Setaria digitata infections in cattle: parasite load, microfilaraemia status and relationship to immune response

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Abstract

A total of 110 cattle were examined in an area endemic for Bancroftian filariasis for the prevalence of infection of the bovine filarial parasite *Setaria digitata*. About 12.5% of cattle were found to harbour both adult worms in the peritoneum and microfilariae (mf) in circulation; 70% of the cattle were amicrofilaraemic but with an adult worm infection. A third group of cattle (16.5%) was free of detectable mf and adult worms. The presence of adult worms and/or mf did not influence the antibody levels to any of the four antigen preparations of *S. digitata*. However, there was a significant inverse relationship between the presence of antibodies to microfilarial sheaths and the absence of circulating mf as shown by the immunoperoxidase assay. Cattle immunoglobulin containing high titres of anti-sheath antibodies cleared circulating microfilariae very effectively in *Mastomys coucha* thus demonstrating the protective nature of anti-sheath antibodies in eliminating circulating microfilariae *in vivo*.

Introduction

Animal models of filariasis have been used widely for understanding the pathogenesis of the disease, protective immunity and for screening potential anti-filarial drugs. Although *Brugia malayi*, a human filarial parasite has been adopted in small laboratory animals such as gerbils and *Mastomys coucha*, these animals do not display clinical features associated with the human disease (Lok & Abraham, 1992). On the other hand, *B. pahangi* infected cats and dogs or *Dirofilaria immitis* infected dogs are naturally existing animal models for filariasis and have been used extensively for addressing issues on pathogenesis and protective immunity in filariasis (Fletcher *et al.*, 1986; Miller *et al.*, 1991; Schreuer & Hammerberg, 1993).

Setaria digitata is a common filarial parasite of cattle. Due to the easy availability of large quantities of parasites from slaughtered animals, the parasite has been increasingly used in recent years for immunological, chemotherapeutic and other biological studies (Mukhopadhyay *et al.*, 1995; Bal & Das, 1996; Wijesundera *et al.*, 1996; Mukhopadhyay & Ravindran, 1997; Dalai *et al.*, 1998). However, detailed investigations such as adult worm load, prevalence of microfilaraemia and relationship to immune responses in cattle naturally infected with *S. digitata* have not previously been undertaken and the current study fills this gap. Furthermore, recent reports have indicated zooprophylaxis in Africa, whereby human populations were found to be protected against *Onchocerca volvulus* infection as a result of exposure to the cattle parasite *O. ochengi* (Wahl *et al.*, 1998). In this context investigations on the epidemiology of *S. digitata* infections in cattle are crucial for evaluating the extent of exposure of humans to infective larvae of *S. digitata* and its effect on human filariasis in endemic areas.

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Materials and methods

Worm load

Fresh adult stage parasites of *S. digitata* were collected in PBS-1% glucose from the peritoneal cavity of cattle slaughtered at the local abattoir in Jadupur, Orissa, India.

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Table 1. The occurrence of adult worms/microfilariae of *Setaria digitata* in naturally infected cattle.

Cattle group*	Number/total (%)	Adult worm load: median (range)		Proportion (%) of cattle infected with adult worms**		
		Females	Males	Females only	Males only	Females and males
I	14/109 (12.84)	5.5 (1–14)	2 (1–5)	3 (21.42)	0 (0)	11 (78.52)
II	77/109 (70.64)	5 (1–30)	2 (1–22)	31 (40.25)	1 (1.29)	45 (58.44)
III	18/109 (16.5)	0	0	0	0	0

* Group I, with adult worms and microfilariae; group II, with adult worms only; group III, absence of adult worms and microfilariae.
** Test for proportion 'Z' test: $P < 0.001$ for unisexual worms in group I vs. group II.

Worm load (both male and female adult parasites) of individual cattle was recorded at the time of collection. The presence of mf in peripheral blood was detected by Giemsa stained thick blood smears and/or mf concentrated by passing 10 ml of blood through 5 μM polycarbonate membranes (Nucleopore, USA).

Serum collection

Blood for sera were collected from all the 110 cattle, allowed to clot at room temperature and the sera separated by microfuging and stored at −20°C.

Preparation of mf slides

Intrauterine microfilariae (mf) of *S. digitata* were obtained from mature adult female worms by dissection *in vitro*. Mature mf were harvested from the circulation of infected cattle by filtration of infected blood through 5 μM polycarbonate membranes (Nucleopore, USA) as described by Ravindran *et al.* (1988). About 25 μl of mf suspension (1500 mf per ml) were spotted onto microscopic slides, air dried, fixed with acetone and stored at 4°C for further use as 'antigen slides' in an immunoperoxidase assay (IPA).

Immunoperoxidase assay

Antibodies to mf sheath in sera were detected by an IPA as described by Ravindran *et al.* (1988). Briefly, acetone-fixed mf on microscopic slides were treated with 1% H_2O_2 in methanol to remove endogenous peroxidase activity and incubated with 5-fold diluted cattle sera for 2 h at 37°C. After washing, the slides were incubated serially with 100-fold diluted rabbit anti-bovine Ig followed by 100-fold diluted swine anti-rabbit Ig-peroxidase conjugate (Dakopatt, Denmark). The slides were stained with diaminobenzidine (0.5 mg ml^{-1} in Tris-HCl buffer with H_2O_2), dehydrated in ethanol and observed using a light microscope to score reactivity. Anti-bovine Ig was raised by immunizing rabbits with three doses (1 mg per dose at 15 day intervals by the IM route, the first dose with complete Freunds adjuvant and the subsequent ones without adjuvant) of bovine Ig affinity purified using protein-A sepharose (Sigma Chem. Co., USA). Sera collected 10 days after the last dose was used for the IPA as described above.

Preparation of antigens

Antibodies to solubilized antigens of adult female and male worms, microfilariae and to excretory-secretory antigens of adult female worms of *S. digitata* were quantified by ELISA. For preparation of antigens, worms (adults and mf) were extensively washed and homogenized in PBS (Polytron homogenizer, USA) and microfuged at 10,000 *g* and the clear supernatant was stored at −20°C. The ES-antigen was prepared by culturing adult female worms in protein-free DMEM overnight at 37°C and the spent medium was microfuged, concentrated 50-fold using Centricon tubes (Amicon, UK) and stored at −20°C.

ELISA

ELISA was performed with modifications for bovine sera as described by Mukhopadhyay *et al.* (1995). Briefly, antigen coated plates were incubated with 500-fold diluted sera (serum titration indicated 1/500 to be optimum for maximum sensitivity of ELISA values) and the bound antibodies were detected using sequentially 500-fold diluted rabbit anti-bovine Ig, swine anti-rabbit IgG-peroxidase conjugate (Dakopatt, Denmark) and O-phenylenediamine and absorbance read in an EIA reader (Bio Rad USA) at 492 nm. The absorbance values were converted into arbitrary ELISA units using a high titre cattle serum pool as an internal standard. For detection of circulating filarial antigen in cattle sera, the *Wuchereria bancrofti* Og_4C_3 kit (Trop Bio Pty. Ltd, Australia) was used as per manufacturer's instructions.

Passive transfer of immunoglobulins

Mastomys coucha (procured from CDRI, Lucknow, India) injected with 5×10^5 *S. digitata* microfilariae by the IP route were used for passive immunization with cattle immunoglobulins (40% ammonium sulphate precipitate of pooled serum, positive or negative for anti-sheath antibodies). Eight days post-infection, animals with mf levels of 1000 ml^{-1} or more were injected for three successive days with cattle immunoglobulins (1 mg per dose by the IP route) in two different groups. The percentage of microfilaraemia was scored with reference to mf counts on the first day of immunoglobulin injection.

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Table 2. ELISA*: antibodies to *Setaria digitata* in infected cattle to different developmental stages.

Cattle group (n)	Category	Adult female antigen	Adult male antigen	Microfilariae	ES product
I (7)	Adult +ve Mf +ve	63.86 ± 16.47	69.96 ± 25.56	59.66 ± 21.85	210.43 ± 51.51
II (30)	Adult +ve Mf −ve	67.88 ± 23.30	70.83 ± 26.49	65.08 ± 26.93	227.01 ± 79.37
III (8)	Adult −ve Mf −ve	74.93 ± 32.80	75.62 ± 32.55	71.13 ± 29.37	183.21 ± 98.61

* ELISA units, mean ± s.d.

Statistical analysis

Statistical analysis of data was performed by the student 't'-test, χ^2 test for consistency and the 'Z' test for testing the proportion of animals in groups.

Results and Discussion

The present study on the prevalence of natural infections of *S. digitata* and immune responses in cattle was undertaken to evaluate the use of this model to address issues on protective immunity in filariasis and to gain insights for future investigations on zooprophylaxis as well as zoonotic infections of human populations with the bovine filarial parasite. A total of 110 cattle was examined for the presence of adult worms of *S. digitata* in the peritoneum and for microfilariae in the blood. The cattle could be classified into three groups based on the presence of two stages of the parasite: group I, those harbouring adult worms and microfilariae; group II, those with only adult worms in peritoneum but without circulating mf; and group III, those with neither of the stages of the parasite (table 1). Group II was the dominant one (about 70% of the animals), the other two (I and III) comprising 13% and 16% respectively. Adult worm loads between groups I and II were comparable, although the percentage of animals with unisexual worms was significantly more in group II than in group I.

Despite a large percentage of infected animals (84% – group I and II together) indicating a high level of transmission in the field, quantitatively the adult parasite load in infected animals was not very high. A median parasite load of 5.5 female worms and 2.0 male worms per infected animal were reported (table 1). Live *S. digitata* adult worms (male worms about 4–6 cm and female worms about 6–12 cm long) are located with relative ease with the naked eye. The worms are found mostly on the peritoneal walls although occasional worms are found on the intestinal folds. There can, however, be marginal error in 'scoring' worm yield. This could not have significantly contributed to very small numbers of worm yield observed in the current study. These findings can be interpreted to indicate the possible existence of concomitant immunity against developing larval stages in animals already harbouring adult worms. Currently, however, there are no methods available that could allow identification of concomitant immunity in filariasis – cattle naturally infected with *S. digitata* can be utilized as a model to develop appropriate indicators for this purpose.

Since 12.8% of cattle were found to be microfilaraemic and the vector, *Armigeres* sp. that transmits *S. digitata* in cattle (Verma *et al.*, 1971) has an anthropophilic biting habit (Mahapatra *et al.*, 1995), it is highly probable that a significant proportion of humans can be exposed to infective larvae of *S. digitata*. However existing tools do not allow specific measurements of immune responses of humans to *S. digitata* infections. Identification of *S. digitata* specific larval antigens could lead to a clearer understanding of zooprophylaxis in human filariasis.

Antibodies to four different filarial antigen preparations of *S. digitata*: (i) adult females; (ii) adult males; (iii) microfilariae; and (iv) ES products of adult females, were quantified by ELISA in all three groups of cattle. Uniformly high titres of anti-filarial antibodies were found in all groups and there was no significant difference in antibody levels between the three groups of cattle (table 2). Interestingly, the filarial antibody levels in the third group of animals (i.e. those without detectable adult worms or microfilariae) were comparable to the other two infected groups (table 2), indicating the possibility that group III could be the bovine counterpart of putatively immune cases observed in human filariasis (Ottesen, 1992).

Immunoperoxidase assay was performed in animals harbouring adult worms (groups I and II), to detect the presence of antibodies to sheaths of mature microfilariae. There was a statistically significant inverse association as shown by the χ^2 test ($P < 0.001$) between the presence of antibodies to microfilarial sheaths and the absence of circulating microfilariae (table 3). Such an association has been found earlier in human Brugian (McGreevy *et al.*, 1981) and Bancroftian (Ravindran *et al.*, 1990) filariasis

Table 3. Immunoperoxidase assay*: correlation between antibodies to microfilarial sheaths and circulating microfilariae in *Setaria digitata* infected cattle.

Cattle group	Antibodies to microfilarial sheaths Number/Total (%)	
	Present	Absent
I (Mf +ve) (n = 13)	1 (7.69)	12 (92.30)
II (Mf −ve) (n = 43)	33 (76.74)	10 (23.25)

* Tested at 1:5 dilution, χ^2 test ($P < 0.001$).

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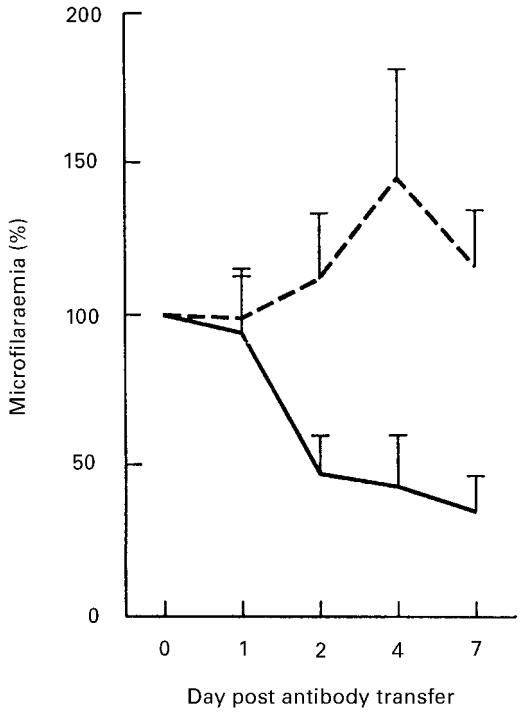

Fig. 1. Percentage of microfilaraemia (mean±SEM) in groups of *Mastomys coucha* infected with 5×10^5 *Setaria digitata* mf by the IP route. Animals with mf levels of 1000 ml^{-1} or more were injected with cattle Ig positive (solid line; n = 8) or negative (broken line; n = 7) for anti-sheath antibodies.

indicating that the nature of antimicrofilarial immunity could be similar in bovine and human filariasis. However, there is no empirical demonstration of the protective nature of anti-sheath antibodies in eliminating microfilariae *in vivo* so far. Administration of three doses of cattle immunoglobulin with high titres of anti-sheath antibodies resulted in significant removal of circulating microfilariae of *S. digitata* in *Mastomys coucha* in comparison to controls injected with immunoglobulins of infected cattle which lack anti-sheath antibodies (fig. 1). Easy access to large quantities of blood as well as parasites in the bovine model and the parasite's adaptability in small rodents (Mukhopadhyay *et al.*, 1995) could thus be utilized for identifying vital filarial sheath antigens involved in transmission blocking immunity.

The Og_4C_3 antigen detection kit used for the detection of circulating filarial antigens in human Bancroftian filariasis was evaluated to detect and quantify a similar antigen (if any) present in *S. digitata*. Titrations of different concentrations revealed the presence of 11520 antigen units per 1 μg of solubilized adult female *S. digitata* and 2300 antigen units per 1 μg of ES antigens. However, the circulating antigen could be detected only in 30% of group I and 20% of group II animals. These results indicate that the Og_4C_3 antigen detection kit is not a useful tool to detect *S. digitata* infections in cattle populations. The peritoneal dwelling nature of adult parasites, the low parasite load seen in infected animals and the high blood volume in cattle could have been the reason for the low sensitivity of the Og_4C_3 kit in detecting circulating filarial antigen in *S. digitata* infected cattle.

The results of the present investigation also indicate the need to study the consequences of *S. digitata* infections in human populations. Zoonotic infections of humans with a variety of animal parasites is known (Orihel & Eberhard, 1998) and several reports on clinical manifestations in humans by the canine filarial worm *D. immitis* further strengthens the need to investigate the clinical consequences of *S. digitata* infections in human populations.

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