species were detected based on the specific DNA fragments obtained after amplification.

3.0 MATERIAL AND METHODS

3.1 General Conditions

3.1.1 Sterilization of glassware and Plastic ware

During the course of this study Corning or Borosil brand of glasswares and plasticwares (M/s Tarsons Ltd.) were used. All the glasswares were cleaned and sterilized as per standard procedures. Plastic wares were also prepared as per standard procedures.

3.1.2 Laboratory chemicals

The laboratory reagents and buffers used in this study were prepared in double glass distilled water using ‘Excellar’ or ‘guaranteed’ reagent quality chemicals. The other chemicals viz., rabbit anti-bovine IgG conjugate, SDS-PAGE standard molecular weight protein marker, 100 bp DNA marker, primers, Taq Polymerase and dNTP’s were procured from Bangalore Genei Private Limited, Bangalore. The reagents Ortho-phenylene
dihydrochloride (OPD) and Ortho-Dianisidine dihydrochloride (ODD) were obtained from Sigma Company (USA). ELISA plates (Titertex) were procured from Flow Laboratories (Netherlands).

3.2 Study area

For immunodiagnosis and molecular diagnosis of Sarcocystosis in cattle, the organs (viz., heart, oesophagus, thigh muscle, tail muscle) and blood samples were collected from cattle slaughtered at Karnataka Meat Products Marketing Corporation Limited (KMPMCL), Bangalore, which were brought mainly from districts such as Bangalore, Tumkur, Kolar, Mandya, Mysore, Gulbarga and Raichur.

3.2.1 Collection of Material

The tissue samples of heart, oesophagus, tongue, thigh, tail and diaphragmatic muscle were collected at the time of slaughter. All the tissue samples were collected in clean plastic bags and brought to the laboratory and stored at -4°C upto 2 days until examination. Later the muscle tissue samples were confirmed as positive or negative for microcyst and macrocyst of sarcocysts by macroscopic and microscopic examination.

3.2.2 Collection of serum samples

For immunodiagnosis three hundred blood samples of cattle were collected in sterile glass test tubes during slaughter. The samples were kept at 4°C for 4-6 hours for separation of serum. The sera were separated and
centrifuged at 1500 rpm for 5 minutes in a refrigerated centrifuge (4°C). They were then stored in small aliquots at -20°C till further use.

A total of 30 known positive serum samples were collected from cattle which revealed *S. bovicanis* microcysts within the tissues. Another 30 known negative serum samples were collected from cattle without showing the presence of microcyst by examination of oesophagus, heart, tongue, diaphragmatic, thigh and tail muscles.

Similarly, thirty known positive and thirty negative serum samples respectively were collected from tissues with macrocyst and tissue without macrocyst of *S. bovifelis* after examination of oesophagus, tongue, thigh and tail muscles.

### 3.3 Morphological Examination of tissue samples for sarcocyst

#### 3.3.1 Macroscopic examination and isolation of sarcocyst of *S.bovifelis*

The organs were collected at the time of slaughter and thoroughly washed in running water after removing superficial fascia and fat. The visible macrocyst of *Sarcocystis* species were dissected out carefully using forceps and B.P. blade and stored in phosphate buffered saline (PBS) (pH 7.2) (appendix). Then the whole organs were thoroughly searched after dissection for the presence of macrocyst and they were removed carefully without rupturing. The macrocyst of *Sarcocystis* species were later confirmed as *S.bovifelis* based on the descriptions of Jain and Shah (1988), Pandit *et al.* (1994) and (Bhatia, 2000).
3.3.2 Microscopic examination of tissues for microcyst of *S. bovicanis*

The different organs including heart, oesophagus, diaphragm, thigh and tail muscles were microscopically examined for the presence of microcyst of *S. bovicanis*. The screening was done by rapid isolation technique and Percoll gradient methods as per Juyal *et al.* (1989) and Tenter (1988) respectively.

3.3.2.1 Rapid isolation technique

About 2-3 cm of suspected tissues were cut and teased in a watch glass containing normal saline solution (0.85%) or distilled water with the help of needle and forceps. The material was then examined under stereoscopic microscope for the presence of microcysts. Then the microsarcocysts were transferred onto a glass slide and examined under low (10x) and high (40x) magnification after placing a coverslip (22mm). The microcysts were identified as *S. bovicanis* based on descriptions of Jain and Shah (1988), Pandit *et al.* (1994) and Bhatia (2000). They were then subjected to percoll gradient method for preparation of antigen. Based on the presence or absence of microcyst by rapid isolation technique the serum samples were considered as known positive and known negative.

3.3.2.2 Separation of microcyst of *S. bovicanis* by Percoll gradient method

The tissue samples with microscopic cysts of *S. bovicanis* were excised and pooled for separation of microcyst. The muscles were finely diced and
stirred vigorously in PBS (appendix) containing an equal volume of glass beads for 45 min. The isolate was washed in PBS, homogenized and layered on top of a discontinuous gradient consisting of 80 and 50 per cent isotonic Percoll (Sigma Co.) stock solution (appendix) in PBS. After centrifugation at 2500 rpm at ambient temperature for 45 minutes a layer consisting of more than 95.0 per cent cystozoites was isolated from the gradient.

3.3.3 Processing of micro and macrocyst of S. cruzi and S. hirsuta

**Microcyst:** The cystozoites obtained from the discontinuous percoll gradient method were washed thrice in PBS and stored at -20°C for further preparation of antigen and DNA isolation studies.

**Macrocyst:** The intact macrocyst were clearly separated from host tissue and washed thrice with PBS. The macrocyst were finally stored at -20°C till further use.

3.4 Preparation of Antigen

3.4.1 Macrocyst antigen of S. bovifelis

The large macroscopic cysts of *S. bovifelis* preserved at -20°C in PBS were used for the preparation of antigen as per Gasbarre *et al.* (1984) with minor modifications. About 10 g of macrocysts were ruptured by mild homogenization using glass tissue homogenizer. The filtrate containing the cystozoites were then disrupted by Sonirep 150 ultrasonication (Sanyo Gallenkamp PLC, UK) 3 times for 20 seconds each at 100 mAmp under
4°C. The suspension was centrifuged at 12000 rpm for 30 minutes in a refrigerated centrifuge (4°C) (Superspin). The supernatant was collected and used as the soluble antigen extract. The protease inhibitor phenyl methyl sulphonyl fluoride (PMSF) was added at a concentration of 2µl/ml of antigen (Appendix). One drop of Sodium azide (0.1%) was added to avoid the fungal contamination. The antigen was aliquoted and stored at -20°C till further use.

3.4.2 Microcyst antigen of *S. bovicanis*

The microcysts and cystozoites of *S. bovicanis* were used for the preparation of soluble microcyst antigen as per Dubey *et al.* (1989). The cystozoites stored at -20°C in PBS was washed thrice again with PBS. The cysts were ruptured by carrying out three cycles of freezing and thawing at -20°C and -60°C respectively. The cystozoites in the suspension were disrupted by Sonirep 150 ultrasonicator (Sanyo Gallenkamp PLC, UK) at 100 mAmp for 60 seconds at 4°C with a gap of 2 minutes at every 20 seconds. The suspension was subjected to centrifugation at 12000 rpm for 30 minutes in a refrigerated centrifuge. The supernatant was collected and PMSF was added as described in section 3.4.1. The soluble antigen was stored at -20°C in aliquots.

3.4.3 Estimation of protein concentration in the Antigen
The protein concentration of macrocyst and microcyst soluble extracts was estimated as per the method of Bradford et al. (1976) using protein estimation kit obtained from Bangalore Genei Co., Bangalore.

3.5 Enzyme Linked Immuno Sorbent Assay (ELISA)

The indirect ELISA was used to detect anti-\textit{Sarcocystis bovicanis} and \textit{S. bovifelis} antibodies as per the method of Shi and Zhao (1987) with minor modifications. The working dilutions of conjugate, antigen and test sera were determined prior to use by checker board titrations.

3.5.1 Determination of working strength of antiglobulin conjugates

To determine the working dilution of antispecies conjugate, 100 µl of normal serum(1:10000) was coated onto 96 well flat bottom polystrene (Titertrek) by diluting with serum coating buffer (Appendix) and incubated at 37°C for 1 hour. The ELISA plate was washed with washing buffer thrice and blocking was carried out by 5% skimmed milk powder. Dilutions of conjugate to be tested were made in blocking buffer and 100µl of each dilution was added to the wells in duplicates and incubated at 37°C for one hour. After washing as above 100 µl of substrate (appendix) was added and colour reaction was monitored in dark area. The reaction in all wells was stopped by addition of 50µl of 2M H$_2$SO$_4$ as stopping solution. The
absorbance of the contents of each well was read in a Multiscan Plus P (Lab systems) ELISA reader at 492 nm.

3.5.2 Determination of optimal antigen coating level

Different dilutions (1μg, 3μg, 5μg/well) of *S. bovicanis* and *S. bovifelis* soluble extracts diluted with carbonate-bicarbonate coating buffer were added to 96 well ELISA plate. The plate was incubated at 37°C for one hour and washed thrice with washing buffer (appendix). The blocking buffer (appendix) was added to block the non-specific reactive sites and incubated at 37°C for one hour. After washing the plates, 100μl of 1:100 dilution of known corresponding serum was added and further steps were followed as described in 3.7.1. Antispecies conjugate (1:5000) diluted with PBST (appendix) was added to all test wells in the microplates. Absorbance values were then taken after adding substrate and stopping solution in the ELISA reader (Multiscan Plus P Lab systems).

3.5.3 Determination of optimum serum dilution

Working strength of antigen was coated onto the plates and incubated at 37°C for 1 hour. Hundred microlitre of different dilutions of known positive serum was added to their corresponding wells in duplicates. The other steps were repeated as described above. The absorbance values were recorded in the ELISA reader.

3.5.4 Determination of Cut off value
To determine the cut off value twenty five known negative serum samples were used. The cut off value was calculated by taking absorbance values of known negative serum plus three standard deviation. Any sera with OD values three times the negative value were regarded as positive.

3.5.5 Indirect ELISA

The flat bottom polystyrene 96 well ELISA plate was coated with 100 µl of antigen with a protein concentration of 5µg/well for *S.bovicanis* and 1µg/well for *S.bovifelis* in antigen coating buffer. The plate was incubated at 37°C for one hour and washed thrice with washing buffer (appendix). The plates were incubated at 37°C for one hour after adding 100µl of blocking buffer (5% skinned milk powder with PBS) and washed thrice with PBS Tween-20. The serum dilution of 1:100 for *S.bovicanis* and 1:200 for *S.bovifelis* with blocking buffer was added in duplicates and incubated for 1 hour at 37°C. The plates were washed as described above and 100µl of 1:5000 diluted anti-species conjugate was added and incubated as above. Then 100µl of substrate chromogen working solution(appendix) was added and color reaction was monitored in dark place. The reaction was stopped by adding 50µl of 2M H₂SO₄. The absorbance values were taken at 492 nm. Positive serum control, negative control and conjugate control was maintained.

3.5.6 Sensitivity and specificity of EITB and ELISA
The sensitivity and specificity of EITB and ELISA was calculated by the following formula:-

\[
\text{Sensitivity} : \frac{\text{True Positive}}{\text{True positive} + \text{false negative}} \times 100
\]

\[
\text{Specificity} : \frac{\text{True negative}}{\text{True negative} + \text{false positive}} \times 100
\]

3.6 Sodium do-decyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted as per description of Laemmli (1970) using discontinuous gel and buffer system. Minidual slab gel electrophoresis (Bangalore Genei) was used to perform the electrophoresis. Two glass plates (one with notch and one without notch), two spacers and comb were washed thoroughly, rinsed in 70 per cent alcohol and dried. Bottom of the glass plates were sealed with 1% agarose. Separating gel (10%) (appendix) was prepared and poured between plates with a gentle continous flow avoiding air bubbles upto about 3/4\(^{th}\) height of the plate. The gel was overlaid with water saturated n-butanol immediately to avoid air contact and the gel was allowed to set at room temperature for 45 minutes. After polymerization the overlay was poured off and separating gel was washed with double distilled water and subsequently dried with the blotting paper. Stacking gel (4.5%) (appendix) was prepared and poured upto the required
height. The comb was placed and the gel was allowed to set for 10 minutes. The comb was removed and the slab was fixed to the electrophoresis apparatus. Running buffer (appendix) was poured into both upper and lower buffer tank.

3.6.1 Preparation of sample and protein marker

The required protein concentration of *S. bovicanis* (27 µg), *S. bovifelis* (18 µg) and host tissue antigen (18 µg) was taken with 15 µl, 5 µl and 4.75 µl of sample buffer in the ratio of 1:4 and 10 µl of tracing dye (appendix). Similarly protein markers obtained from Bangalore Genei of molecular range 29 to 205 kDa was prepared for carrying out electrophoresis (appendix). Once the samples were prepared both samples and protein markers were boiled in water bath at 100°C for 5 minutes. The host tissue antigen was prepared from non-infected cattle heart and used to check the cross reaction between the *Sarcocystis* species of cattle.

The denatured samples were loaded in separate wells and electrophoresis was carried out at a constant current of 10 mA(50v) for stacking gel and 16 mA(100v) for the resolving gel using power pack (Bangalore Genei). When the dye front reached the bottom of the gel, the power pack was switched off. Gel was removed and stained with Coomassie blue stain (Appendix).

3.6.2 Coomassie Brilliant Blue staining procedure
Coomassie Brilliant Blue staining was carried out according to the descriptions of Laemmli (1970). After completion of electrophoresis slowly the spacers and either of the plate was removed. The gel was immediately and carefully transferred to the staining solution (appendix). The gel was allowed in the staining solution overnight at room temperature. Then the gel was transferred to destaining solution (appendix). The destaining solution was changed every 20 minutes till the bands were clearly seen. Gel was transferred to double distilled water and then permanently preserved in 20 per cent glycerol or 7 per cent acetic acid. The protein profile was recorded by gel documentation system (BioRad).

3.6.3 Determination of molecular weights of each peptide

The Relative mobility ($R_f$) of each peptide was calculated using the following formula:-

$$R_f = \frac{\text{Distance migrated by the polypeptide}}{\text{Distance migrated by tracking dye}}$$

The molecular weight of each peptide was calculated by plotting the $R_f$ values of standard molecular weight proteins using the semi log graph paper.

3.7 Enzyme Immuno Transfer Blot (EITB)

3.7.1 Raising of Hyperimmune serum in calves
Two crossbred 9 months old *Holstein Friesian* calves, were used for raising the hyperimmune serum. The methodology for raising the serum was as per Talwar and Gupta (1992). The calves were dosed with albendazole @ 5mg/kg body weight three weeks prior to antigen administration. Approximately 500µg of each *S. bovicanis* (1ml) and *S. bovifelis* (0.6ml) soluble antigen extracts were separately mixed with equal volume of Freund’s complete adjuvant (Sigma Co.) and mixed several times till the uniform suspension was obtained. Then the suspension was injected intramuscularly in to the thigh muscles of calf 1 and calf 2, respectively. Two booster doses were given intramuscularly at 10 days intervals with the same concentration of antigen after mixing with Freund’s incomplete adjuvant. The blood was collected from the jugular vein after 10 days of third injection. Sera were separated and presence of specific antibodies were checked by Agar Gel Precipitation test (AGPT) with their corresponding antigens. The serum samples were aliquoted and stored at –20°C till further use.

### 3.7.2 Titration of Hyperimmune Serum by DOT-Enzyme Linked Immunosorbent Assay (DOT-ELISA)

Dot-ELISA was carried out as per Tenter (1988) with slight modifications. One square centimeter of nitrocellulose membrane (0.45µm porosity) disc were cut and placed in sterile 96 well polystyrene persplex plate. The membranes were placed in the center of the well with the help of
forceps. The soluble antigen extracts of *S.bovicanis* and *S.bovifelis* were diluted in carbonate-bicarbonate coating buffer (appendix).

Two microlitre of *S.bovicanis* and *S.bovifelis* soluble antigens containing 5μg and 1μg of protein were dot blotted on to the dull side of filter discs and incubated at room temperature for one hour. The membrane discs were washed thrice in washing buffer (appendix) with each lasting for 5 minutes. To each well containing the discs 100μl of blocking buffer (appendix) was added and incubated at room temperature for 1 hour. After washing (as similar to above steps) 100μl of different dilutions of hyperimmune serum were added to each well in duplicates to know the end point titre of HIS to be used further for EITB. The membrane discs were incubated for 1 hour at room temperature. Then 100μl of HRP conjugated anti-bovine IgG (1:1000) diluted in 5% skim milk powder was added and incubated at 37°C for 1 hour. After washing, 100μl of substrate working solution (appendix) was added and shaken for 1 minute. Then the plate was incubated at room temperature by monitoring the color reaction. The color reaction was stopped by washing the membrane discs in distilled water and read for positive reactions which appeared as clearly defined brown dots.

### 3.7.3 Enzyme Immuno Transfer Blot (EITB)

The EITB was carried out as per the method of Towbin *et al.* (1979) and Granstrom *et al.* (1990). SDS-PAGE was carried out first as previously
described in section 3.5 using 10 per cent resolving gel. S. bovicanis, S. bovifelis and host tissue soluble antigens containing 27\(\mu\)g, 18\(\mu\)g and 18\(\mu\)g of protein were loaded to each lane along with protein markers in the range of 29 to 205 kDa (appendix). Both antigen and protein marker was prepared as per previous descriptions. Host tissue extract with PBS was included in one lane to verify primary Ab specificity or any cross reactivity with the host tissue antigen. After electrophoresis the marker lane was cut and stained with Coomassie blue staining solution. The remaining gel was used for electro transfer blot.

Before carrying out the transfer step, the gel was washed thrice in transfer buffer (appendix) with a gap of 5 minutes. Nitrocellulose membrane (0.45\(\mu\)m) and blotting paper of size 8 x 7.5 cm was cut and immersed in transfer buffer for 30 minutes. One sheet of blotting paper was transferred onto the electroblot stage and rolled over the blotting paper to remove the excess water or air bubbles. Similarly another two sheets were placed then followed by nitrocellulose membrane gel. Again three layers of blotting paper was placed and trapped air was removed. Transferring was performed with trans-blot semidry electrophoresis transfer cell (BioRad) for 45 minutes at a constant voltage (12v).

Once the transfer was completed the membrane was immersed in blocking buffer (appendix) for overnight at 4\(^\circ\)C. The membrane was washed using washing buffer (appendix) as described in section 3.6.2 and incubated with corresponding HIS at different dilutions with blocking buffer
at room temperature for 1 hour. The cross reactions were also checked by reacting with host antigen. The membrane was washed 3 times in a washing buffer and incubated in 1:1000 HRP-conjugated with anti-bovine antibody (IgG) for one hour at room temperature. After washing, color development was carried out as described in DOT-ELISA. The results were read by comparing with the standard molecular weight protein marker stained with Coomassie blue as described earlier.

3.8 Statistical analysis

The data obtained were analysed by Chi-square test using the software Graph pad prism.

3.9 Random amplified polymorphic DNA Polymerase chain reaction (RAPD-PCR)

3.9.1 Isolation of cystozoites

The cystozoites were isolated as described in section 3.3.2.2 and 3.3.3 from microcyst of S.bovicanis and macrocyst of S.bovifelis. The purified cystozoites stored in PBS at -20°C were used for extraction of DNA.

3.9.2 Extraction of DNA

The purified cystozoites stored in PBS (pH 7.2) at -20°C were used for extraction of DNA as per the method described by MacPherson and Gajadhar (1994) with slight modifications.
The cystozoites of *S. bovicanis* and *S. bovifelis* were washed thrice in PBS. Three cycles of freezing and thawing were carried out at -20°C and -60°C, respectively. To one volume of packed bradyzoites 10 volumes of cold lysis buffer (pH 8.0) (appendix) was added. Proteinase K was added (100μg/ml) and 5μl of 1% aqueous solution (appendix) of sodium do-decyl sulphate (SDS) were added and incubated for 5 hours at 56°C with frequent movements.

The lysate was extracted three times with Tris saturated phenol:chloroform (1:1) (appendix). The DNA present in aqueous supernatant was precipitated by addition of 1/10th volume of 2M sodium chloride and twice the volume of chilled absolute ethanol. The mix was kept at -70°C for one hour. DNA was pelleted at 12000 rpm at 4°C for 15 minutes and the supernatant was carefully discarded. Then it was overlaid with 80% ethanol and centrifuged at 12000 rpm at 4°C for 10 minutes to remove sodium chloride. The supernatant was then discarded and the washing procedure repeated.

The DNA was suspended in 50μl of Tris EDTA buffer (TE) (appendix) with pH 7.4 to each tube after drying at room temperature. The tubes were tapped gently and DNA was stored at -20°C until use.

### 3.9.3 Isolation of DNA from buffy coat

The method was followed as per descriptions of Sambrook *et al.* (1989). Approximately 20ml of fresh blood was collected from cattle in tubes
containing 3.5 ml of acid citrate dextrose solution B (appendix). It was centrifuged at 1300g for 15min and the supernatant plasma discarded. Using a Pasteur pipette the buffy coat was carefully transferred to a fresh tube and recentrifuged. The buffy coat was collected and resuspended in 15ml of extraction buffer and incubated for one hour at 37°C in a water bath. Proteinase K was added to a final concentration of 100µg/ml (4µl) and mixed gently with a glass rod. The suspension of lysed cells was placed in a water bath for 3 hours at 50°C and the viscous solution was swirled periodically. The solution was cooled to room temperature and an equal amount of phenol equilibrated with 1M Tris HCl was added. The two phases was mixed gently by slowly twining the tube end over end for 10 min.

The two phases were separated by centrifugation at 500g for 15min at room temperature. The aqueous phase was transferred to a clean ependorf tube and phenol extraction was repeated twice. The pooled aqueous phase was transferred to a centrifuge tube and precipitated with 1/10th the volume of 2M sodium chloride and 2.5 volume distilled ethanol at room temperature. The tube was kept at –70°C for one hour. The precipitated DNA was collected by centrifugation at 10000g for 10 minutes at 4°C. The DNA was washed with 80% ethanol and dried in a dry block heater at 65°C for five minutes. The DNA pellet was dissolved in 0.5ml of TE buffer and kept at 4°C in aliquots for further use.

3.9.4 Determination of purity and yield of DNA samples
The purity and concentration of the extracted genomic DNA was estimated in a UV-VIS Spectrophotometer (Beckman). Ten microlitre of DNA sample was dissolved in 0.99 ml of sterile milli Q filter water (Fqw). The diluted DNA was transferred into one ml microcuvette and the optical density (OD) was checked at 260 nm and 280 nm in UV spectrophotometer. Sterile triple distilled water was used a blank.

The ratio of 260/280 OD was calculated. A ratio of 1.7 to 1.9 was considered as pure. The concentration of DNA was estimated by the following equation.

\[ 1\text{OD at 260 nm} = 50\mu g/ml \text{ of DNA} \]

**3.9.5 Agarose gel electrophoresis of the DNA sample**

The procedure was followed as per descriptions of Sambrook *et al.* (1989). The edges of a clean, dry, gel tray was sealed with adhesive tape and placed on a horizontal section of the table. Required quantity of agarose was weighed and dissolved in proportionate volume of 1xTBE buffer (appendix) to a final concentration of 0.8% and melted in a microwave oven, the solution was gradually cooled to 55°C. Ethidium bromide at a final concentration of 0.5μg/ml was added to the above agarose (appendix). The comb was positioned above the gel tray as per the manufacturers instructions and agarose was carefully poured into gel tray avoiding air bubbles and allowed to solidfy. On solidification the comb and the seals of either side were removed carefully. The gel tray was held in an
electrophoresis tank and 1x TBE buffer was poured to submerge the gel in the tank. The DNA samples were mixed with 1/6th volume of 6x gel loading dye (appendix) and carefully loaded into the wells with a micropipette. DNA ladder (DNA marker) of 100 bp was also loaded in the first well. The electrophoresis was carried out at 5v/cm (120v) at room temperature till the bromophenol blue indicator reached the end of the gel. After the run the gel was examined under UV-Transiluminator and recorded in gel documentation system.

3.9.6 RAPD Primers

Four primers were used during the present study which had been procured commercially (Bangalore Genei). Nucleotide sequence of RAPD primers is given below.

100μl of sterile double distilled water was used to dissolve each lyophilized primer according to the descriptions of manufacturer and stored at -20°C as stock. Working dilutions were made from the stock solution by mixing 10μl of stock with 90μl of sterile DDW.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Primer Code</th>
<th>Nucleotide sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Primer 1</td>
<td>3¹ AGACCGAAAGTCAACGCGAC 5¹</td>
</tr>
<tr>
<td>2</td>
<td>Primer 2</td>
<td>3¹ GCACGAACGCGCCACAAA 5¹</td>
</tr>
<tr>
<td>3</td>
<td>Primer 3</td>
<td>3¹ CCGGGGAAGAGCGAT 5¹</td>
</tr>
<tr>
<td>4</td>
<td>Primer 4</td>
<td>3¹ AGCCAGGAAGATCAACGCGAC 5¹</td>
</tr>
</tbody>
</table>
3.9.7 Amplification by RAPD-PCR

The amplification reaction was carried out in 0.5 ml microcentrifuge tubes using a programmable thermal cycler (BioRad) as per Guclu et al. (2004). Template genomic DNA (100ng) of *S.bovicanis* and *S.bovifelis* were denatured initially at 95°C for five minutes and then snap cooled in crushed ice pack for 10 minutes. Each 25μl reaction mixture consisted of 2μl genomic DNA, 2μl of primer (10 picomoles), 0.5μl Taq DNA polymerase(0.5U), 2μl of dNTP mix (10mM), 2.5μl of 10x PCR buffer and sterile filtered milli Q water to a final concentration of 25μl was added to each tube.

**The PCR master mix for 100μl reaction was as follows:-**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA</td>
<td>8</td>
</tr>
<tr>
<td>Primer</td>
<td>8</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>2</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>8</td>
</tr>
<tr>
<td>10xPCR buffer</td>
<td>10</td>
</tr>
<tr>
<td>Fqw</td>
<td>64</td>
</tr>
</tbody>
</table>

The amplification was programmed as described in the Table.

**Standardized program parameters for RAPD-PCR**

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C (Denaturation)</td>
<td>1 min</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>36°C (Annealing)</td>
<td>1 min</td>
<td>&quot;</td>
</tr>
<tr>
<td>3</td>
<td>72°C (Extension)</td>
<td>2 min</td>
<td>&quot;</td>
</tr>
<tr>
<td>4</td>
<td>72°C (Final extension)</td>
<td>10 min</td>
<td>-</td>
</tr>
</tbody>
</table>
After completion of PCR reaction 25\(\mu\)l amplified products were subjected to electrophoresis along with 100 bp DNA ladder in 1.2% agarose at 8V/cm. The banding pattern on the gel was visualized under UV light and the image was taken with a video camera in the gel documentation system (BioRad Co.).

### 3.9.8 Evaluation of PCR product by agarose gel electrophoresis

Agarose gel electrophoresis was carried out as described in section 3.9.4 with 1.2 % gel (appendix) using Mini PCR gel electrophoresis unit (Bangalore Genei). After completion of PCR reaction 25\(\mu\)l amplified products were subjected to electrophoresis along with 100 kb DNA ladder (appendix)(Bangalore Genei) at 8V/cm. The bands were visualized on the gel under UV light and the image was taken with a video camera in the gel documentation system (BioRad).

### 3.9.9 Determination of product size

The amplicon sizes were calculated from the standard curve plotted between log values of standard DNA ladder against their mobility.

### 3.9.10 Band sharing Index

Similarity coefficient between the isolate was calculated using the formula:

\[
S_{XY} = \frac{2N_{XY}}{(N_X + N_Y)}
\]
where \( N_X + N_Y \) were the numbers of DNA segments amplified in species ‘x’ and ‘Y’ respectively. \( N_{XY} \) was the number of segments shared by ‘x’ and ‘Y’.

**3.9.11 Scoring of bands**

The scoring of bands was given depending upon its intensity. - = absence of band, ++ = intense band, +++ = bright and thick band, ++++ = very bright and thick band.