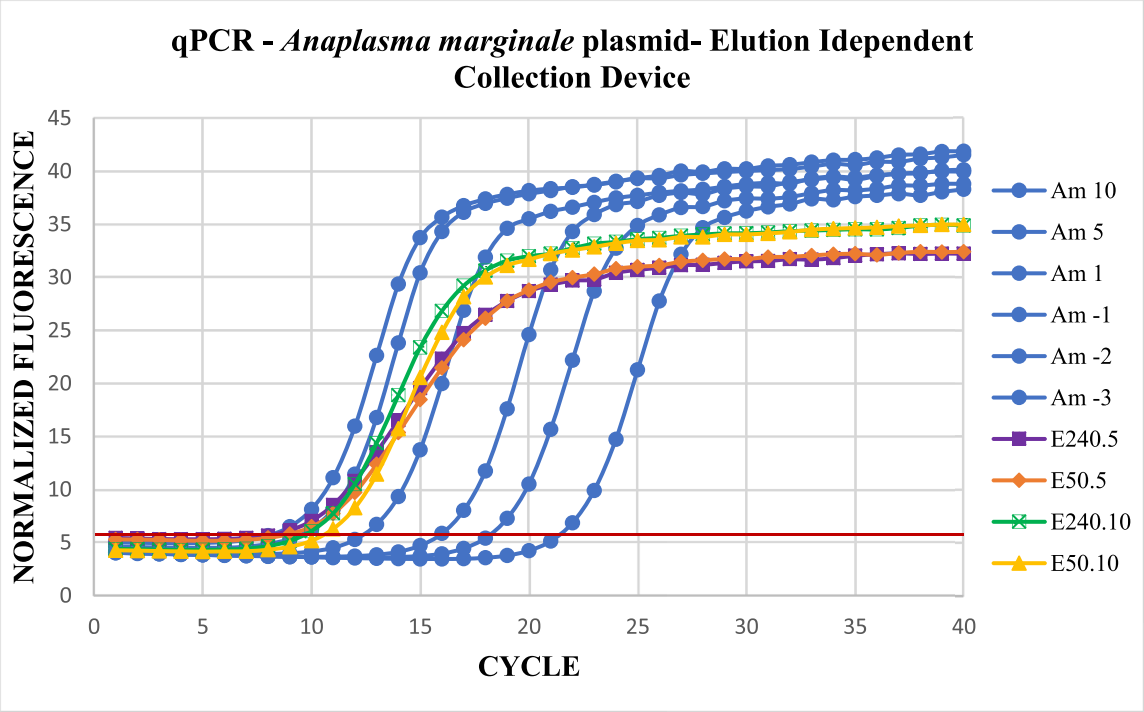

Fig. 2. Quantitative PCR assay using ten-fold serial dilution of *A. marginale* plasmid from 1ng to 1fg and *A. marginale* plasmid filtrated with two types of soluble membranes in EICD prototype. Plasmid concentration: Am 1 = 1 ng/µl, Am -1 = 0.1 ng/µl, Am -2 = 0.01 ng/µl, Am -3 = 1 pg/µl, Am -4 = 0.1 pg/µl, Am -5 = 0.01 pg/µl, Am -6 = 1 fg/µl. Treatments: E240.5 = ASW-240 soluble central membrane and 5 ng/µl of plasmid, E50.5 = ASW-50 soluble central membrane and 5 ng/µl of plasmid, E240.10 = ASW-240 soluble central membrane and 10 ng/µl of plasmid, E50.10 = ASW-50 soluble central membrane and 10 ng/µl of plasmid. Cycle or Ct values ≤ 29 are strong positive reactions indicative of abundant target DNA in the sample (SCM) and cycle or Ct values of 30-35 are positive reactions indicative of moderate amounts of target DNA.

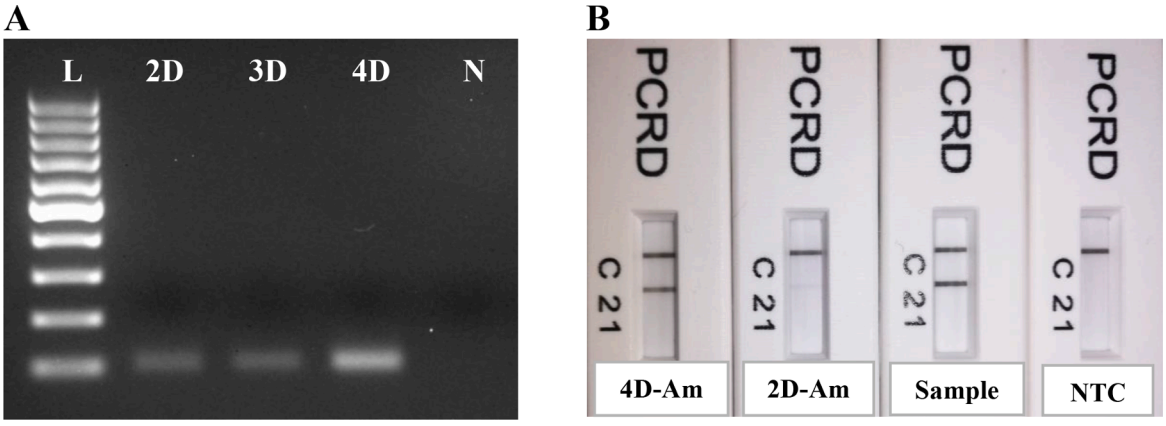

Fig. 3. Number of soluble membrane discs used in *A. marginale* assays. A) Gel-based RPA. Lane L, 100 bp DNA ladder; lane 2D, 2 discs (1 mm diameter each disc); lane 3D, 3 discs (1 mm diameter each disc); lane 4D, 4 discs (1 mm diameter each disc); non-template control (NTC, water). B) Lateral flow RPA. Lane C, flow-check line; Lane 2, detects FAM/Biotin labelled amplicons (*A. marginale*); Lane 1, detects DIG/Biotin labelled amplicons (not included in this assay); DNA-Am, total DNA of *A. marginale;* 2D-Am, 2 discs (1 mm diameter each disc) of soluble membrane; lane 4D, 4 discs (1 mm diameter each disc) of soluble membrane, and NTC, non-template control (water).

for collection of pathogen DNA from blood samples and subsequent molecular detection by qPCR, gel-based RPA and lateral flow RPA (*nfo* RPA).

Anaplasma marginale bacteria exist in red blood cell membranes and need to be lysed in order to release the bacterial DNA (Kocan et al., 2010; Rymaszewska and Grenda, 2008). As such, a DNA extraction method was needed to disrupt the erythrocytic bilayer. One current limitation of using extracted blood products is the need for faster methods for routine extraction and detection (Lievens and Thomma, 2005), particularly in the pre-storage stage of sample preparation. Some studies have reported that Triton X-100 and sodium dodecyl sulfate (SDS) surfactants solubilize cell membrane due to hydrophilic and hydrophobic properties (Butzler et al., 2017; John et al., 2018). Additionally, ammonium chloride buffer has been employed to lysis red blood cells (Chernyshev et al., 2008; Horn et al., 2011). In this study, *A. marginale*-infected blood samples were treated separately by Triton X-100 and ammonium chloride protocols and an optimization process was performed to reduce incubation times, steps, and reagents. Successful amplification occurred using both hemolysis protocols; however, Triton X-100 treatment was selected because it is rapid and simple.

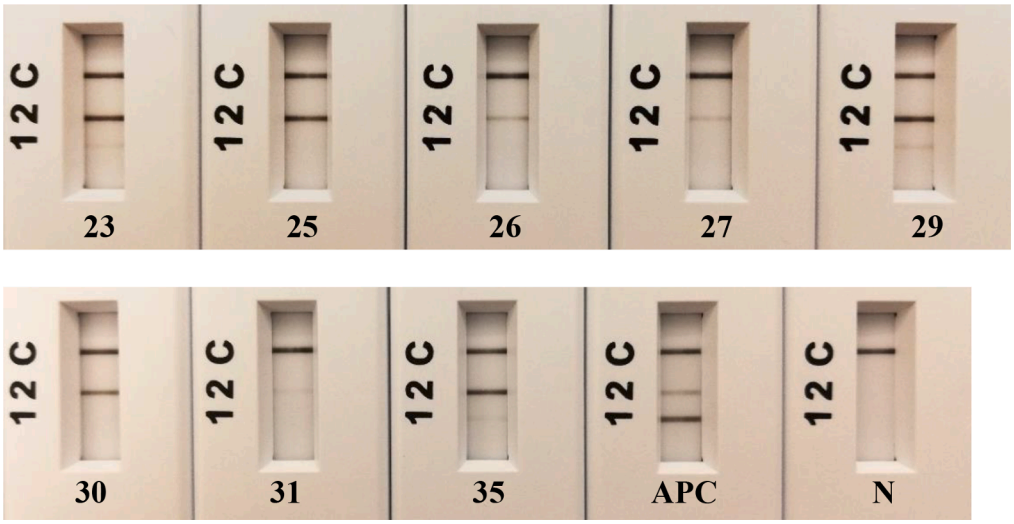

Fig. 4. *Anaplasma marginale nfo* RPA (lateral flow) using EICD samples. Lane C, flow-check line; Lane 2, detects FAM/Biotin labelled amplicons (*A. marginale)*; Lane 1, detects DIG/Biotin labelled amplicons (GAPDH gene); 23, 25, 26, 27, 29, 30, 31, 35, experimental blood samples infected with *A. marginale;* APC, artificial positive control; NTC, non-template control (water).

Triton-X-100 is effective because the non-polar head of the detergent molecule penetrates into the red blood cell membrane and causes lysis of the structure (John et al., 2018). The addition of bovine serum albumin facilitated faster hemolysis (Kitagawa et al., 1977), thus providing increased accurate pre-lysis blood treatment.

The pre-lysis treatment and EICD filtration in this novel system was validated using eight experimental blood samples infected with *A. marginale*. Results demonstrated that the EICD can be adapted to collect genetic material from organisms found in blood samples. However, the internal control test line in multiplex lateral flow RPA did not appear in all blood samples. This is likely because the first membrane in the EICD filters most of the large host molecules, while bacteria and small DNA are mobilized towards the SCM. Therefore, the internal host control (line 1) was either not detected in SCM or faint test line was observed using lateral flow *nfo* RPA method.

One of the limitations for using molecular detection protocols in low resource countries involves the time and cost of obtaining useful genetic material for the molecular assays. The use of the EICD dramatically reduces the time between extraction and testing because only 1mm diameter discs are needed from the SCM, which can be added directly to the reagents used for the molecular detection assay. One of the obstacles that was addressed within this study identified how many discs were needed to achieve positive results in the molecular assays. Early research with plant and insect studies reported that only one or two discs (1- or 2-mm diameter) of sample in each 20 μl of volume reaction was enough for accurate amplification (Josue-Caasi, 2012). However, in the case of our basic RPA and *nfo* RPA- lateral flow dipstick PCRD, four discs of 1-mm diameter were required in 50 μl of volume reaction to obtain intense and clear bands in agarose gel electrophoresis and clear line in lateral flow dipstick. While the number of discs may vary depending on the level of infection in the animal, this information is critical for the development of field-applicable kits for detecting pathogens in blood samples.

While trying to anticipate potential limitations of the system, they will invariably occur. Similar to a limitation for using FTA cards, we found that the percentage of recoverable DNA from the soluble central membrane was only 60% - 70% of the total. Although this was easily adjusted by adding more soluble discs to the reaction, further studies are needed to increase the percentage of DNA recovered from the soluble membrane. Another limitation comes from the potential cross-contamination of samples that could occur via the direct contact with the disc puncher used to cut the SCM (Lievens and Thomma, 2005). To address this concern, we disinfected the puncher after each use using 100% ethanol.

The results of this study highlight the utility of the EICD for molecular detection assays using blood samples. Other methods exist for storing genetic material from blood and other fluid samples from humans and animals. Tang et al. (2017a) developed a paper-based device for DNA extraction and amplification by helicase-dependent isothermal method and detection using lateral flow dipsticks. This device rapidly detected *Salmonella typhimurium* DNA; however, this prototype required a complex electronic design which would not be possible to apply in field-conditions. FTA cards have been used to collect bovine blood samples infected with tick-borne pathogens such as *Babesia bovis, Theileria mutans*, *Anaplasma marginale*, and *Trypansoma* species in field situations (Ahmed et al., 2013; Horn et al., 2011). This method, however, only preserved the samples and required extensive DNA extraction prior to any molecular detection assay could be performed (Hailemariam et al., 2017). The results from the current study demonstrated that the EICD is simple, paper-based, easily assembled in minutes, does not require DNA extraction and can be used directly in molecular assays.

5. Conclusion

This study indicates that the EICD is a credible alternative to traditional nucleic acid extraction protocols from blood. The extraction process using Triton X-100 pre-lysis blood treatment and EICD filtration takes approximately 15 min; in contrast with the 60 or 90 min required by commercial or traditional DNA or RNA extraction processes (Panda et al., 2019). Additionally, the EICD is compatible with gel-based and lateral flow RPA techniques. The low cost of the device, ease of operation due to the use of membranes, papers and inexpensive reagents, the rapid production of adequate inhibitor-free nucleic acids, and easy integration into biomolecular detection techniques such as PCR and RPA had proven the effectiveness of including EICD in field-based studies focused on detecting pathogens from blood of humans and other animals. A Rapid Anaplasma Detection (RAD) kit utilizing this EICD could be used to monitor anaplasmosis expansion in herds which could enhance the detection, diagnosis and treatment of each animal instead of mass treatment of whole herds.

CRediT authorship contribution statement

Andrea Salazar: Conceptualization, Data curation, Investigation,

Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Francisco M. Ochoa-Corona:** Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – review & editing. **Justin L. Talley:** Investigation, Methodology, Project administration, Resources, Writing – review & editing. **Bruce H. Noden:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

Francisco Ochoa Corona, through Oklahoma State University, has a patent for the EICD device (APPARATUS AND METHOD FOR BIOLOGIC SAMPLE RAPID COLLECTION AND RECOVERY DEVICE, AND CONVENIENT STORAGE. United States Patent No. US 9423398 B2. Filed February 8, 2011 and Issued August 23, 2016). The other authors have no competing interests to declare.

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