Diagnosis of bovine sarcocystosis by immunofluorescent antibody technique

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Received: 10 February 2016 / Accepted: 27 January 2017 / Published online: 28 February 2017
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Abstract Studies on sarcocystosis showed an overall seroprevalence of 79.46% by IFAT which included 80.14% in cattle and 78.59% in buffaloes out of 716 animals screened. The antibody titers of six randomly selected positive samples from different age groups of <2, 2–5, 5–10 and >10 years old bovines ranged from 16 to 64, 32 to 256, 32 to 128 and 16 to 64 with an average titer of 32 ± 2.92 , 106.6 ± 34 , 74.6 ± 17 and 34.6 ± 9 , respectively. Significantly, lower rates of infection were observed in the cattle of below 2 years (60%) age and an ascending rate of infection in the age groups of 2–5 years (81.33%), 5–10 years (80.52%) and above 10 years (90.9%).

Keywords Bovine sarcocystosis · Seroprevalence · Detection · Immunofluorescent antibody technique

Introduction

Sarcocystis is an obligatory tissue cyst forming protozoan with a prey predatory life cycle. The presence of *Sarcocystis* spp in meat can lead to a parasitic hazard in humans (Bottner et al. 1987; Li et al. 2002). Antemortem diagnosis is difficult because its symptoms often mimic other diseases. Earlier, the ante-mortem diagnosis of muscular

sarcocystosis was made only by histological examination of muscle collected by biopsy (Tenter 1995) which is always time consuming and always may not be possible to screen large number of cattle before slaughter at abattoirs. For these reasons, serological tests for demonstration of *Sarcocystis* spp specific antibodies are often the primary method of diagnosis and a negative result generally rules out the presence of sarcocystosis (Lunde 1973). The aim of the present study is to standardize immunofluorescence antibody technique as a screening test for the routine diagnosis of bovine sarcocystosis in abattoirs.

Materials and methods

Procurement of Sarcocysts

Heavily infected esophagi with macrocysts of *Sarcocystis* were collected from cattle slaughtered at Chengicherla slaughter house, Hyderabad, A.P., India and carried to the laboratory on ice at 4 °C. The macrocysts were carefully separated from the oesophagus and washed and preserved in PBS pH 7.2 at 4 °C.

Separation of bradyzoites from sarcocyst

The sarcocysts were carefully cut longitudinally with a sharp blade in a sterile beaker containing PBS (pH 7.4). The sarcocysts were neither teased nor triturated to avoid damage to the bradyzoites and to lessen the debris and host material. The cut sarcocysts were agitated for two minutes in PBS (pH 7.4) and filtered through 5 layers of pre-wetted muslin cloth to minimize the debris. The filtered bradyzoites were washed thrice in PBS (pH 7.4) each at 2000 rpm for 5 min. The supernatant was completely

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removed and the final pellet was resuspended in small quantity of PBS (pH 7.4) and adjusted the volume so as to contain 6–8 bradyzoites per high power field of microscope. Such purified bradyzoites were used for further analysis.

Antigen slide preparation for IFAT

Antigen slides were prepared, as per the procedure outlined in the USDHEW manual (1976) with minor modifications. The bradyzoite preparation of *Sarcocystis* spp. were resuspended in a known quantity of PBS (pH 7.4), so as to contain 6–8 bradyzoites per high power field. Approximately, 10–15 µl of the bradyzoite suspension was directly charged and sucked in immediately with the help of a micropipette. This facilitated making a thin smear on the opposite side of the 5 mm diameter area circles (10 No.) each previously using a glass marking diamond pencil on the underside of clean, grease-free glass slides. Once the spots had dried-up, the slides were placed in coupling jar containing chilled acetone and incubated at 4 °C overnight. The antigen coated slides were then air-dried, wrapped in aluminium foil and stored at –20 °C for future use in IFAT.

Immuno-conjugates

Rabbit anti-bovine IgG fluorescence iso-thiocyanate (FITC) for use in IFAT was obtained from Bangalore Genei Pvt. Ltd., India.

Standardization of IFAT

Indirect fluorescent antibody test was performed as described by Renterghem and Nimmen (1976) with minor modifications. Antigen slides were thawed at room temperature for 5 min, rinsed with distilled water and air-dried. The circles on the slides were marked with wax pencil to prevent capillary movement of the well contents. Double dilutions of positive and negative reference sera starting from 1:16 and 1:8, respectively were loaded in antigen wells. Slides were incubated at 37 °C for 30 min in a humid chamber. Such slides were once rinsed with distilled water, followed by 3 washes in PBS (7.6) each for 5 min in a coupling jar with a final rinse in distilled water, and air-dried. Ten microlitres of rabbit anti-bovine IgG FITC (Bangalore Genei) diluted to 1:40 in 1% Evans blue (PBS pH 7.6) were loaded in each well and incubated, followed by washing and air drying, as before. The slides were then immediately examined after mounting in a drop of buffered glycerol under 40 × magnification of a fluorescent microscope (Nikon 80i). Positivity and negativity were determined as per the the guidelines described in USHDEW manual.

Results and discussion

Immunofluorescent antibody technique

In our present study the Immunofluorescent antibody technique was standardized for the diagnosis of bovine sarcocystosis. The primary requirement of the investigation was to collect bradyzoite form of parasites for preparation of particulate antigen for use in serological test. Isolation of host cell free organisms is a basic requirement for antigen preparation. As regard to serological diagnosis the bradyzoites separated from macrocysts of *Sarcocystis* spp were used as source of antigen in IFAT.

Though most of the workers used different procedures, we performed IFAT according to the protocol outlined in the USHDEV manual. In our study, acetone fixed bradyzoites of *sarcocystis* spp. previously smeared on a glass slide and preserved at –20 °C worked very well at 1:16 dilution of positive and negative control sera and 1:40 dilution of rabbit anti-bovine FITC conjugate. The antigenic slide of *Sarcocystis* spp reacted well with sarcocyst positive sera by emitting good fluorescence whereas the same antigenic slide did not react with the bovine anti *Toxoplasma gondii* (RH strain) antisera indicating no cross reactivity. Similar observations were made by Het-tiarachchi and Rajapakse (2008). Whole bradyzoite showing bright yellowish green or distinct yellowish green fluorescence around the entire periphery of the organism were considered as positive (Fig. 1). Similarly the sarcocystis negative sera showed no immune-reactivity with either the antigenic slide of sarcocystis spp or the antigenic slide of *T. gondii* RH strain. Hence, the organisms which did not show any fluorescence but appeared faint reddish or those with polar fluorescence or without any fluorescence

Fig. 1 Photomicrograph of bradyzoite showing *bright yellowish green* fluorescence with sarcocystis positive bovine serum in IFAT (×400)

were declared negative. Non-specific reactions were not observed at higher sera dilutions.

The epidemiological study on bovine sarcocystosis in the sera samples collected from cattle and buffaloes slaughtered at Chengicherla slaughter house, Hyderabad by laboratory standardized Immunofluorescent antibody technique revealed the presence of antisarcocystis antibodies in 323 (80.14%) cattle and 246 (78.59%) buffaloes out of 403 cattle and 313 buffalo sera tested showing an overall prevalence of 79.46% out of 716 animals screened (Fig. 2). Almost similar results were reported by Levine (1973) in cattle in USA. However, various workers (Cerna and Merhautova 1981; Nevole 1982; Bottner et al. 1987; Pana et al. 2001; Sayed et al. 2008) reported higher rate of incidences of sarcocystosis in cattle ranging from 84% to 98% by various methods. The high prevalence in the study may be due to the free movement of large number of stray dogs and access to infected offal accounting for the higher rate of prevalence of infection. Collier et al. (1998) mentioned that a variety of conditions permit such high prevalence of *Sarcocystis* as many definitive hosts are involved in transmission, shedding of large number of sporocysts in external environment for long period, role of invertebrate transport hosts in spreading of infection in addition to little or no immunity to re-shedding of sporocysts after each meal of infected meat.

The highest dilution of serially double diluted bovine sera starting from 1:16 showing immunoreactivity with antigen emitting fluorescence was considered as titer of the serum. Higher average titers were recorded in the age groups 2–5 years (106.6 ± 34), 5–10 years (74.6 ± 17), >10 years (34.6 ± 9) and <2 years (32 ± 2.92) in descending order.

In the present study, the low antibody titers recorded in the young age groups below 2 years could be due to the presence of very young cysts indicated initial stages of infection and proportionate antibody response and above 10 years of age due to very old or ruptured sarcocysts in the muscles due to host immune reaction which was more likely to occur in older animals and may cause progressive

reduction in cyst number resulting in chronic infection over time as opined by Barrows et al. (1982) and Toole (1987). However, the high antibody titers in the age groups 2–5 and 5–10 years provide evidence of acute infection which could be due to continuous intake of infection with actively developed and developing sarcocysts inside the muscles.

In the present study, presence of anti-sarcocystis antibodies in aged animals suggested that the prevalence of sarcocystosis is increases with age. The age wise prevalence of sarcocystosis in cattle indicated low rate of infection in the age group below 2 years (60%) and an ascending rate of infection in the age groups of 2–5 years (81.33%), 5–10 years (80.52%) and in the age group of above 10 years (90.9%). Few cattle were infected below two years of age although the prepatent period is much less than this. Fully formed cysts containing bradyzoites develop in cattle approximately within 70–80 days after ingestion (Dubey 1976). Almost similar findings of age wise prevalence were recorded in buffaloes. The incidence was significantly low in the buffaloes of below 2 years (64%), age group and higher percentage of infection was recorded in 5–10 (86.51%) years of age group followed by in 2–5 (78.94%) year's age group. Our findings were in line with those reports of Huong (1999) and El-Dakhly et al. (2011) who reported an overall prevalence of 79 and 78.9% in buffaloes in Vietnam and Beni-suef, respectively. The above workers also observed a proportionate raise in the incidence with age of the animals between 2 to 7 years of age. However, we recorded a slightly low incidence (77.27%) in buffaloes above 10 years of age which could be due to rupture of *Sarcocystis* which may occur spontaneously or be caused by a host immune reaction, which was more likely to occur in old cysts found in older animals and may cause progressive reduction in cyst number over time as opined by Barrows et al. (1982) and Toole (1987).

Sex wise prevalence of sarcocystosis in bovines indicated a non-significant difference between male and female animals. Our result corroborated with the results of Claveria et al. (1997) who studied the incidence based on the presence of macro and micro sarcocysts in the muscles. However a slight non-significant raise of infection in males (81.87%) than in females (75.19%) could be due to more attention of farmers on milch animals which were productive and maintained in sheds had less proximity to the definitive hosts. In general the male animals were neglected and maintained either for agricultural purposes or for slaughter and kept in open yards, having more accessibility to the definitive host.

The incidence of sarcocystosis in non-descriptive animals was compared with that of cross bred animals in and around Hyderabad. The result showed non-significant difference in the prevalence of sarcocystosis between non-descriptive (79.63%) and cross bred (77.58%) animals. The

Fig. 2 Prevalence of bovine sarcocystosis in cattle and buffaloes by IFAT

reason could be due to an equal accessibility of non-descriptive and cross bred cattle to the faeces of either stray dogs/cats or watch dogs who intern are feed on under cooked or raw beef. In contrary, Claveria et al. (1997) recorded significantly higher incidence of sarcocystosis in imported cattle than in native cattle. It could be due to small sampling of cross bred cattle used in the present study.

For serological examination of the samples we used the IFAT (Pappas et al. 1986). This method revealed more positive animals than the direct demonstration of sarcocysts. The bradyzoites separated from macro cysts of *Sarcocystis* spp. collected from bovine esophagi were used as source of antigen in the diagnosis of bovine sarcocystosis by IFAT. The antigen reacted well with the sera collected from cattle and buffaloes harboring both macroscopic and microscopic sarcocysts which could be due to remarkable degree of cross reaction among *Sarcocystis* species from widely divergent host origins as previously reported by Tadros et al. (1980). Similar results were reported by Habeeb et al. (1996), El-Nazer and Abdil-Azim (2000) who used *S. fusiformis* as the source of antigen in IFAT and same workers and Abdel Rahman (2001) and Sayed et al. (2008) in ELISA for the diagnosis of bovine sarcocystosis.

Our study confirmed that, the visual and microscopic examination of bovine carcass is by no means a satisfactory diagnostic tool and Immunofluorescent antibody technique is the most suited, cost effective, rapid, and reproducible user friendly antimortum diagnostic tool for screening of animals to be slaughtered at abattoirs in large scale.

There is paucity in information on the serological diagnosis of sarcocystosis and prevalence and distribution of sarcocystosis as evidenced by the scanty reports on the subject. A review of available documented studies on sarcocystosis suggested that to date, our findings may represent the first data on the prevalence of bovine sarcocystosis by serological method like immunofluorescent antibody technique in India.

References

- Abdel Rahman SM (2001) Serodiagnosis of two zoonotic parasites (*Toxoplasma* & *Sarcocystis*) in cattle. In: First Congress of Food Hygiene & Human Health, Faculty of Veterinary Medicine, Assiut, Egypt
- Barrows PL, Prestwood AK, Green CE (1982) Experimental *Sarcocystis suicanis* infections: disease in growing pigs. *Am J Vet Res* 43:1409–1412
- Bottner A, Chalerson WAG, Pomroy WE, Rommel M (1987) The prevalence and identity of *Sarcocystis* in beef of cattle in New Zealand. *Vet Parasitol* 24(3–4):157–168
- Cerna Z, Merhautova V (1981) *Sarcocystis* in cattle and sheep at Prague abattoir. *Folia Parasitol* 28:125–129
- Claveria FG, Petersen B, Macabagdal MR, Farolan RJ, Farrol MA, Gonzalvo F, Cadiz R, Ajero R, Roque R, Lozano G (1997) A survey of bovine, bubaline and swine sarcocystosis in the Philippines. *Southeast Asian J Trop Med Public Health* 28(1):173–178
- Collier L, Balows A, Sussman M (1998) *Sarcocystis*, *Isospora* and *Cyclospora*. Topley and Welson's microbiology and microbial infections, vol 5, 9th edn. Oxford University Press, Inc, New York, pp 319–326
- Dubey JP (1976) A review of *Sarcocystis* of domestic animals and of other *Coccidia* of cats and dogs. *J Am Vet Med Assoc* 169(10):1061–1078
- El-Dakhly KM, Khaled A, El-Nesr MS, El-Nahass ES, Hirata A, Sakai H, Yanai T (2011) Prevalence and distribution patterns of *Sarcocystis* spp. in buffaloes in Beni-Suef, Egypt. *Trop Anim Health Prod* 43:1549–1554
- El-Nazer M, Abdil-Azim AH (2000) Seropositivity to *Sarcocystis* antigen in attendants of rheumatology clinic in Sohag University Hospital. *South Val Med* 4(2):145–155
- Habeeb YS, Selim MA, Ali MSM, Mahmoud LA, Abdel-Hady AM, Shfei A (1996) Serological diagnosis of extra-intestinal sarcocystosis. *J Egypt Soc Parasitol* 26(2):393–400
- Hettiarachchi DC, Rajapakse RPVJ (2008) Antigenic analysis of bovine *Sarcocystis* spp in Sri Lanka. *J Nat Sci Found Sri Lanka* 36(3):239–244
- Huong LTT (1999) Prevalence of *Sarcocystis* spp. in water buffaloes in Vietnam. *Vet Parasitol* 86(1):33–39
- Levine ND (1973) *Sarcocystis*, *Toxoplasma* and related protozoa. In: *Protozoan parasites of Animals and Man*, 2nd edn. Burgess Minneapolis, pp 292–294
- Li QQ, Yang ZQ, Zuo YX, Attwood SW, Chen XW, Zhang YP (2002) A PCR-based RFLP analysis of *Sarcocystis cruzi* (protozoa: sarcocystidae) in Yunnan Province, PR China, reveals the water buffalo (*Bubalus bubalis*) as a natural intermediate host. *J Parasitol* 88(6):1259–1261
- Lunde MN (1973) Laboratory methods in the diagnosis of Toxoplasmosis. *Health Lab Sci* 10:328
- Nevole M (1982) Prevalence of sarcocystosis in our cattle. *Veterinafstvi* 32:24
- Pana HF, Ogessawara S, Sinhorini IL (2001) Occurrence of cattle sarcocystosis species in raw kibbe from Arabian food establishments in the city of Sao Paulo, Brazil, and experimental transmission to humans. *J Parasitol* 87:1459–1465
- Pappas MG, Lunde MN, Hajkowski R, McMahon J (1986) Determination of IgM and IgG antibodies to *Toxoplasma* using the IFA test, ELISA, and Dot-ELISA procedures. *Vet Parasitol* 20(1–3):31–42
- Renterghem LV, Nimmen LV (1976) Indirect immunofluorescence in toxoplasmosis: frequency, nature and specificity of polar staining. *Zbl Bakt Hyg I Abt Orig* 235:559–565
- Sayed FG, Shaheen MSI, Arafa MI, Koraa HM (2008) *Sarcocystis* infection in cattle at assiut abattoir: microscopical and serological. *Assiut Univ Bull Environ Res* 11:47–56
- Tadros W, Hazeohoff W, Laarmann JJ (1980) Cross reaction in the indirect fluorescent antibodies among six species of *Sarcocystis*. *Trop Geogr Med* 6:150–153
- Tenter AM (1995) Current research on *Sarcocystis* spp of domestic animals. *Int J Paratsitol* 25:1311–1330
- Toole D (1987) Experimental ovine sarcocystosis: sequential ultrastructural pathology in skeletal muscle. *J Comp Path* 97:51–60
- USDHEW (1976) Manual—a procedural guide for the performance of the serology of toxoplasmosis. Centre for Disease Control Atlanta, (US Department of Health, Education and Welfare), Atlanta