RESEARCH ARTICLE

Micromorphological Identification and Prevalence of *Sarcocystis* Species in Slaughtered Sheep: A Tri-Method Approach

Shetti Ankitha[1]*, Manchukonda Udaya Kumar[1], Banothu Dasmabai[2], Putty Kalyani[3]

Abstract

A study was conducted to determine the prevalence of Ovine Sarcocystosis in sheep slaughtered at various slaughterhouses in and around Hyderabad, Telangana, India. A total of 1,000 muscle samples, comprising oesophagi and hearts were subjected to gross examination. Gross inspection revealed no macrocysts in any of the samples. For microscopic evaluation, 150 samples (90 hearts and 60 oesophagi) were analyzed using squash technique, pepsin-HCl muscle digestion. The squash technique revealed microsarcocysts in 5 out of 90 heart samples (5.5%), while none were detected in oesophageal samples resulting in an overall prevalence of 3.33%. In contrast, the pepsin-HCl digestion technique demonstrated a significantly higher detection rate identifying bradyzoites in 49 heart samples (54.4%) and 18 oesophagi (30.0%) yielding an overall prevalence of 44.6%. Microscopic confirmation of bradyzoites was achieved through Giemsa and Leishman staining which revealed banana-shaped bradyzoites with posterior nuclei under oil immersion. Histopathological examination further confirmed the presence of microsarcocysts in cardiac muscles. This study highlights a high prevalence of microscopic Sarcocystosis in the cardiac muscles of slaughtered sheep in and around Hyderabad, emphasizing the superiority of the digestion technique over squash method and the potential zoonotic and economic implications of subclinical infections in livestock.

Key words: Histopathology, Ovine sarcocystosis, Pepsin-HCl muscle digestion, Prevalence, Squash technique.

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Introduction

Sarcocystis is considered as one of the most prevalent livestock parasites, with potential public health importance acquired due to the consumption of undercooked or raw meat. *Sarcocystis* spp. has been isolated from different animals throughout the world. Sheep are the intermediate hosts of six species of *Sarcocystis*, including *S. gigantea* (syn. *S. ovifelis*), *S. tenella* (*syn. S. ovicanis*), *S. arieticanis, S. medusiformis, S. microps*, and *S. mihoensis*. Among these *S. tenella* and *S. arieticanis* are known to produce tiny cysts and can lead to pathogenic consequences in sheep. This includes acute symptoms like abortion, fever, anaemia and anorexia during the early stages of infection, potentially progressing to chronic disorders. Occasionally encephalitis may occur in sheep after infection with *S. ovicanis* (Formisano *et al*., 2013). On the contrary, *S. gigantea* and *S. medusiformis* are the macro-Sarcocyst forming species and are generally considered non-pathogenic to sheep. However, they can still impact the quality and marketability of sheep meat, resulting in economic losses to farmers.

It's worth noting that sheep in the area are often exposed to a high rate of microscopic infections from these species. This exposure primarily occurs due to the shedding of sporocysts by dogs and possibly wild animals, which serve as definitive hosts. This increased exposure can pose health risks to humans who consume undercooked or raw meat from infected sheep, potentially leading to symptoms like nausea, stomach ache and diarrheoa (Pestechian *et al*., 2021; Shakeri and Adhami, 2022). Small ruminants can become infected with a parasite when they accidentally ingest the infective stage, known as sporocysts, which can be present in contaminated drinking water and feed. The primary sites where muscular *Sarcocystis* predominantly found in the intermediate host include the heart, diaphragm, tongue, oesophagus, and skeletal muscles (Buxton, 1998; Daryani *et al*., 2006; Mirzaei Dehaghi *et al*., 2013). This study was aimed

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to identify micromorphology and know the prevalence of *sarcocystis* species in slaughtered sheep in and around Hyderabad, Telangana.

Materials and Methods

The study was conducted at Department of Veterinary Parasitology, College of Veterinary Science, Rajendranagar, Hyderabad, Telangana (India) in collaboration with various local slaughter houses during the year 2023-24. On-site gross examination of 1000 fresh muscle samples of oesophagi and hearts of slaughtered sheep across various slaughterhouses was performed to check presence of macrocysts of *Sarcocystis* species and lesions. The muscles samples of 90 hearts and 60 oesophagi of sheep were also collected and transported on ice to the laboratory to further check the presence of microsarcocysts by squash and pepsin HCl acid muscle digestion techniques.

Squash Technique

According to the procedure outlined by Singh *et al*. (1990) and Dubey *et al*. (2000), small sections of muscle measuring approximately 5x5x2 mm were squeezed between two glass slides and examined microscopically for cysts of *Sarcocystis* spp.

Pepsin-HCl Muscle Digestion Technique

Suspected muscles (20 g) samples were incubated in 50 mL of acid-pepsin (2.6 g pepsin, 5 g NaCl, 7 mL 1 M HCl, and 993 mL distilled water) for 20 min at 40°C according to the protocol outlined by Motamedi *et al*. (2010). The suspension was filtered through five layers of prewetted muslin cloth, centrifuged at 447.2 x g for 5 min and the sediment was suspended in PBS (pH 7.4). A drop of this solution was then examined for the presence of bradyzoites under a light microscope**.**

Giemsa staining: Smears were made from filtered bradyzoite suspension onto clean, grease-free glass slides, fixed in methyl alcohol for 2-3 min, and then stained in Giemsa stain for 30 min (1 part stock solution to 9 parts distilled water). The stained smears were washed under running water, allowed to air dry and then examined under a microscope under a 100X objective.

Leishman staining: Smears prepared aseptically from the filtered bradyzoite suspension, air dried and were flooded with Leishman stain for 2 min. Then added double the quantity of water to the stain and mixed well by blowing air. Diluted stain was allowed to act for 12 min. After rinsing in running tap water and air drying, the slide was observed under oil immersion (100X) lens of bright field microscope.

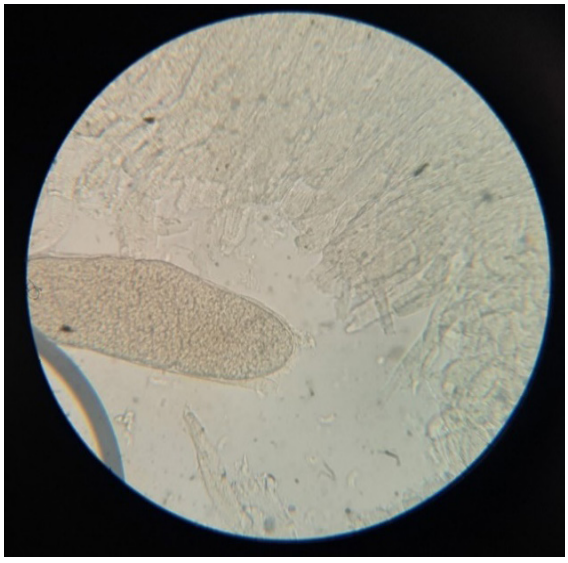
Histopathological Studies

The tissues were collected and fixed in 10 % neutral buffer formalin. The tissues were then split into tiny, representative pieces and washed overnight under running water. The tissues were then dehydrated in progressively stronger alcohol, cleaned in xylol and embedded in paraffin at a temperature of 55 to 56 °C. By using a semi-automatic rotary microtome (Leica; Germany), the paraffin blocks were cut into slices of 5 mm thickness. The cut sections were lifted onto precoated, clean, grease-free glass slides and allowed to dry overnight in the incubator at 37°C and subjected to routine H and E staining (Clayden, 1962). The stained sections were then mounted with DPX mount and examined microscopically.

Results and Discussion

Gross Examination of Muscle Samples

On-site gross examination of one thousand samples of oesophagi and hearts of slaughtered sheep across various slaughterhouses in Hyderabad revealed no macrocysts in the muscles. The muscles were examined by making parallel



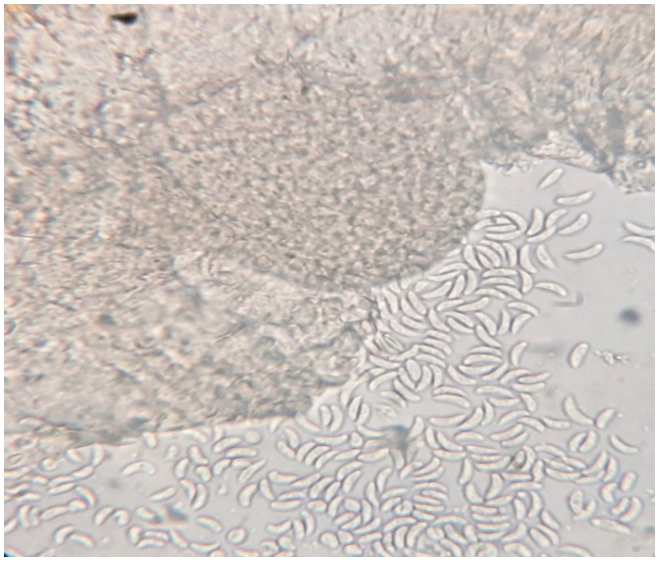


Fig. 1 and 2: Photomicrograph showing ruptured microcyst releasing bradyzoites of *Sarcocystis* spp. in cardiac muscle by squash technique (40X)

incisions in the muscles. Detailed examination revealed an absence of any kind of gross lesions or abnormalities in the muscular tissues of these examined organs. Similar to our results, no macrosarcocysts were observed in sheep by previous workers (Nourani *et al*., 2010; Hajimohammadi *et al*., 2014; Dong *et al*., 2018; Januskevicius *et al*., 2019; Abdullah, 2021; Zainalabidin, 2021). Conversely, macrocysts of *Sarcocystis* were found in 17.08 %, 3.3 %, 5.82 % and 42.70 % of sheep by Farhang-Pajuh *et al*. (2014), Mirzaei and Rezaei (2016), Al-Saadi *et al*. (2020), and Osman *et al*. (2023), respectively.

Microscopic Examination of Muscle Samples

Squash Technique

This technique revealed the presence of microcysts in the muscle fibres specifically within the cardiac muscle. Five samples out of 90 hearts (5.5%) collected revealed the microsarcocysts by squash technique (Fig. 1, 2), whereas no microsarcocyst was found in the oesophagus (0/60), thus giving overall prevalence of 3.33% (5/150). In contrary to this, Dasmabai (2012) found 8% of oesophagi showing bradyzoites in bovine samples by squash technique under light microscopy.

Pepsin-Hydrochloric Acid Muscle Digestion Technique

This method yielded a good number of live bradyzoites showing gliding motion. Bradyzoites were detected in 49/90 heart samples (54.4%) and 18/60 oesophagi (30.0%) yielding an overall prevalence of 44.6% (67/150, Fig. 3). Contrary to our study, Abdullah (2021) detected higher rates of *Sarcocystis* by muscle digestion in oesophagus (95 %) than in diaphragm (90 %) of sheep. Dubey and Livingston (1986) reported 22.4 % and 0.23 % prevalence rates of Sarcocystosis in the diaphragm and oesophagus of sheep, respectively, while Fukuyo *et al*. (2002) and Bahari *et al*. (2014) reported 100 % and 93.2 % prevalence rates of *Sarcocystis* spp., respectively, in the diaphragm. Most positive samples were found in thigh muscles by Zainalabidin (2021).

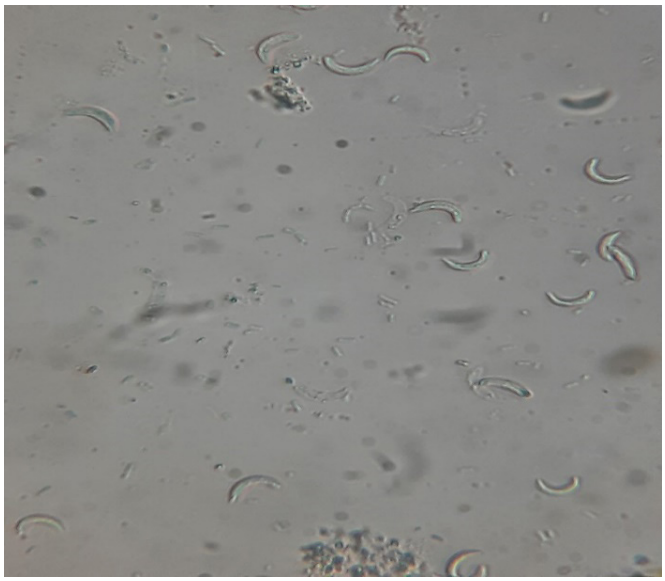


Fig. 3: Photomicrograph showing bradyzoites of *Sarcocystis* spp. by Pepsin HCl acid muscle digestion technique (40X)

Confirmation of Bradyzoites by Staining

Thin smears of suspension of muscle digestion were stained with Giemsa (Fig. 4) and Leishman's stains (Fig. 5). Bradyzoites were visualized under oil immersion objective of a microscope (100X). The stained bradyzoites exhibited a banana-shaped appearance with posteriorly placed nucleus.

Histopathological Findings

Histopathological examination revealed the presence of two distinct types of microsarcocysts within the cardiac muscle tissues displaying noticeable morphological variations in

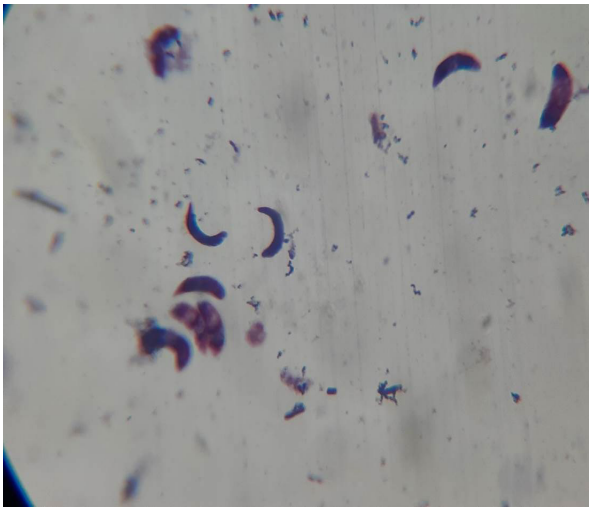


Fig 4: Photomicrograph showing Giemsa stained bradyzoites of *Sarcocystis* spp. (100X)

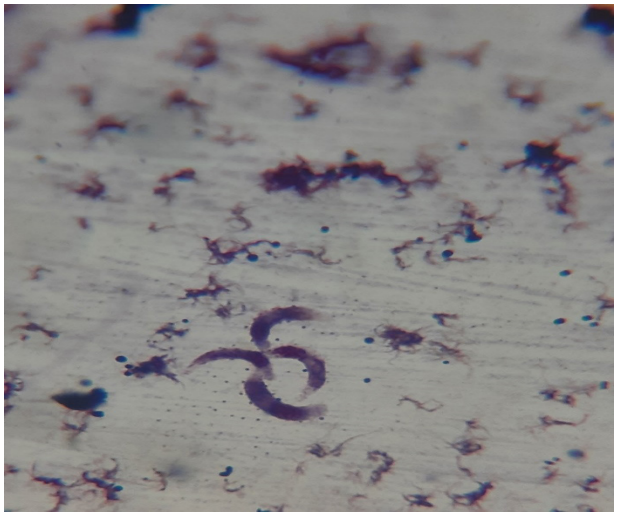


Fig 5: Photomicrograph showing Leishman stained bradyzoites of *Sarcocystis* spp. (100X)

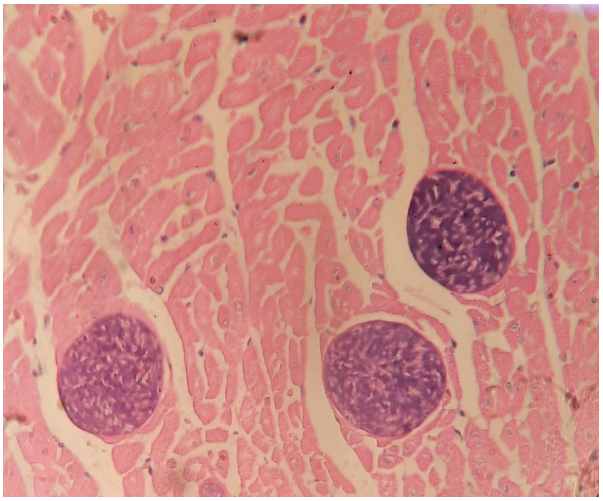


Fig. 6: Photomicrograph of transverse section of heart muscle tissue showing oval to round microcysts containing bradyzoites (H & E stain 40X)

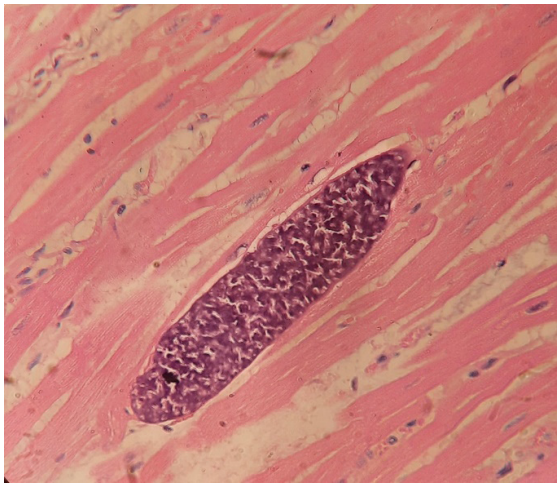


Fig. 7: Photomicrograph of longitudinal section of heart muscle tissue showing elongated microcysts containing bradyzoites (H & E stain 40X)

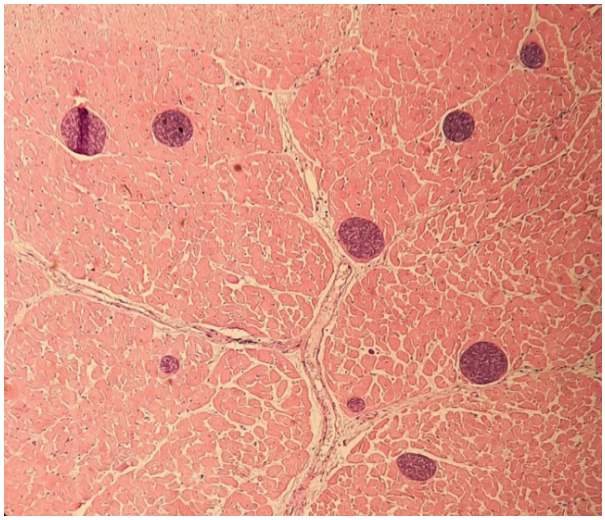


Fig. 8: Photomicrograph of muscle section showing microcysts of variable sizes packed with bradyzoites and encapsulated by fibrous tissue (H & E stain 10X)

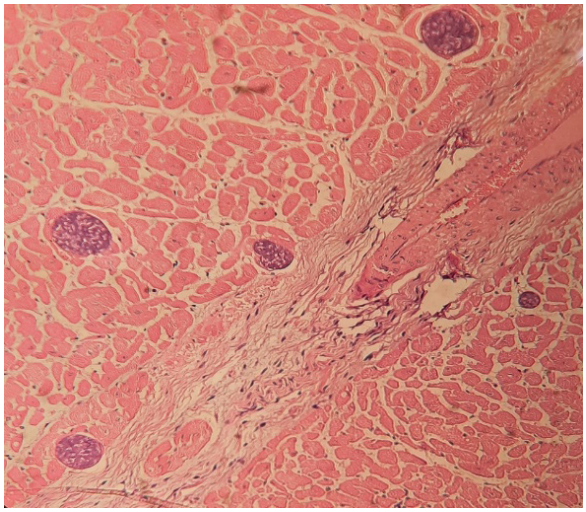


Fig. 9: Photomicrograph of muscle section showing microcysts along with fibrosis around the blood vessels (H & E stain 10X)

their stage of development. One type of Sarcocyst was with a spherical or rounded structure (Fig. 6), while the other exhibiting a fusiform or elongated shape (Fig. 7), showing transverse septal partitions with mild pathological changes around immature and mature cysts (Fig. 8, 9). These diverse forms of cysts resembling potentially pathogenic *Sarcocystis* species specifically, *Sarcocystis tenella* or *S. arieticanis* are known to produce microscopic cysts within the host tissues. Further confirmation of the species requires additional studies employing TEM or molecular studies. Hu *et al*. (2016), El-Morsey *et al*. (2019) and Abdullah (2021) identified the presence of two morphologically distinct microscopic types of Sarcocysts by histopathology. Microsarcocysts in sheep muscle tissues were of thin-walled and thick-walled types, which belonged to *S. arieticanis* and *S. tenella*, respectively.

The changes in results due to various factors such as environmental factors like temperature and humidity, geographical elements such as location and altitude, and managemental practices including grazing methods, animal care and biosecurity measures can impact the prevalence and distribution of *Sarcocystis* infections. Additionally, the density of the host population is a crucial factor, as overcrowding can elevate the chances of transmission. The presence of intermediate host and definitive host and sporocysts also play a significant role in the transmission of sarcocystosis, and their availability in a particular area can influence the overall prevalence.

Conclusion

Overall the Tri-method approach (Squash technique, Pepsin-HCl muscle digestion technique & Histopathology) offers a

practical and accurate strategy for field surveillance. While more advanced techniques like PCR or electron microscopy exist, the approaches we have used are cost-effective, reliable and well suited to detect both macroscopic and microscopic forms of *Sarcocystis* spp. aiding in effective monitoring of Sarcocystosis in sheep. To prevent the spread of Sarcocystosis, it is crucial to maintain good hygiene and sanitation in sheep farms, provide clean feed and water, control potential intermediate hosts, implement quarantine periods for new animals and regularly testing sheep for the presence of parasite. Public awareness about the risks of consuming undercooked or contaminated meat and proper cooking practices is also essential. It is essential to establish surveillance programs to monitor and detect cases of Sarcocystosis.

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