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# Seroprevalence of *Sarcosystis* spp. in Cattle and Buffaloes from the Wet and Dry Zones of Sri Lanka: a Preliminary Study

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With 3 figures and 1 table

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## Summary

An enzyme-linked immunosorbent assay (ELISA) was developed for the detection of antibodies to *Sarcosystis* infection in cattle and buffaloes. Crude antigens derived from cystozoites of *Sarcosystis cruzi* and antibodies from the sera of naturally infected cattle and buffaloes were used. The assay was validated by determination of the specificity, sensitivity and negative and positive predictive values. Using ELISA, a sero-epidemiological survey for sarcosystosis was carried out in cattle ($n = 215$) and buffaloes ($n = 123$) from the wet and dry zones of Sri Lanka. The prevalence of infection in cattle was 69.3%. Prevalence of the infection collectively in both cattle and buffaloes in the dry zone was significantly higher ($P < 0.001$) than that in the wet zone. The number of sero-positive cattle and buffaloes in the dry zone was significantly higher than that in the wet zone ($P < 0.05$). Buffaloes in the dry and wet zones showed a greater prevalence of infection ($P < 0.05$) than cattle.

## Introduction

Cattle and buffaloes are important in the agro-economy of Sri Lanka (Sri Lanka Livestock Statistics, 2000/2001). Cattle are widely used for milk and meat and buffaloes for milk and draught purposes. Sarcosystosis affects both the meat and milk industries adversely (Fayer et al., 1983; Dubey et al., 1989; Zuo, 1992).

Sarcosystosis has been recognized as an important parasitic disease of bovines and is one of the emerging zoonotic protozoan diseases (Kan and Pathmanathan, 1991). Human beings are the definitive host for *Sarcosytis suihominis* and *S. hominis* while pigs and cattle, respectively, are the intermediate hosts. The intermediate hosts acquire the infection by ingesting sporocysts from human faeces. Human beings become infected by eating raw or under-cooked pork or beef. Therefore, intestinal sarcosystiosis caused by these two species of *Sarcosystis* is a zoonosis (Levine, 1988).

The lifecycle of *Sarcosystis* involves two hosts where the definitive host is always a carnivore or an omnivore and the intermediate host a herbivore. Infection in the intermediate host is characterized by anorexia, diarrhoea, and reduced weight gain. Pregnant animals may produce premature stillborn foetuses (Fryer, Johnson and Lude, 1976). Microscopic lesions in the myocardium of the heart, liver, kidneys, brain, lung, spleen, and skeletal muscles have been described (Dubey et al., 1986). Although the acute infection by *Sarcosystis* species might cause disease or death in the intermediate hosts, the symptoms are not species specific (Gunawardena et al., 1996). Pregnancy, lactation, poor nutrition or other stresses may influence the severity of clinical sarcosystosis (Dubey et al., 1989). Sarcosystiosis is economically important, causing abortion, acute fatal illness or poor growth in cattle, sheep and goat (Dubey, 1976) and reduced weight gain by 25%, decrease in milk yield and abortion (Fayer and Elsasser, 1991). Moreover, acute sarcosystiosis can be fatal. Chronic sarcosystiosis is clinically not apparent. Therefore, early diagnosis of the infection is important. The classical method of diagnosing the infection is by visual inspection of meat. Visual observation of carcasses at the Colombo Municipal Abattoir in Sri Lanka is a routine procedure for identifying cysts and currently no other tests are employed. Microscopic examination of tissues at necropsy is also useful in diagnosis. However, diagnosis of acute infection by necropsy is inconclusive because of the absence of microscopic stages in tissues of the intermediate host (Dubey et al., 1989). Additionally, it is laborious, time consuming and trained personnel are required. Furthermore, conventional diagnosis of sarcosystiosis by examining muscle tissue for the presence of cysts or cystozoites is not suitable for use in large-scale screening programmes or for diagnosing infection in live animals. The development of a serological test, is therefore, an essential requirement for the diagnosis of sarcosystiosis (O'Donoghue and Rommel, 1992).

This study describes the development of an enzyme-linked immunosorbent assay (ELISA) to detect antibodies to *Sarcosystis* infection, and a preliminary study conducted on seroprevalence in cattle and buffaloes in the wet and dry zones of Sri Lanka.

## Materials and Methods

### Collection of sera samples

During November 1999, blood samples were collected by heart puncture of 22 slaughtered cattle from the Municipal Abattoir, Colombo. Carcasses of these animals showed heavy infections with *Sarcosystis* manifested by numerous cysts in the musculature. Serum was separated according to standard methodology (Hudson and Hay, 1989). Aliquots of serum samples were stored at −20°C until further use.

Blood was also collected from 76 other slaughtered cattle in which visual inspection did not reveal muscle cysts. A piece each from the heart muscle and oesophagus was also collected

 

from every carcass for histological examination (Bottner et al., 1987). Serum samples from these animals were stored as negative controls following histological examination confirmed by dot–blot analysis. Briefly, a solution of 100 $\mu$g/ml of antigen was prepared in dilution buffer (1% non-fat milk in washing buffer). Twenty-two 1 cm × 1cm squares of nitrocellulose (NC) paper were prepared. Ten microlitres of 10 $\mu$g/ml of cystozoid crude antigen solution was dispensed onto the centre of each square and kept for half an hour in an air-conditioned room for drying. The NC paper squares were blocked with 1% bovine serum albumin (BSA; Sigma Chemicals, St Louis, MO, USA) in dilution buffer for 1 h and washed three times with washing buffer [1% Tween 20 in phosphate-buffered saline (PBS)]. Serum samples were diluted 1 : 50 with dilution buffer and 5 $\mu$l from each sample was dispensed onto an antigen-coated NC paper, in duplicate. These were incubated for 1.30 h at room temperature and then washed three times with washing buffer. Five microlitres of peroxidase-conjugated antibovine IgG solution (Sigma Chemicals) at a dilution of 1 : 5000, was dispensed onto each NC paper square and incubated again for 1.30 h, washed three times and finally made to react with the chromogen substrate, 3-amino-9-ethyl-carbazole (AEC; Sigma Chemicals) for 10 min before washing with distilled water. A stained dot on NC squares indicated the antigen–antibody complex. Fetal calf serum (Sigma Chemicals) diluted in the same manner was used as a negative control. The test was optimized for this system and the optimum concentrations of reagents was as follow. 10 $\mu$l of 10 $\mu$g/ml, antigen, serum concentration 1 : 50, conjugated IgG concentration, 1 : 5000 and chromogen substrate. One per cent BSA (Sigma Chemicals) was selected instead of 5% skimmed milk (non-fat) (Anchor, New Zealand Milk Lanka, Biyagama, Sri Lanka). Of the 22 sera samples, 15 elicited reactions and were selected as known reference positive samples. The 36 sera samples collected from non-infected cattle were subjected to the dot–blot test. The sera samples that were not stained were selected as non-infected. Fifteen of 36 samples were selected as known reference negative samples. Those samples were confirmed negative by stained smears of pepsin-digested beef. Blood samples to be screened by the diagnostic ELISA test for the epidemiological survey were collected randomly from 338 animals from the wet and dry zone herds [cattle ($n = 215$; wet zone, 85 and dry zone, 130), buffalo ($n = 123$; wet zone, 31 and dry zone, 92)]. The wet zone receives an annual average rainfall of 2000 mm or above whereas those areas of the island with less than this belongs to the dry zone (Domros, 1994). Annual average nocturnal humidity between 85 and 90% exists island-wide; humidity during the day is between 70 and 80% (Domros, 1994). Annual average temperature of the dry zone lies between 26.5 and 28°C, whereas the values for the wet zone is below 26°C (Domros, 1994).

**Antigen preparation**

Sarcosysts of *S. hirsutei* were collected from heavily infected cattle from the Colombo Municipal Abattoir by visual observation. Heavily infected pieces of musculature were collected from the oesophagus, breast and thigh muscle and transported at 4°C in containers with ice. In the laboratory, these specimens were stored at 4°C. The extraction of cystozoite crude antigen was carried out using the method described by Savini et al. (1994), with modifications. Briefly, cysts were separated manually by fine forceps and placed in normal saline, washed and stored at 4°C until crude antigen was prepared. Cysts were ground manually and the mixture was sequentially filtered through 500, 250, 100 and 10 $\mu$m metallic sieves. The filtrate was centrifuged at 3500 ***g*** for 10 min and the pellet was washed with normal saline. The washed pellet was suspended in saline solution at a ratio of 1 : 3 and the pH adjusted to 7 with a solution of 10% sodium bicarbonate. The suspension was mixed at a ratio of 1 : 2 with isotonic Percoll solution (Pharmacia Fine Chemicals, St Louis, MO, USA) in distilled water and centrifuged at 2400 rpm for 10 min at room temperature. The pellet containing cystozoites was washed and suspended in normal saline. This was then frozen at −20°C, freeze–thawed four times, and ultrasonicated three times for 20 s each at 100 W. The suspension was centrifuged at 15 000 ***g*** for 30 min. The supernatant collected was used as the antigen. The purity of the prepared antigen was tested by immunoblotting (Sambrook et al., 1989) with peroxidase-conjugated antibovine IgG (Sigma Chemicals), and the bicinchoninic acid protein assay (Sigma Chemicals) quantified the protein content of this preparation. The selected cystozoite crude antigen preparation used for ELISA had a protein concentration of 1.528 mg/ml.

*Selection of reference positive and negative sera*

Serum samples to be used as reference negative were selected as follows: samples of musculature (oesophagus or heart) collected along with blood samples of apparently non-infected animals were digested with pepsin/HCl solution, filtered through 10 $\mu$m filter and then filtrate was washed twice with PBS 7.2 pH. Finally, a smear was prepared on a glass slide and stained with Giemsa. Smears were then examined for the presence of cystozoites of *Sarcosystis*. Samples without cysts were considered negative samples. Of such samples, those that were found negative on dot–blot were selected as negative controls. Sera samples from animals with visual cysts were stored to be used as positive control.

*Development of indirect ELISA for* Sarcosystis *infection using cystozoite crude antigen*

Checkerboard titrations were performed to determine the optimal concentrations of reagents and buffers to give maximum discrimination between known reference positive and negative sera samples.

The ELISA was performed as originally described by Engvall and Perlmann (1972). Hundred microlitres of a 10-$\mu$g protein/ml solution of crude antigen diluted in coating buffer (carbonate–bicarbonate buffer, pH 9.8) was used to coat the surface of 96-well microtitre flat bottom plates (Nunc, Intermed, Roskilde, Denmark). The plates were incubated overnight at 4°C. The contents of the wells were decanted and the plates were flick-washed with washing buffer. Thereafter, each well was filled with 100 $\mu$l of 1% rabbit serum with 1% Tween–PBS as blocking buffer. Plates were incubated for 1.30 h at 37°C, flick-washed and each well was filled with 100 $\mu$l of 1 : 50 test sera in duplicate. Plates were again incubated for 1.30 h at 37°C. Positive and negative reference sera were used as controls. In addition, foetal calf serum was used as an additional negative control. Plates were

flick-washed and freshly prepared goat antibovine peroxidase-conjugated antibody (Sigma Chemicals), at a dilution of 1 : 5000 in dilution buffer was dispensed at 100 $\mu$l per well and incubated for 1.30 h at 37°C. Plates were washed three times with PBS–Tween and once with substrate buffer. Two hundred microlitres of freshly prepared enzyme substrate 3,3′,5,5′-tetramethyl-benzidine dihydrochloride (TMB) in substrate buffer (pH 6) with 0.2% $H_2O_2$ were added. The colour reaction was allowed to proceed for 10 min and stopped by adding 50 $\mu$l of 2 M $H_2S0_4$ to each well. Optical density (OD) was read at 405 nm using an ELISA reader (Cambridge Life Science, Cambridge, UK). Antigen, sera and conjugate controls were included in duplicate in each plate.

A cut-off value for discriminating a negative from a positive reaction on the developed ELISA was defined by adding three standard deviations to the mean OD value of 15 reference negative sera samples thus tested. Test sample OD values higher than this cut-off were considered positive (Deplazes et al., 1990).

A test run was performed with 15 positive and negative sera samples each, to evaluate the ELISA. The results obtained were used to calculate the specificity, sensitivity and, the positive and negative predictive values of the indirect ELISA (Griner et al., 1981).

*Epidemiological survey*

Prevalence of seropositive *Sarcosystis* spp. was studied in cattle and buffalo in the wet and dry zones of Sri Lanka. The proportions of positive animals in each group [wet zone cattle, dry zone cattle, wet zone buffaloes, dry zone buffaloes, cattle in wet and dry zones, buffaloes in wet and dry zones, wet zone livestock (cattle and buffaloes)], dry zone livestock was determined. Differences between proportions were statistically compared as described by Runyon and Habes (1982).

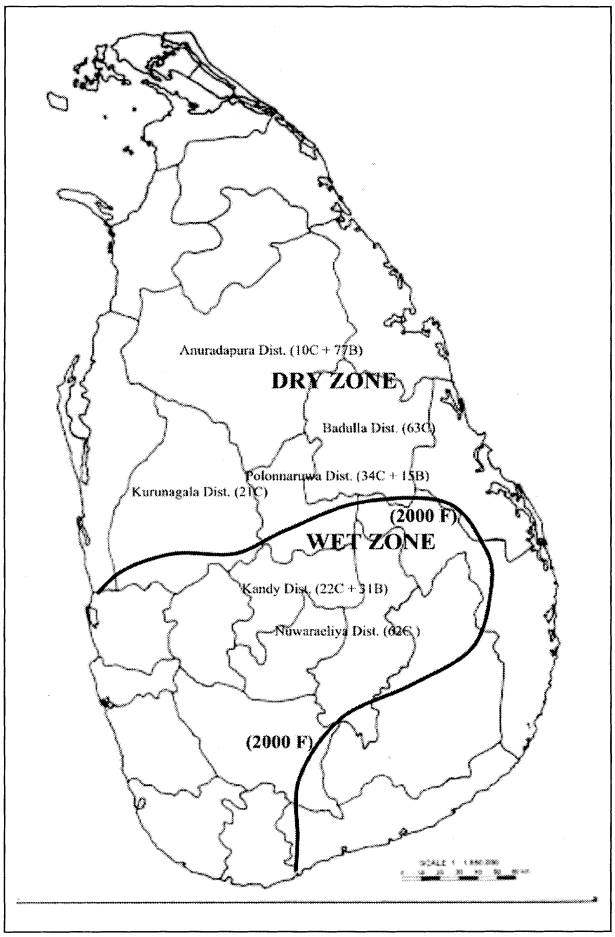


Fig. 1. Showing a two-climatic zone of Sri Lanka. Name the districts where samples were collected from buffaloes (B) and cattle (c).

## Results

Of the 22 known positive sera samples, 15 were randomly selected and confirmed as *Sarcosystis*-positive by the dot–blot test. Of the 76 sera samples collected from animals assumed to be negative, 36 were randomly selected of which 15 were confirmed as negative samples by screening for microscopic cystozoites followed by dot–blot assay.

The results of the preliminary assay performed on the 15 negative sera yielded a mean OD value of 0.179 ± 0.0277 at 405 nm (Fig. 1). Hence, the cut-off value for this ELISA was set at 0.262. This indirect ELISA diagnostic test for *Sarcosystis* infections had 100% specificity, sensitivity, and negative and positive predicted values (Griner et al., 1981).

For the epidemiological survey of sarscocystosis, 215 sera samples from cattle (85 from the wet zone and 130 from the dry zone), and 123 samples from buffalo (31 and 92 from the wet and dry zones, respectively) were collected from areas from the dry zone and areas from the wet zone (Fig. 2). The mean OD values of duplicates of each sera sample screened by the developed ELISA are presented in Fig. 3. Among the cattle tested, 48 samples (56.5%) and 93 samples (71.5%) from the wet and dry zones, respectively, were found positive for sarcosystiosis (Fig. 3; Table 1). Regarding the buffalo samples, 19 of 31 samples (61.3%) from the wet zone and 74 of 92

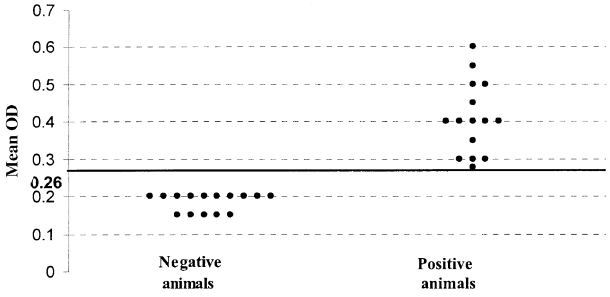


Fig. 2. Plot of OD value of known positive and negative sera showing cut off point of ELISA titre.

(80.4%) from the dry zone, screened positive by this ELISA (Fig. 3; Table 1). Both cattle and buffaloes, 67 (57.6%) originating from the wet zone and 167 (75.6%) from the dry zone tested positive (Fig. 3; Table 1).

Statistical analysis (Runyon and Habes, 1982) showed that the prevalence of sarcosystiosis in bovine in the dry zone (75.2%) was significantly higher ($P < 0.001$) than in the wet zone (57.8%). Collectively, prevalence of sarcosystiosis in buffaloes in both the wet and dry zones (75.6%) was significantly greater ($P < 0.05$) than in cattle (65.6%). When

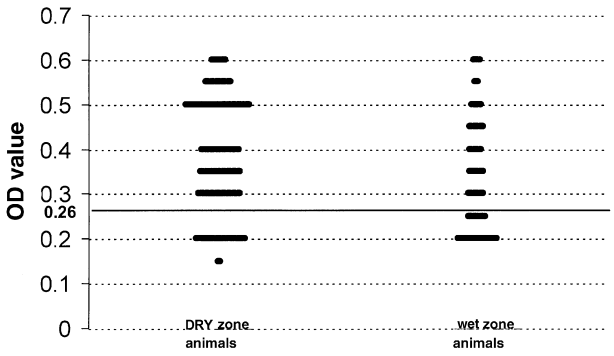


Fig. 3. Showing a OD value of the animals in wet zone and dry zone animals.

Table 1. Sero-prevalence of ELISA antibodies to *Sarcosystis* in cattle and buffaloes in Sri Lanka test group

| Test group | No. of sera samples screened | No. positive | Infection prevalence |
|---|---|---|---|
| Wet-zone cattle | 85 | 48 | 56.5 |
| Wet-zone buffaloes | 31 | 19 | 61.5 |
| Dry-zone cattle | 130 | 93 | 71.5 |
| Dry-zone buffaloes | 92 | 74 | 80.4 |
| Cattle in wet and dry zones | 215 | 141 | 65.6 |
| Buffaloes in wet and dry zones | 123 | 93 | 75.6 |
| Wet zone livestock (cattle and buffaloes) | 116 | 67 | 57.6 |
| Dry zone livestock (cattle and buffaloes) | 222 | 167 | 75.6 |

buffaloes and cattle were considered separately, there was a significantly higher percentage of infection ($P < 0.05$) in dry-zone animals [buffaloes (80.4%) and cattle (71.5%)] than in wet-zone animals [buffaloes (61.3%) and cattle (56.5%)].

## Discussion

In general, trained personnel, expensive chemicals and reagents, specialized testing plates and ELISA readers are required to perform ELISA accurately. The occurrence of aberrant readings caused by plate-to-plate and well-to-well variation should be minimized using optimum techniques.

When a new diagnostic assay is developed, methods must be found to accurately evaluate its performance. A common method of evaluation is to simply compare the results of the new assay with that of a reliable one. This was accomplished here by comparison of results obtained from the developed indirect ELISA with those ensuing from microscopic observations and the dot–blot assay. A 100% specificity and sensitivity was obtained for the developed ELISA using 15 each of reference negative and reference positive sera samples. Foetal bovine serum too served as an additional negative control. However, it would be more appropriate to increase the sample size of both reference negative and positive sera samples and to use a battery of bovine sera from a country free of bovine sarcosystiosis as negative controls.

Cystozoites in the cysts in muscle are restricted to the chronic infection stage (Dubey et al., 1983). Therefore, cystozoite antigens are suitable only for diagnosis of the concealed chronic infection. Therefore, the use of cystozoite crude antigen in the present study is justifiable for developing a diagnostic ELISA for the detection of chronic infections.

Savini et al. (1994) reported that antigens prepared with merozoites are more suitable for the diagnosis of early acute infection and that it was more sensitive than other antigen-coated ELISA. They also showed that cross-reactions occur, when *S. cruzi*-infected cattle serum was tested against *S. tenella* and *S. arieticanis* and it was found that merozoite antigen was more specific to the species. In Sri Lanka, all three species of *Sarcosystis* (*S. cruzi*, *S. hominis* and *S. hirsute*) have been identified in cattle by the cyst morphology (Gunawardena et al., 1996). The developed ELISA was aimed at diagnosis of *Sarcosystis* as a genus. Further studies are required to recognize if there are cross-reactions of cystozoite antigens with other closely related genera such as *Toxoplasma gondii*. However, Savini et al. (1994) has proved that very marginal cross-reactions occur with other related parasites such as *T. gondii* and *N. caninum*, and it can be precluded by using a threefold higher value than the negative reading.

When the protocol originally described by Savini et al. (1994) was used for the preparation of cystozoite crude antigen, host tissue contamination could not be avoided. When bovine tissues contaminate parasite antigen used in ELISA, anti-bovine antibodies conjugated with peroxidase bind non-specifically and yield false-positive results. Therefore, the cysts were manually extracted and were washed to remove contaminating host tissue. The metallic sieve of pore size 250 $\mu$m that Savini et al. (1994) used allowed cyst wall particles to be filtered. In this study, antigens were prepared using a 10-$\mu$m sieve as the final filter, which allowed only cystozoites (bradyzoites) to be filtered. Selection of a 10-$\mu$m sieve was based on the size of individual cystozoites (8–10 $\mu$m in length) measured microscopically. It may be easy to identify 'sero-negative' animals to be used as reference negative controls, in countries where a particular disease has never been recorded. This may not work truly for countries that have endemic diseases. In such conditions, the best assessment of negative animals is by using factors such as other serological test results, knowledge of clinical history of animals, epidemiological factors and so on (Crowther, 1995). In the present study, the factor determining sero-negativity of non-infected animals was based on histological and serological confirmation using microscopic examination of tissues and dot–blotting, respectively.

As the present epidemiological study using the *Sarcosystis* diagnostic ELISA was based on cystozoite crude antigen, this survey concentrated on the secondary prevention level, which occurs after a detectable pathological process. Hence, it is concluded that the positive animals represent diseased animals that are in the post-latent period. Therefore, the true prevalence would probably be greater that than reported here.

The overall prevalence of infection in the dry zone (75.2%) being significantly higher ($P < 0.05$) than that of the wet zone (57.8%) is suggestive of the influence of climatic conditions in the development of the infection. A significantly higher prevalence of the infection in buffaloes in the dry zone over those in the wet zone further indicates that climatic conditions could influence the establishment of infection in buffaloes.

A diagnostic ELISA for *Sarcosystis* infection in cattle and buffaloes was developed and sero-epidemiologically tested, corroborating the reliability of this serological method as an alternative option in diagnosing *Sarcosystis* in bovids of Sri Lanka. However, this baseline study may be improved further to confirm the validity of this assay using a battery of bovine sera from a sarcosystiosis-free country, using larger sample sizes from different climatic zones to evaluate this method further and finally the use of purified *Sarcosystis* antigen for the assay. The ELISA test described in the paper provides an accurate and reliable laboratory procedure for the diagnosis of sarcosystiosis of cattle and buffaloes.

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