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SARCOCYSTIS SPP. IN RELATION TO NON-SPECIFIC AND RHEUMATOID ARTHRITIS DISEASES

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ABSTRACT

The primary objective of this study was to detect antigenicity of *Sarcocystis* extract by ELISA test in order to facilitate development of a diagnostic tool for exploring the relation of non- rheumatoid and rheumatoid arthritis with *Sarcocystis* spp. Sera from control rabbits in order to determine the cut-off values of this test, the best method of inoculation and proper antigen concentration that lead to variable antibody respons representative by variable values of optic density, cut-off levels were considered to be 0.125 for the ELISA. The lower density values which was observed in control rabbits. All of the intramuscular inoculated rabbits serum (100%) were considered moderately positive. The highly positive cases were associated with subcutaneous inoculated rabbits sera (50%) showed high optical density value (0.2) while other (50%) animals had moderate values. Made this method to detection of parasite-specific antibodies in ELISA, soluble somatic extracts of bradyzoites in PBS was used as antigen in this assay. This is the first study for the evaluation of the ELISA for detecting antibodies to *Sarcocystis* spp. in Rhematoid arthritis patients in Iraq. Samples of human was performed on 51 patients attending Misan province Hospitals and private laborataries, Twenty sera from healthy individuals were tested, in order to determine the cut-off values of the ELISA, cut-off levels were considered 0.95 for the ELISA. The highly positive cases were associated with Rheumatoid arthritis patients sera samples (6.6%) showed high absorbance values (2.49-2.70). The over all seropositivity among the examined serum samples was 29.4% When calculated for each group separately, it was 33,3% in the rheumatoid arthritis patients and 23.8% in non- specific arthritis patients.

KEYWORDS: *Sarcocystis* spp. In Iraq, rheumatoid arthritis and non- specific arthritis.

INTRODUCTION

The common cyst-forming coccidian parasites of domestic ruminants are worldwide,the sources of infection for these animals are grassland, feed and water pollution with infective faeces of the definitive hosts (Chabra & Samantaray, 2013). *Sarcocystis* are intracellular protozoan parasites among the most commonly found parasites in domestic ruminants. The parasite ultimately disseminates to skeletal, cardiac, and smooth muscle, that are infectious for a definitive host, acquired by eating undercooked beef or pork. Infection may be asymptomatic or a possible cause of musculoskeletal complaints and can provoke eosinophilic myositis. Rheumatoid arthritis (RA) patients may have high titres of rheumatoid factor in several parasitic diseases (Kiel, 2002; Frank von Sonnenburg, 2011).

Non-specific rheumatic manifestations that occur with non diagnosed cases are common. Some of these are associated with parasitic infections. Muscle sarcocystosis is a possible cause of myositis, rheumatic complaints and idiopathic cardiac diseases (Habeeb *et al*., 1996).

Human muscle sarcocystosis is infection acquired by ingestion of the mature oocysts or sporocysts of the Sarcocystis species. Sarcocystis infection may be an important cause of the non-specific rheumatic diseases associated with myositis (Abdul-Rahman *et al*., 2002; El-Nazer *et al*., 2000).

Due to the microscopic and intracellular localisation of the reproductive forms of these protozoan parasites and the relative difficulty in their laboratory cultivation, Sarcocystis infections confirmation requires the use of the development of immunodiagnostic tools. In the study, of Masoud *et al*(2007) 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. One of the first studies examining the presence of surface antigens associated with *Sarcocystis* species examined *Sarcocystis muris* zoite extracts recovered from infected muscle tissues of mice. The Immunoprecipitates revealed three antigens of varying molecular weights (Abbas and Powell, 1983). Sporozoites and culture derived merozoites of *Sarcocystis cruzi* from mice were found to have similar reacting surface antigens during detection by immunofluorescence (Burgess *et al*., 1988). At least one surface epitope of the sporozoites and merozoites also reacted with antigens of bradyzoites during western blot analysis, while

others showed no reaction. This indicated that epitopes from these three stages of *Sarcocystis* maybe either distinct or similar(Burgess *et al*., 1988). The primary objective of this study was to detect antigenisity of *Sarcocystis extract* by ELISA test in order to facilitate a precise diagnostic tool for exploring the relation of Non-specific and Rheumatoid arthritis with *Sarcocystis* spp.

MATERIALS AND METHODS

Study population

This study was performed on fifty one blood samples of arthritis patients selected from the Misan province Hospitals and Private laboratories. They were considered as three groups:Group (A): Consisted of 30 patients with rheumatoid arthritis, Group (B): Consisted of 21 non rheumatoid arthritis patients, the serum samples of these patients were previously tested for rheumatoid factor in these laboratories. Control group (C): Consisted of 20 healthy persons.

Blood samples for separation of serum were collected from each person in plain tubes, kept at room temperature for 30 min, centrifuged at 3,000 rpm for 15 min. The got serum samples were transferred to Eppendorf tubes and kept at -20°C until used.

Immunological analysis

ELISA test was used in this study for detection of *Sarcocystis* spp. cycst extract antigenisity and the relation of *Sarcocystis* spp.with rheumatoid arthritis (RA).

Soluble antigen source and praparation

Nine hundred and four water buffaloes, aged 0.5-7 years, slaughtered at the Misan province abattoir in southern Iraq, were used in the survey for the presence of *Sarcocystis* during the period from February 2015 to January 2016. The esophagus muscles of the slaughtered animals were collected from 25 water buffaloes infected with *Sarcocystis* spp. and placed in properly labelled plastic bags, then transported to the Parasitology Laboratory of the Education College for Pure Science, Basrah University. The muscle samples were kept in the refrigerator prior to the examination for *Sarcocystis*. The presence of the *Sarcocystis* was evidenced through a naked eye examination of the muscles.

Soluble antigen was obtained from large cysts of *Sarcocystis* spp. 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. The protein content of each allergen extracts was determined by Hudson and Hay method (1989) as summarized below: Three mil of each allergen extract were pipette in quartz cuvattes. The absorbance value was measured spectrophotometrically at (260) and (280) nm. The protein content was calculated by the following equation.

protein concentration mg/ml=1.55 * A280-0.77* A260 .

Detection of *Sarcocystis* extract immunogenicity in animal model

Sixteen rabbits at 3months of age were purchased from local markets. These animals were labelled as R1, R2 and R3(consisted of 4 animals),. After a week of orientation, the R1 and R2 rabbits each group consisted of six animals were inoculated Intramuscularly (IM) and subcutaneously (SC) respectively on 1st, 10th and 20th of February. Freund's complete adjuvant (Sigma Chemicals, St. Luise, USA) was mixed with the *Sarcocystis* extract1:1(v/v) within 2.5 mL syringes, just before inoculation. Blood (2 mL) was drawn from the heart of each rabbit after ten days (3rd of March) in order to select a serum sample with the highest concentration of antibodies. The serum for each animal was stored in an eppendorf tube at –20°C. The ELISA test was used to detect presence of anti *Sarcocystis* spp. antibodies in the collected serum (Table 1).

Table 1: Inoculation of rabbits with *Sarcocystis* antigens

Date of inoculation	Rabbits and type of inoculation		
	R1(IM)	R2(SC)	R3
1st February	*Sarcocystis* antigens + Freund's complete adjuvant (1:1) 0.5ml	*Sarcocystis* antigens + Freund's complete adjuvant (1:1) 0.2ml	Control
10th Februar	*Sarcocystis* antigens 0.2ml	*Sarcocystis* antigens 0.2ml	Control
20th Februar	*Sarcocystis* antigens 0.2ml	*Sarcocystis* antigens 0.2ml	control

Detection of anti *Sarcocystis* spp. antibodies by ELISA

To detect the IgG antibodies response to *Sarcocystis* extract an ELISA protocol, described by Savini *et al*.,(1994) was performed on rabbits and patients sera, with some modifications. The optimal dilution for three reagent serum, antigen and conjugate was determined. by Chequer board ELISA according to the method of Baher *et al*., (1980).

Depending on the results of CB ELISA, the best selected antigens, human sera and conjugate, were used in *Sarcocystis* spp. antigens based ELISAfor detection of antibodies against

Sarcocystis spp. Briefly, Microtiter plates (Linbro-China) were coated with *Sarcocystis* spp. soluble antigen (25 µg/mL) and kept overnight at 4°C. Serum samples (1:100) of rabbits or arthritis patients were added and incubated for 60 min at 37°C. Goat anti- Rabbit immunoglobulin, labelled with horseradish peroxidase (promega)or Anti -human IgG horseradish peroxidase conjugate(promega)was used as enzymatic conjugate at 1:100 dilutions and incubated once more for 60 min at 37°C. The reaction was revealed with a solution of enzymatic substrate *O*-Phenylendiamine dihydrochloride (OPD) (Sigma). The optical density (OD) was determined in a microplate reader (BIO-TEK ELX800-UK) at 405 nm. negative control sera were included in each assay and obtained by mixing equal volumes of sera from 4 R3 rabbits or 20 healthy persons.

The cut-off was calculated as the mean OD values from negative controls plus two standard deviations. The samples were considered seropositive when the OD value of sample was ≥OD of cut-off.

Statistical analysis

Statistical analyses consisted of the limitation of means with a 95% confidence interval of the antibody levels present in the (patient or rabbit) serum samples. The mean antibodies responses of arthritis patients and healthy persons or immunized and control rabbit were compared using Student's t-test. To demonstrate any association between results, the exact Fisher test and Pearson'schi-squared analysis with Yates correctionwere utilized with the limit of significance being set at 5%. Statistical analysis is done by using SPSS software version 11.

RESULTS

When the sera from 4 control rabbits (R3) were tested, in order to determine the cut-off values of the ELISA, they yielded a mean absorbance of 0.115 (SD=0.0053) at 450 nm; therefore, cut-off levels were considered 0.125 for the ELISA. The lower density values which was ranged between 0.109 and 0.120 observed in R3 rabbits. All of the R1positive samples 6 (100%) were considered moderately positive, in which the density values ranged between 0.155 and 0.184. The highly positive cases were associated with R2 (SC inoculated rabbits) sera samples in which3 cases (50%) showed high optical density value (0.2) while moderate values (0.168,0.181 and 0.182) were observed in other 3 animals (Table2). The difference among R1, R2 and R3 density values were considered to be statistically significant ($P< 0.02$).

Both R1 and R2 showed different density value (mean±SD) but R2 had the higher value (0.19 ±0.015) followed by R1 density value (0.169±0.012) comparing to lower density value (0.115±0.005) of R3 rabbits (Table 3). The difference of density value (mean±SD) in the 3 groups of rabbits was considered to be much statistically significant (P<0.0001).

Table 2: ELISA results in rabbits inoculted with *Sarcocystis sp* antigens.

ELISA results Optic density values	Rabbits and type of inoculation n.(%)		
	R1(IC)	R2(SC)	R3
Lower (0.109-0.120)	0	0	4(100)
Moderate (0.155-0.184)	6 (100)	3(50)	0
High (0.2)	0	3(50)	0
Total	6 (100)	6(100)	4(100)

X^2:18.069, DF:8 P< 0.02.

Table 3: Inoculation methods in relation with positive *Sarcocystis sp* antigens based ELISA results of rabbits.

ELISAresults	Rabbits and type of inoculation n.(%)		
	R1(IC)	R2(SC)	R3
Examined n.	6	6	4
Absorbance values mean±SD	0.169±0.012	0.19 ±0.015	0.115±0.005
P value	P<0.0001		

Detection of *Sarcocystis* antibodies in Rheumatoid arthritis patients

Several density values that ranged from 0.56 to 2.70 were detected by the +ve tested patients' serum samples. The higher density values (2.49 and 2.70) were observed in Rheumatoid arthritis patients. Twenty sera from healthy individuals were tested, in order to determine the cut-off values of the ELISA, they yielded a mean absorbance of 0.78 (SD=0.168) at 450 nm; therefore, cut-off levels were considered 0.95 for the ELISA. The lower absorbance values which was ranged between 0.95 and 0.97 observed in 4 positive sample (19.1%) of non Rheumatoid arthritis patients, only one (4.8%) positive sample of non Rheumatoid arthritis patients showed moderate absorbance values (1.04-1.82**)**. The highly positive cases were associated with Rheumatoid arthritis patients sera samples in which 2 patients (6.7%) showed high absorbance values (2.49-2.70). The strength of the reaction of the serum with the *Sarcocystis* spp.antigens was shown in table 4. Statistical analysis revealed that there was a considerable difference (p< 0.05) between the 2 groups (table 5). In table (6)The statistical analysis of ELISA results in Rheumatoid arthritis (A), non Rheumatoid arthritis patients (B) patients and control revealed that there was a significant difference (p< 0.05) between the A

and control groupsand. The difference between B and control groupswas not considered to be statistical significant (p> 0.05).

Table 4: ELISA results of *Sarcocystis* antibodies detection in patients sera.

Serum analysis (Absorbance values)	Patients	
	Rheumatoid arthritis	Non Rheumatoid arthritis
Lower (0.95-0.97)	0	4(19.1)
Moderate (1.04-1.82)	8(26.7)	1(4.8)
High (2.49-2.70)	2(6.7)	0(0)
Negative	20(66.7)	16(76.1)
Total	30(100)	21(100)

Table 5: Statistical analysis of the two groups. Serum analysis cross table between groups Rheumatoid arthritis (A) and Arthritis (B) patients (p value <0.05 sig.).

Serum analysis (Absorbance values)		Group		Total
		A	B	
Lower(0.95-.97)	% within group % of total	0 0 0	4 19.1 7.8	4 7.8 7.8
Moderate (1.04-1.82)	% within group % of total	8 26.7 15.7	1 4.8 1.9	9 17.6 17.6
High (2.49-.70)	% within group % of total	2 6.6 3.9	0 0 0	2 3.9 3.9
Negative	% within group % of total	20 66.7 39.2	16 76.1 31.4	36 70.6 70.6
Total	% within group % of total	30 100 58.8	21 100 41.2	51 100 100

X^2:14, DF:7, P:0.05118.

Table 6: Statistical analysis of ELISA results in Rheumatoid arthritis (A), non Rheumatoid arthritis patients (B) patients and control.

ELISA results	Patients		Healthy (control) C
	A	B	
Examined n.	30	21	20
Absorbance values mean±SD	1.038± 0.517	0.863± 0.117	0.78 ±0.168
P value	P: 0.0367	P:0.0714	

DISCUSSION

To identify the validity of the *Sarcocystis* sp cycst extract for use as antigen can be relied upon in the ELISA test. The rabbits under study were immunized with this extract so as to obtain antibodies that will be used in the ELISA test, which confirmed thier specificity to the injected antigen. an ELISA protocol, described by Savini *et al.* (1994) was performed, with some modifications. Soluble antigens of cycst in PBS was used as antigen in this assay (ELISA). Sera from 4 control rabbits (R3) were tested, in order to determine the cut-off values of the ELISA, the best method of inoculation and proper antigen constration that lead to variable antibody respons representative by variable values of optic density. Therefore, cut-off levels were considered 0.125 for the ELISA. The lower density values observed in control rabbits. All of the intramuscular inoculated rabbits serum samples were considered moderately positive. The highly positive cases were associated with subcoetaneous inoculated rabbits sera. However, the tests were found negative when conducted with sera of the negative control rabbit. Presence of poitive antibody responses in hyperimmune rabbit sera as noticed in the present study is in conformity with the reports made by Reddy *et al*. (1990), Juyal *et al*. (1990) and Kalita *et al*. (2015).

Non-specific rheumatic appearance that occur with undiagnosed cases are ommon. Some of these are associated with *Sarcocystis* spp. infections. Muscle sarcocystosis disease is a possible cause of idiopathic cardiac diseases, rheumatic complaints and myositis (Abdul-Rahman *et al*., 2002; Habeeb *et al*., 1996). Diagnostic limitations have impeded the ability to increase the understanding of the epidemiology and public health significance of *Sarcocystis* infections in humans (Poulsen & Stensvold, 2014). Conventional methods of diagnosing *Sarcocystis* spp. infections, involve time consuming and labour-intensive testes of host muscle for the presence of cysts or cystozoites. Such techniques are neither suitable for utilize in large-scale screening programmes, nor for use in diagnosing infections in livestock. Therefore, serological examination detecting special antibodies to *Sarcocystis* spp. have been assumed to be important in the diagnosis of sarcocystosis (Savini *et al*.,1997).

Many previous studies supported the preference of Indirect detection by serological methods(ELISA) to testing of tissue biopsy specimens in the diagnosis of human muscular sarcocystosis and proved to be accurate and specific. as the study of Poulsen and Stensvold (2014) who mentioned that direct detection of the parasite in cases of human muscular sarcocystosis is hampered by the obvious absence of a predilection site and so indirect

detection by serological methods is preferred to testing of tissue biopsy specimens. Additionely, Metwally *et al*.(2014) recommended the use of ELISA in *Sarcocystis* examine, due to the low sensitivity of both macroscopical and microscopical examinations. Beside that, Habeeb *et al*. (1996) mentioned that there has been no report of cross reactivity with, Toxoplasma, indicating that ELISA have high specificity. Accordingly in the present study serological examinations (ELISA) was used for detection of human Sarcocystis antibodies in the sera of rheumatoid arthritis patients.

Macroscopic cyst of *sarcocystis* spp. was used in the present study as a source of antigen to diagnose the *Sarcocystis* infection in human by ELISA because they are more available since they are macroscopic, easy to be collected and yield a considerable amount of antigen (Abdul-Rahman *et al*., 2002). This Ag. was used by several researchers. Fatma *et al*. (2008); Masoud *et al*., 2007; Habeeb *et al*. (1996) and El-Nazer and Abdel-Azem (2000) used *S. fusiformis* macroscopic antigen in ELISA diagnosis of sarcocystosis in humans. There has been no report of crossreactivity with*Toxoplasma*, indicating that methods in use have high specificity (Habeeb *et al*., 1996). Also, Abdel Rahman (2001) used the same antigen in ELISA test and Western blot for diagnosis of *Sarcocystis* spp. infection in cattle. In this study, antigens derived from bradyzoites of *Sarcocystis* spp. 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 *Sarcocystis* spp. in Rhematoid arthritis patients in Iraq.

In the present study the over all seropositivity among the examined serum samples was 29.4%. %. When calculated for each group separately, it was 33.3% in the rheumatoid arthritis patients and 23.8% in arthritis patients. These results are higher than those obtained by El-Nazer and Abdel-Azim (2000) and Habeeb *et al*. (1996), who recorded 15.6%, 13% among RA patients respectively.

Considering the seropositivity of rheumatoid arthritis patients 33.3%, it was higher than those obtained by Abdul-Rahman *et al*. (2002) who recorded 23.8% in rheumatoid arthritis patients but the current over all seropositivity 29.4% in RA and non RA patients was lower than the over all seropositivity 46% which was reported by those researcher.

The preent low *Sarcocystis* Ag. seropositivity in RA and non RA patients may be due to immunosuppression or use of steroids as said by El-Nazer and Abdel-Azim (2000).

In conclusion the localy prepared antigens can be adapted for the diagnosis of human and animals sarcocystosis by ELISA test. beside that *Sarcocystis* Ag. seropositivity was marked with rheumatoid arthritis.

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