EVALUATION OF AN ELISA FOR THE DIAGNOSIS OF SARCOCYSTOSIS IN WATER BUFFALOES

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Abstract

To investigate the occurrence of sarcocystosis in water buffalo (Bubalus bubalis) in Ahvaz, the Khuzestan province, Iran, and to evaluate an ELISA for the diagnosis of sarcocystosis, serum and oesophagus samples were collected from the 300 water buffaloes, aged 0.5-7 years, and then slaughtered at the Ahvaz abattoir. The oesophagus samples were examined for sarcocysts under microscopic examination, using the digestion method. One hundred and seventy-one (57%) animals were found to be positive for Sarcocystis bradyzoites. One hundred and sixty-three (54.3%) serum samples were positive for sarcocystis antibodies in the ELISA. The prevalence of sarcocystosis was statistically age related, with significantly higher rates in adult buffaloes than young animals (P<0.05). The prevalence did not differ significantly in relation to the gender (P>0.05). The Mc Nemar test revealed a high correlation (94%) between the digestion method and ELISA. The ELISA, with the use of antigens from S. fusiformis, as presented in this study, can be adapted to detect antibodies to Sarcocystis sp., with an acceptable specificity and sensitivity.

Key words: water buffalo, Sarcocystis, sarcocystosis prevalence, Iran.

Sarcocystosis is a zoonotic disease in domestic animals caused by Sarcocystis sp., a cyst-forming coccidian parasite with obligatory two-host life cycle involving carnivorous as definitive hosts and herbivorous or omnivorous as intermediate hosts. Each intermediate and definitive host may harbour more than one Sarcocystis species (5).

The specimens type of at least four species of Sarcocystis have been documented and described in the water buffalo, including two macroscopic species: S. fusiformis, commonly occurring in south-east Asia, and S. buffalonis, and two microscopic species: S. levinei and S. dubeyi (2). Out of these Sarcocystis species infecting domestic animals, a given species usually infects only one species of intermediate host and uses either felids or canids, but not both, as its definitive host (11). Some species of Sarcocystis can cause a reduced weight gain, poor feeding efficiency, anorexia, fever, anaemia, muscle weakness, reduced milk yield, abortion, and death of intermediate hosts such as cattle, sheep, and goats. Certain species can infect humans and can cause digestive disturbances such as nausea, vomiting, and diarrhoea (6). In Iran, S. fusiformis has been reported as commonly occurring in muscles of the oesophagus, throat, and limbs in water buffaloes with cats as the most likely susceptible natural definitive host (4).

In the present study, the ELISA, with a crude antigen prepared from cystozoites of S. fusiformis, was used to study the seroprevalence of sarcocystosis in water buffaloes in Ahvaz. The study also assesses the value of the test in comparison to the digestive method for the diagnosis of sarcocystosis in buffaloes naturally infected with this parasite.

Material and Methods

Three hundred water buffaloes, aged 0.5-7 years, slaughtered at the Ahvaz abattoir, Khuzestan, Iran, were used in the survey for the presence of sarcocysts. The blood samples were collected during slaughter and their sera were separated and then divided into aliquots and stored at -20°C until tested. The oesophagus muscles of the slaughtered animals were placed in properly labelled plastic bags, and then transported to the Parasitology Laboratory of the Veterinary Faculty within 3 h. The muscle samples were kept in the refrigerator prior to the examination for sarcocysts. The presence of the sarcocysts was evidenced through a macroscopic of the muscles and microscopic examination of the digested muscle samples. For the digestion, muscle tissue (50 g) was ground and incubated in 10 volumes of HCL-pepsin solution for 18 h at 37°C (6). The digested material was examined directly after centrifugation at 1 500 rpm for 5 min.
To detect antibodies against *Sarcocystis* sp., an ELISA protocol, described by Savini *et al.* (12) for *S. cruzi* in cattle, with some modifications, was used. Briefly, soluble antigen was obtained from large cysts of *S. fusiformis* by grinding in PBS (pH=7.2), freeze-thawing 6 times, sonication 2 times, each of 20 s, and centrifugation at 15000 rpm for 30 min. Microtitre plates (Nunc) were coated with *S. fusiformis* soluble antigen (15 µg/mL) and kept overnight at 4°C. The plates were blocked by 5% skim milk (Merck) containing 0.05% Tween 20 (Merck) for 2 h at 37°C. Serum samples diluted at 1:100 were added and incubated for 60 min at 37°C. Rabbit anti-bovine immunoglobulin, labelled with horseradish peroxidase (Sigma), was used as enzymatic conjugate at 1:300 dilutions in 5% skim milk and incubated once more for 60 min at 37°C. The reaction was revealed with a solution of enzymatic substrate and chromogen consisting of 0.03% H$_2$O$_2$ (Sigma) and ABTS (Merck) in citrate-phosphate buffer (pH 5.5). The optical density (OD) was determined in a microplate reader (Dynatech, Netherlands) at 405 nm. Positive and negative control sera were included in each assay and obtained by mixing equal volumes of sera from 50 buffaloes with the known results in gross inspection, digestion examination, and previously determined serology (dot-ELISA). The cut-off was calculated as the mean OD values from 50 negative controls plus two standard deviations. The samples were considered seropositive when the OD value of sample was ≥OD of cut-off.

Statistical analysis consisted of the determinations of means with a 95% confidence interval of the antibody levels present in the animal serum samples. Seropositivity rates found for sarcocystosis were analysed in relation to the animal data, such as age and gender, by using the differences between two proportions by Chi square test. The values of P<0.05 were considered as statistically significant.

**Results**

The results of macroscopic examination revealed that 60 (20%) of the 300 buffaloes were infected with *S. fusiformis*. Bradyzoites, and were detected in 57% by the digestion method, and *Sarcocystis* antibodies were detected by the ELISA in 54.6% of the examined buffaloes. It is evident that the results of the macroscopic examination differed significantly from those of the microscopic examinations (P<0.05), and those of the ELISA (P<0.05), whereas no significant differences were observed between the results of the microscopic examinations, and those of the ELISA (P>0.05). The highest prevalence (44.9%) of macroscopic cysts was found in the buffaloes above 6 years and the lowest (2.5%), one in those below 6 months of age. The overall prevalence of sarcocystosis in any assays increased with age (P<0.05) but it did not depend on gender (P>0.05).

Macroscopic cysts, measuring 12-18 mm long and 1-2 mm across, were commonly seen in the oesophageal muscles as fusiform, milky white, opaque bodies, lying between the muscle bundles, and were easily removed from the muscle mass.

When the sera from 50 negative buffaloes were tested, in order to determine the cut-off values of the ELISA, they yielded a mean absorbance of 0.154 (SD=0.019) at 405 nm; therefore, cut-off levels were considered 0.192 ( X + 2SD) for the ELISA. In comparing the results of the ELISA with those of digestion method, similar results were obtained in 282 cases (158 true-positive and 124 true-negative), whereas contradictory results were observed in 18 cases (5 false-positive and 13 false-negative). The accuracy, sensitivity, and specificity of the ELISA were calculated to be 94%, 97%, and 90.5%, respectively. The Mc Nemar test revealed a strong correlation (94%) between the digestion method and ELISA.

**Discussion**

Conventional methods of diagnosing *Sarcocystis* sp. infections, involve time consuming and labour-intensive examinations of host muscle tissue for the presence of cysts or cystozoites. Such techniques are neither suitable for use in large-scale screening programmes, nor for use in diagnosing infections in livestock. Therefore, serological tests detecting specific antibodies to *Sarcocystis* sp. have been assumed to be important in the diagnosis of sarcocystosis (13). Few attempts have been made to evaluate the accuracy of these tests in diagnostics of sarcocystosis in buffaloes. In this study, antigens derived from bradyzoites of *S. fusiformis* were utilised in ELISA procedure. To the best of our knowledge, this is the first study for the evaluation of the ELISA for detecting antibodies to bradyzoites of *S. fusiformis* in naturally infected buffaloes. Our study revealed a good correlation between the results of the ELISA and pepsin digestion technique in the determination of sarcocystosis in water buffaloes. Out of the 163 ELISA-positive samples, 5 samples were negative by digestion analysis, which was most likely due to early stage of infection in these animals.

In the present study, the high prevalence rate of infections, according to microscopic examination (57%) and ELISA (54.3%), indicates the importance of the infection for the intermediate host. The high rates of *Sarcocystis* infection in the water buffalo have also been found in other Asian countries, including Iraq (9), Vietnam (7), Sri Lanka (8), and the Philippines (3), where 99.3%, 79%, 69.3%, and 65% of the analysed buffaloes; respectively, were positive for sarcocystosis. Antibodies to *Sarcocystis* were also detected in 88.6% of buffaloes from Iraq by IFAT (9). In our study, the prevalence of sarcocystosis in water buffaloes showed increasing prevalence with the age of the host, which agreed with the previous findings in other animals (2, 10). This is most likely due to a longer exposure to the infection. However, in agreement with a study of Abo-Shehada (1) on sheep and goats (1), no influence of the
gender on the prevalence of this infection in buffaloes was observed. In conclusion, the ELISA, as presented in this study, can be adapted for the diagnosis of buffalo sarcocystosis, with an acceptable specificity and sensitivity.

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References