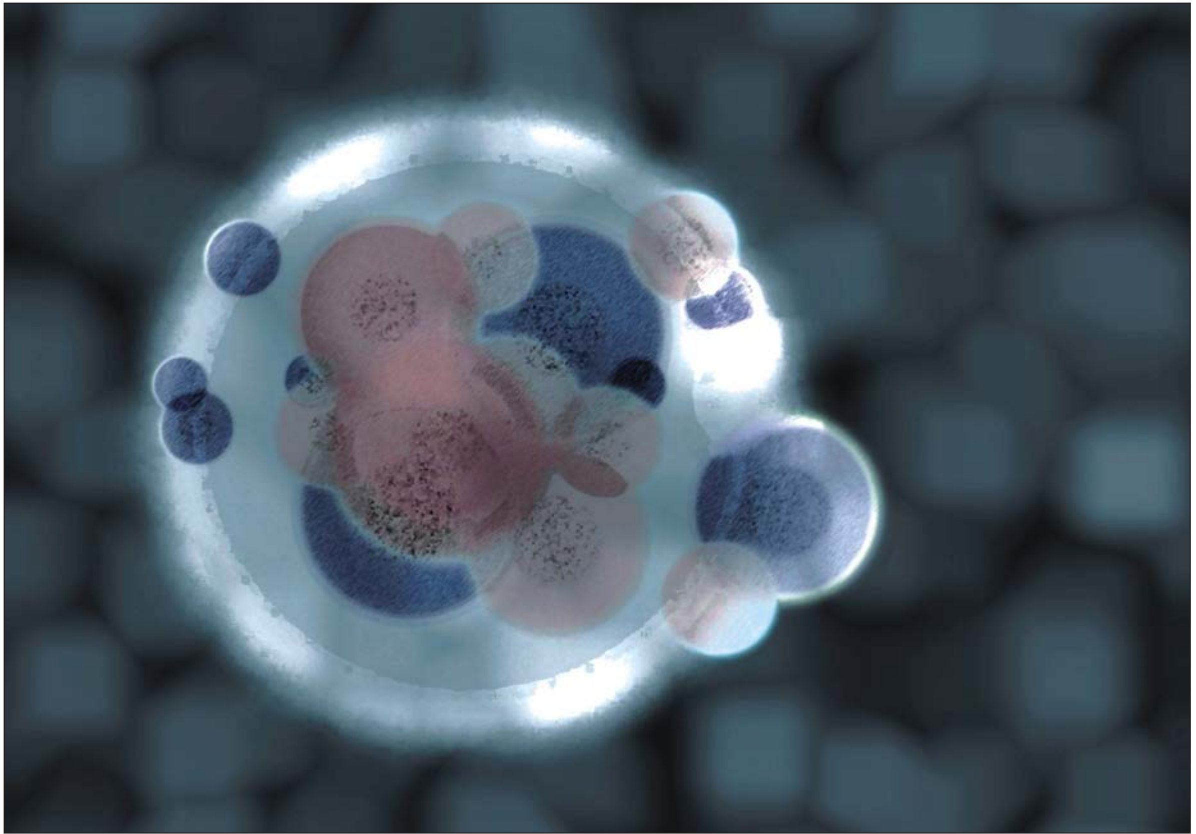

Journal of Biological Sciences

ISSN 1727-3048

science alert

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Journal of Biological Sciences 8 (5): 889-895, 2008
ISSN 1727-3048
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Infection by Cyst Producing Protozoa among Human and Food Producing Animals in Egypt

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Abstract: The infection by cyst-producing protozoa (*Toxoplasmosis*, *Sarcosporediosis* and *Neosporosis*) in human and animals in Egypt was investigated using EITB and micro ELISA technique. Two specific eluted and concentrated protein fractions were used as antigen. The molecular weight (MW) of these fractions is 32 kD for *Toxoplasma gondii* tachyzoites Ag. (TTAg) and that corresponding to 53 kD for *Sarcocystis* bradyzoites Ag. (SBAg). The data cleared a direct relation between contact with animals and the increase in the rate of infection by these parasites among human. The incidence in female and male who work in continuous contact with animals was 36 and 24%, respectively. It was 17 and 19% in females and male patients who are in in-direct contact with animals and visit hospitals complained by mayligia, arthritis and gastro-intestinal disturbances. The low incidence was recorded in non-complained youth (1% in females and 3% in males). The mean rate of infection by the three parasites was high in the examined apparently healthy old buffaloes, cattle and sheep. It was 46.5, 22.5 and 25.6% in the above animals respectively. Sarcosprodiosis is the most common one followed by toxoplasmosis, while the infection by *Neospora* is the lowest one. Toxoplasmosis is high in women's and female animals. Sarcosprodiosis is high in men's while no effect for sex in animals. High sensitivity and specificity of these fractions will improve the diagnostic utilities of these parasites, the matter which improves capability of the related ministries to minimize their distribution in Egypt.

Key words: Human, animal, *Toxoplasma*, *Sarcocystis*, *Neospora*, EITB, Micro, ELISA

INTRODUCTION

Diseases of animal origin remain of great importance in rural areas of developing countries. In such regions people commonly share their home environments with a variety of animal species. This usually present under a conditions of poor sanitation and hygiene. This inevitably led to frequent exposure to each other's pathogens including parasitic one.

Cyst producing protozoa are a wide group of tissue parasites has a mandatory two-host life cycle infect a wide range of domestic, wild animals and man. Their bisexual stages are in the intestine of carnivore (final host) and asexual stages are in the vascular system and tissues of herbivore and man (intermediate host) (Acha and Szyfres, 1991).

Toxoplasma is one of the most common cyst producing protozoa transmitted from cats to all of the surrounding animals. The infection was 34.3% in cows and 28.8-38.1% in buffaloes in north of Egypt. The incidence in apparently healthy women was 37.5%, while it was 71.4% in sheep using ELISA technique (Ibrahim *et al*., 2006).

Sarcosporidiosis is wide spread disease infect muscles of food producing animals in Egypt. Its incidence was 10.6 and 69.5% in buffaloes of 2 and over 7 years old (by macro and microscopic techniques), but it reached to 19.33 and 88.5% in the above 2 age groups, respectively using dot-ELISA technique (Sabry and Shalaby, 2004).

Man can act as final host for some species of *Sarcocystis* as they develop in their intestine (Cook and Zumla, 2003) or as intermediate hosts (Arness *et al*., 1999). The incidence of infection was 37.5% in patients complained with myositis accompanied with eosinophilia as well as 8.33% from patients complained with digestive disturbances (Mamdoh, 2006).

The infection by *Neospora caninum* in Egypt was estimated by El-Ghaysh *et al*. (2003) as 16.2, 36.1, 35.2 and 27.6% in cattle, sheep, goats and stray dogs, respectively, using direct agglutination test versus whole fixed *N. caninum* tachyzoites.

Identification of specific protein fraction able to exclude cross reaction with other related parasite is a way to improve diagnostic utilities for these parasites. In this respect Granstrom *et al*. (1990) determine Seven specific protein bands at molecular weight (MW) of 37, 44, 53, 57, 94, 113 and 215 kD, after fractionation of *Sarcocystis cruzi* bradyzoites using enzyme linked immuno-transfer blot (EITB). Protein fractions of *Toxoplasma* tachyzoites at MW range of 13-48 kD were react specifically versus anti-

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Toxoplasma Ab (IgG) in sera of sheep vaccinated by live tachyzoites (S48 *Toxoplasma*, Toxovax) using EITB technique (Wastling *et al*., 1994). They added that the fractions in the range of 30-32 kD is the most dominated one with no differences between the antigenic structures of the RH strain tachyzoites and the other strains of *Toxoplasma* tachyzoites.

The present study is the second part of the research done during 3 years (2004-2006) project No. 09-04 funded by Developing Scientist Projects Program, Cairo University, Faculty of Veterinary Medicine, El-Giza, Egypt. During the first part of this project, fecal samples from dogs, cats and muscles of slaughtered animals as well as their contact human were investigated. In this part of the project infection by cyst producing protozoa between animals and human was evaluated serologically using specific purified parasitic protein fractions.

MATERIALS AND METHODS

The present study was carried out during the period from October 2005, through September 2006. Human and animal samples were collected from El-Giza and Cairo Governorate, Egypt. The samples were examined at Department of Zoonoses, Faculty of Veterinary Medicine, Cairo University, El-Giza, Egypt.

The used antigen preparation

Sarcocystis and *Toxoplasma* antigen: According to Gasbarre *et al*. (1984) and Hong-Moon (1987), bradyzoites of *Sarcocystis* were extracted from macroscopic cysts of natural infected bovine esophagus (identified as *Sarcocystis bovi-hominis*) by crushing in 0.01 M phosphate buffered saline (PBS) pH 7.4. *Toxoplasma* (RH) strains tachyzoites were collected from the peritoneal fluid of experimentally infected mice. After washing by centrifugation, the bradyzoites and tachyzoites were ruptured separately in few amount of PBS by repeated freezing thawing (3 times). The contents were sonicated using Cole parmer ultrasonic Homogenizer in ice bath. The suspension was centrifuged at 10,000 rpm at 4°C for 1 h. The supernatant was collected and dialyzed overnight in refrigerator against PBS, pH 7.2 using a dialysis membrane (6000 to 8000 molecular weight cut off), its protein contents was measured by the method of Lowery *et al*. (1951) and stored at -70°C until use.

Other antigens: *Cysticercus bovis* and *C. cellulosae* were extracted from freshly slaughtered natural infected cattle and pig heart muscles. After several washing, they used in crude antigens preparation according to Cheng and Ko (1991). By the same way, *Toxocara canis* and *T. vitulorum* antigens were prepared from the anterior parts of fresh worms collected from scarified natural infected dogs and buffaloes according to Kagan *et al*. (1958). *Trichinella spiralis* larval antigens were prepared from the active muscle larvae extracted from experimentally infected albino mice by pepsin digestion according to Soliman and El-Bahy (1998). *Fasciola gigantica* E/S antigens were prepared from living flukes collected from fresh condemn buffaloes livers according to River Marrero *et al*. (1988). The protein content is measured and stored as before.

Hyper-immune sera: Rabbit hyper-immune sera (RHIS) were raised versus the previous prepared antigens according to Langley and Hillyer (1989). The obtained sera were used to estimate the specificity of different fractions using Western blot assay (EITB) technique.

Fractionation and transfer of protein: The extract two antigens (TTAg and SBAg) were fractionated using sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) according to (Laemmli, 1970). Using PROTEAN II Xi cell, BIORAD. The antigens were resolved using 1.5 mm thickness, in 12% polyacrylamide gel slabs in Tris-glycine buffer, pH 8.3 under reducing conditions. The stacking gel consisted of 5% acrylamide prepared in 12.5 mM Tris-HCl buffer (pH 6.7) (Sigma chemical Co.). Prestained low molecular weigh (MW standard was employed (Sigma SDS-100B). The comb was adjusted as one small well for standard and one large for the sample. Electrophoresis transfer of fractionated proteins onto nitrocellulose sheet (NC) was performed according to Towbin *et al*. (1979).

Determination and separation of specific protein fractions: A longitudinal NC strips (15×0.3 cm) were cut out and allowed to react versus sera of experimentally prepared RHIS as well as negative control samples using EITB according to Towbin *et al*. (1979). Specific TTAg or SBAg. fractions is that react positive versus its target anti-bodies in RHIS and in the same time did not cross react with any of the other tested RHIS produced after vaccination of rabbits with TTAg., SBAg., crude Ag of *C. bovis*, *C. cellulosae*, *T. canis*, *T. vitulorum*, *T. spiralis* larval Ag, *F. gigantica* E/S Ag as well as negative rabbit sera.

The tested NC strips were returned back to its original position on NC sheet, where transverse NC strips corresponding to each specific fraction were cut out. The specific protein fraction were extracted by electro-elution using elution tube membrane, 4-6 MW cut off (Spectrum Medical Inc., Los Angeles, CA 900060) and the volume

was reduced by polyethyleneglycol according to Katrak *et al.* (1992). The protein contents were determined and kept at -20°C until use for coating of the ELISA plate. Sensitivity of these fractions was evaluated versus natural infected human and animal sera as well as RHIS using micro ELISA technique.

Micro ELISA technique: The technique was carried out according to Ogunsanmi *et al.* (2000) with slight modifications. Micro-ELISA plates (M129A Dynatech) were coated by the specific protein fractions (1 μg/well) in carbonate buffer pH 9.6 (adjusted after checkerboard titration), 50 μL/well and kept at 4°C over-night prior to use. After washing, tested and control sera were added 50 μL/well in duplicates. After washing, horseradish peroxidase-conjugated was used at 1:2000 dilutions, incubated and washed as before. Ortho-phenylenediamidine-OPD was added (50 μL/well) at a concentration of 340 μg mL^{-1} substrate buffer. The reaction was stopped by addition of 50 μL/well of 1 M H_2SO_4. Absorbency was read at 490 nm using a *Titerteck multiskan* ELISA reader. Specificity and sensitivity of the tested antigenic fractions were calculated according to Rokni *et al.* (2006).

Serum samples: Sensitivity of different antigens was evaluated using selected serum samples. Women and Female animals' sera have history of abortion due to *T. gondii* infection, sera from natural *Sarcocystis* infected buffaloes, high diluted RHIS versus *T. gondii* and *Sarcocystis* were selected for this test.

Incidence of infection was evaluated using random human serum samples in 3 different groups. The first is apparently healthy animal workers. The second are patients live in indirect contact with animals but they come to hospitals complained by myositis, arthritis and or gastro-intestinal disturbances. The last group is non-complained 16-18 years old school students.

Also, two groups of buffalo and cattle over 5 years old and group of sheep over 2 years old were selected as representative to animals available in the area of the project work. Some of these animals were proved to be *Sarcocystis* infected at slaughter house.

The specificity of the selected antigens, were evaluated using sera of known infection history include, surgically proved *Hydatid cyst*, *S. mansoni*, Virus hepatitis C, infected patients and healthy individual were obtained from Institute of Tropical Medicine and Hygiene, Kasr El-Any street, Cairo, Egypt. Also, sera of *Fasciola* infected sheep; *Cysticercus bovis* natural infected buffalo as well as sera of healthy animals were included in this study.

Fecal samples of all tested cases were examined parasitologically using several methods include direct smear method, (WHO, 1983), Concentration floatation technique (Wattal *et al.*, 1986) and successive sieve system (Fluke finder, Moscow, ID) for large size eggs according to Welch *et al.* (1987).

Diagnosis of *Neospora caninum* anti-bodies in sera: Anti-*N. caninum* Ab were diagnosed in animal sera using direct agglutination test versus whole fixed tachyzoites according to El-Ghaysh *et al.* (2003) while that of human sera were diagnosed using the Competitive enzyme-linked immunosorbent assay (cELISA) as an already prepared Kits produced by VMRD, Inc., Pullman, WA 99163, USA. The technique was performed following the manufacture's instructions.

RESULTS AND DISCUSSION

Zoonotic cyst producing protozoa are widely distributed between human and animal in Egypt. The incidence in animals was investigated usually in post slaughtering by non serological methods without interest for diagnosing of infection in living animals. In human, toxoplasmosis is the only parasite investigated serologically in suspected cases.

Persistence of infection by these parasites in Egypt may be related to several factors include: low level of education, absence of restriction on transmission of infected animals between endemic and non endemic localities, variability in the level of control measures applied in each slaughter houses, presence of some wild rodents and animals easily gain access to human and food animal dwellings, in addition to un availability of specific diagnostic technique.

As human and animals act as intermediate hosts (Cook and Zumla, 2003) for these parasites, they did not shed diagnostic stages in their faeces and diagnosis was depends on detection of anti-bodies in their sera. Accuracy of the used serological technique was depends mainly on the specificity and sensitivity of the used antigen fractions. In the present study Treatment of TTAg. and SBAg. antigenic fractions on NC strips versus different rabbits HIS using EITB technique (Fig. 1) revealed presence of 8 KD immunogenic protein fractions in TTA corresponding to molecular weight (MW) of 64-80 kD, 60 , 58, 48, 38, 32, 26 and 14 kD. Testing the specificity of these fractions versus sera of rabbits vaccinated by SBAg, *Cysticercus bovis*, *C. cellulosae* Ag, *Toxocara vitulorum*, *T. canis* Ag. *Trichinella spiralis* Ag, *Fasciola gigantica* excretory secretory (ES), antigen as well as non vaccinated rabbit sera, revealed only 3 kD protein fractions as *T. gondii* specific bands. These bands at MW of 60, 48 and 32 kD.

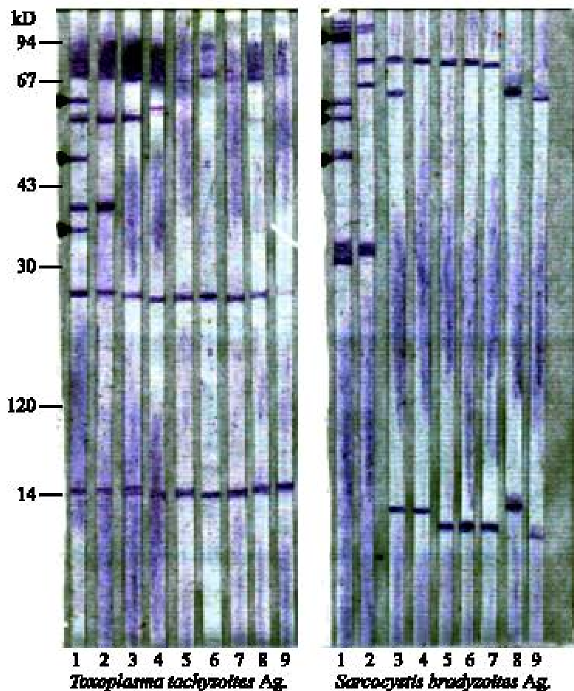

Fig. 1: Recognition of specific *T. gondii* and *Sarcocystis* fractions on NC strips versus Rabbit hyper immune sera using EITB technique. Lane (1) *Toxoplasma*: fractions reacted versus anti- *Toxoplasma* Ab. in rabbit HIS sera. Lane (2)-*Toxoplasma*: Fractions reacted versus anti- *Sarcocystis* Ab. in rabbit HIS sera. Lane (1) *Sarcocystis*: Fractions reacted versus anti-*Sarcocystis* Ab. in rabbit HIS sera. Lane (2) *Sarcocystis*: Fractions reacted versus anti-*Toxoplasma* Ab. in rabbit HIS sera. Lane (3) Fractions reacted versus anti-*Cysticercus-bovis* Ab. in rabbit HIS sera. lane (4) reaction versus anti-*Cysticercus cellulosae* Ab. lane (5) reaction versus anti-*Trichinella spiralis* Ab. lane (6) reaction versus anti-*Toxocara vitulorum* Ab., lane (7) reaction versus anti-*Toxocara canis* Ab. lane (8) reaction versus anti-*F. gigantica* E/S Ab., lane (9) Fractions reacted versus negative rabbit sera

By the same way, treatment of SBAg antigenic fractionation on NC strip versus rabbit HIS vaccinated by SB Ag, revealed presence of 6 immunogenic bands at MW > 94, 94, 57, 53, 44 and 30 kD. From these fractions, four bands at M.W. of 94, 57, 53 and 30 kD react specifically versus RHIS vaccinated by SBA and did not cross reacted with any of the previous mentioned rabbit sera other than of *Sarcocystis* vaccinated rabbits. This was agreed with that mentioned by Wastling *et al.* (1994) and Fatoohi *et al.* (2004).

EITB is one of the most specific sero-diagnostic techniques but it considered non-practical for current field application in comparison with ELISA technique (Ibarra *et al.*, 1998). However, ELISA technique is sensitive serological test, able to analyze many samples simultaneously (Babba *et al.*, 1994).

In the present study micro-ELISA was developed according to Ogunsanmi *et al.* (2000) in order to identify the most sensitive diagnostic fractions from TTA and BSA. Concerning the sensitivity, the results in Table 1 demonstrated that *T. gondii* protein fraction of 32 kD appear as the most sensitive one (93.1%) in diagnosis of *T. gondii* antibodies in tested sera followed by that of 48 kD (79.3%), while that of 60 kD is the lowest sensitive one (48.3%).

The sensitivity of 32 kD fraction is high (100%) versus high diluted (1:400) *T. gondii* vaccinated rabbit sera, while it was 93.3% versus sera of natural *T. gondii* infected sheep and it was (90%) in naturally *T. gondii* infected women. In the same time, all of the tested fractions did not cross reacted with sera of non-infected rabbits, women and sheep.

Similarly, the fraction of 53 KD from SBA appear as the most sensitive one (95.8% sensitivity) followed by that of 57 and 94 kD as the sensitivity was 79.2 and 70.8%, respectively. The fraction of 30 kD is the lowest sensitive one (50.0%). This was recorded after treatment of strips containing the above fractions versus 20 serum samples from macroscopic Sarcocystis naturally infected buffaloes and high dilution of rabbits HIS vaccinated by SBA (Table 2).

The fraction of 32 kD from TTAg. and that at of 53 kD from SBA proved to be sensitive in diagnosis different level of anti-*T. gondii* and anti-*Sarcocystis* anti-bodies in natural infected women, sheep and buffaloes, respectively using micro-ELISA technique. This was agreed with Granstrom *et al.* (1990) and Fatoohi *et al.* (2004) where they mentioned that from large number of soluble *T. gondii* antigens only two fractions in the 20-40 kD range induce a significant humoral response. Also, Elsaid *et al.* (1999) considered *Toxoplasma* antigens at MW of 32 kD from SDS-PAGE, is specific diagnostic one originate from tachyzoites but it is of no value as a vaccine.

The results in Table 3 revealed that the highest incidence of infection (36.0% in women and 24.0% in men) was recorded in peoples that work in close contact to animals. The incidence was 17.0 and 19.0% in women and men outpatients hospitals complained with myaligia, arthritis; gastro-intestinal disturbances and they are in indirect contact with dogs and cat. The incidence of

Table 1: Sensitivity of eluted *T. gondii* specific fractions using micro-ELISA technique

		T. gondii tested fractions					
		60 kD		48 kD		32 kD	
Tested serum samples	No.	No. +ve	%	No. +ve	%	No. +ve	%
T. gondii natural infected women	10	5	50.0	8.0	80.0	9	90.0
T. gondii natural infected sheep	15	8	53.3	13.0	86.6	14	93.3
H.I.R.S. (at 1:200 serum dilution)	2	1	50.0	2.0	100.0	2	100.0
H.I.R.S. (at 1:400 serum dilution)	2	0	0.0	0.0	0.0	2	100.0
Total	29	14	48.3	23.0	79.3	27	93.1

*No reaction could be recorded with sera of negative rabbit, healthy women and sheep of parasite free faeces

Table 2: Sensitivity of eluted Sarcocystis specific fractions using micro-ELISA technique

		Sarcocystis bradyzoites tested fractions							
		94 kD		57 kD		53 kD		30 kD	
Tested serum samples	No.	No. +ve	%	No. +ve	%	No. +ve	%	No. +ve	%
Natural infected buffaloes	20	15.0	75.0	16	80.0	19	95.00	11.0	55.0
H.I.R.S. 1:200	2	2.0	100.0	2	100.0	2	100.00	1.0	50.0
H.I.R.S. 1:400	2	0.0	0.0	1	50.0	2	100.00	0.0	0.0
Total	24	17.0	70.8	19	79.2	23	95.83	12.0	50.0

*No reaction could be recorded with sera of negative rabbit and parasite free healthy buffaloes

Table 3: Incidence of infection by cyst producing protozoa in human and animals

				Infection by								Total infection incidence	
				T. gondii		*Sarcocystis*		*Neospora*		Mixed infection			
Tested serum samples			No.	No.	%	No.	%	No.	%	No.	%	No.	%
Suspected human	Workers in close contact to animals	W	50	7.0	14.00	7	14.0	1	2.0	3	6.0	18	36.0
		M	75	4.0	5.33	14	18.6	0	0.0	0	0.0	18	24.0
	Out-patients clinic have digestive	W	100	9.0	9.00	6	6.0	0	0.0	2	2.0	17	17.0
	disturbances	M	100	5.0	5.00	12	12.0	1	0.5	1	1.0	19	19.0
	Apparently healthy people	W	100	1.0	1.00	0	0.0	0	0.0	0	0.0	1	1.0
		M	100	0.0	0.00	2	2.0	0	0.0	1	1.0	3	3.0
Diseased human	*Hydatid cyst* I.P.		10	0.0	0.00	0	0.0	0	0.0	0	0.0	0	0.0
	S. mansoni I.P		10	0.0	0.00	0	0.0	0	0.0	1	10	1	10.0
	Virus hepatitis C, I.P		5	0.0	0.00	0	0.0	0	0.0	0	0.0	0	0.0
Suspected animals	Buffaloes over 5 years old		200	16.0	8.00	68	34.0	3	1.5	6	3.0	93	46.5
	cattle over 5 years old		200	11.0	5.50	26	13.0	4	2.0	4	2.0	45	22.5
	Sheep over 2 years old		250	21.0	8.40	38	15.2	0	0.0	5	2.0	64	25.6
Diseased animals	*Cysticercus bovis* infected buffaloes		15	0.0	0.00	0	0.0	0	0.0	0	0.0	0	0.0
	Fasciola infected sheep		15	1.0	6.60	0	0.0	1	6.6	0	0.0	1	6.6

No positive results were recorded at testing of ten serum samples from parasite free human, cattle, buffaloes and sheep as negative control, W: Women, M: Man

infection in random samples of group of healthy secondary school's students was reached to 1.0 and 3.0% in females and males respectively, using 32 and 53 kD fractions as antigen for *Toxoplasma* and *Sarcocystis*, respectively (Table 3).

These data was in agreement with Ibrahim *et al.* (2006). Presence of direct relation between level of contact with carnivore and incidence of infection by these parasites was previously mentioned by Ahmadi (2004). However, high incidence of infection was recorded in veterinary workers and other exposed people, this was accepted as they spend an obligatory long times in contact with dogs, cats and other animals. The moderate level of infection was recorded in patients suffering from symptoms related to these parasites as myalgia, arthritis and digestive disturbances. This may be attributed to these groups of patients are indirect or accidentally contact with dogs and cats. *Toxoplasma* is high in females while sarcosporidiosis is high in males. High incidence in human may related to absence of specific symptom for the disease the matter which let onset of infection can miss diagnosed with other more common diseases.

It should be mentioned that the fractions of 32 and 53 kD are high specific as they did not cross react with antibodies in serum samples collected from surgically proved *Hydatid cyst* infected patient and Virus hepatitis C infected individuals, but one case was recorded positive

for *T. gondii* and *Sarcocystis* infection from 10 patient harboring *S. mansoni* eggs in their stool. In the same time no positive results were recorded in ten serum samples collected from healthy people of stool free parasites as negative control. Away from the case which diagnosed from *S. mansoni* infected patient which may be reflect true infection rather than cross reaction. The selected two fractions are able to exclude cross reactions with antibodies of other diseases in sera of natural infected human and animals.

Examination of random samples from apparent healthy old cattle and buffaloes (over 5 years old) as well as sheep over 2 years old in the same localities, revealed high total incidence of infection (46.5%) in buffaloes, 22.5% in cattle and it was 25.6% in sheep. These data was in the contrary with Saleh *et al*. (2006) as low incidence 9.3% was recorded in apparently healthy sheep. This data considered to be lowered than that previously mentioned by Mohamed (1988).

The present study evidenced very low incidence of infection by *Neospora canimum*, this clear that the used 32 and 53 kD fractions did not cross react with anti-Neospora Ab present in these positive sera. This data was in the contrary with Cole *et al*. (1994).

The used fractions considered to be specific versus parasitic infections in animal also as no cross reaction was recorded during testing of serum samples from cattle infected by *Cysticercus bovis*, while one from 10 serum samples of sheep harboring *Fasciola* eggs in their faeces give positive results versus *T. gondii* and *Neospora caninum* antigens. No false positive results were detected versus serum samples of healthy cattle, buffaloes and sheep.

In conclusion, this research demonstrated the fractions of 32 kD from TTAg. and that of 53 from SBAg, considered as a good promising tool for early specific diagnosis of the disease in human and living animals. This will be facilitate slaughtering of infected animals under special control measures and ensure total condemnations of the infected tissues. This will be minimizing the random arrival of the cysts to dogs and control the spread of the disease in the surrounding environment.

ACKNOWLEDGMENTS

The authors express more appreciation for Vice dean research of Cairo university and for Faculty of Veterinary Medicine for funding this work via the project No. 09-04, (2004-2006), Developing Scientist Projects Program, Cairo University, El-Giza, Egypt.

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