

**MOLECULAR CHARACTERIZATION OF *SARCOCYSTIS* spp.
ISOLATED FROM PIGS IN COASTAL DISTRICTS
OF ANDHRA PRADESH, INDIA**

By

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This is to certify that the thesis entitled “MOLECULAR CHARACTERIZATION OF SARCOCYSTIS spp. ISOLATED FROM PIGS IN COASTAL DISTRICTS OF ANDHRA PRADESH, INDIA” submitted in partial fulfillment of the requirements for the degree of MASTER OF VETERINARY SCIENCE of the Sri Venkateswara Veterinary University, Tirupati is a record of bonafide research work carried out by Dr. Gatta Madhuri, GVM/2019-037 under my guidance and supervision. The subject of the thesis has been approved by the Student’s Advisory Committee.

No part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of investigations have been duly acknowledged by the author of the thesis.

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL		FULLFORM
%	:	Percentage
\leq	:	Less than or equal to
\geq	:	Greater than or equal to
μ	:	Micron
μg	:	Microgram
μl	:	Microlitre
A	:	Adenine
bp	:	Base pair
C	:	Cytosine
DNA	:	Deoxyribo Nucleic acid
EDTA	:	Ethylene Diamine Tetra Acetic acid
<i>et al.</i>	:	and others
Fig	:	Figure
G	:	Guanine
gm	:	Gram
HCl	:	Hydrogen Chloride
i.e.	:	That is
M	:	Molar
min	:	Minute
ml	:	Millilitre
mm	:	Millimetre
NaCl	:	Sodium Chloride
Ng	:	Nanogram

no	:	Number
°C	:	Degrees centigrade
OD	:	Optical density
PBS	:	Phosphate Buffered Saline
PCR	:	Polymerase Chain Reaction
pH	:	Negative logarithm of hydrogen ion concentration
pmoles	:	Picomoles
RE	:	Restriction Enzyme
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribo Nucleic acid
rpm	:	Revolutions per minute
SDS	:	Sodium Dodecycl Sulphate
sec	:	Seconds
spp	:	Species
T	:	Thymine
TAE	:	Tris Acetate EDTA
TE	:	Tris EDTA

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ABSTRACT

The present study was carried out to determine the prevalence, morphological and molecular characteristics of *Sarcocystis* species in pigs in coastal Andhra Pradesh by microscopy and polymerase chain reaction using tissue samples collected from 163 slaughtered pigs for a period of one year from January 2021 to December 2021. Data regarding age, sex, breed and area of the animals were noted. Microscopic examination of tissue samples collected from different predilection sites such as heart, tongue, oesophagus, intercostals and diaphragmatic pillar muscles from each of 163 slaughtered pigs by intact cyst isolation and pepsin digestion method revealed sarcocysts in 6 (3.68%) and 20 (12.26%) pigs samples, respectively. Tissue samples positive for sarcocysts were fixed in 10% formalin for histopathological examination. Sarcocysts were microscopic with a smooth and thin cyst wall and were filled with banana-shaped

bradyzoites. No thick walled cysts were identified. The morphological features of the cystic stage were suggestive of *S. suihominis*. Histopathological examination of affected muscle tissues revealed mild leucocytic infiltration, myositis and hyaline degeneration along with fatty degeneration.

DNA was isolated from each pig tissue sample separately and was separately used to amplify partial segment of 18S rRNA gene of *Sarcocystis* spp. with the estimated amplicon size of 915 bp. Out of 163 samples, 34 (20.85 %) samples were found to be positive for *Sarcocystis* species. Subsequent digestion of PCR amplicons with *SspI*, two different patterns were visualized on electrophoresis, one with 915 bp (*S. suihominis*) and other with 650 bp and 265 bp (*S. meischeriana*). Of the 34 positive pigs, 31 (91.2%) were found to be positive for *S. suihominis* infection, one (2.9%) was found to be positive for *S. meischeriana* and two pigs (5.9%) were positive for mixed species infection with *S. suihominis* and *S. meischeriana*. Sequences of *S. suihominis* obtained from pigs demonstrated 90-100% identity with the Indian isolates of *S. suihominis* as single monophyletic group along with Italian isolate of *S. suihominis*.

The prevalence was high in Krishna (36.36%) followed by Guntur (22.22%), Visakhapatnam (20.0%), East Godavari (18.18%), Srikakulam (15.83%), Nellore (14.28%), West Godavari, (8.33%), Prakasam (7.69%) and Vijayanagaram (7.14%) districts of coastal Andhra Pradesh. Age and breed influenced the prevalence of *Sarcocystis* infection being more prevalent in Non descriptive pigs ($P<0.01$) and adults ($P<0.05$). No significant difference was observed between the prevalence of male and female pigs. Overall, the prevalence of *S. suihominis* (20.24%; 33/163) was more compared to that of *S. miescheriana* (1.84%; 3/163) in the study area possessing public health risk.

CHAPTER-I

INTRODUCTION

Globally, pig farming is very common and many people are associated with it either directly or indirectly. According to 20th Livestock census total pig population in India is 9.06 million accounting 1.7% of the total livestock population (Basic Animal Husbandry Statistics, 2019). Because of their association with human being, pigs can act as host to a much wider range of zoonotic parasites such as protozoa *Toxoplasma gondii* and *Sarcocystis* spp., and the helminths *Trichinella* spp. and *Taenia* spp. Among these, *Sarcocystis* species are ubiquitous in nature and are found worldwide. Two hosts are required to continue the life cycle: an intermediate or prey host, in which cysts (sarcocysts) containing infectious zoites infect the muscles, and a definitive, final, or predator host that ingests the cysts, becomes infected with intestinal-stage parasites, and excretes oocysts or sporocysts into the environment. Pigs act as intermediate hosts for three species of *Sarcocystis*: *S. miescheriana*, *S. porcifelis* and *S. suihominis*. In pigs the parasite encysts in muscular tissues but generally without causing pathological changes or symptoms (Avapal *et al.*, 2004). However, only *S. suihominis* can cause an intestinal infection in humans upon consumption of raw pork (Fayer, 2004). Little information on the prevalence of *S. suihominis* infection is available but its distribution is probably worldwide. In pigs, it has been detected in Germany, Japan, Thailand, China and India (Bussieras, 1994; Saito *et al.*, 1998; Bunyaratvej *et al.*, 2007; Hu *et al.*, 2010; Singh *et al.*, 2010; Dubey *et al.*, 2016).

Accurate detection and identification of *Sarcocystis* spp. are essential for designing appropriate control strategies in animal and public health. Routinely,

conventional techniques like intact cyst isolation method, pepsin acid digestion method, histopathology and transmission electron microscopy are used to diagnose sarcocysts in intermediate hosts. These conventional techniques for specific diagnosis of *Sarcocystis* spp. were based on, structure of the sarcocyst wall in the striated muscles of the intermediate host (Jehle *et al.*, 2009). Nevertheless, these conventional methods are not suitable for routine identification of the *Sarcocystis* species that show very little morphological variation. On the other hand, electron microscopy is not a choice for wide and extensive detective studies (McManus and Bowles, 1996). Though, some serological assays including enzyme linked immunosorbent assay (ELISA) and indirect fluorescent antibody test, based on bradyzoites derived from sarcocysts have been assessed for serological diagnosis of sarcocystosis, the substantial cross-reactivity among different *Sarcocystis* spp. infecting the same intermediate host unable to distinguish between pathogenic and non-pathogenic infections (Tenter *et al.*, 1994).

Recently, molecular techniques such as PCR and its variants based on sequence changes have been used to determine genetic diversity among many parasites, phylogenetic and taxonomic studies and in epidemiological mapping (Gonzalez *et al.*, 2006; Maurer, 2011). Molecular studies on *Sarcocystis* spp. have been conducted on the nuclear ribosomal DNA unit, particularly the small subunit (ss or 18S) rRNA gene, and to a much lesser extent on the large subunit (ls or 28S) rRNA gene and the internal transcribed spacer 1 (ITS1) region. The 18S rRNA gene has been identified as a good genetic marker for discriminating certain species of *Sarcocystis* in view of variable regions (Fischer and Odening, 1998; Yang *et al.*, 2000).

Currently, PCR-restriction fragment length polymorphism (PCR-RFLP) based on variable regions of the small subunit ribosomal RNA sequences is considered and

used widely as a rapid, economic and accurate molecular approach to differentiate *Sarcocystis* spp. in different animals including swine (Yang *et al.*, 2002; Gonzalez *et al.*, 2006; Jehle *et al.*, 2009).

Considering the zoonotic importance of *Sarcocystis suis* and unavailability of information on *Sarcocystis* species in pigs from Andhra Pradesh the present work was aimed with the following objectives:

1. To study the prevalence of *Sarcocystis* infection in slaughtered pigs in coastal areas of Andhra Pradesh by polymerase chain reaction.
2. To characterize *Sarcocystis* 18S rRNA gene.
3. To identify the species of *Sarcocystis* using PCR-RFLP.

CHAPTER-II

REVIEW OF LITERATURE

2.1 History of sarcocystosis

Miescher (1843) was the first to observe Miescher's tubules which are long, thin, thread like white cysts in muscles of a mouse. Fayer (1972) noticed that microgametogony, macrogametogony and cyst-like structures in cultured cells revealing the coccidial character of *Sarcocystis*. *Sarcocystis* species have obligatory two-host life cycles, where definitive host get the infection by consumption of parasitized tissues from intermediate host and intermediate host get the infection by ingestion of sporocysts released by the definitive host. *Sarcocystis* infection in intermediate host such as cattle, sheep and horses are of economic importance because they cause illness that results in fever, lethargy, poor growth, poor feed efficiency, reduced milk production, lameness, wool and hair loss, abortion, carcass condemnation at meat inspection (Fayer *et al.*, 2015). Very meagre reports were there regarding the epidemiology of *Sarcocystis* infection in India (Kaur *et al.*, 2015). The majority of *Sarcocystis* species that infect domestic animals are host-specific. Pathogenic *Sarcocystis* species in intermediate hosts can produce acute disease in the early stages of infection and chronic disease in the later stages of infection (Tenter, 1995).

Sarcocystis spp. have been reported in pigs, including *S. meischeriana*, *S. porcifelis* and *S. suihominis*, and dogs, cats and humans serve as their final hosts, respectively (Dubey, 1976; Solaymani-Mohammadi *et al.*, 2006). Sarcocysts can be found in almost every striated muscle in the body, including the tongue, oesophagus and diaphragm, as well as cardiac and smooth muscle to a lesser extent (Fayer, 2004).

Sarcocystis miescheriana is pathogenic for pigs causing clinical signs of weight loss, anorexia, dyspnoea, purpura of the skin, muscle tremors, weakness, thrombocytopenia, fever, abortion and death (Barrows *et al.*, 1982; Reiner *et al.*, 2002). Whereas, similar pathogenic affects attributed by *S. suihominis* (Heydorn, 1977). *Sarcocystis suihominis* is a zoonotic parasite that affects pigs. Contamination from human stools causes infection in pigs, whereas ingestion of contaminated pork causes intestinal disease in humans (Kaur and Bal, 2016). Intestinal sarcocystosis is little understood in India, however there have been multiple instances of muscular sarcocystosis (Beaver *et al.*, 1979; Fayer *et al.*, 2015). In portions of urban slums and rural areas, lack of access to toilets results in environmental contamination with human excrement, and the presence of scavenging pigs helps to sustain the transmission cycle of *S. suihominis* (Kaur *et al.*, 2015). In humans it produces symptoms like diarrhoea, nausea, anorexia, fever, headache, dizziness and rapid pulse (Piekarshi *et al.*, 1978; Li *et al.*, 2007).

Sarcocystis miescheriana is distributed throughout the world (Dubey *et al.*, 2016; Tadros and Laarman, 1982) whereas, *S. suihominis* is exclusively found in Europe, India, Japan, and China (Hu *et al.*, 2010; Dubey *et al.*, 2016). High prevalence of *Sarcocystis* particularly of *S. suihominis* has been reported from different parts of India (Saleque and Bhatia 1991; Avapal *et al.*, 2004; Kaur *et al.*, 2015).

2.2 Zoonotic importance

Chhabra and Samantaray (2013) reviewed the status and emerging perspectives of *Sarcocystis* species and sarcocystosis in India and concluded that there is relatively low prevalence of *S. hominis* in India compared to the prevalence of *S. suihominis*.

During a cross-sectional survey of *Strongyloides stercoralis* in Cambodia, Khieu *et al.* (2017) detected intestinal sarcocystosis infections (*Sarcocystis* oocysts) in 108 (10.0%) individuals on examination of 1081 human stool samples. Males had a significantly higher risk of infection than females. Further, concluded that the occurrence was due to the consumption of raw or insufficiently cooked beef and pork.

Rosenthal (2021) reviewed the status of zoonotic *Sarcocystis* and reported that intestinal sarcocystosis occurs when people consume raw or undercooked beef contaminated with *S. hominis* or *S. heydorni* or undercooked pork contaminated with *S. suihominis*. Most are asymptomatic and humans may also become dead-end hosts for non-human *Sarcocystis* spp. after the ingestion of oocysts accidentally.

2.3 Diagnosis of sarcocystosis

2.3.1 Conventional methods

Hansen *et al.* (1970) reported that the acridine orange stain helps to detect and identify blood parasites more quickly. It is a fluorochrome stain that gives ribonucleic acid a red fluorescence and deoxy ribonucleic acid a green fluorescence which is more sensitive than the routine Giemsa-stained blood smears for diagnosing many haemoprotozoans and haemo rickettsial infections by microscopy.

Pereira and Bermejo (1988) collected diaphragm and oesophageal tissues from 100 pigs and 100 sheep for microscopic and macroscopic examination. Microscopic examination revealed 43 per cent of pigs and 36 per cent of sheep positive for *Sarcocystis* spp. by pepsin digestion technique, while 54 per cent of sheep found to be positive by macroscopic examination.

Saleque and Bhatia (1991) examined muscle samples of 890 slaughtered pigs in Uttar Pradesh by macroscopic and microscopic examination to identify *Sarcocystis* spp. The overall prevalence was 67.98 per cent, two types of microscopic cysts were found by pepsin digestion technique in which 43.41 per cent was *S. miescheriana* and 47.11 per cent was *S. suihominis*.

Freyre *et al.* (1992) reported 57.2 per cent of prevalence of *Sarcocystis* species in slaughtered pigs in Uruguay on examination of 269 slaughtered pigs muscle samples by pepsin digestion technique.

Examination of 893 pig heart muscles by pepsin digestion technique revealed 18.2 per cent prevalence for *Sarcocystis* from Iowa state, USA by Dubey and Powell (1994).

Bose *et al.* (1995) compared the sensitivity of acridine orange and Giemsa staining techniques for the detection of *Babesia* spp. and reported that acridine orange offered a higher sensitivity compared to Giemsa staining (10^7 i.e., one parasite per 10^7 erythrocytes when compared to 10^3 or 10^6).

Saito *et al.* (1998) screened 600 tissue samples of old culled breeding pigs for presence of sarcocysts by scanning electron microscope in Japan. The examination confirmed 0.83 per cent of *S. suihominis* infection based on cyst wall morphology.

Claveria *et al.* (2001) scrutinized skeletal muscles of pigs in Philippines for *Sarcocystis* infection, the examination disclosed 27 per cent of *S. miescheriana* infection.

Avapal *et al.* (2004) collected muscle tissues (gluteals, cardiac, masseter, coccygeal, diaphragm, eye muscles and oesophagus) from 229 pigs slaughtered in

Ludhiana, Punjab to determine the prevalence of sarcocystosis. The overall prevalence recorded was 73.36 per cent in pepsin digestion method and 60.26 per cent in cyst isolation method.

Ravindran *et al.* (2007a) opined that screening large numbers of blood smears for the detection of haemoprotozoans and rickettsiales, the acridine orange staining technique was found to be accurate, simple and fast.

Imre *et al.* (2016) inspected 165 heart samples of pigs by microscopic examination for presence of *Sarcocystis* spp., in which 60.4 per cent of wild boars and 23.4 per cent of domestic pigs revealed positive for *Sarcocystis*.

Zainalabidin *et al.* (2017) collected 150 fresh heart, oesophagus and thigh muscle samples from 50 Yorkshire and Landrace pigs in Perak and observed that 58 per cent of the pigs to be positive for *Sarcocystis* spp. using the pepsin digestion technique.

Gazzonis *et al.* (2019) reported 32 per cent of sarcocystosis in wild boars in Italy by histological examination. The prevalence was high in female and young animals than male and adult animals.

Mounika *et al.* (2019) conducted a study to estimate the prevalence of *Sarcocystis* infection in cattle in Chittoor district, Andhra Pradesh. The results revealed an overall prevalence of 91.33 per cent in which 6.57 per cent were macroscopic and 93.43 per cent were microscopic sarcocysts.

Prakas *et al.* (2020) recorded 87.1 per cent prevalence of *S. miescheriana* species in wild boars from Latvia on examination of different muscle samples by modified compression method.

Abdullah (2021) examined a total of 130 muscle samples from cattle and sheep slaughtered in North Iraq. Samples were subjected for macroscopic and pepsin digestion methods, which revealed higher prevalence rates of 92 per cent and 95 per cent in the esophagus than those of 88 per cent and 90 per cent in the diaphragm for cattle and sheep, respectively. Comparative analysis of detection methods concluded that the pepsin digestion technique is a sensitive, simpler and faster technique than other conventional procedures for investigating the presence *Sarcocystis* spp. in slaughtered animals.

2.3.2 Immunodiagnostic methods

Weber *et al.* (1983) experimentally immunized pigs with subclinical infections with 10^3 of *S. miescheriana* sporocysts and divided growing pigs into groups of immunized and control pigs where they were exposed with 3×10^6 sporocysts at 40, 80, 120, 160 days after post immunization to determine the persistence of protective immunity against acute sarcocystosis which concluded that protective immunity against acute disease persisted only until 80 days post immunization.

Pereira and Bermejo (1988) collected samples of serum from 100 pigs and 100 sheep and examined sera samples using indirect haemagglutination test (IHA) using antigens from *S. gigantea* and results revealed 95 per cent of sheep and 43 per cent of pigs to be positive for *Sarcocystis* spp.

Reiner *et al.* (2002) evaluated differences in susceptibility or resistance against *S. miescheriana* in the European Pietrain (PI) and the Chinese Meishan (ME) pig breeds, based on 25 individuals that were infected orally with 5×10^4 sporocysts of *S. miescheriana*. Significant differences appeared in clinical, serological, haematological and parasitological findings. The major discriminating period post

infection (p.i.) was between days 42 and 45 and concluded that *Sarcocystis* specific differences between the two breeds were in the range of 1–2 standard deviations.

Damriyasa *et al.* (2004) conducted a cross-sectional survey in sows in Germany to estimate the seroprevalences of antibodies of *Toxoplasma gondii*, *Sarcocystis* spp. and *Neospora caninum* of 2041 plasma samples and indicated the risk of infection with *T. gondii* and *Sarcocystis* spp. was high in breeding sows.

Ghorbanpoor *et al.* (2007) detected *Sarcocystis* antibodies in 54.3 per cent of examined buffaloes by ELISA and concluded that there is a significant difference of microscopic examination and ELISA from macroscopic examination and no difference between microscopic examination and ELISA itself.

Panda *et al.* (2020) optimized Dot-ELISA for bovine sarcocystosis using 1ng of bradyzoite antigen and compared its efficiency with indirect fluorescent antibody technique (IFAT) and found that sensitivity and specificity of Dot-ELISA was found to be 86.66 per cent and 88.88 per cent, respectively and for IFAT the sensitivity was 83.33 per cent and specificity was 100 per cent. This study concluded that Dot-ELISA was found to be quite sensitive and inexpensive test to screen the animals at slaughter house or herd level. The standardized Dot-ELISA technique showed overall prevalence of 82.75 per cent for *Sarcocystis* in Hyderabad.

2.3.3 Morphological studies

Saito *et al.* (1986) examined 100 fattened and 200 older culled breeding pigs, out of which 17 were found positive for *S. miescheriana* species in Japan. The cysts were measured about an average of 393.4 x 126.5µm and bradyzoites with an average of 15.49 x 3.92 µm. The cyst wall was radially striated and measures about an average of 4.48µm thickness.

Saleque and Bhatia (1991) examined 890 pig muscles samples in India, the study revealed two types of microscopic sarcocysts *i.e.*, *S. miescheriana* with radially striated perpendicular protrusions of cyst wall and *S. suihominis* with hair like villar protrusions in the cyst wall.

Saito *et al.* (1998) detected *S. suihominis* for the first time in Japan from the heart and diaphragm muscles in pigs. Fresh cysts were measured about 1080-2040 x 106-170 μm in size. The scanning electron microscopic examination revealed 4-6 μm thickness cyst walls with palisade like villar protrusions measuring up to 13 μm length.

Claveria *et al.* (2001) identified *S. miescheriana* sarcocysts in skeletal muscles of pigs in Philippines. The transmission electron microscopic studies of cysts revealed the size of cyst to be 144–180 μm x 20–38 μm with prominent villar protrusions that are 3-4.5 μm long and 0.6-1.2 μm width.

Chen *et al.* (2007) recorded the morphological changes in cysts of *S. suihominis* and *S. miescheriana* from pig muscles in China by light microscope and electron microscope and comparisons were made between fresh cysts and those subjected to frozen storage for periods of 3 days, 20 days and 30 days which resulted the cyst wall of the two *Sarcocystis* species appeared unaffected by storage.

Yan *et al.* (2013) studied ultrastructural characteristics of sarcocysts in pig muscle samples in China. The study manifested thick cyst wall with a number of palisade like protrusions, which suggested the cysts were *S. miescheriana*.

Huang *et al.* (2019) described the morphological characteristics of two distinct sarcocysts in pig muscles by light microscopy. The cyst wall of *S. miescheriana* bounded by finger-like protrusions, whereas sarcocyst of *S. suihominis* cyst wall surrounded by hair like protrusions.

2.3.4 Molecular detection methods

Yang *et al.* (2001) characterized *Sarcocystis* spp. from cattle and water buffaloes based on 18S rRNA gene sequences. The genetic difference in isolates of *S. hominis* like cysts from cattle and water buffaloes were very low suggesting that both cattle and water buffaloes may serve as intermediate host for the same species i.e., *S. hominis*.

Li *et al.* (2002) examined *S. cruzi* like taxa from typical intermediate host, water buffalo in Yunnan, China using PCR-RFLP. The analysis revealed that RFLP patterns for the *S. cruzi* isolates from water buffalo and cattle are identical with all restriction enzymes and proposed that water buffalo acts as natural intermediate host for *S. cruzi* in addition to cattle.

Characterization of *Sarcocystis* species in domestic animals using a PCR-RFLP analysis of variation in the 18S rRNA was carried out by Yang *et al.* (2002).

Gonzalez *et al.* (2006) conducted PCR and PCR-RFLP assays for differentiation of *Taeniid* spp. and *Sarcocystis* spp. cysts isolated from infected pigs and cattle. The results indicated that PCR and PCR-RFLP of the rDNA internal transcribed spacers 1 and 2 (ITS1 and ITS2) found to be specific for the differential diagnosis of taeniids and PCR derived from the 18S rRNA gene and sequencing is specific for identification of *Sarcocystis* spp. The study presumed that the combined application of these three PCR protocols provides a specific diagnosis of *T. saginata*, *T. solium*, *T. hydatigena*, *S. hominis* and *S. suihominis*.

Jehle *et al.* (2009) performed PCR-RFLP of 18S rDNA using *FokI*, *DraI*, *BslI* and *SspI* restriction enzymes to detect infections of different *Sarcocystis* spp. in cattle and water buffaloes slaughtered in the Son La Province of Northern Vietnam. The study

revealed that *Sarcocystis* spp. (*S. hirsuta*, *S. cruzi* and *S. hominis*) infecting cattle were also able to infect water buffaloes.

A PCR using the primers COC1-COC2 that target a conserved region of the small-subunit rRNA gene of Apicomplexa was performed with DNA from paraffin-embedded tissues (heart samples) while diagnosing fatal acute sarcocystosis in a pig breeding herd in an indoor farm in Switzerland (Caspari *et al.*, 2010). An amplification product of about 350 bp was obtained from heart samples. A sequence analysis showed 100% identities with GenBank sequences reported for *S. miescheriana*. The histopathological observations and molecular findings in combination with the clinical signs, and absence of other pathologic agents highly suggested that an acute infection with *S. miescheriana* was the cause of death in this boar.

Sarcocystis isolate collected from the thigh muscle of a wild boar in northern Iran, was subjected to molecular analysis by Kia *et al.* (2011). Polymerase chain reaction for amplification of the 18S ribosomal DNA region yielded an 842 bp product. Analysis of DNA sequencing by BLAST confirmed the isolate as *S. miescheriana*.

More *et al.* (2013) developed and optimized a multiplex real time PCR for differentiation of *Sarcocystis* spp. affecting cattle. Amplification of 18S rRNA genes derived four *Sarcocystis* spp. viz., *S. cruzi*, *S. sinensis*, *S. hirsuta* and *S. hominis*, respectively. *Sarcocystis sinensis* was the most prevalent species among thick walled *Sarcocystis* spp. in Argentinean cattle. Mixed infections were detected in 8.9 per cent of the samples. The study concluded that the diagnostic sensitivity and specificity of the BovSarcoMultiplex realtime PCR relative to previous microscopic examination for thin and thick-walled cyst were 91.5 per cent and 41.7 per cent, 36.3 per cent and 95.9 per cent, respectively.

Tissue samples from 638 sheep slaughtered at Urmia abattoir were randomly collected to determine prevalence of *Sarcocystis* infection and molecular discrimination of *S. gigantea* and *S. medusiformis* infecting domestic sheep (Farhang-Pajuh *et al.*, 2014). Genomic DNA extraction and polymerase chain reaction was performed to amplify a 964 bp fragment of 18S rRNA gene. The PCR products were subjected to digestion with endonuclease *MboII* and/or *MvaI* for discriminating *S. medusiformis* and *S. gigantea*. Results indicated that the overall prevalence of *Sarcocystis* unspecified species was 36.83 per cent (235/638). The PCR-RFLP patterns manifested that fat sarcocysts were *S. gigantea* (29.31%) and thin sarcocysts were *S. medusiformis* (7.52%).

Hamidinejat *et al.* (2014) scrutinized 40 macroscopic and microscopic sarcocysts each in sheep in south-western Iran. The PCR-RFLP patterns of 18S rRNA gene using *HindII*, *TaqI* and *EcoRI* restriction enzymes exhibited *S. gigantea* in both microscopic and macroscopic cysts.

More *et al.* (2014) conducted molecular studies of *Sarcocystis* spp. in beef in Germany by amplification of 18S ribosomal DNA. Sequence studies revealed four species were prevalent i.e., *S. cruzi*, *S. sinensis*, *S. hirsuta* and *S. hominis* in which *S. sinensis* was the more prevalent thick walled *Sarcocystis* species.

Calero-Bernal *et al.* (2015) collected myocardium samples from wild boars in Southwestern Spain and detected sarcocysts and free banana shaped zoites by pepsin digestion method. Amplification of 18S rRNA was carried out by seminested PCR. PCR-RFLP using restriction endonuclease *SspI* revealed presence of *S. miescheriana* and *S. suihominis*.

Coelho *et al.* (2015) observed 73.8 per cent of *S. miescheriana* infection in pigs by histological examination and PCR assays in Portugal. The PCR assay targeting partial sequence of 18S rRNA gene manifested presence of *S. miescheriana* only and no animal was positive for *S. suis*.

Identification of *Sarcocystis* spp. in tissue samples of slaughtered cattle (124) and water buffaloes (147) in Ahvaz, Khuzestan province by PCR-restriction fragment length polymorphism was carried out by Hamidinejat *et al.* (2015). Tissue samples (each 50 g) from heart, esophagus, diaphragm and intercostal muscle were collected from each animal and were examined with digestion method. Genomic DNA of 80 positive samples was extracted and their 18S rRNA gene was amplified. PCR products were digested by restricted enzymes (*FokI*, *SspI* and *DraI*). *Sarcocystis cruzi* in cattle and *S. fusiformis* in water buffaloes were identified.

Kaur *et al.* (2015) provided molecular evidence of *Sarcocystis* cysts present in pigs in Punjab by characterization of *Sarcocystis* 18S rRNA. The results revealed presence of one *S. miescheriana* isolate and six *S. suis* isolates.

Latif *et al.* (2015) examined 1045 tissue samples of skeletal muscles (tongue, heart, diaphragm and esophagus) collected from 209 animals (43 sheep, 89 goats and 77 cattle) from an abattoir in Selangor, Malaysia. Molecular studies targeting 18S rRNA gene confirmed the isolates from sheep as *S. ovis*, goats as *S. capracanis* and cattle as *S. bovis*.

Imre *et al.* (2016) reviewed the molecular epidemiology of *Sarcocystis* spp. affecting cattle and pigs throughout the world. The study disclosed that there is predominance of *S. miescheriana* species than *S. suis* in pigs.

Alina *et al.* (2017) determined the prevalence of *S. suihominis* in pork (n=79) obtained in the traditional households in Alba and Cluj through PCR-RFLP method. The samples were processed first by trichinelloscopic compression method subsequently the positive fragments for *Sarcocystis* spp. were examined through PCR-RFLP method utilising the *AluI* restriction enzyme that successfully differentiated the *S. suihominis* from other *Sarcocystis* spp. prevalent in pork meat. The inspection of compressed muscle tissue has detected the presence of *S. suihominis* in a high percentage of investigated samples. The prevalence *S. suihominis* in the examined samples was 26.58 per cent.

Hooshyar *et al.* (2017) investigated the prevalence and species identification of *Sarcocystis* among of hamburgers (n=200), using PCR-RFLP methods in Kashan, central Iran. The results showed that 58 (29%) of 200 tested hamburger samples were infected to *Sarcocystis* spp. According to PCR-RFLP analysis, 43 (74.1%) of the 58 isolates were *Sarcocystis cruzi*, 12 (20.7%) showed co-infection to *S. cruzi* and *S. hirsuta*, two (3.5%) was mixed infected to *S. cruzi* and *S. hominis*, one (1.7%) showed the pattern of mix infection to three species. This study revealed one-third of industrial hamburger were infected to *S. cruzi* or mixed infection of *S. cruzi* with other bovine sarcocytosis.

Imre *et al.* (2017) investigated the occurrence of *Sarcocystis* infections in wild and domestic boars by genetic characterization with amplification of *Sarcocystis* 18S rRNA. Further analysis of PCR-RFLP using restriction endonucleases revealed single species of *S. miescheriana* in all wild and domestic boars.

Kamber *et al.* (2018) collected a total of 150 beef products in Turkey and examined for the presence of *Sarcocystis* by PCR and microscopy. The results revealed

that 4 per cent and 2 per cent in minced meat and meatballs respectively, were found to be positive for *Sarcocystis* by microscopic examination whereas, 28 per cent, 68 per cent, and 2 per cent in minced meat, meatballs and sausage respectively, were found to be positive by molecular analysis indicating that PCR would provide more conclusive results than that of microscopic examination.

Murata *et al.* (2018) investigated the presence of sarcocysts in 28 horse meat and 121 beef samples collected in Tokyo. Sarcocysts of *S. fayeri* were found in 16 horse meat samples. Sarcocysts of *S. hominis* were not detected in beef samples, while sarcocysts of *S. cruzi* were detected in 60 beef samples. In addition, *S. hirsuta* and *S. bovini* were isolated only from New Zealand beef samples. Molecular analysis of 18S rRNA gene and mitochondrial cytochrome c oxidase subunit 1 (mtDNA *cox1*) gene of identified species revealed that molecular analysis of the mtDNA *cox1* gene is the most useful for identification of *Sarcocystis* species.

A sample of the diaphragm was collected from each of 100 wild boars legally hunted in the Val Grande National Park in north-western Italy by Gazzonis *et al.* (2019) and examined for the presence of *Sarcocystis* infection by histological and molecular methods. In histological sections, thick-walled sarcocysts consistent with those of *S. miescheriana* were detected in 32 wild boars. DNA extracted from diaphragm samples was initially subjected to PCR amplification of the ITS1 region, which revealed *Sarcocystis* infection in 97 wild boars. Selected DNA samples were then subjected to PCR amplification and sequencing of the ITS1 region and the 18S and 28S ribosomal RNA genes of the nuclear ribosomal DNA unit, while all positive samples were subjected to PCR amplification of the *cox1* gene. *Sarcocystis miescheriana* was identified in 97 wild boars (97%), while the zoonotic *S. suis* was identified in one wild boar (1%), which also harboured *S. miescheriana*.

Huang *et al.* (2019) determined sarcocysts in 36.8 (28/76) per cent of tissue samples collected from domestic pigs by light microscopic examination. The organisms were identified as *S. miescheriana* and *S. suihominis* based on their morphological and molecular characteristics. Four genetic markers, i.e., 18S rDNA, 28S rDNA, ITS-1 region (ITS-1), and the mitochondrial COX1 gene (COX1), of the two parasites were sequenced and analyzed. The sequences of the four loci (18S rDNA, 28S rDNA, ITS-1, and COX1) of *S. miescheriana* shared high identities with those of *S. miescheriana* obtained from domestic and/or wild pigs in GenBank, with similarities of 99.6%, 99.6%, 95.9%, and 95.4%, respectively. The 18S rDNA sequences of *S. suihominis* exhibited 99.4% identity with those of *S. suihominis* from domestic and wild pigs. The phylogenetic analysis using 28S rDNA indicated that the two *Sarcocystis* species in domestic pigs had a close relationship.

A total of 117 heart samples from slaughtered cattle in three southwestern Romanian counties (Dolj, Timiș, and Gorj) were analyzed by Imre *et al.* (2019) in order to detect sarcocysts, using microscopic techniques. Subsequently, the isolated sarcocysts and/or cyst fragments from each infected animal were molecularly characterized. Overall, 17.9 per cent (21/117) of the tested animals were found to be *Sarcocystis* spp. positive by microscopy. Genetic characterization of *Sarcocystis* spp. isolates, based on sequence analysis of the 18S rRNA gene, showed the presence of a single species, namely *S. cruzi*. No correlation was found ($p > 0.05$) between *S. cruzi* infection and the origin, age, breed and gender of cattle, but the grazing farming system was positively associated ($p=0.031$) with the pathogen prevalence and can be considered a risk factor (OR = 3.6) in acquiring infection.

Mounika *et al.* (2019) characterized the *Sarcocystis* species in cattle by amplification of genus specific 18S rRNA from representative tissue pieces collected

from slaughtered cattle at Chittor district, Andhra Pradesh. PCR amplicons were subjected to restriction endonucleases revealed three different electromorphs as *S. cruzi*, *S. hirsuta* and *S. fusiformis*.

Chauhan *et al.* (2020) characterized 18S rRNA of *S. sui hominis* in domestic pigs from five North Indian states and compared the sequences with previous published isolates which exhibited 96.9-100% homology. Sequence analysis revealed two new haplotypes of *S. sui hominis* which were not previously recorded and manifested 99.5–100.0% identity within themselves.

The macroscopic (21) and microscopic (42) sarcocysts isolated from slaughtered culled ewes at an abattoir in the Province of Madrid, Central Spain, were characterized by molecular examination (Gjerde *et al.*, 2020). Genomic DNA from 63 sarcocysts were examined at the *cox1*, while selected isolates of each species found were further examined at the 18S and 28S ribosomal RNA genes. The molecular analysis revealed five *Sarcocystis* spp. The slender fusiform and thick macrocysts belonged to *S. medusiformis* and *S. gigantea*, respectively. The microscopic sarcocysts belonged to *S. arieticanis*, *S. tenella* and a *S. mihoensis*-like species with slanting thorn-like cyst wall protrusions.

Prakas *et al.* (2020) examined different muscle samples of wild boar from Latvia were examined for the presence of *Sarcocystis* infection by means of morphological and molecular methods. Sarcocysts were detected in 122 out of 140 (87.1%) wild boars examined. Based on morphology, sarcocysts were identified as cysts of *S. miescheriana*. Twenty-three sarcocysts isolated from the muscles were molecularly characterized at 18S rRNA, ITS1 and *cox1*. The amplified 18S rRNA region was sufficiently variable to separate *S. miecheriana* from *S. sui hominis*. All Latvian isolates were confirmed

belonging to *S. miescheriana*. No genetic variation was detected within 18S rRNA and ITS1. By contrast, the high intraspecific genetic variability of *S. miescheriana* was observed within *cox1* since each newly obtained sequence represented a unique haplotype.

CHAPTER-III

MATERIALS AND METHODS

The present study is carried out to determine the prevalence of *Sarcocystis* species in slaughtered pigs in coastal Andhra Pradesh, to characterize *Sarcocystis* 18S rRNA gene and to differentiate the species of *Sarcocystis* by restriction fragment length polymorphism (RFLP).

3.1 Materials

3.1.1 Laboratory consumables

Laboratory ware such as microcentrifuge tubes, vials, microtips and filtertips were obtained from Eppendorf, USA. All the consumables such as glassware and plastic ware used in the present study were procured from Borosil, Blue star and Tarson. They were washed and sterilized properly as per the standard procedures.

3.1.2 Chemicals and Oligonucleotide primers

All the chemicals used in the present study were procured from Fisher Scientific, Himedia, Merck India, Sigma-Aldrich, SD Fine chemicals and Sisco Research Laboratories. The oligonucleotide primers used in the present study were custom synthesized from Barcode Biosciences, Bengaluru. The restriction enzyme was obtained from New England Biolabs.

3.1.3 Scientific equipment

The present research work was carried out with the following equipment as Thermal cycler (Prima-Duo, Himedia), Gel documentation system (Omega Fluor^{plus}, Aplegen), Nano DropTM 2000/2000c (M/S Thermofisher Scientific), GR 200 Weighing

balancer, Spinwin Microfuge spin apparatus, Refrigerated Centrifuge (M/S Thermofisher Scientific), Horizontal gel electrophoresis system (M/S Bangalore Genei, India), pH meter 720 inoLab (M/S Merck, Bengaluru), YSI-402 Yoriko Autoclave vertical (National Scientific, Hyderabad), Laminar flow cabinet (Deccan Techno corporation), Vortex–mixer (M/S Bangalore Genei, India), Micropipette (M/S Eppendorf, USA), Deep Freezer -20 °C, Olympus Student Dissection Microscope (M/S Annapurna Scientific and Laboratory equipment, Hyderabad) Binocular Microscope (M/S Olympus, Japan), Phase contrast Microscope (M/S Nikon, Towa optics), Ocular Micrometer (M/S National Scientific, Hyderabad), Stage Micrometer (M/S National Scientific, Hyderabad), Hot air oven (M/S Deccan Techno Corporation, Hyderabad), Ice Machine (M/S Sanguine Bio Instruments, Hyderabad) and Yoriko High Precision Water bath (M/S National Scientific), Fluorescent microscope (Olympus BX 40).

3.1.4 Softwares

The softwares used for the present research work was *NEB cutter* V2.0 for PCR-RFLP, Gene Doc 2.7 for Sequence analysis, Mega X for phylogenetic analysis.

3.1.5 Source of samples/Area of study

Andhra Pradesh lies between 12°41' and 19.07°N latitude and 77° and 84°40'E longitude with hot and humid climate. Most of the population (60%) in the state is engaged in agriculture and related activities. In rural areas, ponds, canals, deep wells are the common water resources for domestic and livestock purpose where there is increasing risk of zoonotic infections. Pigs are mainly raised by the people from lower socio-economic backgrounds, marginal livestock farmers where unclean, unhygienic circumstances without veterinary oversight increase the risks of infection. *Sarcocystis*, one of the zoonotically important protozoan species, is not yet highlighted in Andhra

Pradesh, owing to this, the prevalence status of *Sarcocystis* in coastal districts of Andhra Pradesh was undertaken.

The study area included nine coastal districts, Srikakulam, Vijayanagaram, Visakhapatnam, East Godavari, West Godavari, Krishna, Guntur, Prakasam and Nellore of Andhra Pradesh. Fresh tissue samples from different predilection sites such as heart, tongue, oesophagus, intercostal and diaphragmatic pillar muscles (Fig. 2) were collected from 163 slaughtered pigs which were slaughtered at local slaughter shops and Department of Livestock Products Technology (LPT), NTR CVSc, Gannavaram. Approximately 50 g tissue was collected in zip lock self-seal bags and transported to the laboratory in ice. The collected samples were stored at -20 °C until further examination. The particulars of samples regarding age, breed. Gender and area were documented in Table 1.

Table 1. Particulars of tissue samples collected from pigs in different districts of Andhra Pradesh.

DISTRICT	SAMPLES (n)	BREED		AGE		GENDER	
		LWY	ND	≤1 year	≥1year	MALE	FEMALE
Krishna	44	37	7	15	29	31	13
Guntur	27	22	5	12	15	19	8
East Godavari	11	9	2	5	7	7	4
West Godavari	12	8	4	5	6	6	6
Nellore	14	14	-	7	7	9	5
Prakasam	13	13	-	5	8	7	6
Srikakulam	13	13	-	6	7	9	4
Visakhapatnam	15	15	-	7	8	10	5

Vizianagaram	14	14	-	8	6	8	6
TOTAL	163	145	18	70	93	106	57

LWY- Large White Yorkshire; **ND**- Non descriptive.

3.2 Methods

3.2.1 Microscopic examination

3.2.1.1 Detection of sarcocysts by rapid intact cyst isolation

Stored tissue samples were thawed and examined for the presence of sarcocysts by muscle squash examination. First, the tissues were curated by removing fat, connective tissue and fascia, after that approximately 5-10gm of pooled tissues (heart, tongue, oesophagus, intercostal and diaphragmatic pillar muscles) from each sample were transferred in to petri dishes and teased into small pieces (3-5mm) with the help of needles and forceps in PBS solution. The small tissue pieces were compressed between clean glass slides and then examined under low power magnification using a light microscope. The representative tissue pieces were fixed in 10% neutral buffered formalin for histopathological examination.

3.2.1.2 Detection of sarcocysts by pepsin acid digestion technique

Approximately 5 g of pooled tissue samples (heart, tongue, oesophagus, intercostal and diaphragmatic pillar muscles) from each pig irrespective of the presence of sarcocysts were homogenized using mortar and pestle after making into small pieces. The homogenised samples were incubated in 15-20 ml acid pepsin solution for 20 minutes at room temperature under continuous stirring (Jacob *et al.*, 1960) (Fig. 3). The solution was then filtered through a strainer and centrifuged at 2000 rpm for 10 min. The resultant supernatant was decanted and the sediment was washed with PBS (pH

7.2) solution. The sediment was then examined for the presence of bradyzoites under a light microscope. In addition, the bradyzoites were stained with acridine orange according to the procedure of Lauer *et al.* (1981). In brief, thin smears prepared from the sediment were flooded with acridine orange stain for 2 minutes, washed slowly in tap water, dried and examined using a fluorescent microscope. Finally, the pepsin digested sediment was washed with TE buffer (pH 8.0) and stored at -20°C for DNA extraction.

3.2.1.3 Preparation of pepsin acid digestion fluid, TE Buffer and acridine orange stain

Pepsin acid digestion fluid

Ingredients	Quantity
Pepsin (1:1000)	1.3gm
NaCl	2.5gm
HCl	3.5ml
Triple glass distilled water	make volume upto 500ml.

TE Buffer (pH 8.0)

Ingredients	Quantity
Tris HCl (10mM)	0.394 gm
Ethylene Diamine Tetra Acetic acid(EDTA)	0.0093 gm

Triple glass distilled water to make	250 ml
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Composition of Acridine Orange stain (pH 3.5)

Ingredients	Quantity
Acridine Orange powder	20 mg
Sodium acetate	13.6 gm
1N HCl	90 ml
Triple glass distilled water to make	100 ml

3.2.1.4 Histopathological examination

For histopathological examination, tissue samples positive for sarcocysts were fixed in 10% formalin. The formalin fixed tissues with thickness of 2-3mm were processed for paraffin embedding technique and were later washed in running tap water to remove formalin. Subsequently dehydrated in ascending grades of alcohol, cleared in benzene, infiltrated and embedded in paraffin. These embedded tissues were cut into 5 μ thick section using semiautomatic microtome and stained with hematoxylin and eosin (H & E) as per standard procedures (Luna, 1968).

3.2.2 DNA extraction

The genomic DNA was extracted from tissue samples as per Zhang and Hewitt (1998) with minor modifications.

3.2.2.1 DNA extraction procedure

DNA was isolated from the pepsin digested sediment by Phenol Chloroform Isoamyl alcohol method and the steps for the isolation were mentioned as follows:

- i. Pepsin digested sediment was taken into a microcentrifuge tube containing 500 μ l of lysis buffer and incubated at room temperature for one hour.
- ii. Then, 25 μ l of Proteinase - k (20mg/ml) was added to the tube and again incubated at 55-60 °C in water bath for overnight for complete lysis.
- iii. The digested lysate was subjected to an equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1), vortexed and was centrifuged at 12,000 rpm for 10 minutes at room temperature.
- iv. The aqueous phase (approximately 500 μ l) was carefully transferred to a clean Eppendorf tube.
- v. Then, double the quantity of absolute alcohol (1000 μ l) and one-tenth volume of 10 M ammonium acetate (100 μ l) was added to the tube, mixed and centrifuged at 10,000rpm for 10 minutes at 4 °C to precipitate the DNA.
- vi. The precipitated DNA was dissolved with equal volume of chilled 70 percent ethanol and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was discarded and the procedure was repeated twice.
- vii. Finally, the supernatant was discarded and the pellet was left to air dry till free from ethanol. The dried DNA pellet was resuspended in 50 μ l of nuclease free water and was stored at -20 °C until use.

3.2.2.2 Preparation of reagents for DNA extraction

Extraction Buffer/Lysis Buffer (pH 8.0)

Ingredients	Quantity
Tris HCl (10mM)	0.15764 gm
Ethylene Diamine Tetra Acetic acid (EDTA)	3.72 gm
Sodium Dodecyl Sulphate (10 percent)	10 gm
Triple glass distilled water to make	100 ml.

Ammonium acetate:

Ingredients	Quantity
Ammonium acetate (10M)	38.54 gm
Triple glass distilled water to make	50 ml

Proteinase-k:

Ingredients	Quantity
Proteinase-k	20 mg
Triple glass distilled water to make	1 ml.

Phenol: Chloroform: Isoamyl alcohol : 25:24:1

Ethanol (70%) 70 ml of absolute alcohol + 30 ml of triple glass distilled water.

Absolute alcohol

Nuclease free water (used for DNA storage)

3.2.2.3 Quality of genomic DNA

Quality of genomic DNA was detected by using 1% agarose gel electrophoresis, to this Ethidium Bromide at the rate of 0.5 µg/ml is added after cooling the agarose suspension. The gel was casted on electrophoresis plate and left at room temperature. One µl of 6X loading buffer containing bromophenol blue dye along with 2µl of genomic DNA was mixed and loaded into the wells. The DNA samples were run on agarose gel at 80 V for about 45 minutes in 0.5X TAE buffer and gel was visualized under gel documentation (Omega Fluor^{plus}, Aplegen).

3.2.2.4 Quantity of genomic DNA

DNA was quantified by using NanoDropTM 2000/2000c (Thermo Fisher Scientific) using the convention that 1 optical absorbance unit at 260 nm equals 40 µg of DNA per ml. Purity of DNA was judged on the basis of OD ratio of 260:280 and samples having the acceptable purity *i.e.* 1.5 to 2.0 were used for further analysis.

3.2.2.5 Preparation of reagents for gel electrophoresis

Tris Acetate EDTA (TAE) buffer

A stock solution of 50X strength with pH 8.0 was prepared with the following composition.

Tris base	:	242.0 gm
Glacial acetic acid	:	57.1 ml
0.5M EDTA	:	100 ml

Triple glass distilled water to make up the volume 1000ml

The working solution of 1X TAE buffer, prepared by diluting 10ml of 50X TAE buffer with 490 ml of triple glass distilled water was used during agarose gel electrophoresis.

Preparation of 50bp DNA ladder

The Gene ruler 50bp DNA ladder was reconstituted as per the manufacturer's instructions. To prepare working marker DNA ladder, 6X gel loading buffer and nuclease free water was mixed at the ratio of 1:1:4 and was mixed well before loading into the wells.

Agarose preparation for analysis of DNA

Agarose (low EEO) -1% : 0.40 gm

1X TAE buffer : 40 ml

Agarose (0.40 gm) of was dissolved in 40 ml of 1X TAE buffer, heated in microwave for 60 seconds, allowed to cool at 60 °C, and later 2.5µl of ethidium bromide was added.

3.2.3 Polymerase chain reaction

3.2.3.1 Oligonucleotide primers

The amplification of 18S rRNA gene fragment of *Sarcocystis* spp. was carried using the following primers (Rosenthal *et al.* 2010). The primers were synthesized by Barcode Biosciences, Bengaluru, India. These freeze-dried powder form oligonucleotides were reconstituted in nuclease free water to give a final dilution of 10 pmoles/µl.

Primer	Sequence	Product Length
2L Forward	GGATAAACCGTGGTAATTCTATG	915 bp
3H Reverse	GGCAAATGCTTTCGCAGTAG	

3.2.3.2 PCR protocol

For amplification of the 18S rRNA gene of *Sarcocystis* spp. by the PCR assay, the reaction mixture (25 µl) was prepared as below:

Composition of the PCR mix		
Taq DNA Polymerase Master Mix (Ampliqon)	-	12.5 µl
Forward primer	-	0.5 µl
Reverse primer	-	0.5 µl
DNA template	-	2 µl
Nuclease free water	-	9.5 µl
Total	-	25 µl

PCR was performed in a final reaction volume of 25µl with genomic DNA diluted to a final concentration of 450ng/µl with primers that are specific to 18S rRNA gene. Positive and negative control was run simultaneously with the samples at every PCR setup with negative control being the same reaction without the DNA template. In positive control, DNA isolated from sarcocysts isolated from intact cyst isolation method was used as template DNA. To amplify the 18S rRNA gene of *Sarcocystis* spp. the cycling conditions were optimized using the gradient PCR assay and the final PCR cycling conditions were as follows.

3.2.3.3 PCR Amplification Conditions

Step	Optimum temperature	Optimum time
Initial Denaturation	95° C	5 min
Denaturation	95° C	45 sec
Annealing	58° C	45 sec
Extension	72° C	1 min
No. of cycles from 2-4	34	
Final Extension	72° C	5 min

3.2.3.4 Analysis of the PCR product

To determine the amplified DNA fragment of the target 18S rRNA gene, PCR product is detected by agar gel electrophoresis. PCR product of 3µl is loaded in each well along with 50 bp DNA ladder marker and the gel electrophoresis was carried out at 80 volts for 45 minutes. The amplified product was detected in Gel Documentation system for visualization of bands.

Agarose preparation for analysis of PCR amplicons

Agarose (low EEO) -2% : 0.80 gm

1X TAE buffer : 40 ml

Agarose (0.80 gm) was dissolved in 40 ml of 1X TAE buffer and heated in microwave for 80 seconds, the solution was allowed to cool at 60 °C then added 2.5ul of ethidium bromide.

3.2.4 Restriction fragment length polymorphism (RFLP)

PCR-restriction fragment length polymorphism (PCR-RFLP) based on variable regions of the small subunit ribosomal DNA sequences is considered as a rapid, inexpensive and accurate molecular approach to discriminate the *Sarcocystis* species (Hamidinejat *et al.*, 2014).

3.2.4.1 Restriction enzyme digestion

The restriction enzyme (*SspI*) used in the present study were obtained from M/S New England Biolabs. *SspI* restriction enzyme is a good genetic marker for inter specific variations in 18S rRNA gene in pigs (Calero-Bernal *et al.*, 2015).

Enzyme	Recognition sequence
<i>Ssp I</i>	$5' \dots \text{A A T} \begin{array}{c} \downarrow \\ \downarrow \end{array} \text{T T} \dots 3'$ $3' \dots \text{T T A} \begin{array}{c} \uparrow \\ \uparrow \end{array} \text{A A} \dots 5'$

Digestion is accomplished by incubating the target DNA molecule with restriction enzyme that recognize and bind specific DNA sequences and cleave at specific nucleotides within the recognition sequence. These enzymes were selected based on NCBI molecular tool (<http://tools.neb.com/NEBcutter>). The reaction is incubated at a specific temperature required for optimal activity of the restriction enzyme and terminated by heat or inactivated by the use of loading dye. The result of a restriction digestion was evaluated by gel electrophoresis, in which the products of the digestion are separated by molecule length and the pattern formed is determined to distinguish the species.

3.2.4.2 RFLP protocol for 18S rRNA

Restriction enzyme	<i>SspI</i>
RE concentration	8 units
10 X Buffer	1.5 µl
Nuclease free water	9.1 µl
PCR product	4 µl

RFLP was performed in a 15µl reaction and incubated at 37° C for 12 hrs followed by inactivation at 65° C for 20 minutes to terminate the reaction of the enzyme.

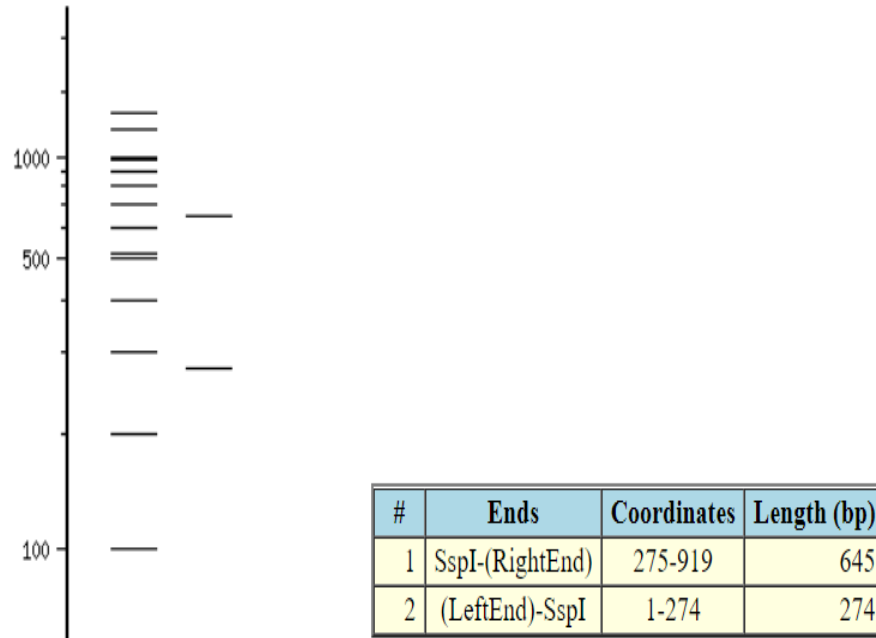
3.2.4.3 PCR-RFLP detection

To determine the *Sarcocystis* species, 7µl of the digested product was separated on 2% agarose gel in 0.5 x TAE buffer at 80 volts, 70 current for 45 minutes. The gels were visualised in gel documentation system (Omega fluor^{plus} Aplegen).

3.2.4.4 PCR-RFLP pattern of 18S rRNA of *Sarcocystis* spp. with *Ssp I* enzyme

After digestion of the PCR product of the 18S rRNA by the *SspI* enzyme, the DNA of 18S rRNA was fragmented into two as 650bp and 265bp in *S. meischeriana*. This enzyme cuts the total length of complete sequence of 18S rRNA of *S. meischeriana* at 5'... A A T ▼ A T T ... 3' and 3'... T T A ▼ T A A ... 5', whereas in *S. sui hominis* do not have any restriction site for the enzyme. The expected RFLP patterns were depicted in (Fig.1) (Vincze *et al.*, 2003).

Figure 1. PCR-RFLP pattern of 18S rRNA of *S. meischeriana* with *SspI* enzyme



3.3 Sequence analysis

The PCR products from samples of different districts of Andhra Pradesh were randomly selected and sequenced using forward and reverse primer to detect variations at the nucleotide level. The sequencing was performed on automated sequencer (ABI prism) using Sangers dideoxy chain termination method at Bioserve Biotechnologies Pvt. Ltd (Bengaluru). The electrophorograms were visualized using codon code aligner programme. The forwarded and reverse sequences were assembled using codon code aligner programme. The resultant sequences were verified using BLAST search (Altschul, 1990). The nucleotide differences between AP isolates (18S rRNA) and various isolates from other geographical locations were carried out by using Gene Doc 2.7 soft ware.

3.4 Phylogenetic analysis

Phylogenetic analysis was performed by using MEGA X (Molecular Evolutionary Genetic Analysis) software. The analysis included *Neospora caninum* as outgroup species to root the trees and phylogeny was tested with the bootstrap method using bootstrap replications. The previously published GenBank accession numbers used for the study was as follows KP732434.1 (Punjab, India); KP732435.1 (Punjab, India); KT736500.1 (U.P, India); KT736503.1 (U.P, India); MH404229.1 (Italy); KT736501.1 (Nainital, India); AF176936.2 (China); JX840465.1 (China); KC709516.1 (India); GU395554.1 (Iran); KP053894.1 (*S. gigantea*); GQ899206.1 (*Neospora caninum*); AF006471.1 (*S. hominis*). A total of 16 partial sequences of 18S rRNA gene were used in the analysis.

3.5 Statistical analysis

The prevalence data was statistically analysed with chi-square (χ^2) test to determine the factors associated with sarcocystosis in pigs.

Figure 2. Tissue samples from different predilection sites of slaughtered pig



Figure 3. Pepsin acid digestion on magnetic stirrer at room temperature



CHAPTER-IV

RESULTS

In the present study the prevalence, morphological and molecular characteristics of *Sarcocystis* species in pigs in coastal Andhra Pradesh was investigated. Tissue samples collected from 163 slaughtered pigs for a period of one year from January 2021 to December 2021 from certain districts of coastal Andhra Pradesh were examined by microscopy and polymerase chain reaction. The findings were documented under the appropriate headings.

4.1 Microscopic examination

4.1.1 Detection of sarcocysts by rapid intact cyst isolation

On examination of tissue samples collected from different predilection sites such as heart, tongue, oesophagus, intercostal and diaphragmatic pillar muscles from each of 163 slaughtered pigs by intact cyst isolation revealed sarcocysts (Fig. 4) in six pigs (3.68%). Sarcocysts were observed only in tissues of pigs collected from Krishna (6.81%), Guntur (7.40%) and East Godavari (9.09%) districts (Table 3).

4.1.2 Detection of *Sarcocystis* by pepsin acid digestion technique

Examination of same tissue samples from each of 163 slaughtered pigs by pepsin-hydrochloric acid digestion method revealed the presence of banana shaped bradyzoites (Fig. 5) in 20 (12.26%) pigs under light microscopic examination. Further staining of thin smears, prepared from the sediment of pepsin digestion technique by acridine orange manifested yellowish green illuminated bradyzoites (Fig. 6) under fluorescent microscope. Some of the tissue samples that were negative for sarcocysts in

intact cyst isolation method also revealed the presence of banana shaped bradyzoites. A total of 13 tissue samples were examined in Srikakulam district, out of which one (7.69%) was found to be positive for *Sarcocystis*, whereas in Visakhapatnam and Vijayanagaram out of 15 and 14 tissue samples, 6.66 (one) and 7.14 (one) per cent of samples respectively, were positive for *Sarcocystis*. Among 11 and 12 tissue samples screened from East Godavari and West Godavari, each one samples were found to be positive with a prevalence of 9.09 and 8.33 per cent, respectively. The prevalence was highest (22.7%) in Krishna district (10 out of 44). Out of 27, 13 and 14 tissue samples screened from Guntur, Prakasam and Nellore districts, three (11.11%), one (7.69%) and one (7.14%), respectively were found positive for *Sarcocystis* infection (Table 3).

4.1.3 Histopathological studies

Histomorphology of selective infected tissues collected from pigs revealed mild to severe infiltration of leucocytes in between the muscle fibres in tongue tissues (Fig. 7). In addition, some tissues also revealed cloudy swelling and focal leucocyte infiltration in between the muscle fibres (Fig. 8). Histological sections of heart tissues manifested myositis and hyaline degeneration (fish flesh like appearance) of muscle fibres (Fig. 9 a and b). Tongue tissue sections exhibited presence of schizont stages in blood vessels, hyaline degeneration and fatty degeneration surrounding the sarcocysts (Fig. 10 a, b and c). Mild to severe infiltration of inflammatory cells were also observed in perivascular tissues. The blood vessels of the tongue tissue revealed schizont and merozoite stages surrounded by mild oedema to severe fibrotic (scarring) changes (Fig. 11 a and b).

4.1.4 Morphometry of sarcocysts

Morphological characters of sarcocysts (shape, size and cyst wall) observed in intact cyst isolation method and histopathological sections were studied. The sarcocysts

were characterised by a definite cyst wall around the numerous bradyzoites which were separated by septa. In all the cysts, the cyst wall was thin and no thick-walled cysts could be identified in the present investigation. The length, width and thickness of cyst wall was (10 sarcocysts) 92.5 µm to 205µm, 30-60 µm and 0.2 to 0.4 µm, respectively. The morphological features of the cystic stage were suggestive of *S. suis*. However, due to inadequate projection of morphological features of the cystic stage, species level differentiation between *S. suis* and *S. miescheriana* cysts was made difficult.

4.2 Detection of *Sarcocystis* spp. by molecular methods

4.2.1 Yield and Quality of DNA

DNA was isolated from all tissue sediments (n=163) collected from pepsin-hydrochloric acid digestion method irrespective of the presence of bradyzoites. DNA was isolated from each pig sample separately (n=163) and was checked by agar gel electrophoresis (Plate 1). The yield of the DNA was quantified by the Biophotometer that was in the range of 132.70 – 1092.25 ng/ µl. The ratio of optical density at 260/280 of DNA for the above samples was ranging from 1.5 to 2.0.

4.2.2 Amplification of 18S rRNA gene by conventional PCR

The isolated DNA from all 163 pigs was separately used to amplify partial segment of 18S rRNA gene of *Sarcocystis* spp. with the primer sets of 2L (forward) and 3H (reverse) by setting different time-temperature combinations for annealing and extension processes. The PCR was optimised at an annealing temperature of 58 °C and extension of 72 °C. The size of the amplified products in 2% agarose gel electrophoresis revealed a single band of approximately 915 bp in positive samples (Plate 2, Table 3). Out of 163 samples tested, 34 samples were found to be positive for

Sarcocystis species. The oligonucleotide primers did not yield PCR product with negative control.

4.2.3 Differentiation of *Sarcocystis* species by PCR-RFLP

The PCR products (915bp) with respect to partial segment of 18S rRNA gene of *Sarcocystis* spp. were subjected for RFLP analysis using *SspI* restriction enzyme to differentiate the *Sarcocystis* species. RFLP analysis revealed two different patterns on electrophoresis, one with 915 bp (*S. suis*) and other with 650 bp and 265 bp (*S. miescheriana*) (Plate 3). Of the 34 positive pigs, 31 (87.4%) were found to be positive for *S. suis* infection, one was found to be positive for *S. miescheriana* and two pigs (12.6%) were positive for mixed species infection with *S. suis* and *S. miescheriana*. Of the 34 positive pigs, 31 (91.2%) were found to be positive for *S. suis* infection, one (2.9%) was found to be positive for *S. miescheriana* and two pigs (5.9%) were positive for mixed species infection with *S. suis* and *S. miescheriana* (Plate 3). Overall, the prevalence of *S. suis* and *S. miescheriana* was 20.24 (33/163) and 1.84 (3/163) per cent respectively, in coastal Andhra Pradesh.

4.3 Comparison of conventional methods with PCR for the diagnosis of *Sarcocystis* species

A total of 163 pigs tissue samples (heart, tongue, oesophagus, intercostal and diaphragmatic pillar muscles) were subjected to conventional and molecular diagnostic methods for detection of *Sarcocystis*. The conventional diagnostic methods such as intact cyst isolation and pepsin digestion methods revealed low prevalence rates i.e., 3.68 and 12.26 per cent respectively, compared to the high (20.85%) prevalence rate of molecular methods (Fig. 14), with a significant difference ($\chi^2=22.341$, $P=0.00$).

4.4 Breed wise, age wise, gender wise and district wise prevalence

The prevalence of *Sarcocystis* species was 15.83, 7.14, 20.0, 8.33, 18.18, 36.36, 22.22, 7.69 and 14.28 per cent in Srikakulam, Vijayanagaram, Visakhapatnam, West Godavari, East Godavari, Krishna, Guntur, Prakasam and Nellore districts of coastal Andhra Pradesh, respectively (Fig. 12, Table 3).

Prevalence with respect of breed, age and gender was assessed and the results were compiled in Table 2. The prevalence was high in Nondescriptive breeds (55.55%) (10/18) per cent compared to Large White Yorkshire (16.55%) with a significant difference ($\chi^2=14.756$, $P=0.00$). The total pigs examined were grouped into two categories according age, as young (\leq one year) and adult (\geq one year). The prevalence was relatively lower in young pigs (12.85%; 9/70) than in adults (26.88%; 25/93) with a significant difference ($\chi^2=4.759$, $P=0.029$). Similarly, the prevalence was higher in female pigs (26.3%; 15/57) than in male pigs (17.9%; 19/106) with out any significant difference ($\chi^2=1.589$, $P=0.209$) (Fig. 13).

4.5 Sequencing and Phylogenetic analysis

The direct sequencing of randomly selected PCR amplicons of 18S rRNA sequences was carried out in both directions with the identical forward and reverse primers used in PCR to determine the nucleotide level differences with the other sequences. A total of three samples were sequenced by Sanger sequencing method to analyze the species variation within the genus of *Sarcocystis* that infect the pigs. Based on the alignment of 18S rRNA sequences, a phylogenetic tree was built (Fig. 16). All of the sequenced samples in this study were from the *Sarcocystis* genus, and two of the 18S rRNA sequences were found to be homologous to *S. suis* and *S. miescheriana* was found in one sample.

The data generated by comparing the sequences of three isolates (two *S. sui hominis* and one *S. miescheriana*) with sequences of *S. sui hominis* and *S. miescheriana* from other geographical locations are presented as identity plots for nucleotides. Multiple sequence analysis was carried out using Clustal W program as implemented in MEGA X software. The data analysis involved 18S rRNA nucleotide sequences of two *S. sui hominis* and one *S. miescheriana* (Fig. 15). All positions containing gaps and missing data were eliminated. All the ambiguous positions were corrected for each sequence pair and were analyzed. Sequence similarity searches in BLAST revealed that the newly generated sequences (AP isolates) were 90-100% identical with the published sequences of *S. sui hominis*. Further, the nucleotide sequence of *S. miescheriana* revealed 90-100% identical with the published sequences.

The maximum parsimony approach with the subtree-pruning regrafting algorithm was used to create a bootstrap consensus tree utilising the sequences of the 18S rRNA gene. The sample no 59 isolate generated in the present study was clustered with the all Indian isolates of *S. sui hominis* (KP732434.1 Punjab; KT736500.1 U.P; KP732435.1 Punjab; KT736503.1 U.P; KT736501.1 Nainital) as single monophyletic group along with Italian isolate of *S. sui hominis* (MH404229). Whereas, the other isolate i.e., sample no. 56 (*S. sui hominis*) deviated from the Indian isolates but shared a same node. The sample number 20 isolate (*S. miescheriana*) manifested as a completely outward group neither clustered with *S. miescheriana* isolates of Indian origin nor other country populations.

Table 2. Breed wise, age wise and gender wise prevalence of sarcocystosis in pigs

DISTRICT	BREED			AGE				GENDER				
	LWY	Positive	ND	Positive	≤1	Positive	≥1	Positive	MALE	Positive	FEMALE	Positive
Krishna	37	11(29.7)	7	5(71.4)	15	5(33.3)	29	11(37.93)	31	9(29)	13	7(53.8)
Guntur	22	3(13.6)	5	3(60)	12	1(8.33)	15	5(33.3)	19	4(21)	8	2(25)
East Godavari	9	1(11.1)	2	1(50)	5	1(20)	7	1(14.2)	7	1(14.2)	4	1(25)
West Godavari	8	-	4	1(25)	5	-	6	1(16.6)	6	1(16.6)	6	-
Nellore	14	2(14.2)	-	-	7	-	7	2(28.57)	9	-	5	2(40)
Prakasam	13	1(7.69)	-	-	5	-	8	1(12.5)	7	1(14.2)	6	-
Srikakulam	13	2(15.38)	-	-	6	-	7	2(28.5)	9	2(22.2)	4	-
Vishakapatnam	15	3(20)	-	-	7	2(28.57)	8	1(12.5)	10	1(10)	5	2(40)
Vizianagaram	14	1(7.4)	-	-	8	-	6	1(16.6)	8	-	6	1(16.6)
TOTAL	145	24(16.55)	18	10(55.55)	70	9(12.85)	93	25(26.88)	106	19(17.9)	57	15(26.3)

LWY- Large White Yorkshire; ND- Non Descriptive. Figures in the parenthesis indicate percent

Table 3. Detection of *Sarcocystis* by conventional (muscle squash and pepsin digestion) and molecular methods

DISTRICT	No. of samples	Muscle Squash		Pepsin Digestion		PCR		Overall prevalence (%)
		Positive	Prevalence (%)	Positive	Prevalence (%)	Positive	Prevalence (%)	
Krishna	44	3	6.81	10	22.7	16	36.36	36.36
Guntur	27	2	7.40	3	11.11	6	22.22	22.22
East Godavari	11	1	9.09	1	9.09	2	18.18	18.18
West Godavari	12	-	-	1	8.33	1	8.33	8.33
Nellore	14	-	-	1	7.14	2	14.28	14.28
Prakasam	13	-	-	1	7.69	1	7.69	7.69
Srikakulam	13	-	-	1	7.69	2	15.83	15.83
Vishakapatnam	15	-	-	1	6.66	3	20	20
Vizianagaram	14	-	-	1	7.14	1	7.14	7.14
TOTAL	163	6	3.68	20	12.26	34	20.85	20.85

Figure 4. Sarcocysts in muscle tissue

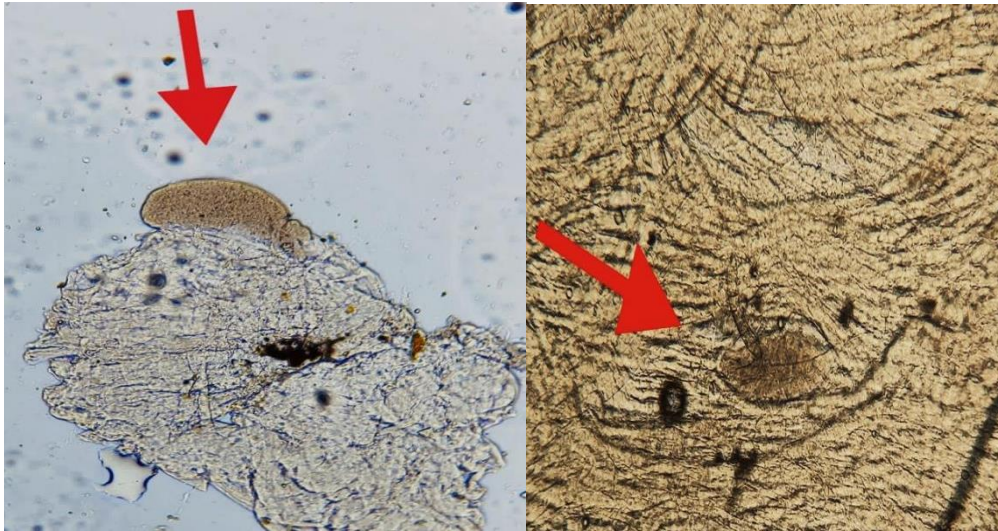


Figure 5. Bradyzoites of sarcocysts unstained (400x)

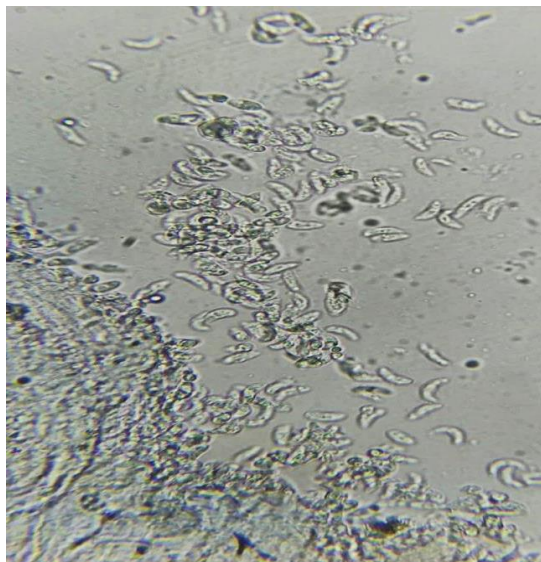


Figure 6. Acridine orange stained bradyzoites (a:100x; b:200x and c:400x)

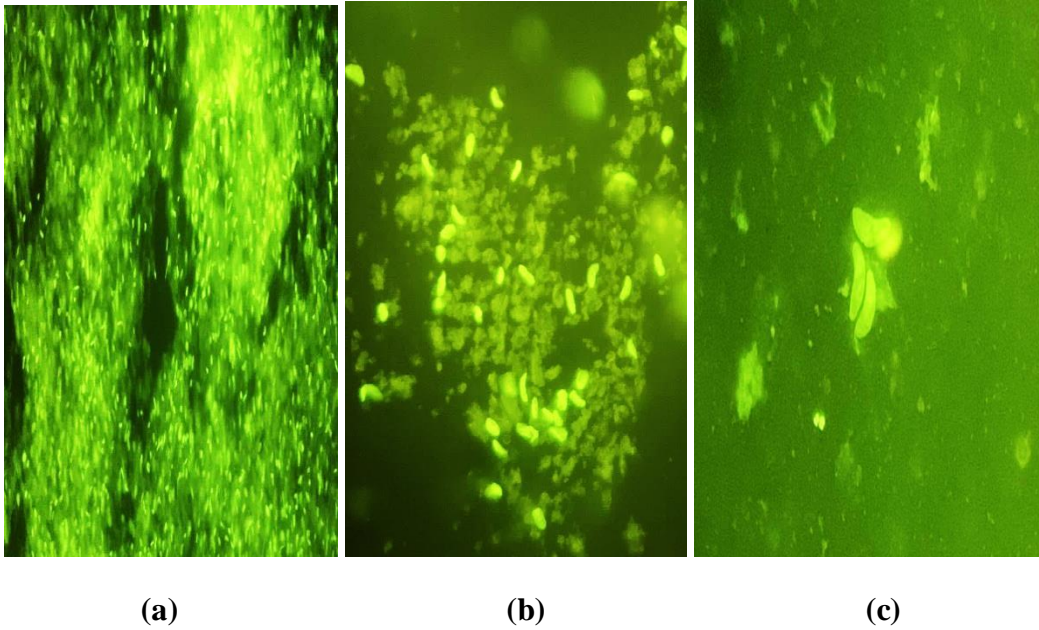


Figure 7. Mild leucocyte infiltration in between the muscle fibres and degenerative changes in muscle fibres (Cloudy Swelling) in tongue muscle (400x)

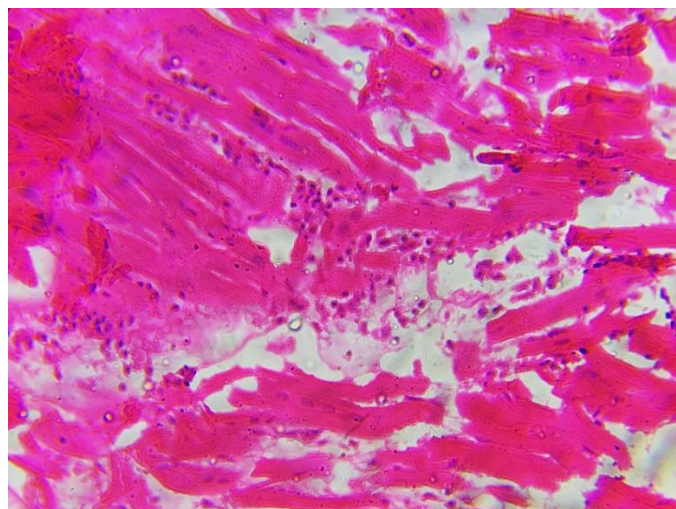


Figure 8. Focal leucocytic infiltration in between the muscle fibres in tongue (400x)

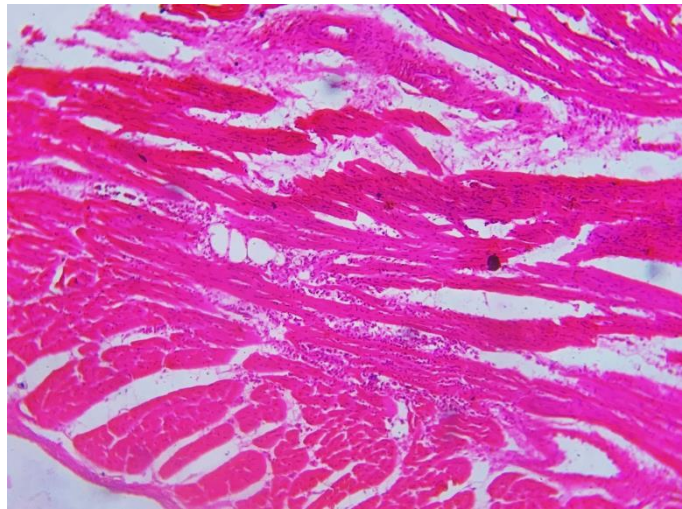
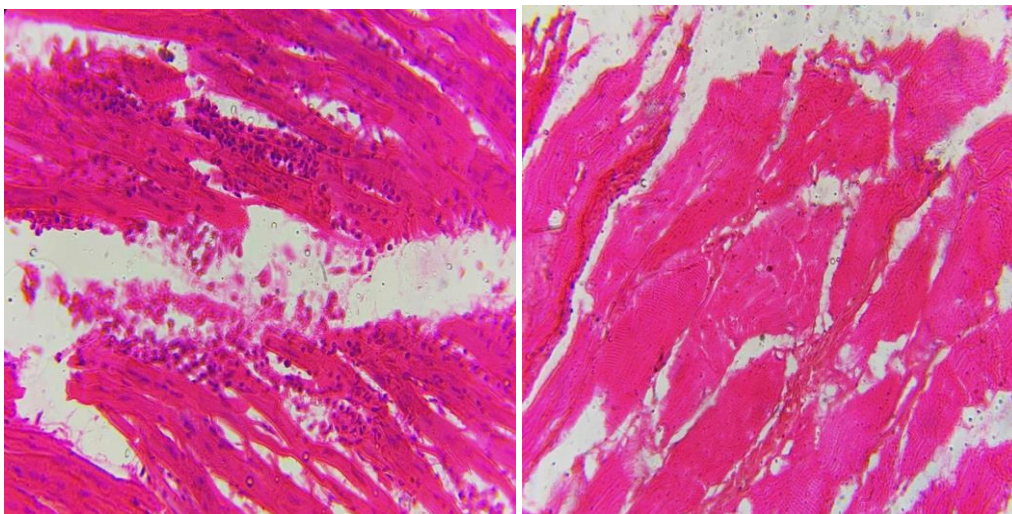


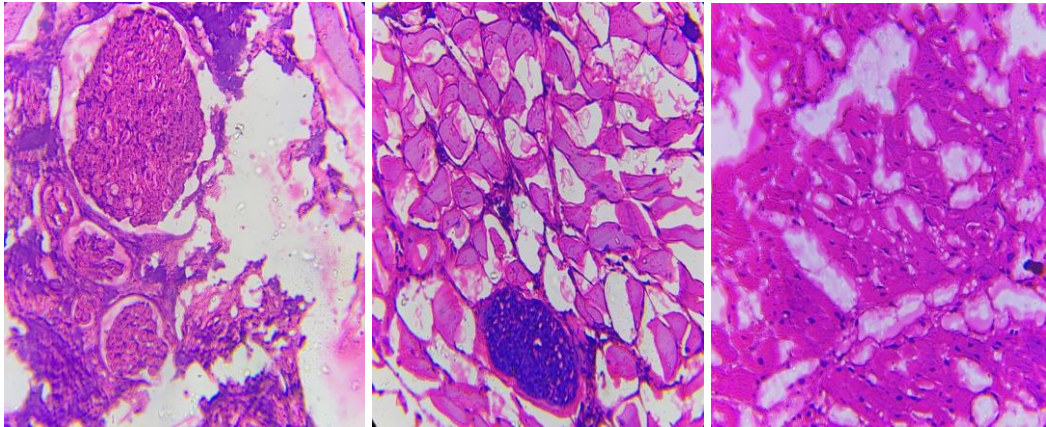
Figure 9. Myositis in heart muscle (a); Hyaline degeneration of tongue muscle (Fish flesh like appearance) (b) (400x in H and E)



(a)

(b)

Figure 10. Schizont stages of *Sarcocystis* in the blood vessel (a); Hyaline degeneration surrounding the sarcocyst in tongue (400x) (b); Fatty degeneration in tongue muscle (400x) (c)

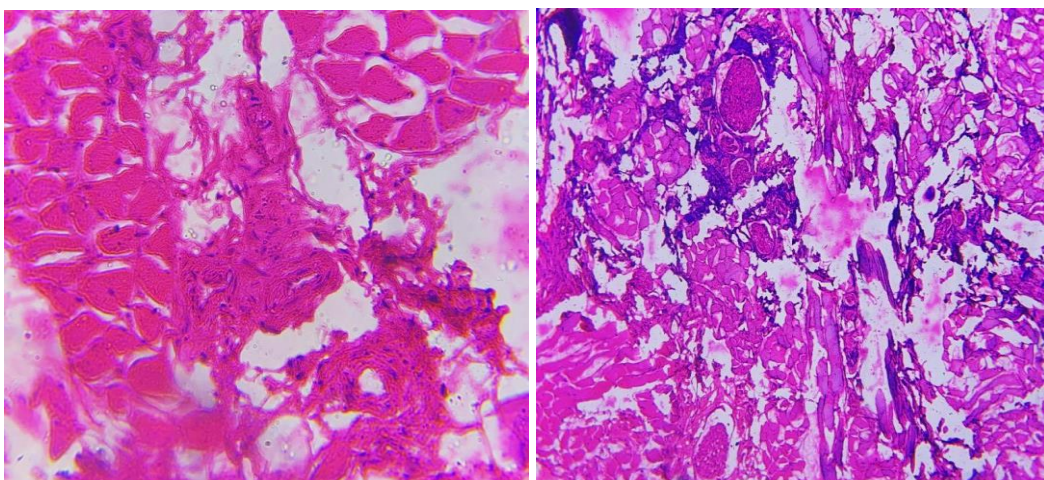


(a)

(b)

(c)

Figure 11. Mild leucocyte infiltration in perivascular areas (a); Severe fibrosis surrounding the blood vessels with schizonts in tongue (100x) (b)



(a)

(b)

Figure 12. Prevalence status of *Sarcocystis* infection in coastal Andhra Pradesh

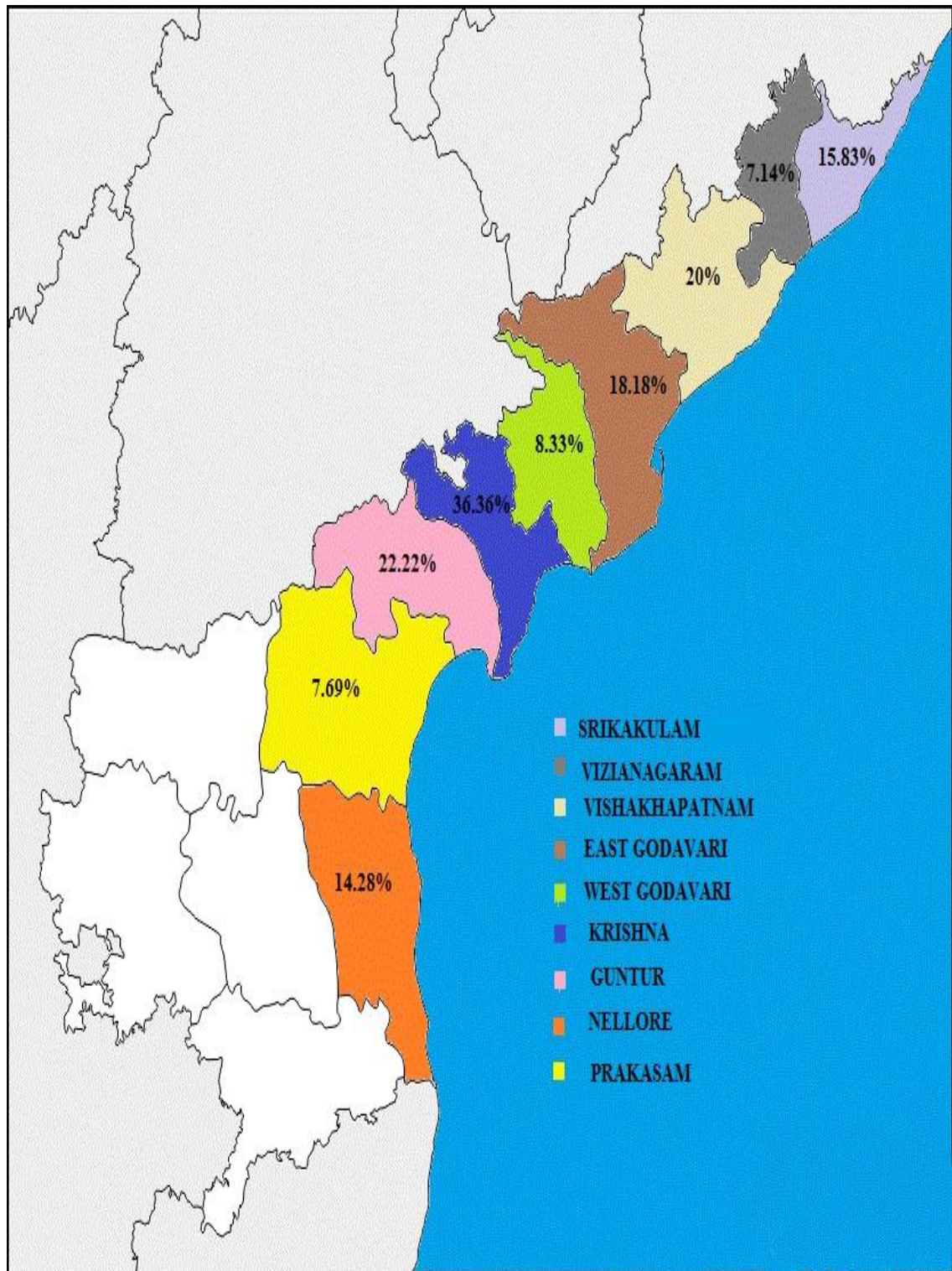


Figure 13. Breed wise, age wise and gender wise prevalence of *Sarcocystis* infection in pigs

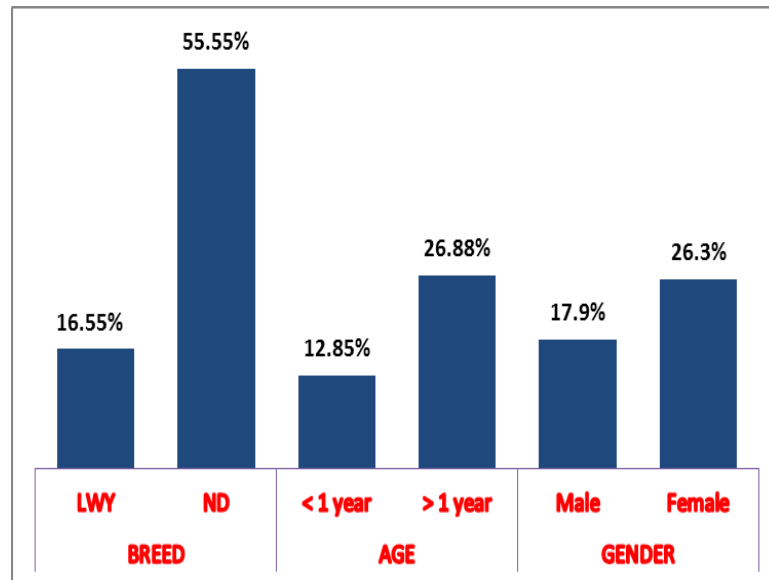


Figure 14. Prevalence of *Sarcocystis* infection in pigs in comparison to conventional and molecular methods

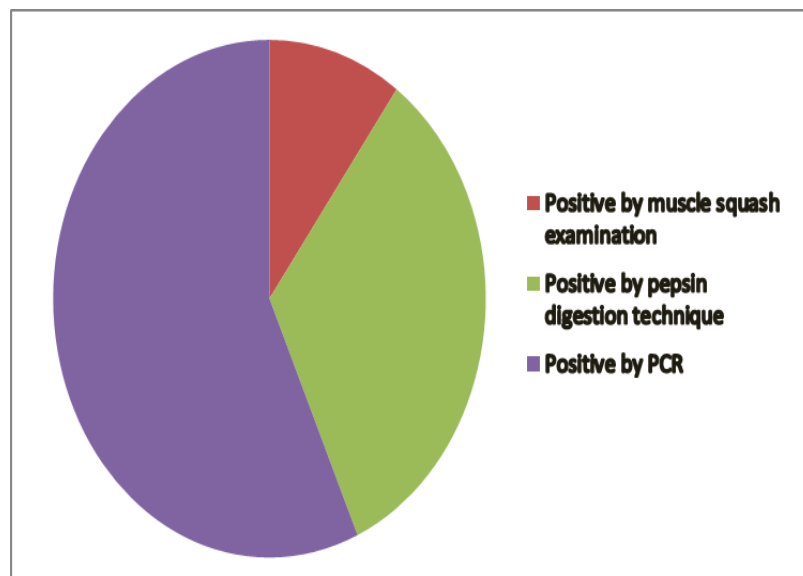


Figure 15. *Sarcocystis sui*hominis (56) and *S. miesheriana* (20) sequences used for the phylogenetic analysis

>Sample_56 (forward and reverse)

TGACAGTTAATACGAATGCCCCCAACTGTCCCTATTAATCATTATTTTCAGTC
 CTAAAAAAAAACCAACAAAATAGAACCGAAATCCTATATTGTTATTCCATGC
 TGCAGTATTCATAGGCAACAAAAGCCTGCTTCAAACACTCTAATTTTCTCAA
 AGTAACATTTCTCCATACTACCAATAATATAGTAGACGCATTATTAGAAAT
 GAATCTAATAATGATCATCTTATATATTGATAATAGGGAGACAATTATGCCG
 GAATTCATCATATTTCCACCCTAATCAAATAGGGCGGACTGATGATTGCTTC
 CAACAGACATCCAACACTACGAGCTTTTTAACTGCAACAACCTTAAATATACGCT
 ATTGGAGCTGGAATTACCGCGGCTGCTGGCACCAGACTTGCCCTCCAATTGT
 TACTCTGAAAGGGGTTTGGATTCCCATCACTCCAATCACTAGAAATCAAAT
 WTCCAGTGTTGTTATTTCTTGTCACTACCTCCCTGAGTCAGGATTGGGTAAT
 TTGCGCGCCTGCTGCCTTCTTAGATGTGGTAGCCGTTTCTCAGGCTCCCTCT
 CCGGAATCGAACCCCTAATTTCCCGTTACCCGCTCACTGCCACGGTAGTCCAAT
 AACTACCGTCGAAAGCTGATAGGTCAGAAACTTGAATGATCTATCGCCAA
 TCATCAAATAATGATAATTATAATGCGATCCGTTTCGGTTACTATGAATCACC
 TTATTTTACATCACTATCATCATAGAATGATAATAATGATGTGTTGGTTCTGT
 ATCTAATAAACACTACTATATCACCACCCTCCCATAAAGA

>Sample_20(forward and reverse)

TTCACCTCTGACAGTTAAATACGAATGCCCCCAACTGTCCCTATTAATCATT
 ATTTTCAGTCCTAAAAAAAAACCAACAAAATAGAACCGAAATCCTATATTGTT
 ATTCCATGCTGCAGTATTCAAGGCAACAAAAGCCTGCTTCAAACACTCTAAT
 TTTCTCAAAGTAACATTTCTCCATACTACCAATAATATAGTAGACGCATTA
 TTAGAAATGAATCTAATAATGATCATCTTATATATTGATAATAGGGAGACA
 ATTATGCCGGAATTCATCATATTCACACCCTAATCAAATAGGGCGGACTGAT
 GATTGCTTCCAACAGACATCCAACACTACGAGCTTTTTAACTGCAACAACCTTAA
 ATATACGCTATTGGAGCTGGAATTACCGCGGCTGCTGGCACCAGACTTGCCC
 TCCAATTGTTACTCTGAAAGGGGTTGGATTCCCATCATTCCAATCACTAGA
 AATCAAATTTCCAGTGTTGTTATTTCTTGTCACTACCTCCCTGAGTCAGGATG
 GCGGAATTTGCGCGCCTGCTGCCTTCTTATATGTGGGGGCGGCTCTCAAG
 CTCCTCTCCGTAATAGAACCCCTTATCCGCCACTAGCCGGTGCCGTGGT
 AGGGTCATCTCACTACAATGCCGCCGAGATATGATAGGCACTATGCTGGA
 AAGGTCTTCTCTCAGAAAAAAAAAAGAGTGATGACGAGGCGATCCGTTG
 GGATGAATACAGCAGCCTGAATTAACATC

Figure 16. Phylogenetic analysis of A.P isolates based on partial 18S rRNA sequence of *Sarcocystis* species with representative sequences received from GenBank-NCBI and *Neospora caninum* was used as outgroup

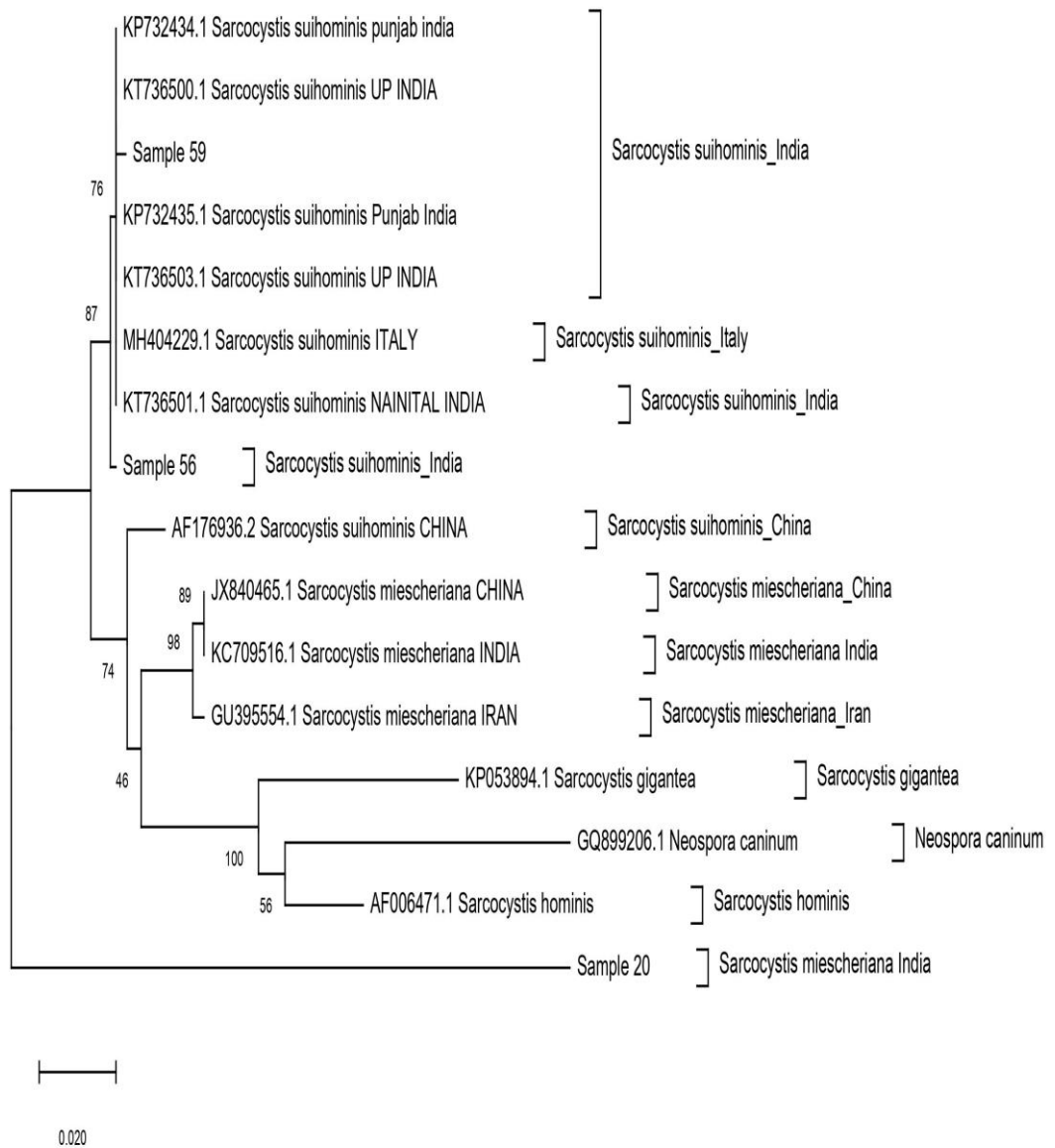


PLATE 1: Agarose gel showing quality of genomic DNA

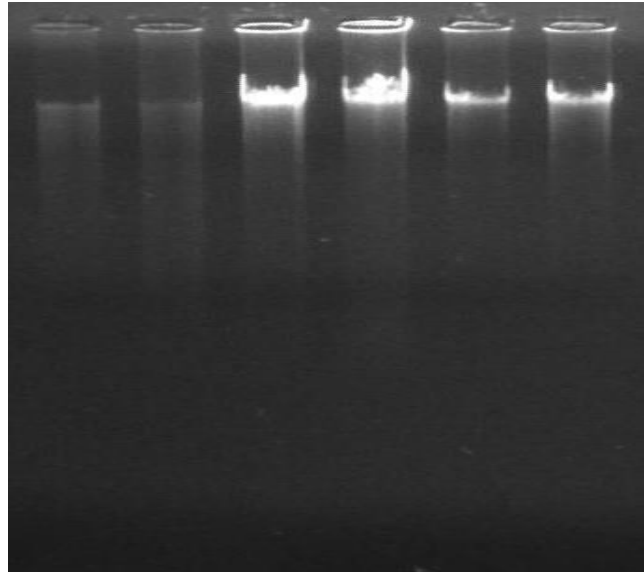
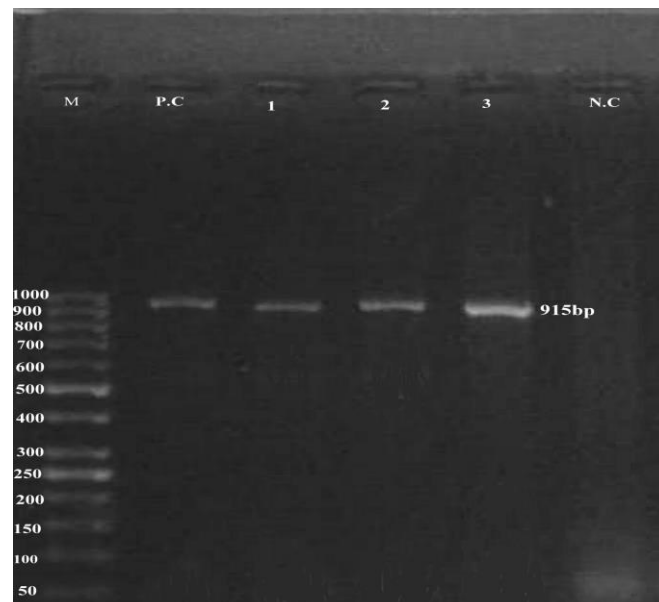
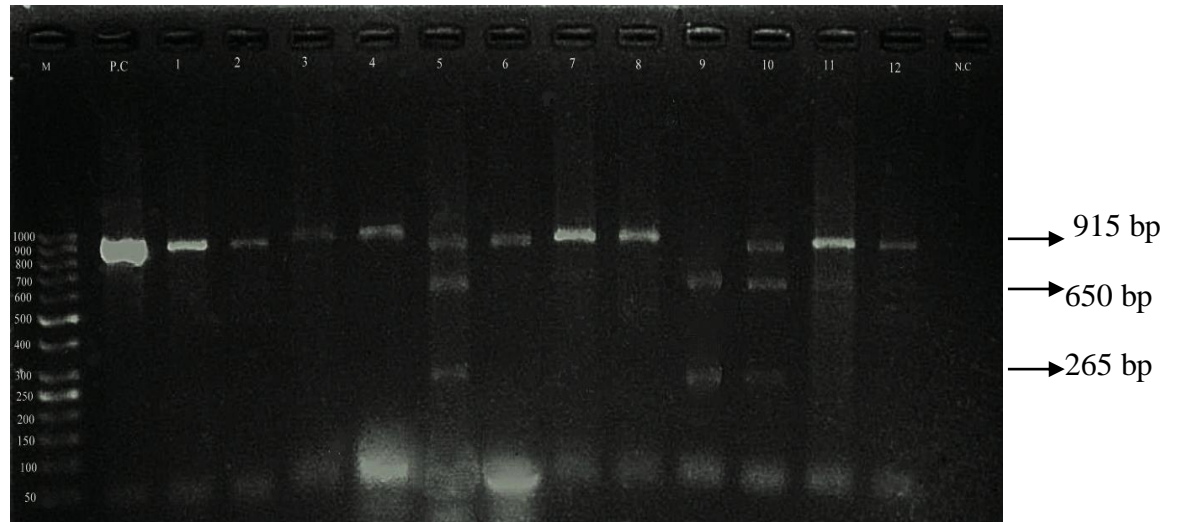


PLATE 2: PCR amplification product of 18S rRNA of *Sarcocystis* spp. in pigs



M: 50bp ladder; P: Positive control; N: Negative control; Lane 1-3: *Sarcocystis* positive samples

PLATE 3: 18S rRNA/*SspI* pattern of sarcocysts from pigs showing *S. miescheriana* and *S. suihominis*



M: 50bp ladder, P: Positive control (PCR product without restriction enzyme), N: Negative control, Lane 1-4, 6-8, 11-12: *S. suihominis* (915bp); Lane 9: *S. miescheriana* (650bp, 265bp); Lane 5 and 10: Mixed infection

CHAPTER-V

DISCUSSION

In India, pigs are an important livestock species because they are raised by people from lower socio-economic backgrounds. As compared to other livestock species, they have a greater potential to contribute to a faster economic return to farmers due to high fecundity, higher feed conversion efficiency, early maturity, and a short generation gap. In Andhra Pradesh, pigs are typically raised in semi-intensive and free-range environments on domestic garbage, farm refuse and night soil, which make them more prone for risk of infections. Particularly, parasite infections are most common in pigs because of their coprophagic tendencies. The existence of stray pigs and a lack of system for waste disposal make the situation even worsen (NAP on pig, 2019).

Sarcocystis is a tissue protozoan parasite that involves carnivores as definitive hosts and domestic animals as intermediate host in which sarcocysts develop. Infection of intermediate occurs through food and water contamination and diagnosis of sarcocystosis based on clinical signs is highly difficult. Moreover, sarcocysts of different species have similar morphological characters making it hard to differentiate species. Domestic pigs, as intermediate hosts, can harbour tissue cysts of three *Sarcocystis* species namely *S. miescheriana*, *S. suihominis* and *S. porcifelis*. Out of three, *S. suihominis* is a serious food-borne zoonosis, second only to *Cysticercus cellulosae* infection, both of which are transmitted by the consumption of raw or undercooked pig products (Banerjee *et al.*, 1994). *Sarcocystis* infection is common in pigs all around the world, including India (Dubey *et al.*, 1989; Saleque and Bhatia, 1991; Omata *et al.*, 1993; Prasanth and Bhatia 1996; Claveria *et al.*, 2001; Avapal *et al.*, 2002). Hence, the present work was designed to study the prevalence rate of *Sarcocystis*

species and its molecular characterization based on 18S rRNA sequence in pigs in coastal districts of Andhra Pradesh.

5.1 Microscopic examination

5.1.1 Histopathological studies

Histopathology of sarcocysts infected tissues revealed mild to severe infiltration of leucocytes in between the muscle fibres in tongue tissues along with cloudy swelling and focal leucocyte infiltration in between the muscle fibres. Heart tissue sections manifested myositis and hyaline degeneration. The histomorphological alterations were in accordance with the descriptions of Barrows *et al.* (1982), Avapal *et al.* (2004), Yan *et al.* (2013), Calero-Bernal *et al.* (2015) and Chauhan *et al.* (2020).

5.1.2 Morphometry

Proper identification and differentiation of various *Sarcocystis* species is necessary for thorough understanding of parasite epidemiology and systematics. Each *Sarcocystis* species cyst wall has unique ultra-structural characteristics which cannot be distinguished under light microscopy. A total of 37 distinct type of sarcocyst walls have been reported (Markus *et al.*, 2004). The sarcocysts can change their appearance in the host musculature depending on their age and location (Frenkel *et al.*, 1979; Yan *et al.*, 2013). So, the conventional method of distinguishing different *Sarcocystis* spp. by observing the structure of the cyst wall under light microscope are no longer appropriate for routine diagnosis of *Sarcocystis* species with little morphological variants or host range investigations. Sometimes mixed infections may also be encountered in single animal (Dubey *et al.*, 2016). Furthermore, natural variation within the *Sarcocystis* species may lead to the erroneous classification of new taxa that closely resemble recognised species and their inclusion as new species (Yang *et al.*, 2002).

Therefore, PCR assays and sequencing of potential genetic markers have been adjudged more pragmatic, precise, authentic, and reliable methods for delineation of coccidian infection, including *Sarcocystis*, than traditional methods based on morphology (Gjerde, 2013). The 18S rRNA gene have been identified as a good genetic marker for distinguishing species of *Sarcocystis* (Fischer and Odening, 1998; Yang *et al.*, 2000) and has recently been widely used in domestic pigs, wild boars, cattle, buffaloes, camels, and cervids to identify the *Sarcocystis* spp. (Yang *et al.*, 2001; Caspari *et al.*, 2011; Kia *et al.*, 2011; Motamedi *et al.*, 2011; Gjerde, 2012), besides that it also useful for determining genetic variations between isolates (More *et al.*, 2013; Yan *et al.*, 2013). To distinguish *Sarcocystis* spp., the PCR-RFLP approach provides a simple, quick, suitable, and cost-effective method compared to sequencing.

5.2 PCR-RFLP

All the samples that were processed by pepsin-HCl digestion method were analyzed in PCR-RFLP. The partial 18S rRNA sequence was amplified to determine the species. The amplification resulted into approximately 915 bp specific for the *Sarcocystis* spp. The results were in agreement with the findings of Calero-Bernal *et al.* (2015); Kaur *et al.* (2015); Imre *et al.* (2017) and Chauhan *et al.* (2020) who amplified the 18S rRNA by PCR and got the final product length 915 bp.

In the current investigation, the predicted PCR-RFLP pattern of partial 18S rRNA sequence (915bp) with *SspI* restriction enzyme using *NEB cutter V2.0* was 650bp and 265bp in *S. meischeriana*, where as in *S. sui hominis* the enzyme did not have any restriction site (Vincze *et al.*, 2003). Calero-Bernal *et al.* (2015) and Imre *et al.* (2017) analyzed the 18S rRNA sequence and proposed that the PCR-RFLP with *SspI*

restriction enzyme could be helpful for the molecular taxonomic identification of *Sarcocystis* spp.

RFLP analysis on 18S rRNA gene of 34 sarcocystosis infected pigs revealed presence of two species of *Sarcocystis* namely, *S. suis* and *S. miescheriana* in coastal Andhra Pradesh. Out of 34 samples, 31 pigs were positive for *S. suis*, two were with mixed infection of *S. suis* and *S. miescheriana* and one was positive for *S. miescheriana*. Results revealed predominance of *S. suis*, a zoonotic species. Contrary, Imre *et al.* (2017) identified only *S. miescheriana* in feral pigs in USA and pathogenic species *S. suis* could not be identified.

5.3 Prevalence of *Sarcocystis* spp. by different diagnostic methods

Examination of tissue samples from 163 slaughtered pigs to detect the *Sarcocystis* in coastal districts of Andhra Pradesh by intact cyst isolation, pepsin digestion method and molecular methods revealed 3.68, 12.26 and 20.85 per cent prevalence of sarcocystosis, respectively with a significant difference ($\chi^2=22.341$, $P=0.00$). The previous studies also reported higher rate of prevalence by molecular method (76.4%) compared to intact cyst isolation method (58.4%) and pepsin digestion method (67.6%) and stated that the prevalence estimates are strongly influenced by methodology (Calero-Bernal *et al.*, 2015; Kaur *et al.*, 2015).

Over all 34 (20.85%) pigs were found to be positive for sarcocystosis. The current prevalence rate was comparatively low with the previous reports as 53.5 per cent of *S. miescheriana* in Bihar (Sahi *et al.*, 1982), 68.8 per cent at Hisar (Gupta and Gautam 1984), 68.98 per cent in western Uttar Pradesh (Agnihotri *et al.*, 1987; Saleque and Bhatia 1991), 84 per cent in Madhya Pradesh (Solanki *et al.*, 1991), 76.1 per cent in Guwahati, Assam (Devi *et al.*, 1998), 61.48 per cent in Andhra Pradesh

(Srinivasa Rao and Hafeez 2002a), 73.36 per cent in Ludhiana (Avapal *et al.*, 2003a, 2004), *S. suis* (48.9 %) and *S. miescheriana* (40.2 %) in Western Uttar Pradesh (Prasanth, 1995), 72.8 per cent in Punjab (Kaur *et al.*, 2015). The difference in rate of infection with *Sarcocystis* spp. might be related to the hygiene and management measures used in pig farming in the research area (Agnihotri *et al.*, 1987; Saleque and Bhatia, 1991; Solanki *et al.*, 1991; Prasanth 1995; Avapal *et al.*, 2004; Kaur *et al.*, 2016). In addition, factors such as study design, sampling strategy, detection method, and geographical circumstances of the investigated locations also influence the prevalence rate (Imre *et al.*, 2017).

5.4 Breed wise, age wise, gender wise and district wise prevalence

Age wise, highest prevalence was recorded in adult pigs (\geq one year) (26.88%) compared to young animals (\leq one year) (12.85%) with a significant difference ($\chi^2=4.759$, $P=0.029$) that might be due to frequent exposures adult animals harbour heavy parasitic loads. The study results were in agreement with the earlier findings (Hinaidy and Supperer, 1979; Barrows *et al.*, 1981; Avapal *et al.*, 2003; Calero-Bernal *et al.*, 2015; Coelho *et al.*, 2015). *Sarcocystis* in relation to gender was highest in female pigs (26.3%) than in the male pigs (17.9%) with out any significance ($\chi^2=1.589$, $P=0.209$). Possibly the difference in the prevalence rates might be due to regular gestational stress in females that lowers immunity makes them more susceptible for infections (Calero-Bernal *et al.*, 2015).

Breed wise non descriptive breed revealed highest prevalence rate (55.55%) compared to Large White Yorkshire (LWY) (16.55%) with a significant difference ($\chi^2=14.756$, $P=0.00$) which was contradictory to the findings of Calero-Bernal *et al.* (2015) and Imre *et al.* (2017). Naturally, the occurrence of *Sarcocystis* infections in pigs are frequently undetectable, owing to natural immunization from low-dose infections,

which are more common in free-ranging pigs. The prevalence of individual species, 20.24 per cent of *S. suihominis*, 1.84 per cent of *S. meischeriana* and 1.22 per cent of mixed infection revealed that *S. suihominis* was predominant. Similar observations were recorded in Switzerland (Caspari *et al.*, 2011), China (Yan *et al.*, 2013) and India (Kaur *et al.*, 2015). This could be related to pigs being reared in backyards with access to scavenging human excrement in the slums, as well as unhygienic slaughter, handling, and ingestion of pork by slum inhabitants, as reported by Solanki *et al.* (1991), Saleque and Bhatia (1991), Prasanth (1995), Shah (1995) and Kaur and Bal (2016). Occurrence of mixed infections might be due to consumption of contaminated feed and water with excreta of humans and carnivore species which acts as definitive hosts for *S. suihominis* and *S. meischeriana*, respectively.

Regarding the district wise, the study manifested highest prevalence in Krishna (36.36%) followed by Guntur (22.22%), Visakhapatnam (20.0%), East Godavari (18.18%), Srikakulam (15.83%), Nellore (14.28%), West Godavari (8.33%), Prakasam (7.69%) and Vijayanagaram (7.14%). High prevalence of infection in Krishna indicates poor management of pigs.

5.5 Phylogeny

The small subunit ribosomal RNA (18S rRNA) gene has been widely used to distinguish between apicomplexans and other eukaryotic species in recent years due to its abundance in the genome and the availability of hypervariable regions interspersed within highly conserved DNA (Neefs *et al.*, 1991; Jeffries *et al.*, 1997; Fischer and Odening 1998). Is also utilized solely for molecular identification and phylogenetic study of several *Sarcocystis* spp. that infect animals (Hu *et al.*, 2016). However, there is a scarcity of genetic information on *Sarcocystis* spp. infecting pigs in India,

necessitating comprehensive research to better understand the genus evolution and genetic diversity (Chauhan *et al.*, 2020). Phylogenetic studies will ascertain evolutionary relatedness of our isolates with the other geographical isolates within the genus *Sarcocystis* and other members of the family sarcocystidae.

The phylogenetic analysis on three sequences from different regions of coastal districts of Andhra Pradesh based on 18S rRNA gene revealed two sequences (59 and 56 samples) were homologous with *S. sui hominis* with low genetic variability and one with *S. meischeriana* (20 sample). *Sarcocystis sui hominis* samples clustered with Indian isolates of *S. sui hominis* isolates (KP732434.1 Punjab; KT736500.1 U.P; KP732435.1 Punjab; KT736503.1 U.P; KT736501.1 Nainital) together with Italian isolate of *S. sui hominis* (MH404229). *Sarcocystis gigantea* (KP053894.1) and *S. hominis* (AF006471.1) of same genus constituted a separate sub branch. For the analysis *Neospora caninum* (GQ899206.1) was also clustered as a separate sub branch. The present phylogenetic analysis of 18S rRNA gene sequences of *S. sui hominis* were completely in agreement with the previous reports of Chauhan *et al.* (2020).

In the present analysis *S. meischeriana* was found to be an out group. Two species within the same genus are likely to share a recent common ancestor. Hence, no concrete statement can be made with respect to the evolutionary status of *S. meischeriana* in the present study. Construction of phylogenetic tree using sequence derived by cloning will lead to a reliable result.

The present study concludes that as the zoonotic significance of *S. sui hominis*, comprehensive epidemiological scrutiny and constant surveillance are required. In addition, disruption of the life cycle is foremost thing through preventing eating of raw flesh from slaughtered/dead animals (Bhatia *et al.*, 2010) by definitive hosts simultaneously taking care of contamination of pasture by their faeces.

CHAPTER- VI

SUMMARY

Considering the zoonotic importance of *Sarcocystis* species, the present study was carried out to determine the prevalence and morphological and molecular characteristics of *Sarcocystis* species in pigs in coastal Andhra Pradesh by microscopy and polymerase chain reaction using tissue samples collected from 163 slaughtered pigs for a period of one year from January 2021 to December 2021. Demographic data regarding age, sex, breed and area of the animals were noted.

Microscopic examination of tissue samples collected from different predilection sites such as heart, tongue, oesophagus, intercostal and diaphragmatic pillar muscles from each of 163 slaughtered pigs by intact cyst isolation and pepsin digestion method revealed sarcocysts in six (3.68%) and 20 (12.26%) pigs samples, respectively. Some of the tissue samples that were negative for sarcocysts in intact cyst isolation method also revealed the presence of banana shaped bradyzoites.

Tissue samples positive for sarcocysts were fixed in 10% formalin for histopathological examination. Sarcocysts were microscopic with a smooth and thin cyst wall and were filled with banana-shaped bradyzoites. Cysts of different sizes were observed. Pathological changes like mild leucocytic infiltration, myositis and hyaline degeneration along with fatty degeneration were observed in muscle tissues. Changes like mild to severe infiltration of inflammatory cells were also observed in perivascular tissues. The tongue tissue also revealed schizont and merozoite stages surrounded by mild edema to severe fibrotic changes. Morphometric analysis of sarcocysts (shape, size and cyst wall) in histopathological sections revealed only thin walled cysts with a length, width and

thickness of 92.5 µm to 205µm, 30-60 µm and 0.2 to 0.4 µm, respectively. No thick walled cysts were identified. The morphological features of the cystic stage were suggestive of *S. suihominis*.

DNA was isolated from all tissue sediments (n=163) collected from pepsin-hydrochloric acid digestion method irrespective of the presence of bradyzoites. DNA was isolated from each pig sample separately and was separately used to amplify partial segment of 18S rRNA gene of *Sarcocystis* spp. with the estimated amplicon size of 915 bp. Out of 163 samples tested, 34 (20.85 %) samples were found to be positive for *Sarcocystis* species. The oligonucleotide primers did not yield PCR product with negative control. The PCR was more sensitive than the conventional methods in detection of sarcocystosis with a significant difference ($\chi^2=22.341$, $P=0.00$). Subsequent digestion of PCR amplicons with *SspI*, two different patterns were visualized on electrophoresis, one with 915 bp (*S. suihominis*) and other with 650 bp and 265 bp (*S. miescheriana*). Of the 34 positive pigs, 31 (91.2%) were found to be positive for *S. suihominis* infection, one (2.9%) was found to be positive for *S. miescheriana* and two pigs (5.9%) were positive for mixed species infection with *S. suihominis* and *S. miescheriana*. Overall, the prevalence of *S. suihominis* and *S. miescheriana* was 20.24 (33/163) and 1.84 (3/163) per cent respectively, in costal Andhra Pradesh.

Randomly selected PCR amplicons of 18S rRNA were sequenced. The sequencing results after nucleotide sequencing were aligned with other *Sarcocystis* sequences deposited in the GenBank database, using NCBI BLAST software, which revealed that the 915 bp long sequences of *S. suihominis* obtained from pigs demonstrated 90-100% identity with the Indian isolates of *S. suihominis* (KP732434.1 Punjab, KT736500.1 U.P, KP732435.1

Punjab, KT736503.1 U.P, KT736501.1 Nainital) as single monophyletic group along with Italian isolate of *S. suihominis* (MH404229).

Overall, the prevalence of sarcocystosis was high in Non descriptive breeds (55.55%) compared to Large White Yorkshire (16.55%) with a significant difference ($\chi^2=14.756$, $P=0.00$). The total pigs examined were grouped into two categories according age, as young (\leq one year) and adult (\geq one year). The prevalence was relatively lower in young pigs (12.85%; 9/70) than in adults (26.88%; 25/93) with a significant difference ($\chi^2=4.759$, $P=0.029$). Similarly, the prevalence was higher in female pigs (26.3%; 15/57) than in male pigs (17.9%; 19/106) without any significant difference ($\chi^2=1.589$, $P=0.209$). The prevalence was high in Krishna (36.36%) followed by Guntur (22.22%), Visakhapatnam (20.0%), East Godavari (18.18%), Srikakulam (15.83%), Nellore (14.28%), West Godavari, (8.33%), Prakasam (7.69%) and Vijayanagaram (7.14%) without any significant difference.

Conclusions

Prevalence of *Sarcocystis* species in pigs from coastal Andhra Pradesh was found to be 3.68, 12.26 and 20.85 per cent by intact cyst isolation method, pepsin digestion method and conventional PCR, respectively indicating higher sensitivity of the molecular assay.

Age and breed influenced the prevalence of *Sarcocystis* infection being more prevalent in Non descriptive pigs ($P<0.01$) and adults ($P<0.05$). No significant difference was observed between the prevalence of male and female pigs.

Sarcocystis suihominis (20.24 %) was more prevalent than *S. miescheriana* (1.84 %) in coastal Andhra Pradesh possessing public health risk. Sarcocystosis is prevalent in pigs slaughtered for human consumption in Andhra Pradesh. Hence, it is imperative to

ensure that meat is well-cooked to prevent possible infection of the definitive hosts, human beings.

CHAPTER- VII

LITERATURE CITED

- Abdullah S H 2021 Investigation of *Sarcocystis* spp. in slaughtered cattle and sheep by peptic digestion and histological examination in Sulaimani Province, Iraq. *Veterinary World*, 14(2), 468.
- Agnihotri R K, Juyal P D and Bhatia B B 1987 Microsarcocysts in pigs in UttarPradesh, India. *J. Veterinary Parasitology*, 1: 69–70.
- Alina, Tabaran A, Sorin Daniel D A N, Reget O, Cordis I, Cordea D and Mihaiu M 2017 Molecular Identification and Prevalence of *Sarcocystis suihominis* in Pork Meat Intended for Public Consumption. *Bulletin UASVM Veterinary Medicine*, 74, 1.
- Altschul S F, Gish W, Miller W, Myers E W and Lipman D J 1990 Basic local alignment search tool. *Journal of Molecular Biology*, 215(3), 403-410.
- Avapal R S, Bhatia B B, Sharma J K and Juyal P D 2002 Seroprevalence of *Sarcocystis* in swine. *Journal of Parasitic Diseases*, 18: 75-76.
- Avapal R S, Sharma J K and Juyal P D 2003a Comparison of techniques to detect *Sarcocystis* infection in slaughtered pigs. *Journal of Veterinary Parasitology* 17:65–66.
- Avapal R S, Sharma J K and Juyal P D 2004 Pathological changes in *Sarcocystis* infection in domestic pigs (*Sus scrofa*). *Veterinary Journal (London, England: 1997)*, 168(3), 358-361.
- Banerjee P S, Bhatia B B and Pandit B A 1994 *Sarcocystis suihominis* infection in human beings in India. *Journal of Veterinary Parasitology* 8: 57-58.

- Barrows P L, Prestwood A K, Adams D D and Dykstra M J 1982 Development of *Sarcocystis suicanis* Erber, 1977 in the pig. *The Journal of Parasitology*, 674-680.
- Barrows P L, Smith H M Jr, Prestwood A K and Brown J 1981 Prevalence and distribution of *Sarcocystis* spp. among wild swine of southeastern United States. *Journal of American Veterinary Medical Association*, 179(11): 1117-8.
- Basic Animal Husbandry statistics 2019 Ministry of Agriculture, Government of India. pp. 13 (www.Faostat.Org).
- Beaver P C, Gadgil R K, Morera P 1979 *Sarcocystis* in man: a review and report of five cases. *American Journal of Tropical Medicine and Hygiene*, 28:819- 844.
- Bhatia B B, Pathak K M L, Juyal P D 2010 Text book of Veterinary Parasitology, 3rd edn, New Delhi, pp 497–506.
- Bose R, Jorgensen W K, Dalgliesh R J, Friedhoff K T and De Vos A J 1995 Current state and future trends in diagnosis of babesiosis. *Veterinary Parasitology*, 57: 61–74.
- Bunyaratvej S, Unpunyo P and Pongtippan A 2007 The *Sarcocystis*-cyst containing beef and pork as the sources of natural intestinal sarcocystosis in Thai people. *Medical journal of the Medical Association of Thailand*, 90(10), 2128.
- Bussieras, J 1994 An example of holozoonosis: human coccidiosis due to *Sarcocystis* spp. *Bulletin de l'Academie nationale de medecine*, 178(4), 613-22.
- Calero Bernal R, Perez Martin J E, Reina D, Serrano F J, Frontera E, Fuentes I and Dubey J P 2015 Detection of zoonotic protozoa *Toxoplasma gondii* and *Sarcocystis suihominis* in wild boars from Spain. *Zoonoses and Public Health*, 63(5), 346-350.

- Caspari K, Grimm F, Kuhn N, Caspari N C, and Basso W 2011 First report of naturally acquired clinical sarcocystosis in a pig breeding stock. *Veterinary Parasitology*, 177(1-2), 175-178.
- Caspari K, Grimm F, Kuhn N, Caspari N C, Basso W 2010 First report of naturally acquired clinical sarcocystosis in a pig breeding stock. *Veterinary Parasitology*, 177: 175-178.
- Chauhan R P, Kumari A, Nehra A K, Ram H, Garg R, Banerjee P S, Karikalan M and Sharma A K 2020 Genetic characterization and phylogenetic analysis of *Sarcocystis suis hominis* infecting domestic pigs (*Sus scrofa*) in India. *Parasitology Research*, 119 (10), 3347- 3357.
- Chen L Y, Zhou B J, Yang Z Q, Li C Y, Attwood S W, Wang W L, Lei L, Sun X D and Zhang Z X 2007 Effects of frozen storage on the structure of sarcocysts in pig muscle and implications in taxonomic studies. *Experimental Parasitology*, 115(4), 393-398.
- Chhabra M B, and Samantaray S 2013 *Sarcocystis* and sarcocystosis in India: status and emerging perspectives. *Journal of Parasitic Diseases*, 37(1), 1-10.
- Claveria F G, De La Pena C, and Cruz-Flores M J 2001 *Sarcocystis miescheriana* infection in domestic pigs (*Sus scrofa*) in the Philippines. *Journal of Parasitology*, 87(4), 938-939.
- Coelho C, Gomes J, Inacio J, Amaro A, Mesquita J R, Pires I, Lopes A P, and Vieira-Pinto M 2015 Unraveling *Sarcocystis miescheriana* and *Sarcocystis suis hominis* infections in wild boar. *Veterinary Parasitology*, 212(3-4), 100-104.
- Damriyasa I M, Bauer C, Edelhofer R, Failing K, Lind P, Petersen E, Schares G, Tenter, A M, Volmer R, and Zahner H 2004 Cross-sectional survey in pig breeding farms in Hesse, Germany: seroprevalence and risk factors of infections with

- Toxoplasma gondii*, *Sarcocystis* spp. and *Neospora caninum* in sows. *Veterinary Parasitology*, 126(3), 271-286.
- Devi P P, Sarmah P C and Saleque A 1998 *Sarcocystis* spp infection in carcasses of domestic pigs in Guwahati, Assam. *Journal of Veterinary Parasitology* 12(1): 56-57.
- Dubey J P 1976 A review of *Sarcocystis* of domestic animals and of other coccidia of cats and dogs. *Journal of the American Veterinary Medical Association*, 169(10), 1061-1078.
- Dubey J P, and Powell E C 1994 Prevalence of *Sarcocystis* in sows from Iowa. *Veterinary Parasitology*, 52(1-2), 151-155.
- Dubey J P, Calero-Bernal R, Rosenthal B M, Speer C A, Fayer R 2016 *Sarcocystosis of animals and humans*, 2nd edn. CRC Press, BocaRaton.
- Dubey J P, Speer C A and Fayer R 1989. *Sarcocystosis of animals and man*. CRC Press, Inc. Boca Raton, Florida.
- Farhang-Pajuh F, Yakhchali M, and Mardani K 2014 Molecular determination of abundance of infection with *Sarcocystis* species in slaughtered sheep of Urmia, Iran. In *Veterinary research forum: an international quarterly journal* (Vol. 5, No. 3, p. 181). *Faculty of Veterinary Medicine, Urmia University, Urmia, Iran*.
- Fayer R 1972 Gametogony of *Sarcocystis* sp. in cell culture. *Science*, 175(4017), 65-67.
- Fayer R 2004 *Sarcocystis* spp. in human infections. *Clinical Microbiology Reviews*, 17(4), 894-902.
- Fayer R, Esposito D H and Dubey J P 2015 Human infections with *Sarcocystis* species. *Clinical Microbiology Reviews*, 28(2), 295-311.

- Fischer, S. and K. Odening 1998. Characterization of bovine *Sarcocystis* species by analysis of their 18S ribosomal DNA sequences. *Journal of Parasitology*, 84: 50–54.
- Frenkel J K, Heydorn A O, Mehlhorn H and Rommel M 1979 Sarcocystinae: Nomina dubia and available names. *Zeitschrift fur Parasitenkunde*, 58(2), 115-139.
- Freyre A, Chifflet L and Mendez J 1992 Sarcosporidian infection in pigs in Uruguay. *Veterinary Parasitology*, 41(1-2), 167-171.
- Gazzonis A L, Gjerde B, Villa L, Minazzi S, Zanzani S A, Riccaboni P, Sironi G and Manfredi M T 2019 Prevalence and molecular characterisation of *Sarcocystis miescheriana* and *Sarcocystis suihominis* in wild boars (*Sus scrofa*) in Italy. *Parasitology Research*, 118(4), 1271-1287.
- Ghorbanpoor M, Hamidinejat H, Nabavi L, Khadjeh G H and Jalali M R 2007 Evaluation of an ELISA for the diagnosis of sarcocystosis in water buffaloes. *Bulletin-Veterinary Institute in Pulawy*, 51(2), 229.
- Gjerde B 2012 Morphological and molecular characterization and phylogenetic placement of *Sarcocystis capreolicanis* and *Sarcocystis silva* n. sp. from roe deer (*Capreolus capreolus*) in Norway. *Parasitology Research*, 110(3), 1225-1237.
- Gjerde B 2013 Phylogenetic relationships among *Sarcocystis* species in cervids, cattle and sheep inferred from the mitochondrial cytochrome c oxidase subunit I gene. *International Journal for Parasitology*, 43(7), 579-591.
- Gjerde B De la Fuente C, Alunda J M and Luzon, M 2020 Molecular characterisation of five *Sarcocystis* species in domestic sheep (*Ovis aries*) from Spain. *Parasitology Research*, 119(1), 215-231.

- Gonzalez L M, Villalobos N, Montero E, Morales J, Alamo Sanz R R, Muro A, Harrison L J S, Parkhouse R M E, Garate T 2006 Differential molecular identification of *Taeniid* spp. and *Sarcocystis* spp. cysts isolated from infected pigs and cattle. *Veterinary Parasitology*; 142(1-2), 95–101.
- Gonzalez L M, Villalobos N, Montero E, Morales J, Sanz R A, Muro A, Harrison L J S, Parkhouse R M E and Garate T 2006 Differential molecular identification of *Taeniid* spp. and *Sarcocystis* spp. cysts isolated from infected pigs and cattle. *Veterinary Parasitology*, 142(1-2), 95-101.
- Gupta S L and Gautam O P 1984 *Sarcocystis* infection in pigs of Hissar, Haryana, India and its transmission to dogs. *Veterinary Parasitology* 16: 1-3.
- Hamidinejat H, Jalali M H R, Gharibi D and Molayan P H 2015 Detection of *Sarcocystis* spp. in cattle (*Bos taurus*) and water buffaloes (*Bubalus bubalis*) in Iran by PCR–RFLP. *Journal of Parasitic Diseases*, 39(4), 658-662.
- Hamidinejat H, Moetamedi H, Alborzi A and Hatami A 2014 Molecular detection of *Sarcocystis* species in slaughtered sheep by PCR–RFLP from south-western of Iran. *Journal of Parasitic Diseases*, 38(2), 233-237.
- Hansen W D, Hunter D T, Richards D E and Allred L 1970 Acridine orange in the staining of blood parasites. *Parasitology* 56: 386–87.
- Heydorn A O 1977 Lifecycle of Sarcosporidia. IX Developmental cyclus of *Sarcocystis suihominis* n. spec. *Berl Munch Tierarztl Wochenschr* 90(11):218-24.
- Hinaidy, H.K. and Supperer, R 1979 Sarkosporidien befall des Schweines in t3sterreich. Wien. Tieraerztl. Monatsschr., 66:281-285.
- Hooshyar H, Abbaszadeh Z, Sharafati-Chaleshtori R and Arbabi M 2017 Molecular identification of *Sarcocystis* species in raw hamburgers using PCR–RFLP

- method in Kashan, central Iran. *Journal of Parasitic Diseases*, 41(4), 1001-1005.
- Hu J J, Liu T T, Liu Q, Esch G W, Chen J Q, Huang S and Wen T 2016 Prevalence, morphology, and molecular characteristics of *Sarcocystis* spp. in domestic goats (*Capra hircus*) from Kunming, China. *Parasitology Research*, 115(10), 3973-3981.
- Hu J J, Meng Y, Chen X W, Zuo Y X, Wu P 2010 Research progress on human sarcocystosis. *Chinese Journal of Parasitology and Parasitic Diseases*, 28:460–465.
- Huang Z, Ye Y, Zhang H, Deng S, Tao J, Hu J and Yang Y 2019 Morphological and molecular characterizations of *Sarcocystis miescheriana* and *Sarcocystis suihominis* in domestic pigs (*Sus scrofa*) in China. *Parasitology Research*, 118(12), 3491-3496.
- Imre K, Darabus G, Tirziu E, Morariu S, Imre M, Plutzer J, Boldea M and Morar A 2019 *Sarcocystis* spp. in romanian slaughtered cattle: Molecular characterization and epidemiological significance of the findings. *BioMed Research International*, 2019.
- Imre K, Morar A, Imre M, and Sala C C 2016 Molecular epidemiology of the neglected meat-borne pathogen *Sarcocystis* spp. in pigs and cattle: an update. *Scientific Papers Animal Science and Biotechnologies*, 49(1), 241-246.
- Imre K, Sala C, Morar A, Imre M, Ciontu C, Chisalita I, Dudu A, Matei M and Darabus G 2017 Occurrence and first molecular characterization of *Sarcocystis* spp. in wildboars (*Sus scrofa*) and domestic pigs (*Sus scrofa domesticus*) in Romania: Public health significance of the isolates. *Acta Tropica*, 167, 191-195.

- Jacobs L, Remington J and Melton M 1960 A survey of meat samples from swine, cattle and sheep for the presence of encysted *Toxoplasma*. *Journal of Parasitology* 46: 23-28.
- Jeffries A C, Schnitzler B, heydorn, A O, Johnson A M and Tenter A M 1997 Identification of synapomorphic characteris in the genus *Sarcocystis* based on 18S rDNA sequence comparison. *Journal of Eukaryotic Microbiology*, 44(5), 388-392.
- Jehle C, Dinkel A, Sander A, Morent M, Romig T, Luc P V, De T V, Thai V V and Mackenstedt U 2009 Diagnosis of *Sarcocystis* spp. in cattle (*Bos taurus*) and water buffalo (*Bubalus bubalis*) in Northern Vietnam. *Veterinary Parasitology*, 166(3-4), 314-320.
- Kamber U, Arslan M O, Gulbaz G, Tasci G T and Atila A K C A 2018 Identification of *Sarcocystis* spp. by polymerase chain reaction and microscopic examination in various beef products (minced meat, meatballs, fermented sausage). *Turkish Journal of Veterinary and Animal Sciences*, 42(1), 1-6.
- Kaur M, Singh B B, Sharma R and Gill J P S 2015 Pervasive environmental contamination with human feces results in high prevalence of zoonotic *Sarcocystis* infection in pigs in the Punjab, India. *The Journal of Parasitology*, 102(2), 229-232
- Kaur M and Bal M S 2016 Overview of zoonotic porcine *Sarcocystis* infection in India. *International Journal of Advanced Research*, 4, 1836-1840.
- Khieu V, Marti H, Chhay S, Char M C, Muth S and Odermatt P 2017 First report of human intestinal sarcocystosis in Cambodia. *Parasitology International*, 66(5), 560-562.

- Kia E B, Mirhendi H, Rezaeian M, Zahabiun F and Sharbatkhori M 2011 First molecular identification of *Sarcocystis miescheriana* (Protozoa, Apicomplexa) from wild boar (*Sus scrofa*) in Iran. *Experimental Parasitology*, 127(3), 724-726.
- Latif B, Kannan Kutty M, Muslim A, Hussaini J, Omar E, Heo C C, Rossle N F, Abdullah S, Kamarudin M A and Zulkarnain M A 2015 Light microscopy and molecular identification of *Sarcocystis* spp. in meat producing animals in Selangor, Malaysia. *Tropical Biomedicine*, 32(3), 444-452.
- Lauer B A, Reller L B and Mirrett S 1981 Comparison of acridine orange and Gram stains for detection of microorganisms in cerebrospinal fluid and other clinical specimens. *Journal of Clinical Microbiology*, 14(2), 201-205.
- Li J H, Lin Z, Du J F and Qin Y X 2007 Experimental infection of *Sarcocystis suihominis* in pig and human volunteer in Guangxi. *Chinese Journal of Parasitology and Parasitic Diseases*, 25(6), 466-468.
- Li Q Q, Yang Z Q, Zuo Y X, Attwood S W, Chen X W and Zhang Y P 2002 A PCR-based RFLP analysis of *Sarcocystis cruzi* (Protozoa: Sarcocystidae) in Yunnan Province, PR China, reveals the water buffalo (*Bubalus bubalis*) as a natural intermediate host. *Journal of Parasitology*, 88(6), 1259-1261.
- Luna L G 1968 Manual of histologic staining methods of the Armed Forces Institute of Pathology.
- Markus MB, Van Der Lugt J J, Dubey J P 2004 Sarcocystosis. In: Coetzer JAW, Tustin RC (eds) *Infectious diseases of livestock*, vol 1, 2nd edn. Oxford University Press, Cape Town, pp 360–375.
- Maurer J J 2011 Rapid detection and limitations of molecular techniques. *Annual Review of Food Science and Technology*, 2, 259-279.

- McManus D P and Bowles J 1996 Molecular genetic approaches to parasite identification: their value in diagnostic parasitology and systematics. *International Journal for Parasitology*, 26(7), 687-704.
- Miescher F 1843 Uber eigenthimliche Schliuche in den Muskeln einer Hausmaus. Bericht der Verhandlungen der Naturforschenden Gesellschaft 5: 198-202.
- More G, Pantchev A, Skuballa J, Langenmayer M C, Maksimov P, Conraths F J, Venturini M C and Schares G 2014 *Sarcocystis sinensis* is the most prevalent thick-walled *Sarcocystis* species in beef on sale for consumers in Germany. *Parasitology Research*, 113(6), 2223-2230.
- More G, Schares S, Maksimov A, Conraths F J, Venturini M C and Schares G 2013 Development of a multiplex real time PCR to differentiate *Sarcocystis* spp. affecting cattle. *Veterinary Parasitology*, 197(1-2), 85-94.
- Motamedi G R, Dalimi A, Nouri A and Aghaeipour K 2011 Ultrastructural and molecular characterization of *Sarcocystis* isolated from camel (*Camelus dromedarius*) in Iran. *Parasitology Research*, 108(4), 949-954.
- Mounika K, Chennuru S, Ravipati V, Tumati S R and Krovvidi S 2019 Studies on prevalence and histomorphology of *Sarcocystis* species infecting cattle in Andhra Pradesh, India. *Journal of Parasitic Diseases*, 42(1), 77-80.
- Murata R, Suzuki J, Hyuga A, Shinkai T and Sadamasu K 2018 Molecular identification and characterization of *Sarcocystis* spp. in horsemeat and beef marketed in Japan. *Parasite*, 25.
- National Action Plan on Pig 2019 Department of Animal husbandry and Dairying (dahd.nic.in).

- Neefs J M, Y Van De Peer, P De Rijk, A Goris and R De Wachter 1991 Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Research*, 19: 1987-2016.
- Omata, Y, Heydorn A O, Heidrich H G, Igarashi I, Saito A, Toba H and Suzuki N 1993 Survey of *Sarcocystis* spp. infection in slaughtered pigs in East Hokkaido Japan. *Journal of Proteome Research*, 3: 29-30.
- Panda R, Kumar M U, Murthy G S and Reddy Y N 2020 Serodiagnosis of bovine sarcocystosis by dot-elisa. *Indian Journal of Animal Research*, 54(6), 734-738.
- Pereira A and Bermejo M 1988. Prevalence of *Sarcocystis* cyst in pigs and sheep in Spain. *Veterinary Parasitology*, 27(3-4), 353-355.
- Piekarski G, Heydorn A O, Aryeetey M E, Hartlapp J H and Kimmig P 1978 Clinical , parasitological and serological investigations on human sarcosporidiosis (*Sarcocystis sui hominis*). *Immunitat-und-infection*, 6:4, 153-59.
- Prakas P, Kirillova V, Dzerkale A, Kirjusina M, Butkauskas D, Gavarane I, Lukosiene E R and Sulinskas G 2020 First molecular characterization of *Sarcocystis miescheriana* in wild boars (*Sus scrofa*) from Latvia. *Parasitology Research*, 119(11), 3777-3783.
- Prasanth H and Bhatia B B 1996 On the species of *Sarcocystis* of pigs in Uttar Pradesh, India with a description of a sarcocyst of hitherto unrecognized species. *Journal of Veterinary Parasitology*, 10: 57-61.
- Prasanth Hema 1995 *Sarcocystis* infection in domestic pigs in Uttar Pradesh. *Journal of Veterinary Parasitology*, 9:119–123
- Ravindran R, Bindu L, Sreekumar C, Lalitha J, Gomathinayagam S and Mishra A K. 2007a Acridine orange staining for quick detection of blood parasites. *Journal of Veterinary Parasitology* 21(1): 85–86.

- Reiner G, Eckert J, Peischl T, Bochert S, Jakel T, Mackenstedt U, Joachim A, Dausgchies A and Geldermann H 2002 Variation in clinical and parasitological traits in Pietrain and Meishan pigs infected with *Sarcocystis miescheriana*. *Veterinary Parasitology*, 106(2), 99-113.
- Rosenthal B 2010 *Sarcocystis*, *Molecular Detection of Food borne Pathogens*. Pp 735-740 (ed) Liu D CRC press, Boca Raton, USA.
- Rosenthal B M 2021. Zoonotic *Sarcocystis*. *Research in Veterinary Science*.
- Sahi B N, Singh S P, Sahay M N, Srivastava P S and Juyal P D 1982 Note on the incidence and epidemiology of *Sarcocystis* infection in cattle, buffaloes and pigs in Bihar. *Indian Journal of Animal Sciences*.
- Saito M, Nakajima T, Watanabe A and Itagaki H 1986 *Sarcocystis miescheriana* infection and its frequency in pigs in Japan. *The Japanese Journal of Veterinary Science*, 48(6), 1083-1090.
- Saito M, Shibata Y, Ohno A, Kubo M, Shimura K and Itagaki H 1998 *Sarcocystis suihominis* detected for the first time from pigs in Japan. *Journal of Veterinary Medical Science*, 60(3), 307-309.
- Saleque A and Bhatia B B 1991 Prevalence of *Sarcocystis* in domestic pigs in India. *Veterinary Parasitology*, 40(1-2), 151-153.
- Shah H L 1995 Sarcocystosis as a zoonoses with special reference to India. *Journal Veterinary Parasitology* 9(2): 57-61.
- Singh B B, Sharma R, Sharma J K and Juyal P D 2010 Parasitic zoonoses in India: an overview. *Rev Sci Tech*, 29(3), 629-37.
- Solanki P K, Shrivastava H O P and Shah H L 1991 Prevalence of *Sarcocystis* innaturally infected pigs in Madhya Pradesh with an epidemiological

- explanation for the higher prevalence of *Sarcocystis suis hominis*. *Indian Journal of Animal Sciences*, 61: 820-821.
- Solaymani-Mohammadi S and Petri Jr W A 2006 Zoonotic implications of the swine-transmitted protozoal infections. *Veterinary Parasitology*, 140(3-4), 189-203.
- Soulsby E J L 1982 Helminths. *Arthropods and Protozoa of domesticated animals*, 291.
- Srinivasa Rao K, Hafeez Md 2002a Incidence of porcine sarcocystosis in Andhra Pradesh. *Journal of Parasitic Diseases*, 26:69-71
- Tadros W and Laarman J J 1982 Current concepts on the biology, evolution and taxonomy of tissue cyst-forming eimeriid coccidia. *Advances in Parasitology*, 20, 293-468.
- Tenter A M 1995 Current research on *Sarcocystis* species of domestic animals. *International Journal of Parasitology*, 25: 1311-1330.
- Tenter A M, Luton K and Johnson A M 1994 Species-specific identification of *Sarcocystis* and *Toxoplasma* by PCR amplification of small subunit ribosomal RNA gene fragments. *Applied Parasitology*, 35(3), 173-188.
- Vincze T, Posfai J and Roberts R J 2003 NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acids Research*, 31(13), 3688-3691.
- Weber M, Weyreter H, O'Donoghue P J, Rommel M and Trautwein G 1983 Persistence of acquired immunity to *Sarcocystis miescheriana* infection in growing pigs. *Veterinary Parasitology*, 13(4), 287-297.
- Yan W, Qian W, Li X, Wang T, Ding K and Huang T 2013 Morphological and molecular characterization of *Sarcocystis miescheriana* from pigs in the central region of China. *Parasitology Research*, 112(3), 975-980.
- Yang Z Q, Li Q Q, Zuo Y X, Chen X W, Chen Y J, Nie L, Wei C G, Zen J S, Attwood S W, Zhang X Z and Zhang Y P 2002 Characterization of *Sarcocystis* species

in domestic animals using a PCR-RFLP analysis of variation in the 18S rRNA gene: a cost-effective and simple technique for routine species identification. *Experimental Parasitology*, 102 (3-4),212-217.

Yang Z Q, Zuo Y X, Ding B, Chen X W, Luo J and Zhang Y P 2001 Identification of *Sarcocystis hominis*-like (Protozoa: Sarcocystidae) cyst in water buffalo (*Bubalus bubalis*) based on 18S rRNA gene sequences. *Journal of Parasitology*, 87(4), 934-937.

Yang Z, Zuo Y, Chen X, Ding B, Luo J and Zhang Y 2000 18S rRNA gene of *Sarcocystis hominis* cyst from water buffalo and cattle. *Zoological Research*, 21(2), 133-138.

Zainalabidin F A, Noorazmi M S N, Bakri W N W O, Sathaya G and Ismail M I 2017 Prevalence of Muscular Sarcosporidiosis in Slaughtered Domestic Pigs in Perak, Peninsular Malaysia. *Tropical Life Sciences Research*, 28(1), 161.

Zhang D X and Hewitt G M 1998 Isolation of animal cellular total DNA. In *Molecular tools for screening biodiversity* (pp. 5-9). Springer, Dordrecht.