STANDARD OPERATING PROCEDURE

Title: Detection of ssurRNA gene of *Theileria equii* by Polymerase Chain Reaction (PCR).

Reference: Heim *et al.*, 2007

1.0 INTRODUCTION

1.1 PURPOSE/SCOPE OF THIS SOP

Detection of ssurRNA gene of *Theileria equii* with the help of PCR in DNA extracted from whole blood. Gel based PCR method is one of the most sensitive technique for DNA detection currently available. With reference of specific primers as detailed in Heim *et al*., 2007 a one-step PCR method has been developed.

2.0 MATERIALS:

CONSUMABLES	**SUPPLIER**
2ml disposable syringe	BD
EDTA vacuutainer	BD
1.5/2 ml centrifuge tubes	TARSON
0.2ml PCR tubes	GENAXY
Micro tips (.5-10 µl, 10-100 µl, 20-200 µl, 100-1000 µ)	GENAXY
Conical Flask 250 ml	TARSON
Gel Casting tray and combs	TARSON
Agarose Powder (Molecular Grade)	Merck
TAE/TBE Buffer (50X)	THERMO SCIENTIFIC
Parafilm	TARSON
DNeasy Blood and Tissue kit	QUIAGEN® KIT, CATALOGUE NO. 69504
DyNAzyme II PCR Master Mix	THERMO SCIENTIFIC

Nuclease Free Water	THERMO SCIENTIFIC
Taq. Polymerase	THERMO SCIENTIFIC
Primers	MOLBIOGEN
6X DNA Loading Dye	THERMO SCIENTIFIC
100 BP DNA Ladder	THERMO SCIENTIFIC
Ethidium Bromide	SRL
EQUIPMENT	**SUPPLIER, MODEL**
Class 2 safety microbiological safety cabinet	LABCHEM & LABORTENIK INSTRUMENTS
Micro-centrifuge	TARSONS
VERITI 96-well Thermal cycler	APPLIED BIOSYSTEMS
Immuno Electro Phoresis Apparatus	APPLIED BIOSYSTEMS
Gel Documentation system	DNR MINI LUMI, APPLIED BIOIMAGING
Micropipette (0.5-10 µl, 10-100 µl, 20-200 µl, 100-1000 µl).	GENAXY (Nichipette)

3.0 PROCEDURES:

3.1 EXTRACTION OF DNA FROM WHOLE BLOOD.

DNA Extraction was carried out using the DNeasy Blood and Tissue kit (Quiagen® Kit, Catalogue No. 69504) as per manufacturer's protocol. About 100µl anticoagulated blood taken individually from each of the sample in a 2ml microfuge tube was lysed in 20µl proteinase K and the volume adjusted to 220 µl by adding Phosphate Buffered Saline (PBS; Appendix: I) before adding another Lysis buffer (Buffer AL). The tubes were then incubated at 56°C in water bath for 10 minutes. Final elution was done with 100 µl elution buffer (Buffer AE) after following all the mid-step protocols and the templates were kept at -20°C, until further use.

3.2 POLYMERASE CHAIN REACTION (PCR):

Amplification of the targeted gene was done using the following reaction condition and primer sets (Table. 1).

TABLE: 1 DESCRIPTION OF THE PRIMERS USED IN THE PRESENT STUDY.

Parasite	**Primer sequence pair**	**Product Size**	**Reference**
Theileria equii.	Ema 1F: GAGTCCATTGACCACCTCACC Ema 1R: GTGCCTGACGACAGTCTTTGG	800 bp	Heim *et al.*, 2007.

3.2.1 REACTION MASTER MIXTURE:

The targeted gene of *T. equii.*was amplified using following reagents-

TABLE: 2 DETAILS OF THE PCR REACTION MIXTURE

Total reaction volume : 25µl	
Reagents/Ingredients	**Volume**
Nuclease free water	5.5µl
PCR Master mix	12.5µl
Forward primer (10pmol)	1 µl
Reverse primer (10pmol)	1 µl
DNA template	5µl

The contents were mixed by brief vortexing and placed all the tubes in a thermal using following thermal cycling conditions:

TABLE: 3 DETAILS OF THE THERMAL CYCLER CONDITION

Number of cycles	**Temperature**	**Time**
1 cycle	95^0C	15minutes
35 cycles	94^0C	15 seconds
	60^0C	30 seconds
	72^0C	45 seconds
1 cycle	72^0C	10 minutes
	4^0C	Stored until use

3.4 CONFIRMATION OF PCR AMPLICONS:

The amplified PCR products was confirmed by agarose gel electrophoresis using 1.5 percent agarose gel containing ethidium bromide in 1X tris acetate EDTA(TAE) buffer at 60 volt for 60 minutes.

- Briefly, gel casting tray was set up by placing the comb in the slot containing 8 wells.
- A volume of 40ml agarose gel was prepared by adding 0.60g of agarose to 40 ml of 1X TAE.
- The agarose was melted in microwave oven (Haier, India) for 2 minute.
- Thereafter, the melted agarose was allowed to come down to 56^0C and 2 µl of ethidium bromide (10mg/ml) was added.
- After proper mixing it was poured on the gel casting tray and allowed to solidify without disturbing the tray.
- Then 1X TAE (490 ml DW + 10 ml 50x tae BUFFER) was poured on the gel casting tray till the gel was submerged and the comb was removed carefully.
- Then 5 µl of each PCR amplified products was mixed with 2 µl of 6X loading dye and loaded into the wells.
- Along the PCR amplicon a 100bp DNA ladder was loaded and run parallel to the PCR amplicons.
- Electrophoresis was carried out at 60V for 1hour. The gel was then visualized on a UV transilluminaor.
- The PCR amplicons showed a product size of **800 bp**.