

## Antigenic cross-reactivity between *Necator americanus* and *Ascaris lumbricoides* in a community in Papua New Guinea infected predominantly with hookworm

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### Abstract

Sero-epidemiological data are presented in which antigenic cross-reactivity between *Necator americanus* and *Ascaris lumbricoides* has been investigated in a community in Papua New Guinea infected predominantly with *N. americanus*. It is our contention that the antigenic cross-reactivity which undoubtedly exists between these species accounted for (i) a peak in antibody levels against *N. americanus* in 10-13 years old children (driven by infection with *A. lumbricoides*), and (ii) the maintenance of apparent antibody levels against *A. lumbricoides* in older age groups (driven by infection with *N. americanus* in the absence of overt infection with *A. lumbricoides*). Cross-reactivity was analysed further, and apparently *N. americanus*-specific epitopes identified, by immunoblotting. These observations could have considerable bearing on the interpretation of data from sero-epidemiological studies which failed to take account of concurrent infection with these parasites.

### Introduction

The antigenic cross-reactivity which undoubtedly exists between *Necator americanus* and *Ascaris lumbricoides* (BALL *et al.*, 1971; NEPPERT & WARNS, 1974; TURNER *et al.*, 1980), and their widespread co-existence in human populations (SCHAD *et al.*, 1975; SHIELD *et al.*, 1981; ELKINS *et al.*, 1986), have the potential to complicate the interpretation of immunological data obtained during field investigations into the epidemiology of these gastrointestinal nematodes.

Therefore, a detailed investigation of serological cross-reactivity between these species was considered essential for the accurate evaluation and interpretation of data collected from future sero-epidemiological studies and an analysis of antibody levels in sera collected during a recent field study in Papua New Guinea (PRITCHARD *et al.*, 1990) was conducted. As the respective prevalences of *Necator* and *Ascaris* in the study population were 92% and 11%, it was possible to investigate immunological cross-reactivity at the molecular level, and to look for species-specific molecules, by selecting for further analysis a subset of sera from individuals free from infection with *A. lumbricoides*.

### Materials and Methods

Fieldwork was carried out in Kebasob village on Karkar Island, Papua New Guinea, between July and

August 1988, and has been described in detail elsewhere (PRITCHARD *et al.*, 1990). 202 individuals were selected to form the study group.

### Parasitology

Faecal samples of known weight (1-2 g) were collected and preserved in 10% formalin (v/v). These were processed using a formalin-ether concentration technique (HALL, 1981), and eggs per gram (epg) values calculated. Each subject was then treated with the anthelmintic pyrantel pamoate (Combantrin®; Pfizer, Sandwich, Kent), administered at a single dose of 10 mg/kg. Following anthelmintic treatment, faeces were collected for 48 h. Faeces were washed through 2 grades of sieve (1 mm and 300µm) with unlimited water and the sediment preserved in 10% formalin. Worms were recovered from the sediment after careful searching by eye, and hookworms were identified to species by microscopical examination after clearing by lactophenol. Full 48 h post-treatment faecal samples were obtained from 123 individuals. Between 6 and 12 d after chemotherapy, a further faecal sample was obtained from 25 individuals to assess treatment efficacy (see PRITCHARD *et al.*, 1990, for further details).

### Serology

At the time of anthelmintic treatment, venous blood samples (4 ml) were collected into tubes coated with ethylene diaminetetraacetic acid and centrifuged and the plasma collected. Samples were frozen within 2 h of collection, and sent by air-freight to Nottingham on dry ice; they were still frozen on arrival.

Adult *N. americanus* excretory-secretory (ES) products were prepared according to the methods of CARR & PRITCHARD (1987). Adult *A. lumbricoides* pseudocoelomic fluid (PCF) was collected in the field from freshly expelled live worms. For enzyme-linked immunosorbent assay (ELISA), 50 µl of the appropriate antigen preparation was diluted to 2.5 µg/ml in 0.1 M carbonate-bicarbonate buffer, pH 9.6, then added to each well of 96-well plates for incubation overnight at 4°C. After washing (×5) in phosphate-buffered saline containing Tween 20® (0.05%), 100 µl of 5% skimmed milk solution was added for 1 h at room temperature to block potential non-specific binding sites. Following a further 3 washes, sera were added in triplicate (50 µl of a 1:250 dilution). Following 2 h incubation, sera were removed by washing (×3), and 50 µl of an alkaline-phosphatase conjugated goat anti-human immunoglobulin serum added (1:350) for 2 h. The plates were

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finally washed ( $\times 3$ ) before the addition of 100  $\mu$ l of *p*-nitrophenyl phosphate substrate. Absorbance at 410 nm was determined 30 min later on an automated ELISA reader. Absorptions were performed *in situ* according to the method of LYNCH *et al.* (1988), using 150  $\mu$ g PCF/well, and the efficiency of absorption monitored by ELISA against PCF. (It had been shown in previous experiments to standardize this assay that concentrations of PCF in excess of 2  $\mu$ g/ml were capable of removing anti-*Ascaris* activity completely whilst retaining activity against hookworm antigens. Therefore, 150  $\mu$ g PCF/well can be considered to be in vast excess.)

#### Immunoblotting

For immunoblotting, 750  $\mu$ g of each parasite antigen were loaded across the full stacking gel surface (apart from 1 marker lane) of a 5–20% sodium dodecyl sulphate-polyacrylamide electrophoresis gel, then separated overnight at constant voltage (80V). The proteins were transferred to nitrocellulose according to the method of TOWBIN *et al.* (1979). Individual strips were incubated overnight with a 1:200 dilution of serum (in 5% skimmed milk in tris-buffered saline (TBS), pH 8.2). Strips were washed ( $\times 5$ ) in TBS, and [ $^{125}$ I] protein A added (3.7 kBq per lane) for 2 h. Following a further 5 washes, strips were placed on Kodak X-OMATS® film for 24 h at  $-70^{\circ}\text{C}$  in the presence of an intensifying screen. For absorption, 25  $\mu$ l of sera were incubated at  $4^{\circ}\text{C}$  overnight on 3 successive occasions with an equal volume of *A. lumbricoides* PCF (13mg soluble protein/ml).

## Results

### Parasitology

The prevalence of hookworm infection (individuals with worms and/or eggs in faecal samples were counted as infected) was 92%. Prevalence increased rapidly with age, reaching 100% in the 14–23 years old age class (Fig. 1). The arithmetic mean hookworm burden was  $25.3 \pm 4.0$  (standard error) worms/host (range 0–263 worms/host), and hookworm burden increased significantly with host age (PRITCHARD *et al.*, 1990). All hookworms identified were *N. americanus*.

The prevalence of *Ascaris* infection was 11%, with peak prevalence in the 10–13 years old group (Fig. 1). The mean *Ascaris* burden was  $0.12 \pm 0.04$  worms/host, with a maximum burden of 2 worms. There was no correlation between *Ascaris* burden and hookworm burden (Kendall's rank correlation test,  $\tau = 0.092$ ,  $n = 123$ , not significant).

*Enterobius vermicularis* and *Trichuris trichiura* were also present in the population, at prevalences of 53% and 3% respectively.

### Serology

The relationship between ELISA values against *Necator* and *Ascaris* antigens and host age is shown in Fig. 2. Both anti-*Ascaris* activity and anti-*Necator* activity before absorption showed a clear peak in the 10–13 years old age class; the peak in activity against *Necator* was largely removed by absorption with *Ascaris*. Absorption with *Ascaris* clearly reduced the ELISA value against *Necator*: mean ( $\pm$  standard error) values were  $1.03 \pm 0.04$  before absorption and  $0.74 \pm 0.03$  after absorption (paired *t* test on log-transformed data:  $t = 10.6$ ,  $n = 108$ ,  $P < 0.001$ ).

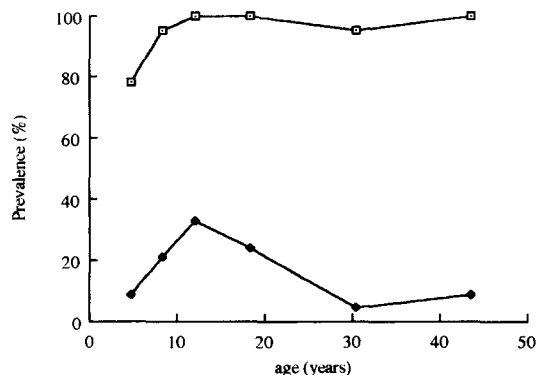


Fig. 1. The age-prevalence profile of gastrointestinal nematode infection, assessed by counts of expelled worms and faecal egg counts, in Kerasob, Papua New Guinea.  $\square$ , *Necator americanus*,  $\blacklozenge$ , *Ascaris lumbricoides*.

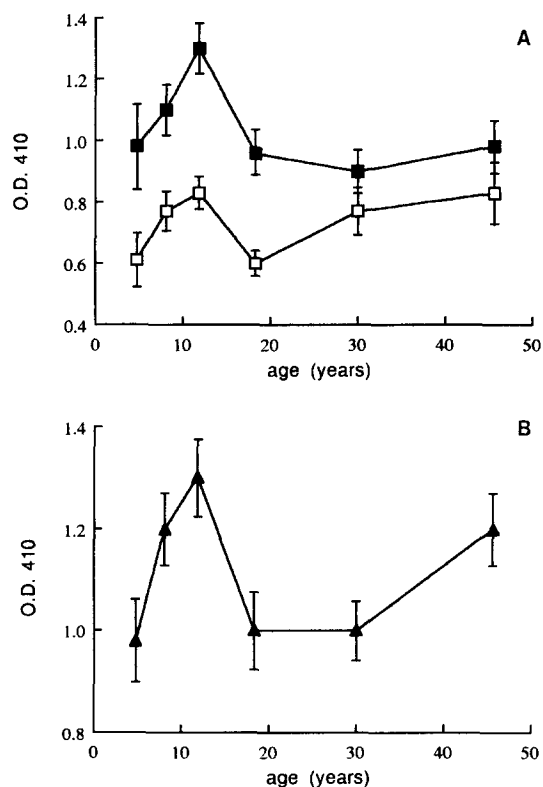


Fig. 2. The relationship between antibody levels and host age. A. Anti-*Necator* excretory-secretory antigens antibodies before ( $\blacksquare$ ) and after ( $\square$ ) absorption with *Ascaris* pseudocoelomic fluid. B. Anti-*Ascaris* pseudocoelomic fluid antibodies ( $\blacktriangle$ ). All values are arithmetic mean  $\pm$  standard errors of optical density of 410 nm (O.D.410).

There was a significant correlation between ELISA values against *N. americanus* and *Ascaris*, and a significant correlation between ELISA values against *N. americanus* ES itself, both before and after absorption (Table).

**Table.** The relationship between antibody levels against *Necator* excretory-secretory antigens (ES), before and after absorption with *Ascaris* pseudocoelomic fluid (PCF), and against *Ascaris* PCF, in 108 individuals from Karkar Island, Papua New Guinea (correlation coefficients between log-transformed data)

|                                      | Anti- <i>Necator</i> ES |                    | Anti- <i>Ascaris</i> |
|--------------------------------------|-------------------------|--------------------|----------------------|
|                                      | Unabsorbed              | Absorbed           | PCF                  |
| Anti- <i>Necator</i> ES (unabsorbed) | 1                       | —                  | —                    |
| Anti- <i>Necator</i> ES (absorbed)   | 0.676 <sup>a</sup>      | 1                  | —                    |
| Anti- <i>Ascaris</i> PCF             | 0.516 <sup>a</sup>      | 0.468 <sup>a</sup> | 1                    |

<sup>a</sup> $P < 0.001$ .

range of antigens in *A. lumbricoides* PCF (9, 13, 23, 28, 36, 40, 47, 56, 65, 74, 88, 97 and 109 kDa). Activity against *Ascaris* was removed by absorption with PCF (Fig. 3). However, activity was retained against *Necator* ES products (Fig 3), indicating the presence of *N. americanus*-specific epitopes on a range of molecules. (The relatively poor resolution of the *Ascaris* blots is a common experience in our hands and is not a reflection of the poor resolution of protein bands.)

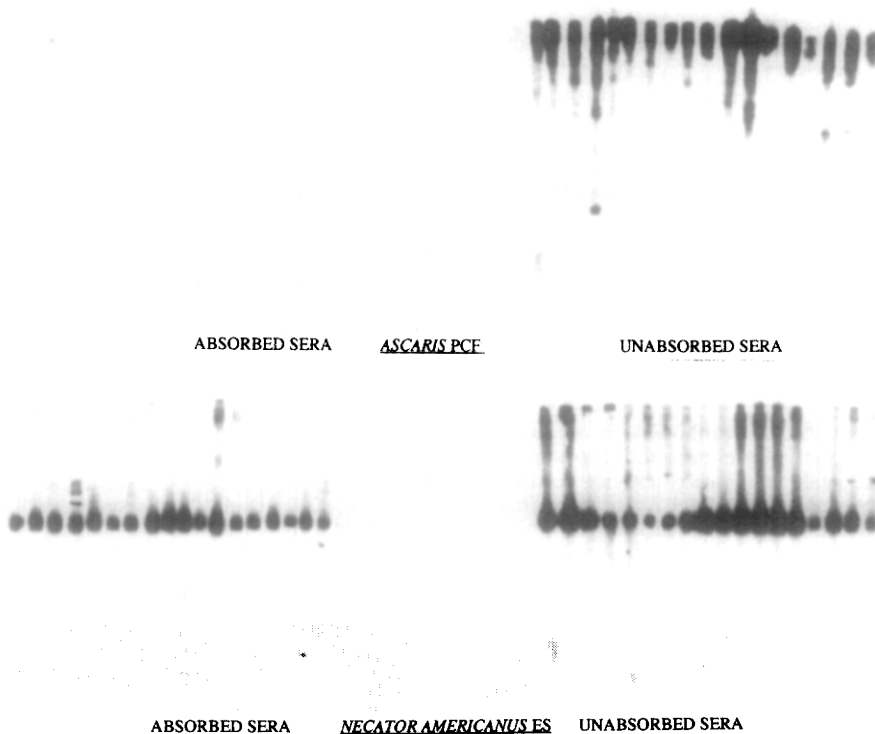


**Fig. 3.** Western blots. Upper panels: *A. lumbricoides* pseudocoelomic fluid (PCF), probed with absorbed and unabsorbed sera. Lower panels: *N. americanus* excretory-secretory antigen (ES), probed with absorbed and unabsorbed sera. The first (left) lane of each panel was probed with normal human serum, followed by the 18 Papua New Guinea sera.

To assess the nature of the molecules recognized in each antigen preparation before and after absorption, immunoblotting was performed using sera from 18 individuals infected with *N. americanus*, but free from infection with *A. lumbricoides*, at the time of sampling. This sub-sample would be expected to react against cross-reactive epitopes in *Ascaris* PCF before absorption, but against *Necator*-specific epitopes after absorption.

Before absorption, all sera were tested against *N. americanus* ES and *A. lumbricoides* PCF (Fig. 3) by immunoblotting. All sera (apart from normal human serum) reacted predominantly against a 33 kDa ES product of *N. americanus* (with minor recognition at 40, 43, 53, 92, 108 and 120 kDa), and against a wide

## Discussion

The results of this study show a high degree of cross-reactivity between immunoglobulin (Ig) G, A and M antibody responses to *Necator* ES and *Ascaris* PCF antigens. Cross-reactivity has previously been reported by a number of authors (BALL *et al.*, 1971; NEPPERT & WARNS, 1974; TURNER *et al.*, 1980), but the molecular basis of the cross-reactivity has not previously been investigated. Our results suggested that cross-reactivity is due to epitopes carried on a range of *Ascaris* antigens, and that a number of *Necator*-specific antigens exist. Following the absorption of sera with PCF, residual antibody was directed predominately against what has been reported to be an *N. americanus*-specific epitope, resolving at

33 kDa (PRITCHARD *et al.*, 1991). This may be homologous to the *N. americanus*-specific allergens identified by iso-electric focusing ( $pI < 5.1$ ) by TURNER *et al.* (1980), and it will now be of interest to assess the reactivity of IgE antibodies against this molecule.

Absorption significantly reduced binding in ELISA, suggesting that cross-reactivity is more extensive at the level of IgG, IgA and IgM isotypes, as only marginal reductions in IgE titres were seen following absorption in a previous study (TURNER *et al.*, 1980). It is possible, therefore, that levels of specific IgE antibody against *N. americanus* will most accurately reflect the degree or presence of hookworm infection in the individual (GANGULY *et al.*, 1988).

Serological analysis revealed a strong positive correlation between levels of anti-*Necator* and anti-*Ascaris* antibodies. Taking into account the respective prevalence and intensity of infection with each parasite species, the data suggest that *Ascaris* PCF could almost be used as a surrogate antigen in the analysis of antibody responses to *Necator*. This correlation persisted even after absorption of sera with *Ascaris* PCF, due perhaps to incomplete absorption or perhaps to variation between individuals in absolute levels of antibody production. It could not be attributed to correlation between *Necator* and *Ascaris* worm burdens. The retention of a correlation between ELISA values against *N. americanus* ES before and after absorption with PCF supports the contention that the removal of cross-reactive antibodies does not affect the recognition of epitopes specific to *N. americanus*.

The level of anti-*Ascaris* antibodies in our study population must partly have reflected exposure to *Ascaris* infection: peak anti-*Ascaris* activity occurred in the same age-class as peak *Ascaris* prevalence. However, the high levels of anti-*Ascaris* activity in the older age-classes are associated with a very low prevalence of *Ascaris* and the maintenance of anti-*Ascaris* activity could be due to a number of mechanisms; (i) pre-exposure to *Ascaris* generates long-lived antibody responses, (ii) continued exposure to larval stages of *Ascaris* maintains anti-PCF levels, or (iii) anti-PCF activity is maintained by cross-reactive epitopes expressed by *N. americanus*.

However, most of the published data on the cross-reactivity between these species indicates that it is probably more likely that the response is due to cross-reactivity with *Necator* antigens, and is not genuinely anti-*Ascaris*. In contrast, there is a strong *Necator*-specific response in all age-classes.

To conclude, a study has been conducted (using sera from individuals characterized with respect to gastrointestinal nematode infection) to re-assess antigenic cross reactivity between *A. lumbricoides* and *N. americanus*. The results must serve as a timely reminder to present and future field parasitologists that cross-absorption of sera might be essential for the collection of accurate species-specific serological data. Although cross-reactivity was confirmed, and further defined by Western blotting, molecules specific to *N. americanus* were also observed; the evaluation of the immunological recognition of epitopes expressed on these molecules may form the basis for future field studies of the epidemiology of hookworm infection.

## Acknowledgements

Thanks are extended to the Wellcome Trust, Christensen Research Institute, Madang Provincial Government, Papua New Guinea Institute of Medical Research, the people of Kebasob, and Drs Katharine Trenholme, Terry Reardon, Matthew Jebb, Rosella Uwedo and Michael Alpers. In addition, the study would not have been possible without the considerable contributions of Gideon Philip and Taita Watt of the Papua New Guinea Institute of Medical Research.

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Received 27 September 1990; accepted for publication 19 February 1991