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Environmental friendly rapid extraction method of nucleic acid for diagnosis of tropical theileriosis

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Abstract
Background: Tropical theileriosis is a haemoprotozoan disease causing huge economic losses in livestock sector. PCR is the most sensitive and specific for theileriosis. For PCR, DNA extraction is an important prerequisite step. A good DNA extraction method must be rapid, safe and yield good quality DNA. All of these criteria are met by DNA extraction method using Chelex-100. Chelex-100 is ionic resin that chelates ions like magnesium and other PCR inhibitors from sample yielding good quality and stable DNA that can be used for PCR.
Method: In present study, a method using Chelex-100 was standardized for DNA extraction from blood. A novel PCR assay was standardized for *Theileria annulata* using primers designed for TAMS1 gene. For comparison, DNA was extracted from 20 blood samples from theileriosis affected cows using both the Chelex-100 method and commercial kit method. Each DNA sample was used for detection of tropical theileriosis using standardized PCR assay.
Result: The average yield of DNA was 61.36 ng/µL and 99.7 ng/µL by Chelex-100 and commercial kit method, respectively. The amount of blood required for extraction was 25 µL and 200 µL in Chelex method and kit method, respectively. The DNA samples extracted by both method yielded positive results in PCR.

Keywords: Chelex-100, tropical theileriosis, PCR, *Theileria annulata*, DNA extraction

Introduction
Haemoprotozoan diseases are the serious threat to the economy of livestock sector. Theileriosis is the most important among them which is prevalent in tropical and subtropical regions (Minjauw and McLeod, 2003) [12]. Clinical tropical theileriosis is characterized by anorexia, persistent fever, swollen lymph nodes, diarrhea and anemia (Radostits *et al*., 2010) [16]. Mortality, treatment cost and production loss are the major factor for economic loss in clinical disease. Exotic and crossbred cattle are more susceptible to clinical disease and have high mortality rates (Kundave *et al*., 2015) [9]. But in indigenous cattle being resistant show subclinical disease or remain carrier. Such animals remain source of infection to susceptible animals. Also, subclinical infection has been associated with poor body condition and production loses (Kolte *et al*., 2017) [8]. This subclinical disease often goes undiagnosed and poses major hindrance in disease control and profitability. PCR is the most reliable test for diagnosing subclinical carrier. It is the most sensitive and specific test in diagnosis of theileriosis as compared to microscopic examination and serological tests (Nourollahi-Fard *et al*., 2015; Ranjan *et al*., 2015) [14, 17].
For PCR, DNA isolation from blood is prerequisite. The good DNA extraction method should be efficient, easy, inexpensive, rapid and eco-friendly (Chen *et al*., 2010) [4]. Various methods of DNA isolation are available like phenol–chloroform-isoamyl alcohol-based extraction (PCI), commercial extraction kits based on silica membranes or magnetic separation and Chelex based method (IP *et al*., 2015; Mann *et al*., 2015) [7, 11]. Traditional PCI method involves use of hazardous chemicals and commercial DNA extraction kits are expensive. Chelex based extraction is rapid, inexpensive single tube method which is free from harmful chemicals and yield high quality DNA suited for molecular techniques like PCR.
Chelex- 100 is an ion exchange resin which has a structure containing styrene-divinylbenzene skeleton having iminodiacetic acid as functional group which chelates polyvalent metal ions Mg+2 (Samczynski, 2006) [19]. Since magnesium serve as cofactor for deoxyribonucleases, unavailability of Mg+2 helps in preventing DNA degradation (Singh *et al*., 2018) [23]. Use of Chelex for DNA extraction was first reported in 1991 by (Walsh *et al*., 1991) [26]. The method is used widely in DNA extraction from plants (Hwang-Bo *et al*., 2010) [6], insects (Lienhard

and Schaffer, 2019) [10], blood (Hailemariam *et al*., 2017; Strom *et al*., 2014) [5], tissues (Al-Griw*et al*., 2017) and forensic materials (Becker *et al*., 2004; Walsh *et al*., 2013; Willard *et al*., 1998) [2, 27]. So, in present study we developed an easy, fast and safe method of DNA isolation from blood for diagnosis of tropical theileriosis by PCR.

Materials and methods

Sample collection

Samples were collected from cattle presented at Medicine Section, Veterinary Clinical Complex (VCC), Lala Lajpat Rai University of Veterinary & Animal Sciences (LUVAS), Hisar. Fresh blood samples from suspected clinical cases (having ticks or fever or enlarged lymph nodes or anaemia) were used for preparation of thin blood smear which is fixed by methanol and stained by Giemsa stain as standard procedure (Benjamin, 1978). Stained blood smears were examined under 100x magnification in microscope for the presence of different forms of *Theileria annulata*. For testing the efficacy of DNA extraction methods blood samples from 20 clinically affected cattle which are positive for theileriosis by blood smear examination were collected in EDTA vials.

Extraction of DNA by commercial kit

DNA was extracted from blood by DNeasy blood and tissue kit (Qiagen, Germany) following the manufacturer's protocol. Aliquots of extracted DNA were quantified using NanoDrop™ 2000 spectrophotometer and then stored at -20 °C until further use.

Eco friendly extraction method of DNA from blood (Figure 1)

Chelex solution (10%) is prepared in 1x Tris EDTA buffer and autoclaved. 25 µL of blood is mixed with 100 µL of 10% Chelex and 1 µL of proteinase K. It is then mixed properly and incubated at 56 °C for 40 min. after incubation mixture is boiled at 100 °C for 8 min and vigorously vortexed for 10 seconds and centrifuged at 10,000 rpm for 5 min. After centrifugation, beads and debris settles at bottom and supernatant contain DNA. Transfer the supernatant to fresh tube. The extracted DNA samples were quantified using NanoDrop™ 2000 spectrophotometer and then stored at –20 °C until further use.

Polymerase chain reaction

The PCR assay for *Theileria annulata* was performed for all samples to confirm and evaluate the extracted DNA. PCR primers were designed from TAMS1 gene of *T. annulata* using Primer 3 plus software. The sequences of forward and reverse primers are 5'-CTACTTCTATACCGGTGACTCAAGG-3' and 5'-CGAACATGGGTTTTAAAGGAAG-3', respectively with product size of 229 bp. The specificity of primers was evaluated by using the BLASTN from the NCBI database. Each 12.5 µL reaction mix contains 6.25 µL of 2X Top Taq master mix (Qiagen), 0.4 µL of each forward and reverse primer and nuclease free water to make 12.5 µL. In case of DNA extracted from kit template volume is 1 µL but while using Chelex extracted DNA template volume is 3 µL. The PCR conditions includes initial denaturation at 95 °C for 5 min; followed by 30 cycles of 94 °C for 30 s (denaturation), 60 °C for 30 s (annealing), and 72 °C for 40 sec (extension); with a final extension step of 72 °C for 10 min. Amplified PCR products were examined by loading 3 µL of product in 1.5% agarose gel along with 100 bp ladder (GeneDirex 100bp RTU).

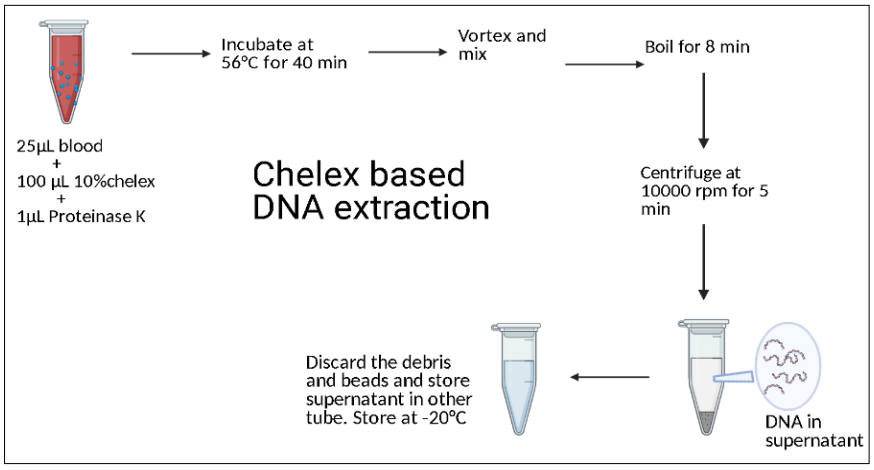

Fig 1: Steps for Eco friendly extraction of DNA from blood

Results and Discussion

The average yield of DNA by Chelex-100 method was 61.36 ng/µL (range 42.3-88.3 ng/µL) while with kit method it was 99.7 ng/µL (range 48.3-145.5 ng/µL). The amount of blood required for extraction in Chelex was 25 µL while in kit method it was 200 µL. The final volume extracted was approximately 100 µL in both the methods. The average yield of DNA extraction in Chelex method was lower than that of extraction by commercial kit method. The reason for that use of lower blood sample volume *i.e.*, 25µL but the final volume yield after extraction is 100 µL. But in commercial kit, initial sample volume used is 200 µL extracted in final volume of 100 µL. Since in latter method requires very small volume (25 µL), it can be used in conditions where very low sample volume is available.

All the 20 samples positive in blood smear method showed the amplification of product of 229 bp in PCR while no amplification in negative control. DNA extracted from positive blood smear samples by both methods were showing amplification in PCR (Figure 2). Thus, both methods are effective in extracting DNA which is suitable for PCR. Since, all the blood smear positive samples were amplified, it indicates the PCR primer designed were specific for *Theileria annulata*. In present study we found that both methods are suitable for DNA extraction from blood for PCR. It has been reported earlier that DNA solution obtained after Chelex

extraction can be directly used for PCR (Strøm*et al*., 2014; Zhang *et al*., 2010) [29]. The use of this extraction method has been reported for extracting genomic DNA from blood (Simon *et al*., 2020; Singh *et al*., 2018) [22, 23], identification of blood parasites like Plasmodium (Mann *et al*., 2015; Schwartz *et al*., 2015), *Babesia gibsoni* (Tani*et al*., 2008), *Plasmodium gallinarum* (Pattaradilokrat *et al*., 2015) and other haemoprotozoan (Hailemariam *et al*., 2017) [5, 11, 21, 15].

Other methods like DNA extraction kits or PCI method involve multiple steps, making these methods technically difficult. Multiple steps also involve opening of tubes several times resulting in increased chances of contamination. On other hand, Chelex based extraction having only three steps is easy to perform and have less chances of contamination (Nagdev *et al*., 2010; Sajib *et al*., 2017) [18]. Also, as it uses single tube, this method reduces plastic waste making it environment friendly method. Comparative cost of extraction by this method is very less as compared to commercially available kits (Lienhard and Schaffer, 2019) [10]. (Lienhard and Schaffer, 2019) [10]. Compared the cost of Chelex extraction method with commercially available kits and found Chelex costs 0.02€ commercial kit costs from 1.4 to 3.5€. Unlike conventional methods of extraction, it does not involve use of any hazardous chemicals (Nagdev*et al*., 2010).

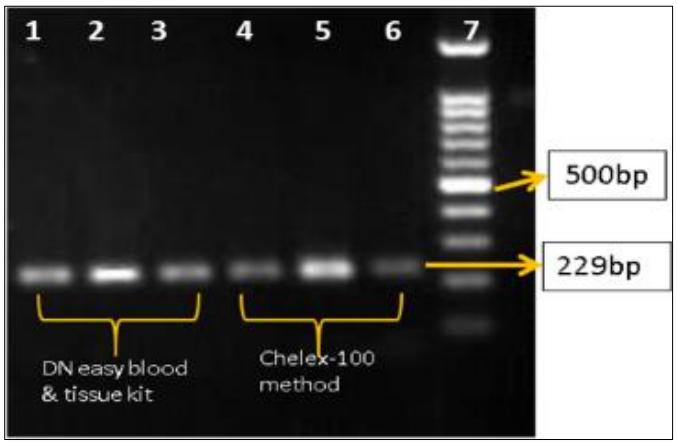

Fig 2: Agarose gel electrophoresis result of PCR assay for detection of *Theileria annulata.* Lane 1, 2, 3 showed PCR product DNA of sample a, b and c, respectively extracted by DNeasy blood and tissue kit. Lane 4, 5, 6 showed PCR product DNA of sample a, b and c, respectively extracted by Chelex extraction method. Lane 7 represents 100 bp ladder

Conclusion

The resin Chelex-100 was found effective, economical and eco-friendly method for DNA extraction that can be used for molecular detection of theileriosis and other haemoprotozoan diseases. No hazardous chemicals such as phenol, chloroform and isoamyl alcohol were used for isolation of DNA. The method is quick with minimum chances of cross contamination in multiple sample handling due to single tube extraction protocol.

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