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Immunological Investigation of Sarcocystosis in Cattle of Basrah Province / Iraq

Abstract

Sarcocyst infection rates in slaughtered cattle were the focus of this investigation. In 300 cattle esophageal muscles and serum samples, the ELISA test was utilized to identify Sarcocystis antigens and Sarcocystis IgG antibody. ELISA results for sarcocystis antigens were positive in 160 cattle (53.3%). According to the anti-sarcocystis IgG ELISA, 182 cattle (60.7%) had positive results. The statistical significance of differences between these two ELISA test results was not established (P>0.05). According to the cattle's age, Higher positive sarcocystis antigens- ELISA results were detected in 138 older cattle (61.3%) than in 22 younger cattle (29.3%). The mean optic density (OD) values of sarcocystis antigens were higher in older animals (1.204±0.296) than in younger animals (0.576±0.207). There is a statistically significant (P<0.05) association between the mean of OD values and age groups. Anti-sarcocystis IgG antibody detection by ELISA revealed that the percentage of Sarcocystosis rose with age, with a remarkable 66.7% of Sarcocystosis in older animals compared to 42.7% in younger ones. The mean OD values were higher in older cattle (1.297 ± 0.480) than in younger cattle (0.490 ± 0.182). A statistically significant (P<0.05) correlation is found between age groups and the mean of OD values. The results of the two types of ELISA tests showed a significant seasonal difference(P<0.05) in the distribution of Sarcocystosis in cattle. Sarcocystosis was most common in the spring, with a 70.5% percentage in both ELISA tests based on sarcocystis antigens and anti-sarcocystis IgG.

Key words: Cattle Sarcocystosis, ELISA, Antigen, Antibody

Article Info.

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Introduction

Red meat consumption is on the rise worldwide since meat serves as many consumers' main source of animal protein. Therefore, abattoirs are essential for managing and controlling a variety of zoonotic infections, such as *Sarcocystis* , a parasite that causes foodborne illnesses that people can get by eating raw or undercooked meat (1).

Significant *Sarcocystis* species use cattle as intermediate hosts. Currently, a total of eight species have been identified as causing infections in cattle: *S. cruzi*, *Sarcocystis hirsuta*, *S. bovifelis*, *S. hominis*, *S. bovini*, *S. heydorni*, *S. sigmoideus*, *and S. rommeli* (2; 3). Although there is a considerable amount of information about sarcocystosis, it remains incomplete and requires further research. For food producers, the spread of bovine sarcocystosis is a financial burden due to the disease's ability to reduce livestock productivity(4). To address the problems of food insecurity and malnutrition, more oversight is required in the domains of veterinary parasitology and food biotechnology.

A connection between sarcocystosis and inflammatory processes of striated muscles called bovine eosinophilic myositis is indicated (2). Although myositis does not have clinical manifestations, it is detected after cutting the carcass. Agriculture suffers financial losses since it is impossible to sell meat from an infected animal. In the acute phase of infection, clinical symptoms and decreased performance are reported in domestic animals, particularly those that are infected with *S. cruzi* (5). After a postmortem inspection, sarcocystosis is usually detected by examining the muscle tissues of the diaphragm, tongue , and esophagus of infected animals for its macroscopic appearance (6). Sarcocystosis is difficult to diagnose antemortem because it can be asymptomatic or mimic other illnesses. According to (7), it has subclinical or chronic courses, characterized by anorexia, muscle weakness, generally low meat productivity, and occasionally even death. Time-consuming methods include the indirect fluorescent antibody test (IFAT), the Dot-blot test, and the histological analysis of muscle biopsies. Furthermore, even with specialized equipment, accurately diagnosing a majority of animals before slaughter is challenging (8). Numerous serological assays can quantify Sarcocystis antibodies, including the ELISA (9), dot-ELISA (10). The sensitivity and specificity of these tests might fluctuate according to the antigenic types, but they have been shown to be simple and precise. Since ELISA is simple to use, widely accessible, and requires little equipment or work, it is frequently employed for detecting antibodies against a wide variety of pathogens (11–16). Despite being widely utilized, the ELISA based on *Sarcocystis spp*. cystozoites have a number of disadvantages, including antigenic cross-reactivity and limited sensitivity (17). The cyst walls regarding pathogenic protozoa include large amounts of glycolipid glycosylphosphatidylinositol as well as glycoproteins (18). Because such glycans are often immunodominant, they could be utilized to identify parasites including *Sarcocystis neurona*,

Babesia bovis, and *Neospora caninum* (19). The present work's objective was to use ELISA to identify Sarcocystis antigens as well as antibody against Sarcocystis antigens in samples of esophageal muscle and cattle sera.

Materials and Methods

Ethical approval

The research ethics committee of the University of Basrah Veterinary College conducted this study in compliance with international standards for the use and care of animals.

Study animals

To determine the risk factors and distribution for Sarcocystis infection as cattle age and monthly distribution, 300 cattle (*Bos taurus*) were screened using immunological and macroscopic cyst tests. At Basrah University, each sample was analyzed in the Central Research Laboratory of the Veterinary College.

Sampling

The survey for the presence of Sarcocystis infection in three hundred cattle, aged one to seven years, was used. These animals were slaughtered at the Qurna abattoir in Basra, Iraq, between January and November of 2024. A total of five milliliters of blood have been extracted throughout the slaughter process, and the sera were separated, sorted into aliquots, and stored at a temperature of -20°C until testing. Plastic bags labeled appropriately were used to hold samples regarding the animals' esophageal muscles. The muscle samples were refrigerated before being examined for macrocystis.

Macroscopic cyst examination

The obtained esophageal muscles samples were examined closely with the unaided eye. As demonstrated by (20), each muscle tissue was cut into tiny pieces (3-5 mm) using a sterile blade in order to view macroscopic cysts.

***Sarcocystis* antigens detection by ELISA**

Bovine Sarcocystis ELISA kit (Sun Long Biotech; China) was used for the qualitative determination of Sarcocystis in Bovine muscle samples. Bovine Sarcocystis ELISA was used in accordance with the instructions that are provided by the manufacturer.

Preparation of tissue samples

PBS (pH 7.4) has been added to the tissue samples, and they were then homogenized. It is recommended to operate samples at 4°C. Centrifugation was performed for 20min at 2,000rpm–3,000rpm, and the supernatant was collected carefully. For the ELISA test, the supernatant was separated.

ELISA procedure

To put it briefly, the appropriate micropores regarding the sample have been counted in the antibody-coated microplate. One empty well served as a blank control, 2 wells served as a positive

control, and 2 wells served as the negative control. 50µl of each of the positive and negative controls was applied to the corresponding control wells. 10µ of sample and 40µ of sample dilution buffer were added to the sample wells and gently shaken to mix. Following sealing the plate with the Closure plate membrane, it has been incubated for 30min at 37ºC. Three hand washes with the wash buffer were performed on the plate. Except for the blank control well, every well received 50µl of HRP-Conjugate reagent. The wells have then been incubated at 37°C for 30 minutes. As before, the wells have been cleaned. Each well received 50µl of Chromogen Solution A and 50µl of Chromogen Solution B. The mixture was gently shaken and incubated for a period of 15min at 37ºC. 50µL of stop solution has been used to halt the chromogenic process, and a plate reader (Micro ELISA auto reader, Biotech, USA) was used for reading the optical density readings at 450 nm. The next computation was used for determining ELISA results: The average value regarding negative control + 0.15 is the crucial value (CUT OFF). If the OD value is < CUT OFF, making a negative judgment; if the OD value is ≥ CUT OFF, making a positive judgment.

Detection of anti *Sarcocystis* IgG antibody

Parasite collection and antigen preparation

To isolate macroscopic form of S. fusiformis, fresh tissue samples from the esophageal muscles regarding the infected buffaloes have been dissected. To get rid of the associated skeletal tissues, the cysts have been washed three times using sterile phosphate-buffered saline (PBS) at pH 7.2. A sterile scalpel was used to cut the cysts into a cream-colored suspension. A manual glass homogenizer was then used for homogenizing the mixture in PBS, and the mix was centrifuged for 20min at 13,000rpm and 4ºC. After being aliquoted, the *S. fusiformis* whole cyst antigen preparation has been kept at a temperature of -20ºC. With the use of the next equation, the protein concentration was determined spectrophotometrically at 260 and 280 Nm in accordance with Hudson and Hay's approach (21): Mg/ml of protein equals 1.55×A 280-0.77 × A260.

ELISA procedure

With slight changes, *S. fusiformis* whole cyst crude antigen was used to analyze the diagnostic potential of this antigen regarding in diagnosing bovine sarcocystosis using an IgG-based ELISA test(22). Checkerboard titration has been used to find the ideal antigen concentration as well as serum dilution levels. In short, 40 µg/ml of *S. fusiformis* entire cyst antigen was applied to polystyrene ELISA plate with a flat bottom. The plate has been incubated at 4°C for the entire night before being rinsed three times. After adding the serum samples at a 1:100 dilution (100µl/well), they have been incubated at a temperature of 37°C for 90 minutes. Following washing, 100 µl/well of anti-bovine horseradish peroxidase conjugate (1:1000) was applied to the designated wells as well as incubated for one hour at 37°C (Sigma Chem. Co. USA). An ELISA reader (Bio Tek, Germany) was used to read the plates at 450 nm following the addition of the substrate, ortho-phenylenediamine (Sigma Chem. Co., USA). Through combining equal quantities regarding sera from 10 cattle with the known results in ELISA based antigens, negative

and positive control sera were added in each assay. The mean OD values from ELISA negative controls depending on Sarcocystis antigens plus two standard deviations were used to determine the cut-off. In the case when a sample's OD value was more than the cut-off, it was deemed seropositive.

Statistical analysis

To evaluate the correlation between data, the chi-square and t-tests have been used, with a significance threshold of 5%. SPSS software (version 11) was used for analyzing the data.

Results

Macroscopical examination

A total of 300 cattle slaughtered in the Qurna slaughterhouse had their esophageal muscle samples examined under a microscope, and the results showed no obvious cyst infection.

Detection of S*arcocystis* antigens

Table (1) displayed the results of the ELISA used to detect Sarcocystis antigens and anti-Sarcocystis IgG antibody. ELISA results based on sarcocystis antigens showed that 160 out of 300 cattle (53.3%) tested positive for sarcocystis infection. Anti-sarcocystis IgG antibody-based ELISA results showed that 60.7% of the cattle (182/300) were infected with sarcocystis. *Sarcocystis* infection percentage differences between the two ELISA types weren't considered statistically significant (P>0.05).

According to the cattle's age, the distribution of Sarcocystosis infections was displayed in Table (2). ELISA results showed that the percentage of sarcocystis infection rose as animals aged, with a higher percentage of sarcocystis infection (61.3%) in older animals than in younger ones (29.3%). Older animals had a higher mean for the OD values of sarcocystis antigens (1.204±0.296) than younger ones (0.576±0.207). A statistically significant (P<0.05) correlation is found between age groups and the mean of OD values.

Anti-sarcocystis IgG antibody detection by ELISA

The IgG-ELISA antibodies cut-off values were determined by testing the sera of 12 control cattle with sarcocystis antigens-based ELISA negative results. The results showed a mean absorbance of 0.044 (SD=0.028) at 405nm; thus, cut-off levels have been taken into consideration at 0.1. Sarcocystosis prevalence in relation to age in screened cattle revealed that the percentage of sarcocystis infections rose with age, with a remarkable 66.7% of sarcocystis infections in older animals compared to 42.7% in younger ones. The mean OD values were higher in older cattle (1.297 ± 0.480) than in younger cattle (0.490 ± 0.182). A statistically significant (P<0.05) correlation is found between age groups and the mean of OD values. (Table 3)

Table (1): Detection of Sarcocystis antigens and anti-sarcocystis IgG antibody by ELISA.

Examined No.	Sarcocystis infected N.	%	Sarcocystis non-infected N	%	Chi-squared	95% Confidence Intervals	P-value
300	160 182	53.3 60.7	140 118	46.7 39.3	1.89	-3.07 to 17.69	0.168

Table (2): Optical Density (OD) values of Sarcocystis antigens-based ELISA according to age of 160 infected cattle

Age	Sarcocystis infected N.(%)	Mean ±SD	95% Confidence Intervals	t-statistic	P-value
<2year	22 (29.3)	0.576 ± 0.207	0.503 to 0.757	9.81	< 0.0001
≥2 year	138 (61.3)	1.204± 0.296			

Table (3) Optical Density (OD) values of anti-sarcocystis IgG antibody-based ELISA according to age of 182 infected cattle.

Age	Sarcocystis infected N.(%)	Mean ±SD	95% Confidence Intervals	t-statistic	P-value
<2 year	32(42.7)	0.490 ± 0.182	0.637- 0.977	9.351	0.0000
≥2 year	150(66.7)	1.297 ± 0.480			

Monthly and seasonal distribution of *Sarcocystis* infection

Monthly and seasonal distribution of *Sarcocystis* infection was determined according to the results of two different ELISA tests based on anti-sarcocystis IgG antibody and sarcocystis antigens. Comparison between the results of these two tests revealed that significant seasonal difference in the distribution of *Sarcocystis* infection in examined cattle (Chi-square: 25.49; degrees of freedom: 7; p-value: 0.0006) (Table 4).

According to the findings of the Sarcocystis antigen-based ELISA, the largest proportion of cattle infected with *Sarcocystis* was found in February (83.3%), April (81.5), and November (81.1%). In contrast, September had the lowest *Sarcocystis* infection rate (25%). According to the seasonal distribution of *Sarcocystis* infection, the rate of prevalence has been highest in the spring (70.5%)

and lowest in the summer (36.2%). A statistically significant seasonal difference was observed (p-value: 0.0047; degrees of freedom: 3; chi-square: 12.98) (Table 4).

The most frequent months for Sarcocystis-infected cattle's ELISA results based on anti-sarcocystis IgG antibody were February (83.3%), April (81.5%), and November (75.7%). The lowest Sarcocystis infection rate has been recorded in September (15%). The seasonal distribution of *Sarcocystis* infection showed that spring had the maximum prevalence rate at 70.5%, followed by winter at 63.5%. The summer season rate was the lowest (42.7%). There has been a statistically significant difference among the seasons (p-value: 0.026; degrees of freedom: 3; chi-square: 9.265) (Table 4).

Table (4) Seasonal and monthly distribution of *Sarcocystis* infection among cattle according to ELISA results.

Months	Examined N.	Sarcocystis antigens ELISA	%	Anti-sarcocystis IgG antibody-ELISA	%
January	37	12	32.4	17	45.9
February	30	25	83.3	25	83.3
March	29	14	48.3	19	65.5
Winter	96	51	53.1	61	63.5
April	27	26	81.5	22	81.5
May	22	13	59.1	13	59.1
June	12	4	33.3	8	66.7
Spring	61	43	70.5	43	70.5
July	12	4	33.3	7	58.3
August	15	8	53.3	10	66.7
September	20	5	25	3	15
Summer	47	17	36.2	20	42.7
October	35	9	25.7	16	45.7
November	37	30	81.1	28	75.7
December	24	10	41.7	14	58.3
Autumn	96	49	51.7	58	60..4
Total	300	160		182	

Discussion

One of the most prevalent parasites affecting a variety of livestock, *Sarcocystis* parasites are apicomplexan protozoan organisms that could lead to serious disorders in certain hosts, including cattle (23). Additionally, because certain parasite species produce clinical symptoms, they are significant for public health. The results of the present investigation demonstrated that no obvious cysts were found in the screened cattle esophagus muscle samples. The current result was supported by earlier research (5;, 13). In contrast, one research conducted in Iraq (24) reported the observation of macroscopic sarcocysts in the esophagus of cattle. In addition, a small number of macroscopic sarcocysts were discovered in Iranian(25) and Egyptian cattle(26 The lack of macroscopic cysts in this study was previously explained by (27), who noted that cattle are killed before the cysts mature and that Sarcocystis species macrocysts take years to develop. *S. fusiformis* antigen was utilized in this research to diagnose cattle sarcocystosis by ELISA since it has been

demonstrated that *Sarcocystis* species of cattle could infect buffaloes (28) and that the macroscopic *S. fusiformis* of buffaloes produces a significant amount of antigen that is difficult to obtain from microscopic cysts of cattle. The detection of antibodies against *Sarcocystis fusiformis* using an enzyme-linked immunosorbent test was used in a number of investigations to diagnose *Sarcocystis* species infection (29-31;12). ELISA was used in this work to determine the prevalence of the *Sarcocystis* species infection in slaughtered cattle at the Qurna abattoir in Basra, Iraq, which was 60.7%. According to a recent Iraqi study, the infection rate in the central Iraqi province of Diyala was almost the same at 65% (32). Additionally,(33) used ELISA to report a comparable rate of infection in cattle (69.3%). While other regions of the world reported varying percentages, (31) used ELISA to report a greater frequency (92.31%) of *Sarcocystis* infection in slaughtered cattle. Along with the availability of definitive hosts, frequent subjects including the breeding system, tissue type, climatic conditions, and anatomic location of analysis may be responsible for this notable variation in infection rates (34, 35). A statistically significant correlation between the age of cattle and the prevalence of *Sarcocystis* infection. Higher percentages among older age groups were observed in the current study Recent Iraqi study (36) supported the present result, in which there were significant age-group differences, with cattle older than four years exhibiting the highest infection rate (100%). Additionally, this result was consistent with reports of Sarcocystis infection in cattle from other countries (37, 34, 13). In contrast, (38, 5) demonstrated, that the rate of bovine sarcocystosis infection across age groups was statistically insignificant.The breeding system, climatic variables, and variations in management conditions, along with the availability of definitive hosts, may be responsible for the notable discrepancy between the current results and those of other studies (34, 35). The highest seasonal proportion was recorded in the spring (70.5%), while the largest monthly proportion was noted in February, April, and November. The results of this study demonstrated a significant connection between the prevalence of sarcocystosis and seasonality. In contrast, the parasite frequency did not significantly change by season (39 ; 13) . Besides that (40) mentioned that the parasite outbreak was lowest in April (4%), and the highest in June (16.13%). Furthermore. ,(13) reported a higher frequency of occurrence in the autumn (97.2%). The longer grazing periods may be the cause of the differences between the current results and those obtained by others. Another element impacting the seasonal changing of the infection distribution, which is arranged according to climatic conditions like temperature, humidity, and rainfall, is the survival and viability of sporocysts in the environment (13). Consistent with current findings. (35) found that the highest incidence of Sarcocystis infection (91.7%) occurred in the spring.

Conclusion

There was a significant correlation between the seasonality and prevalence of sarcocystosis, and the ELISA, as described in this study, can be adapted for the diagnosis of cattle sarcocystosis.

Conflicts Of Interest

The authors declare that there is no conflict of interest.

Ethical Clearance

This work is approved by The Research Ethical Committee.

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التحقق المناعي لداء المتكيسات العضلية في ماشية محافظة البصرة/ العراق

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الخلاصة

ركزت هذه الدراسة على معدلات الإصابة بالمتكيسات العضلية في 300 عينة من عضلات المريء ومصل الأبقار المذبوحة، استُخدم اختبار الممتز المناعي المرتبط بالانزيم (ELISA) لتحديد مستضدات المتكيسات العضلية والأجسام المضادة لها من نوع IgG، كانت نتائج اختبار الممتز المناعي المرتبط بالأنزيم (ELISA) لمستضدات المتكيسات العضلية إيجابية في 160 بقرة (53.3%). ووفقًا لاختبار الممتز المناعي المرتبط بالأنزيم (ELISA)، أظهرت 182 بقرة (60.7%) نتائج إيجابية للأجسام المضادة (IgG) للصنوبريات. لم تُحدد الدلالة الإحصائية للاختلافات بين نتائج اختبار الممتز المناعي المرتبط بالأنزيم (ELISA) (P>0.05). وفقًا لعمر الماشية، سُجِّلت نتائج إيجابية أعلى لاختبار مستضدات المتكيسات العضلية- اختبار الممتز المناعي المرتبط بالأنزيم (ELISA) لدى 138 من الماشية الأكبر سنًا (61.3%) مقارنةً بـــ 22 من الماشية الأصغر سنًا (29.3%). وكان متوسط قيم الكثافة البصرية لمستضدات المتكيسات العضلية أعلى لدى الماشية الأكبر

سنًا (1.204±0.296) مقارنةً بالحيوانات الأصغر سنًا (0.576±0.207). ويوجد ارتباط ذو دلالة إحصائية (P<0.05) بين متوسط قيم الكثافة البصرية والفئات العمرية. اظهر الكشف عن الأجسام المضادة IgG للمتكيسات العضلية باستخدام اختبار الممتز المناعي المرتبط بالأنزيم (ELISA) أن نسبة الإصابة بالمتكيسات العضلية ارتفعت مع التقدم في السن، حيث بلغت66.7 % لدى الحيوانات الأكبر سنًا، مقارنةً بـ 42.7% لدى الحيوانات الأصغر سنًا. وكان متوسط قيم الكثافة البصرية أعلى لدى الماشية الأكبر سنًا (0.480 ± 1.297) منه لدى الماشية الأصغر سنًا (0.182 ± 0.490). وُجد ارتباط ذو دلالة إحصائية (P<0.05) بين الفئات العمرية ومتوسط قيم الكثافة البصرية ، أظهرت نتائج النوعين من اختبار الممتز المناعي المرتبط بالأنزيم (ELISA) اختلافًا موسميًا ذا دلالة إحصائية (P<0.05) في انتشار العدوى بداء المتكيسات العضلية في الأبقار. كانت عدوى داء المتكيسات العضلية أكثر شيوعا في فصل الربيع، بنسبة 70.5 % في كل من اختبارات الممتز المناعي المرتبط بالأنزيم (ELISA) المعتمدة على المستضدات و الاجسام المضادة IgG للمتكيسات العضلية.

الكلمات المفتاحية: داء المتكيسات العضلية في الماشية ، اختبار الإليزا، المستضد، الجسم المضاد.