HELMINTHS AND ALLERGIC DISEASE IN VIETNAM

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**To my wife, Susanne, and my sons Carl, Jonas, and Moritz,
who accompanied me on this journey. Without them this project
would not have been possible.**

**And to my parents for their love, great encouragement,
and support over the years.**

ABSTRACT

Background:
Allergic disease is uncommon in developing countries, especially in rural areas. A protective effect of helminth infection has been implicated as a potential explanation.

Objectives:
To determine whether reduced exposure to helminth infection is associated with a higher risk of allergen skin sensitisation and allergic disease, and whether such an association could be explained by a helminth-induced up-regulation of certain cytokines, in particular anti-inflammatory IL-10.

Methods:
We invited 1,742 rural Vietnamese schoolchildren to take part in a cross-sectional baseline survey followed by a randomised, double blind, placebo-controlled trial of anti-helminthic therapy at 0, 3, 6, and 9 months to compare the change in exercise-induced bronchospasm (primary outcome), wheeze, rhinitis, eczema, and allergen skin sensitisation (secondary outcomes) at 12 months. 244 secondary schoolchildren also had venous blood taken to measure helminth induced IL-10, IFN-γ, IL-5, and IL-13. Out of these 244 children, 144 were infected with hookworm and had bloods taken again at 12 months.

Results:

- **Baseline survey**

1,601 schoolchildren (92% of those eligible) in grades 1-9 aged 6-18 participated in the baseline survey. 0.4% (6/1601) of children had a fall in peak flow after exercise of at least 15%. Doctor-diagnosed asthma was equally rare (0.4%, 6/1601), while 5.0% (80/1601) of children had experienced wheezing over the past 12 months. 6.9% (110/1601) of parents reported that their children had suffered of hay fever in the past 12 months, and in 2.6% (41/1601) of cases this diagnosis was confirmed by a doctor. 5.6% of children (89/1601) reported an itchy rash over the past 12 months. 0.9% (14/1601) had a history of flexural involvement and on examination 0.5% (8/1601) proved to have flexural eczema on the day of the survey. Skin prick test positivity was commoner than allergic disease. 33.5% (537/1601) of children had at least one positive skin prick test (dustmites 14.4%, cockroach 27.6%). The cross-sectional analysis yielded only significant results for allergen skin sensitisation.
In univariate analysis, sensitisation was less frequent in children with hookworm or *Ascaris* infection, and increased in those with better santitation, including flush toilets and piped drinking water. In multivariate analysis, the risk of allergen skin sensitisation to house dust mite was reduced in those with *Ascaris lumbricoides* infection (adjusted OR=0.28, 95% CI 0.10-0.78) and in children with higher hookworm burden (adjusted OR for 350+ versus no eggs per gram

faeces=0.61, 0.39-0.96), and increased in those using flush toilets (adjusted OR for flush toilet versus none/bush/pit=2.51, 1.00-6.28). In contrast, sensitisation to cockroach was not independently related to helminth infection but was increased in those regularly drinking piped or well water rather than from a stream (adjusted OR=1.33, 1.02-1.75).

- **Intervention study**

1,566 children in grades 1-8 completed the baseline survey and all consented to be randomised to either anti-helminthic treatment or placebo. 1487 children (95%) completed the intervention study. There was no effect of therapy on the primary outcome, exercise-induced bronchoconstriction (within-participant mean % fall in peak flow from baseline after anti-helminthic treatment 2.25 (SD 7.3) vs placebo 2.19 (SD 7.8, mean difference 0.06 (95% CI -0.71-0.83), p=0.9), or on the prevalence of the secondary clinical outcomes questionnaire-reported wheeze (adjusted OR=1.16, 0.35-3.82), rhinitis (adjusted OR= 1.39, 0.89-2.15), or flexural eczema (adjusted OR=1.17, 0.39-3.49). However, anti-helmithic therapy was associated with a significant allergen skin sensitisation risk increase in the treatment compared to the placebo group (adjusted OR=1.31, 1.02-1.67). In post-hoc analysis this effect was particularly strong for children infected with *Ascaris lumbricoides* at baseline (adjusted OR=4.90, 1.48-16.19), the majority of whom were co-infected with hookworm.

- **Cytokine profiles**

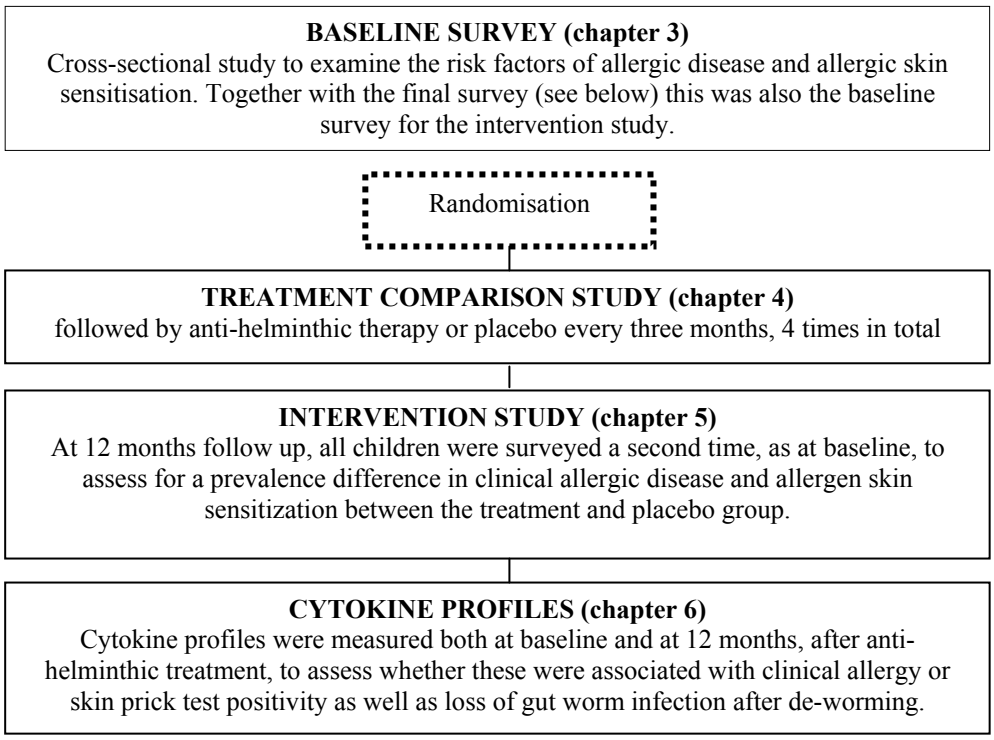
Hookworm-induced IL-10 was inversely related to allergen skin sensitisation (any positive skin prick test) at baseline, but this result missed conventional statistical significance (univariate OR=0.70, 0.48-1.03; adjusted OR=0.72, 0.44-1.18). No other cytokine response was associated with skin prick test positivity at baseline (univariate OR IFN-γ=1.15, 0.71-1.85; univariate OR IL-5=0.84, 0.53-1.33). Similary, no significant changes in any of the cytokine profiles were observed following anti-helminthic therapy in the treatment compared to the placebo group (p=0.3 for all three cytokines).

Conclusion

The baseline study suggested that hookworm and *Ascaris* infection, sanitation and water supply independently reduce the risk of allergic sensitisation. The intervention study confirmed that helminth infection and allergic sensitisation are inversely related and that the effect of *Ascaris* and hookworm infections on skin prick test responses is additive. However, we found little evidence to suggest that this effect was mediated by IL-10. There was also insufficient evidence to suggest that loss of exposure to gut worms for 12 months results in an increase in clinical allergic disease. The effect of more prolonged de-worming warrants further research.

THE PhD ROAD MAP

To aid the reader just a few words of orientation at the beginning. I have followed the conventional background, methods, results, and discussion format. However, since the PhD consists of three main studies, a cross-sectional baseline survey (chapter 3), a randomised double blind placebo-controlled trial to indentify the most suitable anti-helminthic agent for the main intervention study (chapter 4), and the main intervention study (combination of baseline and final survey after anti-helminthic treatment, chapter 5), which all employ essentially the same methods, I have described the methods in detail in one dedicated chapter (chapter 2) and only briefly summarise them again in chapters 3 to 5. As for the cytokine methods and results, both are found in chapter 6. An overview flow chart of the studies is found below:



FOREWORD

PJ Preston was the first to speculate in a paper presented at a symposium on allergy held at the Royal Naval Hospital in Haslar in 1970 that the high prevalence of allergic diseases in western countries might be related to improved hygiene and loss of exposure to helminth parasites. Based on his personal clinical experience with 12 hayfever patients who had become symptom-free while infected with *Ascaris lumbricoides* on trips abroad, he wondered: "…Is the atopic syndrome a consequence of good hygiene? … Could it then be that the biological advantage associated with an efficient IgE producing mechanism is related to the maintenance of the balance between host and parasite in worm infestations, …?" (Preston, 1970). Subsequently, JA Turton wrote in *The Lancet* in 1976: "…, I infected myself with 250 *N. americanus* larvae of Nigerian origin …, and the most pertinent finding in the context of the discussion on IgE, parasites, and allergy was that during the summer of 1975 and 1976 I remained completely free from all symptoms of hayfever." (Turton, 1976) The discussion of the potential links between helminth parasites and allergic disease has continued ever since.

Certainly in evolutionary terms a protective effect of gut worm infection on allergic disease would make sense. Humans have evolved exposed to gut worms for millions of years. It is striking that infection with *Ascaris lumbricoides* and hookworm, two host invasive parasites, tends to be asymptomatic and chronic, commonly lasting for years.

Indeed, it is known that adult hookworms have substances in their saliva that can tone down the human host immune response directed at them, thus prolonging their own survival inside the host (Pritchard and Brown, 2001). In addition, *Ascaris lumbricoides* and schistosomiasis infections are associated with an increased expression of certain anti-inflammatory host cytokines, such as interleukin 10, which have been implicated as being protective against allergic skin sensitisation and clinical allergic disease (Yazdanbakhsh et al., 2002).

This PhD thesis explores the relationship between helminth infection and allergic disease in a paediatric population in a rural setting in central Vietnam, first in a cross-sectional survey and then also in an intervention study with an antihelminthic agent to see whether helminth infection reduces the risk of clinical allergic disease and allergen skin sensitisation.

Most of the work in this thesis is already in print. The background chapter, chapter 1, is partly based on two systematic reviews (Flohr, 2003, Flohr et al., 2005). The cross-sectional survey results presented in chapter 3 have been published in the *Journal of Allergy and Clinical Immunology* (Flohr et al., 2006). The randomised controlled trial to identify the most suitable anti-helminthic agent for the intervention study described in chapter 4 has appeared in the *American Journal of Tropical Medicine and Hygiene* (Flohr et al., 2007a), and the intervention study results (chapter 5) together with the cytokine analysis (chapter 6) has been submitted to *The Lancet*. All papers already in print have been included in the appendix.

STATEMENT OF AUTHOR'S ROLE

I was responsible for designing the study. I was the Principal Investigator on all research grant applications. I also supervised the fieldwork in Vietnam and the data entry. I performed the laboratory work for cytokine profiles, first at the Pasteur Institute Nha Trang and then at the Oxford University Clinical Research Unit in Ho Chi Minh City, where I was based throughout the project. The statistical analysis of the baseline and intervention study was conducted by myself and supervised by Prof Sarah Lewis, Professor of Medical Statistics, University of Nottingham. Dr Rupert Quinnell, Lecturer, Institute of Integrative and Comparative Biology, Faculty of Biological Sciences, University of Leeds supervised the data analysis of the randomised controlled trial to identify the most efficacious anti-helminthic treatment and the analysis of the cytokine data. I wrote this PhD thesis and the manuscripts of the papers that have already been published from this work.

ACKNOWLEDGEMENTS

I would especially like to thank my supervisor Prof Hywel C Williams (Professor of Dermato-Epidemiology, Centre of Evidence-Based Dermatology) and my co-supervisor, Prof John Britton (Professor of Epidemiology, Division of Epidemiology & Public Health), for their support and mentorship. It was Prof Williams' idea to work on helminth-allergy links, and he helped to established the fruitful collaboration with Profs John Britton and Sarah Lewis (Professor of Medical Statistics, Division of Epidemiology & Public Health) and Prof David Pritchard (Chair of Parasite Immunology, School of Pharmaceutical Sciences) here in Nottingham, all of whom gave scientific advice both in the run up to the study and also from the distance, once I was working in the field in Vietnam.

I would like to thank Prof Sarah Lewis for her invaluable statistical support throughout the project. Dr Rupert Quinnell (Lecturer, Institute of Integrative and Comparative Biology, University of Leeds) came out to Vietnam to show me how to perform whole blood cultures for cytokine assays in the field, and he was also my anchor when I needed parasitological advice and help with the analysis of the cytokine data. He also helped with proof-reading of the cytokine chapter.

Since my medical student elective at the Oxford University Clinical Research Unit in Ho Chi Minh City in 1997, I had hoped to return to Vietnam for a period of research. Over the years, Prof Jeremy Farrar, back then and now Director of the Oxford University Clinical Research Unit and my supervisor in Vietnam, was a source of great

encouragement and support. Dr Cameron Simmons, Head of the Oxford Research Unit's immunology lab, gave important scientific advice. I am particularly grateful to Ms Bich Chau for teaching me the art of cytokine ELISAs. The Oxford Unit's research microbiologist Mr Jim Campbell kindly oversaw the training in parasite stool analysis in the field.

My Vietnamese collaborators, Prof Tran Tinh Hien (Vice-Director, Hospital for Tropical Diseases, Ho Chi Minh City), Dr Luc Nguyen Tuyen (Director, Khanh Hoa Provincial Center for Malaria and Filariasis Control, Nha Trang City), and Dr Truong Tan Minh (Director, Khanh Hoa Provincial Health Service, Nha Trang City) all made crucial contributions to the conduct of the project and gave invaluable advice on how to conduct research projects in a rural setting in Vietnam. I am indebted to the children, parents, and fieldworkers in Khanh Son, central Vietnam, for their participation and enthusiasm for this study.

I would also like to thank Prof Richard Powell (Dept. of Immunology, Queen's Medical Centre, Nottingham) for training in skin prick testing. I was very grateful for the logistic support through Dr Alan Brown, Ms Marilyn Antoniak, and Mr Steve Cockbill (all University of Nottingham) and the staff of the Oxford University Clinical Research Unit, Ho Chi Minh City. Prof Terence Ryan (Emeritus Professor of Dermatology, Oxford University), Prof Laura Rodrigues (London School of Hygiene and Tropical Medicine), Mr Ron Marchand and Dr Do Hoang Huy (both Medical Committee Netherland Vietnam) as well

as Dr Antonio Montresor (WHO Hanoi) gave additional scientific advice.

My project would not have been possible without salary support from Oxford University (John Radcliffe Travelling Research Fellowship in Medical Sciences, University College, University of Oxford) and the Wellcome Trust UK (grant code: WT077078) as well as a research grant from Asthma UK (Applicants: C Flohr (PI), J Britton, DI Pritchard, HC Williams (co-applicants); project code: 04/045) and the Bastow Award from the Special Trustees of Nottingham University Hospitals (Applicant: C Flohr, project code: STR 03/M/B3).

Nottingham, September 2007

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ABBREVIATIONS

CI – confidence interval

Epg - eggs per gram faeces

ELISA – enzyme linked immunoabsorbent assay

IgE – Immunoglobulin E

IL – interleukin

IFN – interferon

MAb – monoclonal antibody

OR – odds ratio

PHA - phytohemagglutinin

SPT – skin prick test

STH – soil-transmitted helminths

Th cells – T helper cells

CHAPTER 1: BACKGROUND

About 1 in 5 children in industrialised countries suffer of asthma, allergic rhinitis, and eczema, the so-called 'allergic diseases' (The ISAAC Steering Committee, 1998). While a family history plays an important role in allergic disease susceptibility, genetics alone cannot explain the increase in allergic disease prevalence in the western world over past decades, the urban-rural prevalence gradient in less developed countries, and the positive association with higher social class (Heinrich et al., 1998, Williams et al., 1994d, Golding and Peters, 1987). Equally, migrant studies point towards a role for the environment (Waite et al., 1980, Burrel-Morris and Williams, 2000, Neame et al., 1995). Current epidemiological research has therefore focused on identifying possible environmental factors that are associated with increased allergy risk with a particular emphasis on lifestyle differences between urban and rural communities.

1.1 THE HYGIENE HYPOTHESIS

Based on data collected from 17,414 British children born during one week in 1958 and followed up until the age of 23 (the National Child Development Study), Strachan found that eczema and hay fever were less common in children growing up with larger numbers of siblings (Strachan, 1989). At the time, he speculated that this sibling effect might be due to increased opportunities for cross-infection in children growing up in larger families. Since then, the sibling effect has been

confirmed by a large body of epidemiological studies (Strachan, 2000, McKeever et al., 2001, Karmaus and Botezan, 2002).

It has been suggested that allergic disease occurs when the developing immune system is deprived of the obligatory stimulation through microbes, especially if this occurs during the first few months of life (Bach, 2002, Rook and Brunet, 2002). Immunologically, there is some evidence to suggest that this may partly be due to an imbalance between type 1 and type 2 T helper (Th) cells, ie that the immune system at birth fails to mature postnatally and achieve immune tolerance, manifested in an imbalance between both types of T helper cells due to a lack of microbial stimulation. This may lead to overshooting Th2-mediated responses to environmental antigens and therefore a higher risk of clinical expression of allergic diseases. In addition, bacterial and viral infections are well-known inducers of type 1 Th cells, and it has been suggested that early infection with such pathogens may lead to the expression of Th1-mediated immune responses and suppress allergic disease later on in life (Yazdanbakhsh and Rodrigues, 2001).

Indirect epidemiological evidence to support the ‘hygiene hypothesis’ comes from studies that compare the risk of allergic disease in children who regularly attend day care facilities with children who are being looked after at home, assuming that day care facilities provide a broader exposure to pathogens than a home environment. Most but not all of these studies have shown a reduced risk of developing allergic disease in children who attend dare care facilities during the first year

of life (Hagerhed-Engman et al., 2006, Celedon et al., 2003, Benn et al., 2004). While a number of earlier studies have shown negative associations between allergic disease, allergen skin sensitisation and individual pathogens, such as Hepatitis A (Matricardi et al., 1997, Linneberg et al., 2003, Matricardi et al., 2002), *Toxoplasma gondii*, *Helicobacter pylori* (Linneberg et al., 2003, Matricardi et al., 2000, Matricardi et al., 2002), measles (Shaheen et al., 1996), and tuberculosis (Shirakawa et al., 1997), such associations have not been confirmed in other settings, and it may not be a specific pathogen but microbial burden *per se* that has an impact on allergy development (Flohr et al., 2005, Farooqi and Hopkin, 1998, von Mutius et al., 1999, McKeever et al., 2002, Olesen et al., 2003, Benn et al., 2004, Bodner et al., 1998, Paunio et al., 2000, Gibbs et al., 2004).

This view is supported by the fact that children growing up in anthroposophic communities have less allergies compared to children from a non-anthroposophic background (Floistrup et al., 2006, Alm et al., 2002, Alm et al., 1999).[1] The anthroposophic lifestyle includes a diet rich in lactobacilli and restrictive use of antibiotics. In fact, a number of studies, not only among children from anthroposophic communities, have shown a positive association between antibiotic prescribing and allergy risk (Floistrup et al., 2006, Flohr et al., 2005, von Mutius et al., 1999, Farooqi and Hopkin, 1998, Droste et al., 2000, Wickens et al., 1999, Celedon et al., 2002). Antibiotics lead to a

[1] The anthroposophic movement ('anthroposophy': Greek for 'wisdom of man') was founded by the Austrian Rudolf Steiner in the early twentieth century. Anthroposophical philosophy has been applied to many domains of life, including agriculture and medicine.

reduction in microbial burden, and they are also known to alter the gut microflora and thus could have an effect on the child's immune system at the level of the gut mucosa.

In addition, farm children have less allergic disease than peers who are not exposed to a farm environment, and this may well be due to the consumption of unpasteurised farm milk rich in lactobacilli and mycobacteria and higher endotoxin exposure (Ege et al., 2006, Alfven et al., 2006, Flohr et al., 2005, Von Ehrenstein et al., 2000, Riedler et al., 2000, Benn et al., 2004, Kilpelainen et al., 2000, Braback et al., 2004, Perkin and Strachan, 2006).

In particular the positive association between an anthroposophic lifestyle, antibiotic use and the reduced allergy risk in farm children suggests that the key to a better understanding of the increase in allergic disease in industrialised countries and urban centres of developing nations may lie in environmental factors that prime the infant's immune system perinatally (Warner, 2004, Chung et al., 2007), especially at the level of the gut mucosa (Figure 1.1).

Indeed, the gut is the largest mucosal surface area of the human body interacting with the environment and therefore probably represents the most important source of antigenic stimulation that drives maturation of the immune system postnatally (Hooper and Gordon, 2001). Some but not all studies have suggested that the microflora of children with allergic diseases is less often colonised with lactobacilli. Instead, more coliforms and *Staphylococcus aureus* have been found in such children, and these changes can occur already a few months post-

natally and precede the clinical manifestation of eczema (Bjorksten, 2006, Bjorksten, 2004, Bjorksten et al., 1999, Bjorksten et al., 2001, Flohr et al., 2005, Watanabe et al., 2003, Kalliomaki et al., 2001, Bottcher et al., 2000). This has led to promising new therapeutic strategies, such as diet supplementation with *Lactobacillus* or formula feed that enhances growth of bifidobacteria and lactobacilli, also marketed as pro- and pre-biotics (Noverr and Huffnagle, 2005, Boehm et al., 2005, Moro et al., 2006, Bjorksten, 2006, Flohr et al., 2005). The current epidemiological and immunological understanding has thus moved beyond the earlier and rather simplistic Th1/Th2 imbalance paradigm (Yazdanbakhsh and Rodrigues, 2001, Sheikh et al., 2003). It is now believed that microbial antigens, such as the normal gut microflora, play a crucial role during early infancy in the switch between atopic and nonatopic phenotypes, possibly through stimulation of dendritic cells at the level of the gastrointestinal mucosa and then mediated upstream through regulatory T cells and the induction of anti-inflammatory cytokines, such as interleukin 10 (IL-10, Figure 1.1). This is also where helminths come into the picture.

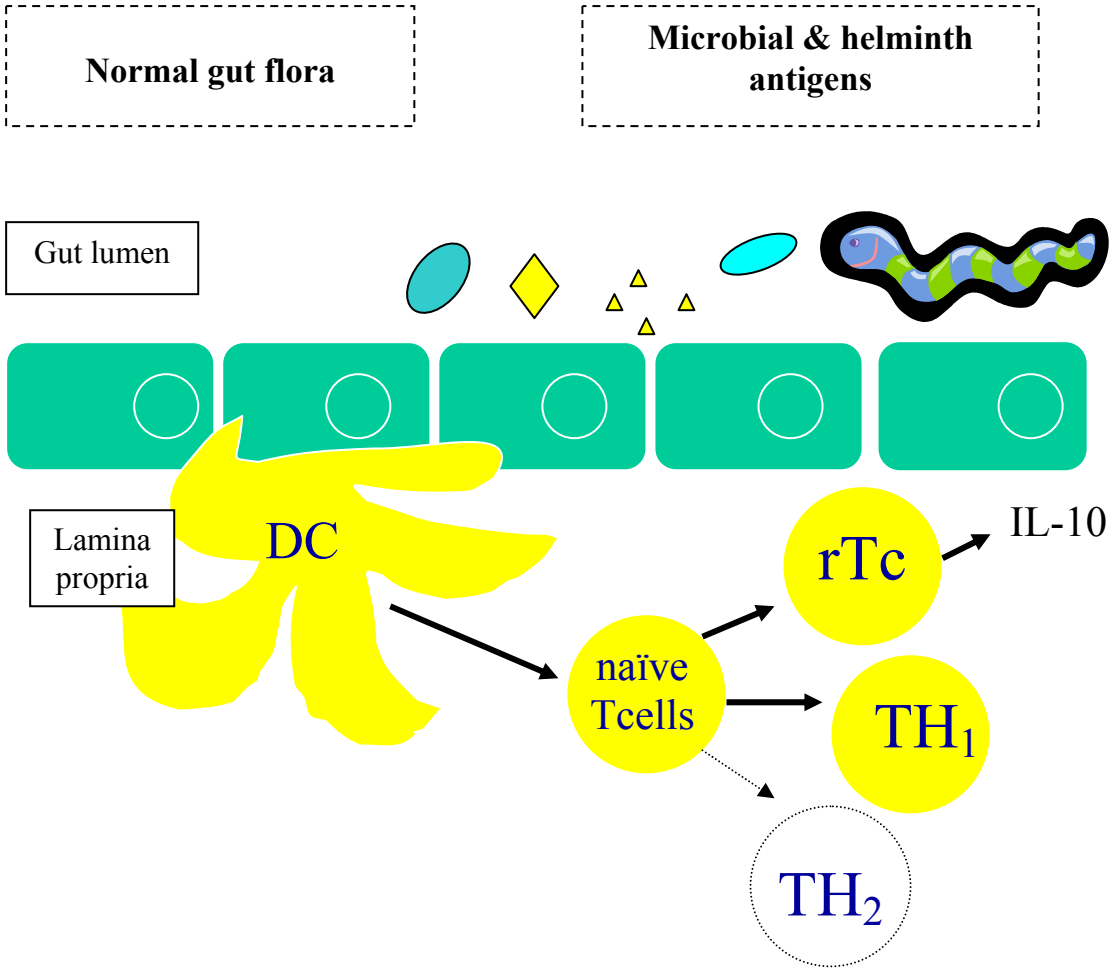


Figure 1.1 The postulated link between allergic disease and the gut microflora. Chronic stimulation of gut-associated lymphoid tissue through normal gut flora and other microbial antigens might ensure the development of a Th1-/regulatory T cell-dominated cytokine milieu and therefore prevention of allergic disease. Regulatory T cells may have a role in this through the production of anti-inflammatory cytokines, such as IL-10.

DC, dendritic cell; TH, T helper cell; rTc, regulatory T cell.

Source: (Flohr et al., 2005)

1.2 SOIL-TRANSMITTED HELMINTHS AND ALLERGIC DISEASE

Worldwide more than 2 billion people are chronically infected with soil-transmitted helminths (STH): schistosomiasis, *Ascaris lumbricoides*, the hookworms (*Ancylostoma duodenale* and *Necator americanus*), and *Trichuris trichiura* (WHO, 2002). The hookworms, *A. lumbricoides*, and schistosomiasis have a systemic phase inside the human host, while *Trichuris* does not migrate beyond the lumen of the gut. Poor sanitation and hygiene are the main factors that predispose to all four infections. While *Ascaris* and *Trichuris* are transmitted faecal-orally, hookworm larvae and schistosomal cercariae enter the host via the skin (Figure 1.2).

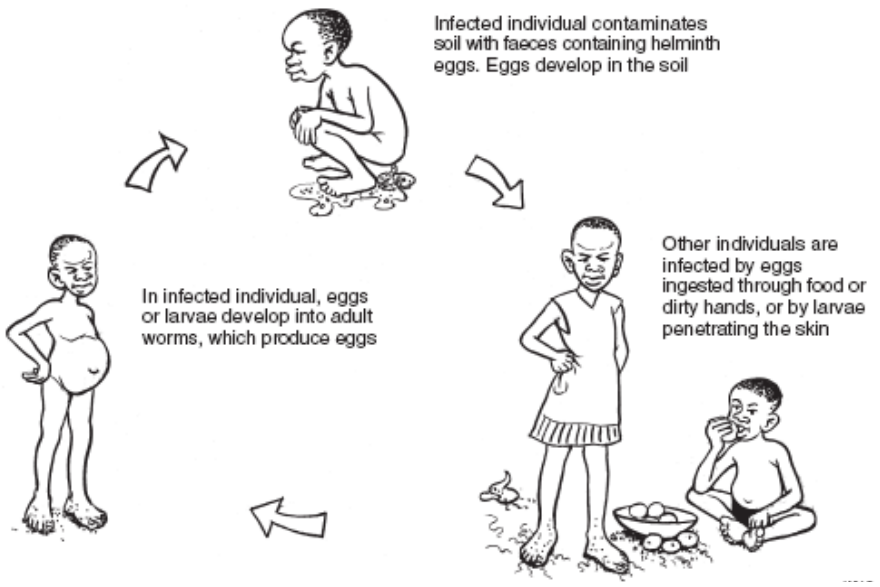


Figure 1.2 Lifecyle of intestinal helminths. *Ascaris lumbricoides* and *Trichuris trichiura* are transmitted faecal-orally, while hookworm larvae and schistosomal cercariae penetrate the skin (Montresor et al., 2002).

In endemic areas without existing de-worming campaigns infection with individual worms commonly lasts for years, recurs and is largely asymptomatic. Humans and helminths have evolved together over millions of years, and it is a commonly held view that human host immune responses designed to expel the parasite, such as soil-transmitted helminths, are in evolutionary terms among the earliest allergic responses generated by the human immune system. Indeed, Th2-dominated immune responses leading to an up-regulation of IL-4, IL-5, and IL-13-mediated IgE and eosinophil production and mast cell degranulation are hallmarks of both allergic and helminth diseases, and IgE-driven acute sequelae of helminth infection, for example eosinophilic pneumonitis associated with Ascariasis, are well-known disease complications (Pritchard and Brown, 2001, Markell et al., 1999). Allergic diseases now so common in the western world may represent a phenotype with particularly strong Th2-driven responses, which originally conveyed a higher chance of survival in an environment where helminthic infestations were endemic. However, where endoparasites are uncommon and other microbial stimuli equally lacking, the human immune system may fail to develop immune tolerance postnatally and may consequently be more prone to allergies.

While IgE-mediated host responses are associated with the acute stages of helminth infections, parasites have developed methods to modulate the host immune system, presumably to prolong survival within the host (Pritchard and Brown, 2001). Despite the immunological

similarities between endoparasitic infections and allergies, helminth-infested individuals appear to be protected from mast cell degranulation and inflammatory responses in affected tissues, and it has been suggested that this is due to a protective immunomodulatory network generated by parasite-induced T-cells and their cytokines, including IL-10, ultimately leading to prevention of allergic tissue inflammation (Yazdanbakhsh et al., 2002, Yazdanbakhsh and Matricardi, 2004, Pritchard and Brown, 2001).

1.2.1 Early exposure to parasite antigens

Similar to the physiological gut microflora, enteric exposure to helminths may provide important signals to the neonatal immune system that promote maturation and immune tolerance (Cooper, 2004). There is no doubt that fetal exposure to helminth antigens can occur already *in utero* through maternal infection. For instance, cord blood lymphocytes from babies born to helminth-infected mothers in Kenya produce parasite antigen-specific IgE, and filarisis-specific IgE has been demonstrated in cord blood from infants delivered in India (Weil et al., 1983, King et al., 1998). Another source of helminth antigen exposure of the neonate may be though breastmilk as well as early postnatal infection, although the latter is probably less important in children who are not yet able to walk.

It has been suggested from work on lymphatic filariasis that such early exposure may lead to lasting immune tolerance to parasite antigens and prevent children from developing acute, overshooting tissue

inflammation in response to acute infection and re-infection, and that this may be particularly advantageous in highly endemic areas (Cooper, 2004, Das et al., 1997, Terhell et al., 2002, Steel et al., 1994). If this also holds true for helminth infection, perinatal exposure to high levels of parasite antigen may prove sufficient to induce long-term immune system hypo-responsiveness, and through the cross-reactivity between parasite antigens and environmental allergens, such as house dust mites and cockroach, this may also convey protection against atopy and allergic disease (Arruda and Santos, 2005).

1.2.2 Helminths and skin sensitisation to environmental allergens

Following Hagel et al's suggestion that frequent and heavy helminth infection might protect against allergic skin sensitisation (in this thesis also refer to as 'atopy') based on observations made among Venezuelan slum children, Cooper et al formally demonstrated cross-sectionally in rural Ecuadorian paediatric populations that a heavier gut worm burden has a significantly stronger protective effect on skin prick test positivity than light infestation (Hagel et al., 1993a, Cooper et al., 2003b, Cooper et al., 2003a). While protective effects on atopy have been shown for all helminths, including *Ascaris lumbricoides*, *Trichuris trichuria*, hookworm, and schistosomiasis, the effect sizes have been varied, not always significant, and, in the case of *Trichuris* infection, an increase in atopy risk was found in one study (Dagoye et al., 2003). For a comprehensive summary of previous study results,

based on a systematic MedLine search conducted in June 2007, see Table 1.1.

Table 1.1 Cross-sectional studies on the link between helminths and atopy

Type of helminth	Odds ratio (95% CI)	Effect
Any helminth		
(Cooper et al., 2004)	0.65 (0.47-0.91)	↓
(Davey et al., 2005)	0.75 (0.58-0.97)	↓
(Nyan et al., 2001)	0.30 (0.11-0.80)	↓
Hookworm		
(Cooper et al., 2003a)	0.67 (0.33-1.37)	NS
(Cooper et al., 2003b)	0.39 (0.18-0.85)	↓
(Dagoye et al., 2003)	1.30 (0.80-2.20)	NS
(Davey et al., 2005)	0.74 (0.55-0.99)	↓
(Scrivener et al., 2001)	1.70 (0.88-3.27)	NS
Ascaris		
(Cooper et al., 2003a)	0.65 (0.54-0.78)	↓
(Cooper et al., 2003b)	0.74 (0.60-0.91)	↓
(Dagoye et al., 2003)	1.00 (0.70-1.40)	NS
(Obihara et al., 2006)	0.57 (0.23-1.40)	NS
(Schafer et al., 2005)	0.74 (0.60-0.92)	↓
(Scrivener et al., 2001)	1.52 (0.81-2.87)	NS
Trichuris		
(Cooper et al., 2003a)	0.69 (0.56-0.86)	↓
(Cooper et al., 2003b)	0.82 (0.67-1.01)	Borderline ↓
(Dagoye et al., 2003)	1.70 (1.10-2.40)	↑
(Scrivener et al., 2001)	1.10 (0.56-2.16)	NS
Schistosomiasis		
(Araujo et al., 2000)	0.14 (0.03-0.63)	↓
(van den Biggelaar et al., 2000)	0.32 (0.16-0.63)	↓

↓ = reduced risk, ↑ = increased risk, NS = non-significant association

1.2.3 Helminths and clinical allergic disease

While the majority of studies point towards an inverse relationship between skin prick test sensitisation and helminth infection, the picture is less clear for clinical allergic disease, namely asthma, eczema, and hay fever.

1.2.3.1 Rhinitis

In 1976, a British researcher reported having been completely symptom-free from hitherto treatment-resistant hay fever, while he remained experimentally infected with *Necator americanus* larvae (Turton, 1976). However, few have studied the association between hay fever and helminths since. Lynch et al. looked at urban-rural prevalence differences of allergic disease and helminth infection among 811 Venezuelan children. Allergic rhinitis was significantly more common in the urban population while parasitosis with *Ascaris lumbricoides* was equally prevalent among both urban and rural participants (Lynch et al., 1984), suggesting that other exposures were responsible for urban-rural prevalence differences in rhinitis. Equally, a large cross-sectional questionnaire-based survey conducted in a paediatric population in rural Ecuador found no significant association between rhino-conjunctivitis symptoms and *Ascaris lumbricoides* infection [adjusted OR=1.00, 0.55-1.79, (Cooper et al., 2004)], as did a population-based cross-sectional survey in Cape Town, South Africa [OR=1.04, 0.22-4.82, (Obihara et al., 2006)]. However, a study conducted among 3,107 primary school children in Taipei, Taiwan, suggested that *Enterobius vermicularis* may be protective against physician-diagnosed rhinitis (adjusted OR=0.61, 0.45-0.84).

1.2.3.2 Eczema

As for eczema, there is evidence from observational studies that it is common in populations with low parasite burden (Gerrard et al., 1976,

Larrick et al., 1982, Lynch et al., 1983, Lynch et al., 1998, Flohr, 2003). In 2005, Schäfer et al. confirmed this anecdotal evidence, reporting an independent inverse association between *Ascaris lumbricoides* and eczema on physical examination in a population-based study of 4,169 East German children [adjusted OR=0.45, 0.33-0.60, (Schafer et al., 2005)]. This effect was even more pronounced in sensitised children (adjusted OR=0.31, 0.18-0.56). Further supporting evidence comes from a small birth cohort study among 103 mother-infant pairs in Uganda, where eczema risk was decreased by 74% until age 15 months with maternal helminth infection during pregnancy and at birth [adjusted OR=0.26, 0.08-0.83 (Elliott et al., 2005)]. This birth cohort study included the randomised administration of either single oral dose albendazole or placebo to pregnant mothers, and anti-helminthic treatment was associated with an increased eczema risk in infants in univariate analysis. However, this effect did not reach statistical significance after adjustment (adjusted RR=2.40, 0.77-7.48), most likely due to small participant numbers and resulting low statistical power. However, apart from these supportive studies, there are also a number of cross-sectional studies that have not found a significant association between eczema risk and helminth infection (Haileamlak et al., 2005, Huang et al., 2002, Cooper et al., 2003a).

1.2.3.3 Asthma and allergic wheeze

Herrick was the first to recognise that helminths can trigger asthma attacks (Herrick, 1913). Following his work in the early twentieth

century, little attention was paid to the potential links between endoparasitic infestations and asthma until the early 1970s. Reports that asthma was commonly caused by endoparasites could, however, not be confirmed (Tullis, 1970, Van Dellen and Thompson, 1971) and subsequent cross-sectional work pointed more towards an inverse relationship between asthma and helminth infection. Nevertheless, two reviews suggested that further evidence was needed to either support or disprove the hypothesis that parasites protect against asthma (Masters and Barrett-Connor, 1985, Weiss, 2000). Further six years down the line, a systematic review recently concluded following meta-analysis of 30 cross-sectional studies that the strongest inverse relationship between asthma and endoparasites was seen for hookworm infection (predominantly *Necator americanus*), and that this effect was infection-intensity related. *Ascaris lumbricoides* appeared to increase asthma risk, while *Trichuris trichiura* had no significant effect (Weiss, 2000, Leonardi-Bee et al., 2006, Masters and Barrett-Connor, 1985).

1.2.3.4 Evidence from prospective studies

Despite mounting cross-sectional evidence that STHs, in particular hookworm, can protect against skin prick test positivity to aeroallergens, asthma and potentially rhinitis and eczema, this observation does not prove causality. Confounding of hitherto unmeasured exposures is an alternative explanation. Reverse causality is equally plausible, ie that atopics have an immune system that reduces parasite burden and only prospective birth cohort and

intervention studies are able to differentiate between causality, confounding and reverse causality.

Two previous studies in children, one an observational study built into a helminth eradication programme in 375 Venezuelan children (Lynch et al., 1993a), the other a single blind trial of 317 children in Gabon (van den Biggelaar et al., 2004), have reported evidence of an increase in allergic skin sensitisation following anti-helminthic therapy. Clinical *improvement* of established asthma following de-worming was reported in a small study among 89 Venezuelan adults and children with asthma (Lynch et al., 1997), while a cluster randomised trial of helminth therapy in a much larger sample of Ecuadorian children was recently reported to show no increase in atopy or allergic disease (Cooper et al., 2006). However, the predominant parasite infections in these study populations were *Ascaris lumbricoides* and *Trichuris trichiura.* It is therefore important to test this hypothesis in a population in which the predominant pathogen is hookworm, and this is why the fieldsite in Khanh Son, central Vietnam, was chosen. Please refer to the Methods chapter for a detailed description of the study site and study population.

1.3 HELMINTHS IN VIETNAM

Soil-transmitted helminths are an important cause of ill health in developing nations, especially in children. Anaemia, growth impairment, poor school attendance and delay in intellectual development are well established consequences of infection, resulting in a significant burden on health care and financial resources (WHO, 2002).

Since a worldwide gut worm control programme was launched by the World Health Assembly in 2001, the World Health Organization together with a number of research institutions has collected prevalence data on STHs from developing countries across the globe, including Vietnam (Figure 1.2).

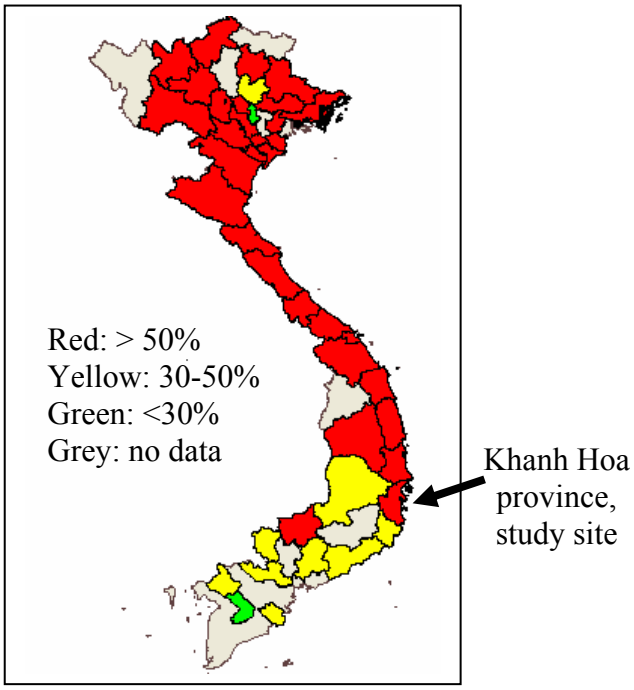


Figure 1.3 STH prevalences in Vietnam

In Vietnam, around 40 million people are infected with a STH (van der Hoek et al., 2003), out of which 6.2 million are children age 6-11 (Montresor, A. personal communication 30.07.2004). While there is no schistosomiasis in Vietnam, *Ascaris lumbricoides* and *Trichuris trichiura* prevalences are highest in the north of the country, and hookworm tends to be more evenly distributed throughout the country.

STH infections are particularly common in peri-urban and rural areas where sanitation is poor, such as our study site in Khanh Hoa province. However, only a small number of unpublished surveys had been conducted in Khanh Hoa province prior to our project (Table 1.2) and none of these in our study communes. A pilot study therefore became necessary in the run up to the main project (see Chapter 2 for more details).

Table 1.2 Helminth infection surveys conducted in Khanh Hoa province prior to our study. All unpublished data. Source: Montresor, A. personal communication 30.07.2004.

Urban/ rural	Study size (n)	Ascaris (n)	%	Trichuris (n)	%	Hookworm (n)	%	Year	Place of survey, Institution
Across province	3696	638	17%	143	4%	1744	48%	1990	Across province, IMPE Quy Nhon
Urban	118	9	8%	2	2%	24	20%	1995	Cam Ranh City, National Institute of Nutrition Hanoi
Semi-urban	210	62	30%	3	1%	10	4%	1997	To Hap town, Khanh Son District Health Centre
Rural	667	230	35%	8	1%	385	58%	1995	Khanh Vinh commune IMPE Hanoi

IMPE = Institute of Malariology, Parasitology and Entomology

1.4 ALLERGIC DISEASE IN VIETNAM

Vietnam is a developing country undergoing rapid demographic change with stark contrasts in lifestyle between urban and rural areas and, hence, an ideal location to study the aetiology of allergic diseases. Hanoi, the northern capital, and Ho Chi Minh City are Vietnam's largest and most rapidly growing cities with around 2.5 and 7 million inhabitants respectively (Gubry and Huong, 2002). Anecdotally, increasing urbanisation and demographic change have been associated with an increase in allergy prevalences, and, as a consequence, a number of allergy surveys have been conducted (Table 1.3).

Table 1.3 Allergy surveys conducted in Vietnam to date

Author	Year	Place	Methods	Sample size (n)	Age (years)	Prevalence (%)
ASTHMA						
NN Huong*	1990	Hanoi	Questionnaire	4,058	6-15	2.1
NN Huong*	1991	Hanoi	Questionnaire	57,876	<15	3.3
LT Tuyet*	1991 1995	Hanoi	Questionnaire	2,000 1,864	6-14 6-14	3.3 4.3
Med Univ Hanoi*	1993	Hanoi	Questionnaire	7,600	11-15	6.5
PD Linh*	1996	HCM City	Questionnaire	7,998	15-19	7.5
(Nga et al., 2003)	1999	Hanoi	ISAAC Questionnaire	969	5-11	14.9 (wheeze past year) 13.9 (asthma ever)
ISAAC Phase I survey*	2001	HCM City	ISAAC Questionnaire	3,884 4,235	6-7 13-14	17.0 (wheeze past year) 4.5 (asthma ever) 29.1 (wheeze past year) 5.0 (asthma ever)
RHINITIS						
(Nga et al., 2003)	1999	Hanoi	ISAAC Questionnaire	969	5-11	10.7 (rhinitis past year) 11.2 (hay fever ever)
ISAAC Phase I survey*	2001	HCM City	ISAAC Questionnaire	3,884 4,235	6-7 13-14	35.1 (rhinitis past year) 22.6 (hay fever ever) 67.5 (rhinitis past year) 27.5 (hay fever ever)
ECZEMA						
(Chai et al., 2004, Nga et al., 2003)	1999	Hanoi	ISAAC Questionnaire	969	5-11	3.3 (eczema past year) 3.2 (eczema ever)
ISAAC Phase I survey*	2001	HCM City	ISAAC Questionnaire	3,884 4,235	6-7 13-14	3.0 (eczema past year) 3.8 (eczema ever) 2.6 (eczema past year) 6.8 (eczema ever)

*unpublished data, HCM City= Ho Chi Minh City

The latest surveys suggest that allergic disease prevalence levels in urban areas are comparable to industrialised countries. Our study was the first to provide prevalence data for a rural area of Vietnam.

As for more detailed risk factor analyses, Chai et al. studied the association between atopic symptoms and a number of known risk factors for asthma, including birth order, educational status, smoking, immunisations, breastfeeding, animal exposure, farming environment, and history of TB in schoolchildren in Hanoi (Chai et al., 2004). Intriguingly and in contrast to the hygiene hypothesis, past history of TB (adjusted OR=3.09, 95% CI 1.10-8.79), household contact with a TB case (adjusted OR=1.94, 1.11-3.39), and tobacco smoke (adjusted OR=1.73, 1.12-2.66) were all positively associated with physician-diagnosed asthma, and the only variables retained in the final logistic regression model. Japanese researchers have recently published cross-sectional results from three questionnaire-based studies conducted among Vietnamese schoolchildren and adolescents in and around Ho Chi Minh City on the role of helminth infection, obesity, and diet in allergy. Apart from a small positive effect for obesity on rhino-conjunctivitis [adjusted OR=1.03, 1.01-1.06; (Irei et al., 2005b)], only riboflavin intake was significantly negatively related to allergic disease [p=0.038; (Quyen et al., 2004, Irei et al., 2005a)]. No associations between gut parasites and allergic disease were found.

The clear limitation of the currently available data on allergic disease in Vietnam is that surveys relied on questionnaire tools rather than using

more objective markers, such as exercise testing for asthma, physical examination for eczema, and skin prick testing for allergic sensitisation.

CHAPTER 2: METHODS

This chapter describes the methods used in the pilot studies and the three main studies that make up this PhD, the cross-sectional baseline survey (chapter 3), the randomised controlled trial to identify the best anti-helminthic agent (chapter 4), and the main intervention study (chapter 5). At the start, however, a description of the study setting and study population.

2.1 THE STUDY SETTING AND STUDY POPULATION

Khanh Son district in Khanh Hoa province in central Vietnam (Figure 2.1 and Figure 2.2) was chosen as field site due to its high hookworm (*Necator americanus*) burden. Also advantageous for an epidemiological study were Khanh Son's well-defined borders as a highland plateau (around 800m altitude), its demographic stability and ethnically uniform population.

80% out of the around 18,000 people living in Khanh Son belong to the Raclay tribe, one of 53 officially recognised ethnic minorities in Vietnam (Plant, 2002). The remaining 20% are Kinh people, ethnic Vietnamese. The Raclay live a simple subsistence lifestyle. Their houses have traditionally been built on stilts made of wood, bamboo and palm leaves. However, recently the central government has provided new housing in an attempt to improve living conditions in this poorly developed rural area.

Figure 2.1 Map of Vietnam

Figure 2.2 Khanh Hoa province with individual districts (field site Khanh Son marked with arrow).

Today, many houses are built of brick with concrete flooring and corrugated iron roofs. Nevertheless, local ownership of goods remains very limited, especially among the Raclay. Chemical fertiliser use is rare with the majority of farmers using animal dung, but not human night soil. There are a number of factors that predispose to high gut parasite prevalences. Since piped water supply is rare (4% of households have been connected), only 1% of houses have a flush closet. 17% of people use a self-made pit for defaecation, while 82% defaecate outside their houses in bushes or fields.

Poor sanitation and walking with open or no footware clearly predispose to helminth infections, especially hookworm, since hookworm larvae need to penetrate the skin of the lower legs to invade the human host (Fig. 1.2). Other endoparasitic disease is uncommon. For instance, there is no schistosomiasis or lymphatic filariasis (unpublished internal report National Institute of Malariology, Parasitology, and Entomology (NIMPE), Hanoi, March 2006), and the point prevalence of malaria is low. Further contributing to a high gut worm burden in Khanh Son are the very limited availability of anti-helminthics and the absence of previous gut worm control campaigns.

As study population the primary and secondary schoolchildren in four out of seven communes (Thanh Son, Son Lam, Son Binh and Ba Cum Nam) in Khanh Son were targeted. The communes were chosen, partly based on their population size but also because the other communes have for a number of years been supported by a Dutch non-governmental organisation through a community-based development programme, which includes building of wells, and it was likely that these activities would interfere with the study.

The population of Khanh Son district is medically looked after by a 50-bed district health centre as well as communal health stations. A microbiology laboratory for simple tests, such as quantitative and qualitative parasite stool analysis, with experienced laboratory technicians was in place at the district health centre. Doctors, physician assistants, as well as nurses and community health care workers were readily available locally for recruitment as fieldworkers.

2.2 THE PILOT STUDIES

2.2.1 Pilot study 1

A pilot study was necessary to assess the prevalence of allergic disease symptoms in our study communes and the local burden of helminth infection. We targeted the four communes that we planned to use for the main cross-sectional survey and the intervention study. Four primary schools, one in each commune, were selected by lottery method. All 263 children enrolled at these four schools were eligible to take part. On the day prior to the survey, the children were visited by local health care workers, specifically trained for the project to explain the purpose of the pilot study and to gain consent, and also to give children a container which was to be brought into school with a fresh stool sample the next morning. 24 hours later, the sample was collected and information on children's age, sex and allergy symptoms was gathered from a close relative through an interviewer-led questionnaire (for further details on the questionnaire see section 2.3.1 and the appendix). All stool samples were transported to the local district hospital where they were examined for parasite eggs both qualitatively and quantitatively in terms of eggs per gram faeces (epg) by salt flotation method with McMaster counting chambers within a maximum of 6 hours after collection (for further details see section 2.3.1.2).

97% (254/263) of those eligible took part. 51% (129) were boys. The mean age was 10 years (range 9-12). As expected from previous surveys in Khanh Hoa province (Tab. 1.2), hookworm was the predominant helminth parasite (n=164, 65%), while both *Ascaris lumbricoides* and *Trichuris trichiura* infections were rare (n=4/2% and n=1/0.4% respectively). Dual helminth infection was found in only 1% (n=3/254) of children. Whereas symptoms of allergic respiratory disease were common (18% (46/254) reported "wheeze over the past 12 months"), only few children had a diagnosis of asthma (6%, 16/254) or eczema (2%, 4/254; Table 2.1). Allergy symptom frequencies were different between the communes, and consequently the randomisation for the main intervention study was stratified by school (Table 2.2).

Table 2.1 Results pilot study 1

	N (%)
Pupils eligible to take part	263
Participants	254 (97%)
Raclay ethnic minority	197 (78%)
Kinh (Vietnamese)	57 (22%)
Male	129 (51%)
Female	125 (49%)
Age	9-12, mean age 10
Hookworm	164 (65%)
Ascaris	4 (2%)
Trichuris	1 (0.4%)
Dual helminth infection	3 (1%)
Wheeze ever	100 (39%)
Wheezing past 12mth	46 (18%)
Asthma ever	16 (6%)
Rhinitis symptoms ever	89 (35%)
Rhinitis symptoms past 12mth	53 (21%)
Allergic rhinitis ever	31 (12%)
Flexural itchy rash past 12 mth	23 (9%)
Eczema ever	4 (2%)

Table 2.2 Results pilot study 1 stratified by commune

School/Area	**Children per school N (%)**	**Hookworm N (%)**	**Wheeze past 12 mths N (%)**	**Asthma N (%)**	**Eczema ever N (%)**
Son Lam	55 (22)	24 (44)	18 (33)	2 (4)	2 (4)
Son Binh	98 (39)	64 (65)	11 (11)	7 (7)	1 (1)
Ba Cum Nam	47 (19)	26 (55)	14 (30)	6 (13)	1 (2)
Thanh Son	54 (21)	50 (94)	3 (6)	1 (2)	0
Total	**254**	**164 (65)**	**46 (18)**	**16 (6)**	**4 (2)**

2.2.2 Pilot Study 2

A separate pilot study was conducted to assess the prevalence of sensitisation to common environmental allergens in this rural community. For logistic reasons, we invited 100 healthy parents of children admitted to the district hospital in Khanh Son district to take part. All participants were skin prick tested to American cockroach (*Periplaneta americana*), house dust mite (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), grass mix, weed mix, dog, cat, animal hair mix, *Aspergillus fumigatus*, *Cladosporium herbarum*, and *Penicillium notatum* (all Merck, see section 2.3.2.2.3 for further details).

As for skin prick testing, overall 30% (30/100) of participants had at least one positive skin prick test, most commonly to American cockroach (25%), followed by *Dermatophagoides pteronyssinus* (16%) and *Dermatophagoides farinae* (13%). Reactions to other allergens were rare and in almost all cases accompanied by reactions to one of the three commonest allergens (Table 2.3).

Table 2.3 Results pilot study 2

	N=%
Total number of participants	100
Atopy in whole sample (≥ one pos SPT)	30
Atopy among Raclay (≥ one pos SPT)	32
Periplaneta americana	25
Dermatophagoides pteronyssinus (Dp)	16
Dermatophagoides farinae (Df)	13
Dp and Df	7
Grass mix	2
Grass mix alone	None, also Dp+ Df pos
Weed mix	1
Weed mix alone	None, also Dp+Df pos
Dog	2
Dog alone	None, also Dp+Df pos
Cat	2
Cat alone	None, also Dp+Df pos
Animal hair mix	2
Animal hair mix alone	1
Aspergillus	None
Cladosporium	2
Caldosporium alone	1
Penicillium	1
Penicillium alone	None, also Dp+Df pos

2.2.3 Summary pilot study results

Overall, the pilot studies suggested that Khanh Son district was suitable for our project. The participation rate in pilot study 1 was high (97%), as was the prevalence of helminth infection (66%), clearly dominated by hookworm (65%). The Vietnamese study questionnaire performed well, although some doubt remained as to the specificity of the allergy terminology given the prevalence differences, for instance, between 'wheeze ever' (39%) and 'asthma ever' (6%). The questionnaire-derived prevalences for allergic disease symptoms were

used for the power calculation of the main study, since use of exercise testing and physical examination was logistically too difficult at the pilot stage. Finally, the skin prick test pilot (pilot study 2) allowed us to select *Periplaneta americana*, *Dermatophagoides pternonyssinus* and *Dermatophagoides farinae* as the locally most relevant environmental allergens for the main study.

2.3 METHODS BASELINE SURVEY, TREATMENT COMPARISON STUDY, AND INTERVENTION STUDY

Where possible we used objective exposure and outcome measures for the main study and supplemented these with questionnaire-derived information. In overview:

1. BASELINE SURVEY (CROSS-SECTIONAL DESIGN)

Exposures:
- allergy risk factors (questionnaire)
- helminth infection (stool analysis)
- past malaria (questionnaire), current malaria (thick smear)

Outcomes:
- exercise-induced bronchospasm (Burr protocol)
- flexural eczema (ISAAC protocol, physical examination)
- allergic disease symptoms (ISAAC questions)
- allergic sensitisation (skin prick testing)
- differences in cytokine profiles atopics vs non-atopics (ELISAs)

2. TREATMENT COMPARISON STUDY (RCT)

Intervention: several anti-helminthics vs placebo to find optimal trial regimen
Outcome: reduction in hookworm prevalence/epg (treatment vs placebo groups)

3. INTERVENTION STUDY (RCT)

Intervention: anti-helminthic treatment vs placebo, 4x, every 3 months
Outcomes: outcome measures identical to baseline survey
Primary outcome: prevalence of exercise-induced bronchospasm
Secondary outcomes: prevalence of flexural eczema
allergy symptoms, and skin prick test positivity
(anti-helminthic treatment vs placebo group)

2.3.1 Exposure measures

2.3.1.1 The risk factor questionnaire (baseline survey and final survey intervention study)

For the baseline survey and the final survey of the intervention study, we collected information on children's age, sex, ethnic group, and other demographic and lifestyle factors, including number of siblings, parental smoking, housing style (roof, walls, flooring), sleeping mattress material, symptoms of allergic disease, current medication, toilet facilities, drinking water supply, animals in the home, fuel use, and previous helminth and malaria infections (for full questionnaire see appendix). The English questionnaire was initially translated into Vietnamese, then pilot-tested among 254 volunteers (see chapter 2.2.1), and finally back-translated into English to ensure accurate translation of key terms.

2.3.1.2 Stool analysis pilot study 1, baseline survey (chapter 3) and final survey intervention study (chapter 5), as well as treatment comparison study (chapter 4)

On the day before each survey (this was identical for pilot study 1, baseline survey, treatment comparison study, and intervention study), all participating children and their parents were given a disposable container with clear instructions on how to provide a fresh stool sample the next day. Local health care workers, specifically trained for this project, collected the specimens next morning. Immediately after collection, the specimens were transported to the local district hospital

where they were examined for parasite eggs both qualitatively and quantitatively in terms of epg by salt flotation method (http://www.rvc.ac.uk/Review/Parasitology/EggCount/Purpose.htm). Stool samples were examined within a maximum of 6 hours post collection. A salt flotation fluid was used to separate eggs from faecal material in a counting chamber (McMaster) with two compartments. The technique detected 50 or more epg. 4 gram of faeces were weighed and then placed in a plastic beaker. 56ml of flotation fluid (sodium chloride 400gr, water 1000ml, specific gravity 1.18-1.20) were then added and mixed with the faeces through thorough stirring with a tongue depressor. The mixture was put through a tea strainer into a second container. While stirring the filtrate, a sample was taken with a Pasteur pipette. Both McMaster counting chambers were filled with the sample. The slide was allowed to stand for 5 minutes and then examined under the microscope at 10x10 magnification, counting all eggs within the area of the two compartments. The number of eggs per gram of faeces was calculated by counting the eggs in the two chambers together, followed by multiplication of the total by 50. This gave the epg (example: 12 eggs seen in chamber 1 and 15 eggs seen in chamber 2 = (12+15) x 50 = 1350 epg). All negative samples were examined a second time, using the same method, to increase sensitivity. Please refer to the appendix for the written instructions given to the laboratory technicians.

2.3.1.3 Validation stool analysis method

McMaster salt flotation is a comparatively quick, hygienic, easy to teach, low cost method that does not require much special equipment and is therefore ideal for a rural developing country setting. However, since a salt solution of high specific gravity is used, there is a theoretical risk of eggs popping if left for too long in salt solution. This would reduce the sensitivity of this method. The accepted standard diagnostic method in developed country settings is formol-ether sedimentation (Cheesbrough, 2000). While potentially more sensitive, the method uses toxic substances, is time-consuming and less suitable for field studies. So far no validation studies have compared McMaster salt flotation and formol-ether sedimentation.

We therefore used the opportunity of the treatment comparison study (chapter 4) to compare both stool analysis methods among 180 adults in a village in our study area with very high hookworm infection burden. While McMaster salt flotation was performed as usual on all samples, we also preserved one further gram of faeces from 151 of these participants (insufficient sample from the other 29 participants) in 10% formalin in the field. Samples were then transported to the National Institute of Malariology, Parasitology, and Entomology (NIMPE) Ho Chi Minh City for formol-ether sedimentation. The laboratory technicians at NIMPE were blind to the results with salt flotation. 89% of samples (134/151) were positive for hookworm by salt flotation, compared to 91% (137/151) by ether sedimentation, and 95% (144/151) by either method. Arithmetic mean egg counts were

1332 epg for salt flotation and 1723 epg for ether sedimentation. These results suggest that the two egg counting techniques were comparable, and that we did not miss many hookworm infections due to egg collapse.

2.3.1.4 Malaria infection (baseline survey only)

Thick smears were prepared directly from fingerprick venous blood samples from all children by two trained fieldworkers. The slides were left to dry and then transported to the Khanh Hoa Provincial Center for Malaria and Filariasis Control in Nha Trang City for further analysis. Blood films were stained with 10% Giemsa solution and examined by an expert microscopist with 15 years experience. The microscopist was blind to the clinical diagnosis. The initial film was considered negative if no parasites were seen in at least 100 high-power fields. A high-quality (Olympus) microscope with an incandescent light source was used. Each film required approximately 20 minutes to read. All positive and a randomly selected 10% of the negative slides were independently read by another experienced microscopist from the same institute for quality control, who was blind to clinical diagnosis and the previous microscopy results.

2.3.2 Outcome measures

2.3.2.1 Questionnaire-derived allergy symptoms (pilot study 1, baseline survey and final survey intervention study)

We used the ISAAC Phase Two core questions to measure wheeze, rhinitis, and eczema in pilot study 1 and in the baseline and final surveys of the main study (http://isaac.auckland.ac.nz/PhaseOne/Translation/TransFrame.html). Parents or a close relative answered with the child present. The questionnaire was administered in Vietnamese (see appendix for full version of the study questionnaires).

2.3.2.1.1 Wheezing and asthma

Parents were asked whether their child ever had "wheezing or whistling in the chest at any time in the past". If the answer was "yes", they were also asked whether their child had "wheezing or whistling in the chest in the past 12 months", how many attacks of wheezing they recalled during that period, and how often on average their child's sleep had been disturbed due to wheezing. In addition, the questionnaire enquired whether the child had ever had asthma and, if yes, whether this diagnosis had been made by a doctor.

For the intervention study, parents were asked after 12 months of anti-helminthic or placebo treatment (they were not aware of their child's

treatment allocation) whether any of the above symptoms had occurred "since the first treatment was given".

2.3.2.1.2 Rhinitis

Parents were also asked whether their child had ever had "a problem with sneezing or a runny nose or a blocked nose when he/she did not have a cold or flu". If the answer was "yes", parents were asked whether this problem had occurred over the past 12 months and whether it had ever been accompanied by itchy-watery eyes. Furthermore, it was asked whether the child had "ever had allergic rhinitis", and, if yes, whether this had been diagnosed by a doctor. At 12 months post treatment, parents were asked whether any of the above symptoms had been observed since the first treatment was given.

2.3.2.1.3 Eczema

The questions relating to eczema were mainly taken from the ISAAC Phase Two core questionnaire, but we also asked for age of onset, flexural involvement, and generally dry skin, in keeping with the UK diagnostic criteria for eczema (Williams et al., 1995).

2.3.2.2 Objective allergy outcome measures (baseline survey and final survey intervention study)

2.3.2.2.1 Exercise-induced bronchospasm

Exercise-induced bronchoconstriction, measured as the fall in peak expiratory flow induced by a period of vigorous exercise, has been used in a number of epidemiological studies as a measure of asthma (Barry et al., 1991, Burr et al., 1989, Burr et al., 1974, Burr et al., 1994, Priftanji et al., 2001, Jones et al., 1996, Keeley et al., 1991, Addo Yobo et al., 1997, Cooper et al., 2006, Addo-Yobo et al., 2007). Peakflow measurement is particularly suited for studies in developing countries, since it is a simple and safe method that can easily be explained to children.

Children were surveyed class by class. First, children were taught how to use a peak flow meter by a trained fieldworker in the classroom, and each child was assigned his or her own peak flow meter. After initial instructions were given to the whole group, children were then allowed to practice in groups, until good technique had been achieved (blowing through the peakflow meter from the peak of deep inspiration with maximum effort and without any air leaks from around the mouth piece until three readings less than 5% apart had been obtained). Following these instructions and practice, peak flow rates were measured in each child three times immediately before, and 5 minutes after a 6 minute period of free outdoor running at jogging pace

(Taussig et al., 1980). The maximum of each set of readings was then used to calculate the percentage fall in peak flow after exercise. The pulse rate was measured for 15 seconds immediately after completion of the run, as an indicator of the intensity of the exercise (Priftanji et al., 2001).

2.3.2.2.2 Diagnostic criteria for flexural eczema

Whilst there are a number of diagnostic criteria for atopic dermatitis (Hanifin and Lobitz, 1977, Hanifin and Rajka, 1980, Bos et al., 1998), only the UK diagnostic criteria have been extensively validated and field tested (Popescu et al., 1998, Williams et al., 1994b, Williams et al., 1994c, Williams et al., 1994a, Williams et al., 1995, Williams, 1996, Gu et al., 2001). A manual, photographic images, and a test (Williams, 1997) were especially developed for the training of fieldworkers for large epidemiological studies, such as ISAAC Phase Two (http://www.nottingham.ac.uk/dermatology/eczema/index.html). Since the validity of a purely questionnaire-based diagnosis of childhood eczema is uncertain, especially in a Vietnamese context, where translation of key terms may be difficult, we examined all children physically for flexural eczema, using the ISAAC Phase Two photographic protocol (see appendix). In this way, we ensured high specificity of the diagnosis, which was the most important consideration for the intervention study part of this project. All fieldworkers were trained in the recognition of flexural eczema by the main investigator, using the ISAAC field manual and had to pass the

standardised test prior to commencing fieldwork. In addition, the main investigator personally examined all children in whom the fieldworkers reported an itchy rash in one of the five designated flexures to confirm the diagnosis of flexural eczema.

2.3.2.2.3 Allergen skin hypersensitivity

The ISAAC Phase Two skin prick test protocol to measure environmental sensitisation was used throughout. This method has been shown to be reproducible under field conditions, and that it is simple, safe, and acceptable to children (http://isaac.auckland.ac.nz/Phasetwo/Modules/ChilCont/ChilFrame.html). Allergen skin prick testing was performed to house dust mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) and American cockroach (*Periplaneta americana*), and to normal saline (negative) and histamine 1.7mg/ml (positive) controls (all Merck, supplied by Thalassa Medical Ltd, Hong Kong). The allergens used had previously been identified as being those most prevalent in the local population in pilot study 2 (see also section 2.2.2).

All skin prick tests were performed between 08:00 and 12:00 local time, since there is a circadian rhythm in the size of skin prick reactions to allergens and histamine (Taudorf et al., 1985). The fieldworkers were instructed to ensure that the site of testing was free of eczema or other skin disease. A tape with numbers indicating the sequence of allergen extracts was then placed in the middle of the volar aspect of the left forearm, 3 cm distal to the elbow crease. One drop of

each skin prick solution was placed on the forearm beside the tape. A separate skin prick test lancet was pricked vertically through each drop. All drops were dapped dry immediately afterwards, taking good care not to contaminate prick points with a different allergen extract. Reactions to each skin test solution were measured 15 minutes later. The contours of each wheal were outlined with a fine filter tip pen. The contours were then transferred to the record sheet with magic tape. The size of each wheal was documented as the mean of the longest diameter (a) and the diameter perpendicular to it at its mid-point (b): ie (a+b)/2. Measurements of each diameter were made to the nearest millimetre above. A positive skin prick test was defined as a wheal diameter at least 3mm greater than the saline control.

Two local hospital nurses were trained in this method. Pilot study 2 (see section 2.2.2) was used to improve the nurses' skills under direct supervision by the principal investigator. Skin prick test reproducibility was tested with at least three series of 16 skin prick tests with histamine on the volar aspect of the forearm of a volunteer, until the coefficient of variation (standard deviation as a % of the mean) of the last series was less than 20%. This was done at the beginning, in the middle and at the end of the main two surveys (see appendix for fieldworker instructions on skin prick testing).

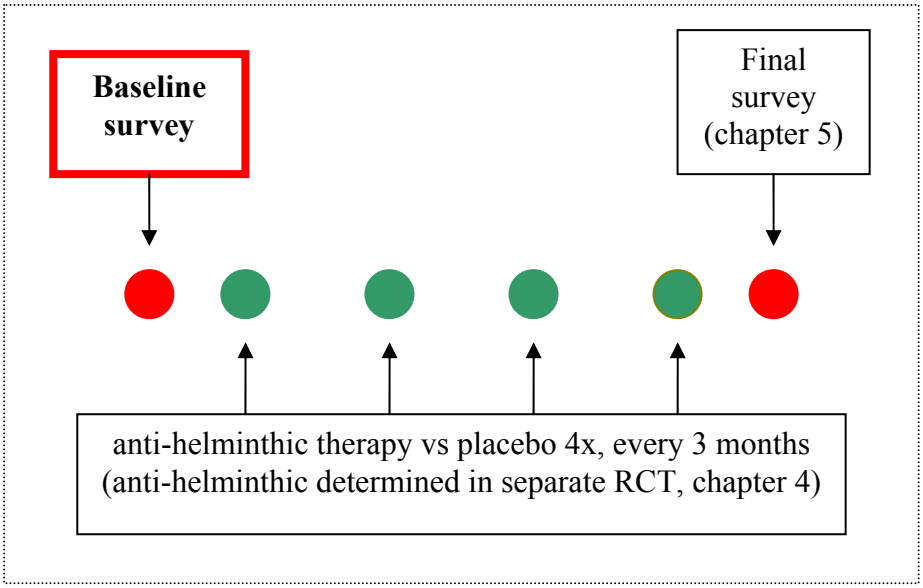
2.4 SAMPLE SIZE, DATA MANAGEMENT, AND STATISTICAL ANALYSIS

This is described separately in each chapter (baseline cross-sectional survey chapter 3, treatment comparison study chapter 4, intervention study chapter 5, cytokine profile analysis chapter 6).

2.5 ETHICAL APPROVAL

Ethics approval for all studies was granted in Nottingham (Nottingham Research Ethics Committee 2, ref. Q2010305) and in Vietnam (Scientific Committee of the Provincial Health Service, Nha Trang, Khanh Hoa Province, Vietnam).

CHAPTER 3: THE BASELINE SURVEY



3.1 METHODS

The cross-sectional baseline survey was conducted in April and May 2005. The methodology used in the baseline survey has already been described in detail in chapter 2. In brief, we invited all 1,742 primary and secondary schoolchildren from four neighbouring rural communes in Khanh Son, Khanh Hoa province, central Vietnam, to take part. On the day before the survey, the children were visited by local health care workers, specifically trained for the project, to explain the purpose of the study, to gain parental consent, and also to give children a container which was to be brought into school with a fresh stool sample the next morning. The next day, the sample was collected and information gathered on children's age, sex, ethnic group and other demographic and lifestyle factors, including number of siblings,

parental smoking, housing style, symptoms of allergic disease, current medication, toilet facilities, drinking water supply, animals in the home, fuel use and previous helminth and malaria infections. We measured allergic skin sensitisation to two house dust mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) and to American cockroach (*Periplaneta americana*) with normal saline (negative) and histamine 1.7mg/ml (positive) controls (all Merck, supplied by Thalassa Medical Ltd. Hong Kong). The ISAAC skin prick test protocol, which defines SPT positivity as a skin wheal diameter of at least 3mm greater than the saline control, was used. Fresh stool samples were analysed within a maximum of 6 hours post collection to determine helminth status qualitatively and in terms of epg, using McMaster salt flotation. Furthermore, all children were tested for carriage of malaria parasites with a standard thick blood smear read by an expert malaria microscopist at the Khanh Hoa Provincial Centre for Malaria and Filariasis Control.

All data were double entered by two data entry clerks, using SPSS Data Entry Station 4.0 and then analysed by logistic regression in SPSS version 14.0. Age, sex, and area were included in the multivariate model as *a priori* confounders, and initially all those variables that were significant in univariate analysis. Variables for which there was significant heterogeneity across categories or trend through ordered categories were retained in the model, taking $P < 0.05$ as statistically significant. In the case of collinear variables, each was

fitted in the absence of the other, and the variable with the strongest effect was retained in the model.

Our sample size was determined by the needs of the intervention study (chapter 5), but a retrospective power calculation based on 1,601 participants, of which 14% were sensitive to dustmite, indicates that there was 80% power to detect an odds ratio of 0.66 for the protective effect of an exposure such as hookworm, which occurs in 65% of the population.

3.2 RESULTS

At baseline, data was collected from 1,601 (47.7% male) participants, comprising 92% of those eligible. The mean age of study subjects was 8.7 (range 6-18), and the majority (79.5%) belonged to the Raclay ethnic group. 19.1% were from ethnic Vietnamese (Kinh) families and the remaining 1.4% came from mixed backgrounds.

3.2.1 Symptoms of allergic disease and atopy

In keeping with the pilot study results, symptoms for allergic disease were uncommon. Only 5.0% (80/1601) of children had experienced wheezing during the preceding 12 months. Asthma and doctor-diagnosed asthma were even rarer with prevalences of 0.7% (11/1601) and 0.4% (6/1601) respectively. 6.9% (110/1601) of parents reported that their children had suffered of hay fever in the past 12 months, and in 2.6% (41/1601) of cases this diagnosis was confirmed by a doctor.

Eczema symptoms were even less common than asthma and rhinitis symptoms. 5.6% of children (89/1601) reported an "itchy rash over the past 12 months". 0.9% (14/1601) had a history of flexural involvement, and in only two cases the diagnosis of eczema was confirmed by a physician. On examination, 0.5% (8/1601) of children proved to have flexural eczema as per study protocol.

Skin prick test positivity was commoner than allergic disease symptoms. 230 (14.4%) children had a positive skin prick test to either *Dermatophagoides pteronyssinus* or *Dermatophagoides farinae*, and 442 (27.6%) were sensitised to American cockroach. Since the risk factor analysis yielded different patterns of association for house dust mite and cockroach sensitisation, results are presented for both allergens separately. As for the other outcomes, the risk factor analysis did not produce statistically significant results (data not shown). None of the participating children were taking antihistamines or corticosteroids at the time of the survey.

3.2.2 Demographic and socioeconomic factors

In univariate analysis, sensitivity to house dust mite, but not to cockroach, increased with age, and was more common, though not significantly so, in girls (Table 3.1). Allergic sensitisation was generally more common in ethnic Vietnamese (Kinh) and least common in those of Raclay descent ($OR_{\text{house dust mite}}$=2.56, 1.88-3.49, $OR_{\text{cockroach}}$=2.78, 2.15-3.61, Table 3.1). Higher levels of parental education and ownership of goods (a marker of socio-economic status)

were both positively associated with sensitisation. There was no association between sensitisation and number of older siblings.

3.2.3 Household exposures

A large number of domestic exposures previously shown to be associated with allergic sensitisation, including breastfeeding, parental smoking, insecticide use, crowding, housing style (roof, walls, flooring), sleeping mattress material, cooking fuel, and animal ownership were examined. In univariate analysis, sensitisation was less common in those exposed to smoking in the household ($Ptrend_{house\ dust\ mite}$=0.003 and $Ptrend_{cockroach}$=0.003), and in those using wood for fuel ($OR_{house\ dust\ mite}$=0.40, 0.26-0.63 and $OR_{cockroach}$=0.41, 0.28-0.60, Table 3.2). Sensitisation was more common in those using gas as a domestic fuel ($OR_{house\ dust\ mite}$=2.81, 1.80-4.39 and $OR_{cockroach}$=2.45, 1.63-3.66), and with exposure to animals, especially cat ($OR_{house\ dust\ mite}$=1.48, 1.06-2.05 and $OR_{cockroach}$=1.40, 1.07-1.83). However, none of these exposures were independently related to sensitisation to dustmite or cockroach in multivariate analysis.

Table 3.1 Demographic and socioeconomic factors

	N (%)	Der p 1 or Der f 1 sensitive N (%)	Crude OR (95% CI)	P value	Cockroach sensitive N (%)	Crude OR (95% CI)	P value
Total	1601	230 (14.4)	-	-	442 (27.6)	-	
Age							
6-7	540 (33.7)	73 (13.5)	**1**	**< 0.001**	156 (28.9)	1	0.6
8-9	585 (36.5)	66 (11.3)	**0.81 (0.57,1.16)**	**P_{trend}**	144 (24.6)	0.80 (0.62,1.05)	P_{trend}
10-11	290 (18.1)	40 (13.8)	**1.02 (0.68,1.55)**		85 (29.3)	1.02 (0.75,1.40)	
12 +	186 (11.6)	51 (27.4)	**2.42 (1.61,3.63)**		57 (30.6)	1.09 (0.76,1.56)	
Sex							
Male	764 (47.7)	100 (13.1)	1	0.2	196 (25.7)	1	0.09
Female	837 (52.3)	130 (15.5)	1.22 (0.92,1.62)		246 (29.4)	1.21 (0.97,1.50)	
Ownership of goods							
None	403 (25.2)	40 (9.9)	**1**	**0.001**	110 (27.3)	1	0.09
1 or 2 items	745 (46.5)	108 (14.5)	**1.54 (1.05,2.26)**	**P_{trend}**	186 (25.0)	0.89 (0.67,1.17)	P_{trend}
3 or more items	453 (28.3)	82 (18.1)	**2.01 (1.34,3.01)**		146 (32.2)	1.27 (0.94,1.70)	
Ethnicity							
Raclay	1271 (79.5)	148 (11.6)	**1**	**<0.001**	294 (23.1)	**1**	**<0.001**
Vietnamese (Kinh)	305 (19.1)	77 (25.2)	**2.56 (1.88,3.49)**		139 (45.6)	**2.78 (2.15,3.61)**	
Other	23 (1.4)	5 (21.7)	**2.11 (0.77,5.76)**		9 (39.1)	**2.14 (0.92,4.99)**	
Parental education							
Illiterate	393 (24.5)	43 (10.9)	**1**	**< 0.001**	80 (20.4)	**1**	**<0.001**
Primary	819 (51.2)	103 (12.6)	**1.17 (0.80,1.71)**	**P_{trend}**	215 (26.3)	**1.39 (1.04,1.86)**	**P_{trend}**
Higher	389 (24.3)	84 (21.6)	**2.24 (1.51,3.34)**		147 (37.8)	**2.38 (1.73,3.27)**	
Area							
Thanh Son	370 (23.1)	42 (11.4)	**1**	**< 0.001**	88 (23.8)	**1**	**0.003**
Son Lam	604 (37.7)	116 (19.2)	**1.86 (1.27,2.71)**		177 (29.3)	**1.33 (0.99,1.79)**	
Son Binh	418 (26.1)	49 (11.7)	**1.04 (0.67,1.61)**		135 (32.3)	**1.53 (1.12,2.09)**	
Ba Cum Nam	209 (13.1)	23 (11.0)	**0.97 (0.56,1.65)**		42 (20.1)	**0.81 (0.53,1.22)**	
Older siblings (N)							
0	417 (26.0)	61 (14.6)	1	0.3	121 (29.0)	1	0.3
1	340 (21.2)	53 (15.6)	1.08 (0.72,1.61)	P_{trend}	95 (27.9)	0.95 (0.69,1.30)	P_{trend}
2 or 3	530 (33.1)	81 (15.3)	1.05 (0.73,1.51)		145 (27.4)	0.92 (0.69,1.23)	
4 or more	314 (19.6)	35 (11.1)	0.73 (0.47,1.14)		81 (25.8)	0.85 (0.61,1.18)	

Bold implies significance at the 5% level.

Table 3.2 Household exposures

	N (%)	Der p 1 or Der f 1 sensitive N (%)	Crude OR (95% CI)	P value	Cockroach sensitive N (%)	Crude OR (95% CI)	P value
Smoking in the household (N)							
0	263 (16.4)	48 (18.3)	**1**	**0.003**	74 (28.1)	1	**0.003**
1	600 (37.5)	96 (16.0)	**0.85 (0.58,1.25)**	**P_{trend}**	202 (33.7)	1.30 (0.94,1.78)	**P_{trend}**
2 or more	738 (46.1)	86 (11.7)	**0.59 (0.40,0.87)**		166 (22.5)	0.74 (0.54,1.02)	
Cooking fuel							
Wood No	112 (7.0)	31 (27.7)	**1**	**< 0.001**	52 (46.4)	**1**	**<0.001**
Yes	1489 (93.0)	199 (13.4)	**0.40 (0.26,0.63)**		390 (26.2)	**0.41 (0.28,0.60)**	
Gas No	1498 (93.6)	199 (13.3)	**1**	**<0.001**	394 (26.3)	**1**	**< 0.001**
Yes	103 (6.4)	31 (30.1)	**2.81 (1.80,4.39)**		48 (46.6)	**2.45 (1.63,3.66)**	
Animal ownership							
Cat No	1288 (80.4)	172 (13.4)	**1**	**0.02**	338 (26.2)	**1**	**0.01**
Yes	313 (19.6)	58 (18.5)	**1.48 (1.06,2.05)**		104 (33.2)	**1.40 (1.07,1.83)**	
Dog No	493 (30.8)	55 (11.2)	**1**	**0.01**	135 (27.4)	1	0.8
Yes	1108 (69.2)	175 (15.8)	**1.49 (1.08,2.06)**		307 (27.7)	1.02 (0.80,1.29)	
Chicken/duck No	638 (39.9)	74 (11.6)	**1**	**0.01**	179 (28.1)	1	0.7
Yes	963 (60.1)	156 (16.2)	**1.47 (1.10,1.98)**		263 (27.3)	0.96 (0.77,1.21)	
Waterbuffalo No	379 (23.7)	59 (15.6)	1	0.4	119 (31.4)	1	0.06
Yes	1222 (76.3)	171 (14.0)	0.88 (0.64,1.22)		323 (26.4)	0.79 (0.61,1.01)	
Pig No	1113 (69.5)	159 (14.3)	1	0.9	316 (28.4)	1	0.3
Yes	488 (30.5)	71 (14.5)	1.02 (0.76,1.38)		126 (25.8)	0.88 (0.69,1.12)	

Bold implies significance at the 5% level.

3.2.4 Associations with parasites and hygiene

65% of all children were found to have hookworm infection, and 7% *Ascaris lumbricoides*. Infection intensity for both helminths was relatively low (hookworm egp: range 0-7300, mean epg 432; Ascaris epg: range 0-13850, mean 432). Other helminth infections, such as *Trichuris trichiura*, were rare (1% or lower) and therefore not analysed further. In univariate analysis, hookworm ($OR_{house\ dust\ mite}$=0.57, 0.43-0.75 and $OR_{cockroach}$=0.78, 0.62-0.97) and *Ascaris* infections ($OR_{house\ dust\ mite}$=0.21, 0.08-0.59 and $OR_{cockroach}$=0.61, 0.37-0.99) were both associated with a reduced risk of sensitisation, which for hookworm was intensity-related ($Ptrend_{house\ dust\ mite}$<0.001 and $Ptrend_{cockroach}$=0.006, Table 3.3). Allergen sensitisation was more common in those with flush toilet facilities vs none/bush/pit ($OR_{house\ dust\ mite}$=4.61, 1.92-11.07 and $OR_{cockroach}$=4.36, 1.80-10.59), and in those regulary drinking piped or well water rather than from a stream ($OR_{house\ dust\ mite}$=1.39, 1.03-1.86 and $OR_{cockroach}$=1.68, 1.33-2.11). Past malaria infection was protective against both house dust mite and cockroach sensitisation ($OR_{house\ dust\ mite}$=0.64, 0.48-0.86 and $OR_{cockroach}$=0.71, 0.56-0.89), while current malaria increased sensitisation risk, but this did not reach conventional statistical significance ($OR_{house\ dust\ mite}$=1.38, 0.39-4.88 and $OR_{cockroach}$=2.65, 0.99-7.11). Both effects were not retained in the final logistic regression model. Anti-helminthic treatment within the past 6 months had no effect on any of these estimates (data not shown). As for other measures related to infections, data was also collected on routine

childhood vaccinations, previous TB and measles, as well as past antibiotic use. Among these variables, only BCG vaccination showed a significant and weakly protective effect against house dust mite sensitisation in univariate analysis (Table 3.3, only significant results shown), but this was not retained in the final logistic regression model.

Table 3.3 Parasites and hygiene

	N (%)	Der p 1 or Der f 1 sensitive N (%)	Crude OR (95% CI)	P value	Cockroach sensitive N (%)	Crude OR (95% CI)	P value
Hookworm							
No	566 (35.4)	108 (19.1)	**1**	**< 0.001**	175 (30.9)	**1**	**0.03**
Yes	1035 (64.6)	122 (11.8)	**0.57 (0.43,0.75)**		267 (25.8)	**0.78 (0.62,0.97)**	
Hookworm epg							
Low (50-199)	409 (25.5)	56 (13.7)	**0.67 (0.47,0.96)**	**<0.001** P_{trend}	110 (26.9)	**0.82 (0.62,1.09)**	**0.006** P_{trend}
Med (200-349)	228 (14.2)	27 (11.8)	**0.57 (0.36,0.90)**		71 (31.1)	**1.01 (0.72,1.41)**	
High (350+)	398 (24.9)	39 (9.8)	**0.46 (0.31,0.68)**		86 (21.6)	**0.62 (0.46,0.83)**	
Ascaris							
No	1492 (93.2)	226 (15.1)	**1**	**0.001**	421 (28.2)	**1**	**0.04**
Yes	109 (6.8)	4 (3.7)	**0.21 (0.08,0.59)**		21 (19.3)	**0.61 (0.37,0.99)**	
Malaria infection (past)							
No	535 (33.4)	97 (18.1)	**1**	**0.002**	173 (32.3)	**1**	**0.003**
Yes	1066 (66.6)	133 (12.5)	**0.64 (0.48,0.86)**		269 (25.2)	**0.71 (0.56,0.89)**	
Malaria infection (current)							
No	1585 (99.0)	227 (14.3)	1	0.6	434 (27.4)	1	0.05
Yes	16 (1.0)	3 (18.8)	1.38 (0.39,4.88)		8 (50.0)	2.65 (0.99,7.11)	
BCG vaccination							
(with scar)							
No	336 (21.0)	60 (17.9)	**1**	**0.04**	94 (28.0)	1	0.9
Yes	1265 (79.0)	170 (13.4)	**0.71 (0.52,0.99)**		348 (27.5)	0.98 (0.75,1.28)	
Toilet facilities							
None/bush/pit	1580 (98.7)	221 (14.0)	**1**	**0.001**	429 (27.2)	**1**	**0.001**
Flush toilet	21 (1.3)	9 (42.9)	**4.61 (1.92,11.07)**		13 (61.9)	**4.36 (1.80,10.59)**	
Drinking water source							
Stream	655 (40.9)	79 (12.1)	**1**	**0.03**	142 (21.7)	**1**	**<0.001**
Well or piped	946 (59.1)	151 (16.0)	**1.39 (1.03,1.86)**		300 (31.7)	**1.68 (1.33,2.11)**	

Bold implies significance at the 5% level.

3.2.5 Multivariate analysis

In a multivariate model, hookworm burden and *Ascaris* infection were the strongest independent predictors of sensitivity to house dust mite. The effect of hookworm infection was intensity-related, and those in the highest category of hookworm egg counts (≥350 epg) had a 39% reduction in risk of sensitivity to house dust mite (epg 1-199 adjusted OR=0.79, 0.54-1.16, epg 200-349 adjusted OR=0.75, 0.46-1.23, epg 350+ adjusted OR=0.61, 0.39-0.96, P_{trend}=0.03, Table 3.4). Those with *Ascaris* infection had a 72% reduction in risk (adjusted OR=0.28, 0.10-0.78, Table 3.4), but there were too few children infected to determine whether this effect was related to infection intensity. There was also a significant effect of having better toilet facilities (adjusted OR flush toilet vs none/bush/pit = 2.51, 1.00-6.28), but with no independent effect of drinking water source. Sensitivity to house dust mites was independently related to ethnicity (p=0.02). These effects were not appreciably changed by adjustment for parental education or ownership of goods.

Cockroach sensitisation was increased in individuals who had regular access to well or piped drinking water (adjusted OR well/piped water vs stream=1.33, 1.02-1.75, Table 3.5). The protective effect of *Ascaris* infection was of borderline statistical significance in this model (adjusted OR=0.66, 0.35-1.05). Cockroach sensitisation was also independently related to ethnicity (p<0.001, Table 3.5), but there was no significant effect of having better toilet facilities.

Table 3.4 Multivariate model for dust mite sensitivity

	Mutually adjusted OR (95% CI)*	**P value**
Ethnic group		
Raclay	1	
Kinh	1.73 (1.17,2.55)	0.02
Other	1.57 (0.56,4.39)	
Hookworm		
None	1	
1-199	0.79 (0.54,1.16)	0.03 (P_{trend})
200-349	0.75 (0.46,1.23)	
350+	0.61 (0.39,0.96)	
Ascaris		
No	1	
Yes	0.28 (0.10,0.78)	0.01
Toilet facilities		
None/bush/pit	1	
Flush toilet	2.51 (1.00,6.28)	0.05

* The results are adjusted for age, sex, and area as *a priori* confounders.

Table 3.5 Multivariate model for cockroach sensitivity

	Mutually adjusted OR (95% CI)*	**P value**
Ethnic group		
Raclay	1	
Kinh	2.89 (2.12,3.94)	<0.001
Other	2.04 (0.87,4.81)	
Drinking water source		
Stream	1	
Well or piped	1.33 (1.02,1.75)	0.04

*The results are adjusted for age, sex, and area as *a priori* confounders.

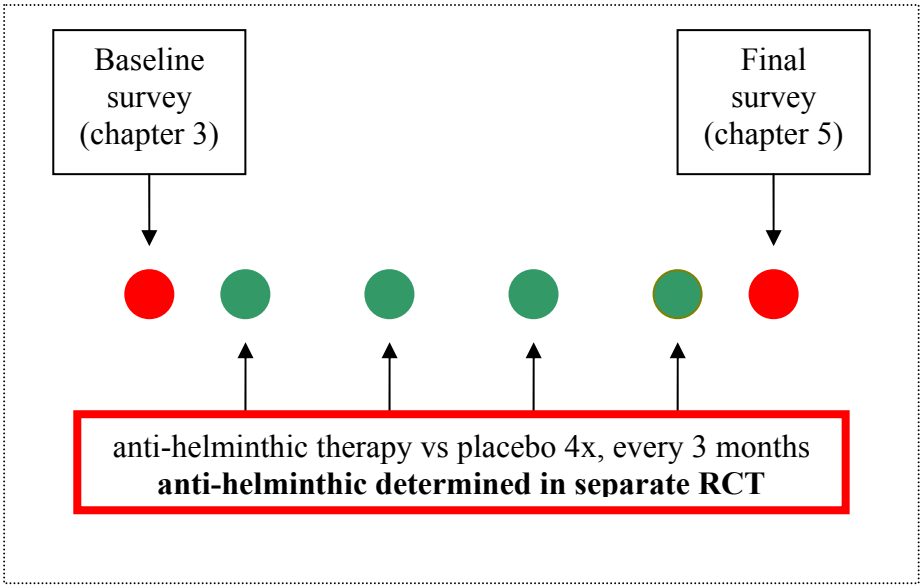
3.3 SUMMARY

This study demonstrated independent protective effects of hookworm and *Ascaris lumbricoides* infection, markers of poor sanitation and of ethnic group on allergic skin sensitisation to dustmite, and effects of drinking water supply and ethnic group on skin sensitisation to cockroach allergen. Overall, these findings support the hypothesis that

gastrointestinal infection, with either helminths or other microorganisms, protects against allergic sensitisation and perhaps clinical allergy.

As argued in chapter 1, only a carefully designed prospective intervention study can prove or disprove whether the observed inverse relationship between skin prick test positivity to house dust mite and helminth infection is causal. The next two chapters describe the treatment comparison study to find the most appropriate anti-helminthic treatment (chapter 4) and then the intervention study itself (chapter 5).

CHAPTER 4: TREATMENT COMPARISON STUDY



4.1 METHODS

Following the baseline survey, children were randomised to receive either single dose oral mebendazole 500mg (Phardazone®, Pharbaco, Hanoi) or placebo, since Phardazone® is currently being used by the World Health Organization-led national helminth control programme in Vietnam in areas where hookworm is the main helminth parasite (Flohr et al., 2007a). This treatment was scheduled to be given four times, three months apart. Each treatment was assigned at random in permuted blocks of 10 stratified by school, given in double blind fashion. Tablets were first chewed and then swallowed under direct observation. Both mebendazole and placebo were packaged in envelopes marked with a computer-generated randomised letter code at the Oxford University Clinical Research Unit in Ho Chi Minh City.

To ensure that our study intervention was effective in reducing worm burden, we assessed the effectiveness of single dose Phardazone® 500 mg in a small randomised controlled trial (Study 1), which was integrated into the main study. To our surprise Phardazone® only reached a 25% reduction in infection intensity compared to placebo, and we consequently carried out a further randomised, double blind, placebo-controlled trial in so far untreated adults (Study 2), this time comparing the effectiveness of multiple dose mebendazole with single or multiple dose albendazole, to find the best suitable treatment for the last three treatments in the main study.

All data were double entered by two data entry clerks, using SPSS Data Entry Station 4.0. The primary endpoint for both studies was parasite intensity as measured by percent decline in mean epg after treatment, relative to mean post-treatment placebo. A secondary endpoint was cure from hookworm infection, ie loss of hookworm eggs from faeces in those infected at baseline. Cure rate was analysed as a binary variable by logistic regression. To control for any change in mean epg in the placebo group, reduction in egg count was calculated as the percentage mean reduction compared to post-treatment egg counts in the placebo group. Egg counts were highly overdispersed, and were therefore analysed by generalised negative binomial regression. This approach assumes a negative binomial error structure for the dependent variable and a log link, and allows both the mean and the negative binomial parameter (ln(alpha)) to be dependent on the explanatory variable (treatment group). The dependent variable was

the number of eggs counted, with the weight of faeces examined included as an offset. Post-treatment egg counts were analysed in those with pre-treatment egg count above zero. Differences between treatment and placebo groups were analysed by simple linear contrasts. For analysis of the effect of pretreatment burden on efficacy in the second study, pretreatment burden was coded as 0=light (<2000 epg), 1=medium (2000-3999 epg) or 2=heavy (>3999 epg), according to WHO guidelines (WHO, 2002). The analysis was then repeated, including burden class as a continuous covariate, to examine for interaction between burden class and treatment. All analysis was performed using Stata 9.1. An intention-to-treat analysis was planned in the event of study dropouts.

4.2 STUDY 1

Directly after the first treatment round with Phardazone®, we analysed data from all 309 children enrolled in the study in Ba Cum Nam commune. In these children, a second stool sample was collected 2 weeks post treatment in addition to the one collected at baseline. Stool analysis was performed in the same manner as for pre-treatment samples (see chapter 2 for a more detailed method description).

Results study 1

Out of the 271 children who participated (87.7% of those eligible), 134 received placebo and 137 mebendazole. The prevalence of hookworm infection in the study group was 62%, and the mean infection intensity

was 175 epg (range 0-3800 epg, Table 4.1 and Figure 4.1). In addition, we found 16 children (6%) with *Ascaris lumbricoides* infection. No participants were lost to follow-up and all were entered in the final analysis.

Figure 4.1 Flow diagram Study 1

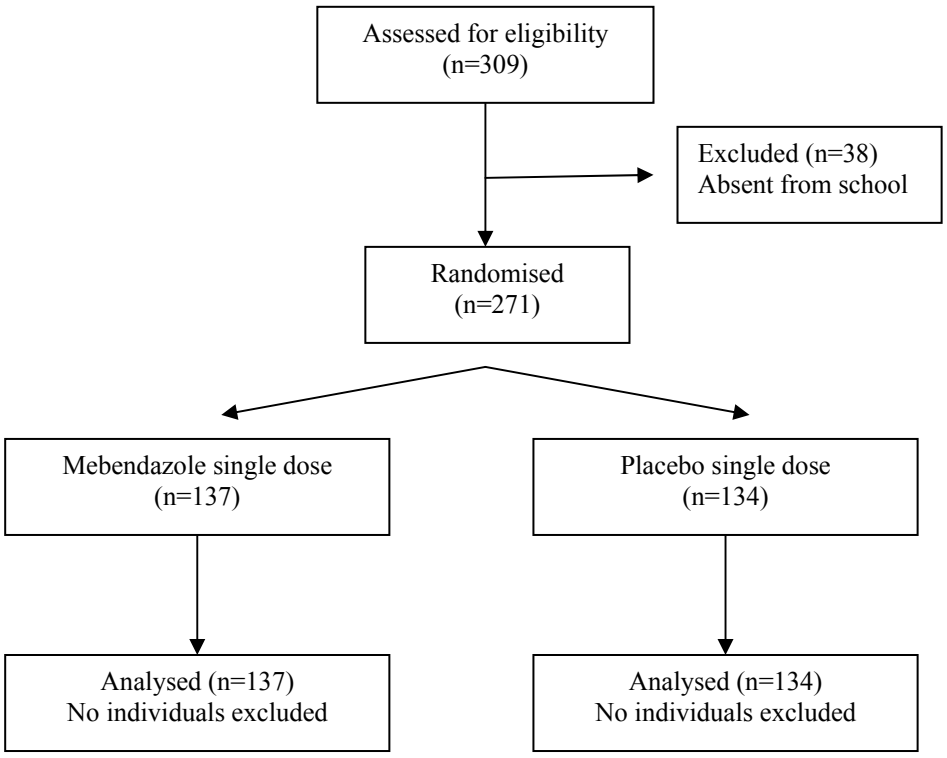


Table 4.1 Baseline characteristics, and baseline and post treatment hookworm epg by treatment group, and estimated percentage efficacy relative to placebo for both trials

	STUDY 1 (CHILDREN)			STUDY 2 (ADULTS)				
	Placebo	**Single dose mebendazole**	**P value**	**Placebo**	**3 doses mebendazole**	**Single dose albendazole**	**3 doses albendazole**	**P value††**
N treated	134	137		54	54	54	47	
Male/female	57/77	65/72		25/29	25/29	25/29	28/19	
Mean age **Age range**	9 7-11	9 7-11		37 18-68	35 16-75	37 16-65	33 17-65	
Hookworm positive at baseline	58% (78/134)	66% (90/137)	0.2	94% (51/54)	93% (50/54)	87% (47/54)	91% (43/47)	0.6
Hookworm negative post treatment†	33% (26/78)	38% (34/90)	0.5	35% (18/51)	26% (13/50)	45% (21/47)	79%*** (34/43)	<0.001
Baseline mean hookworm epg†	306	263	0.3	1068	2210**	1120	1173	0.004
Fitted mean post-treatment hookworm epg, adjusted for baseline*	164	124	0.2	717	279**	224**	97**	0.001
Estimated efficacy relative to placebo % (95% CI)		25 (-18,52)			61 (29,79)	69 (35,85)	87 (55,96)	

† in those positive for hookworm at baseline
†† test for difference between groups
* using a generalised linear model adjusted for baseline egg count, and calculated at mean baseline egg count for total population in each study
** significantly different from placebo ($p<0.005$); *** ($p<0.001$)

After 2 weeks, there was no significant difference between treatments in the proportion of infected children cured (33% (26/78) in the placebo group and in the mebendazole group (38% (34/90), p=0.5). In terms of reduction in mean epg, mebendazole 500 mg single dose achieved a 25% (95% CI -18% to 52%) greater reduction than placebo (Table 4.1).

4.3 STUDY 2

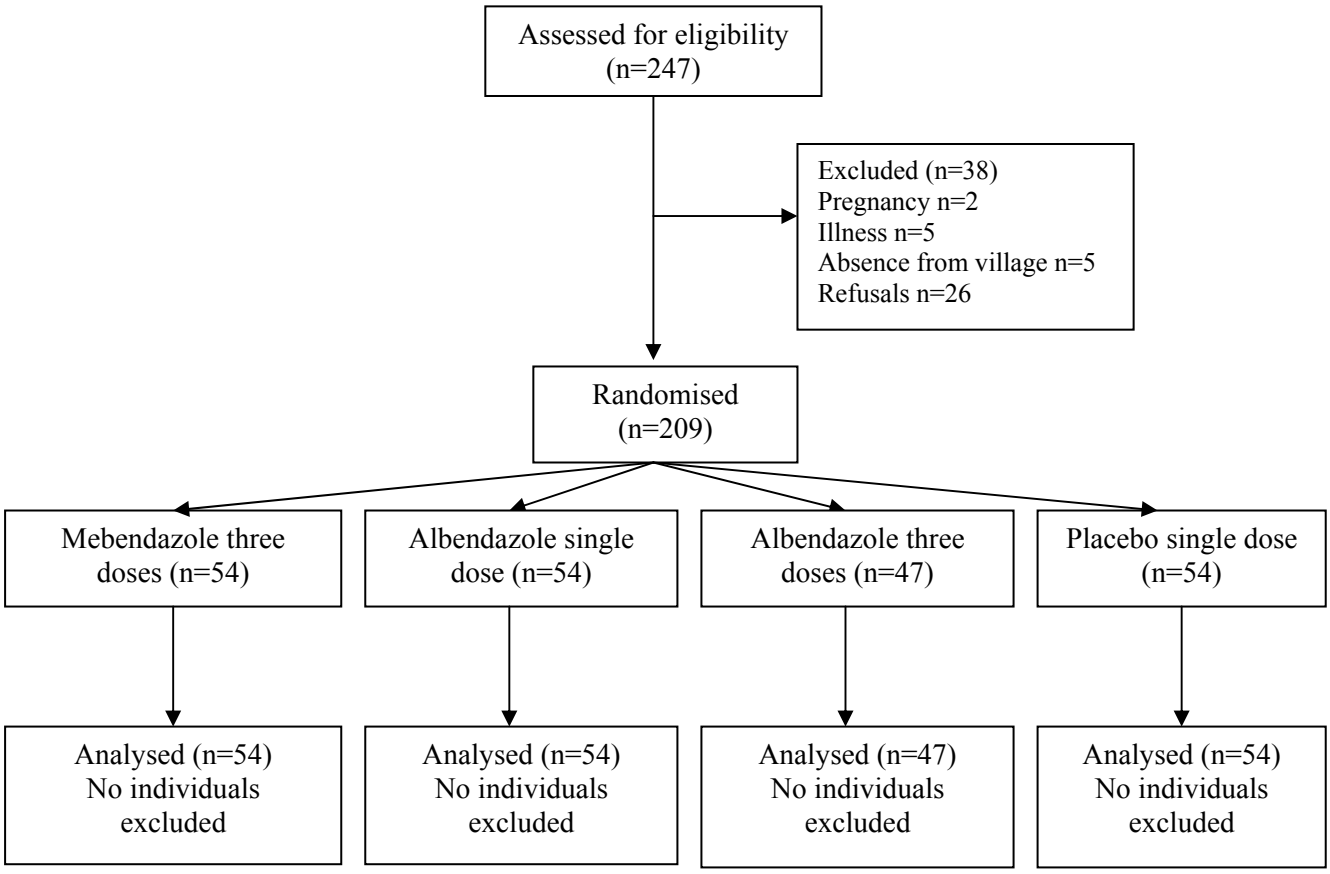
Study 1 indicated that treatment efficacy was not sufficient, and we consequently invited all 247 adults (age 16 and above) living in one village in the same geographical area to participate in a similar study, comparing treatment with either single dose mebendazole placebo, mebendazole (Phardazone®) 500 mg daily for three days, albendazole 400 mg single dose (Mekozetel®, Mekophar Chemical Pharmaceutical Joint Stock Company, Ho Chi Minh City), or albendazole 400 mg daily for three consecutive days. Faecal egg counts were carried out just before and two weeks after therapy as in Study 1.

Results study 2

Of the 209 adults who took part in the second study (85% of those eligible), 54 received three doses of mebendazole for three days, 54 single dose albendazole, 47 three doses of albendazole and 54 single dose mebendazole placebo tablets (Table 4.1 and Figure 4.2). 91% of participants were infected with hookworm at baseline, with a mean intensity of 1283 epg (range 0-17850 epg). 14 individuals had heavy

infections [>3999 epg; (WHO, 2002)]. Six participants (3%) had *Ascaris lumbricoides* or *Trichuris trichiura* as well as hookworm infection. No participants were lost to follow-up and all were included in the analysis.

Figure 4.2 Flow diagram Study 2



There was a highly significant effect of treatment regimen on cure rate ($p < 0.001$), with only albendazole given for three days having a significantly higher cure rate than placebo. In terms of reduction in epg, all treatments were significantly more effective than placebo. The estimated reduction in mean epg relative to placebo varied between 61% for 3 days mebendazole and 87% for 3 days albendazole. Adjustment for age and sex did not alter any of these findings. All drugs, single or triple dose were equally well tolerated.

4.4 SUMMARY

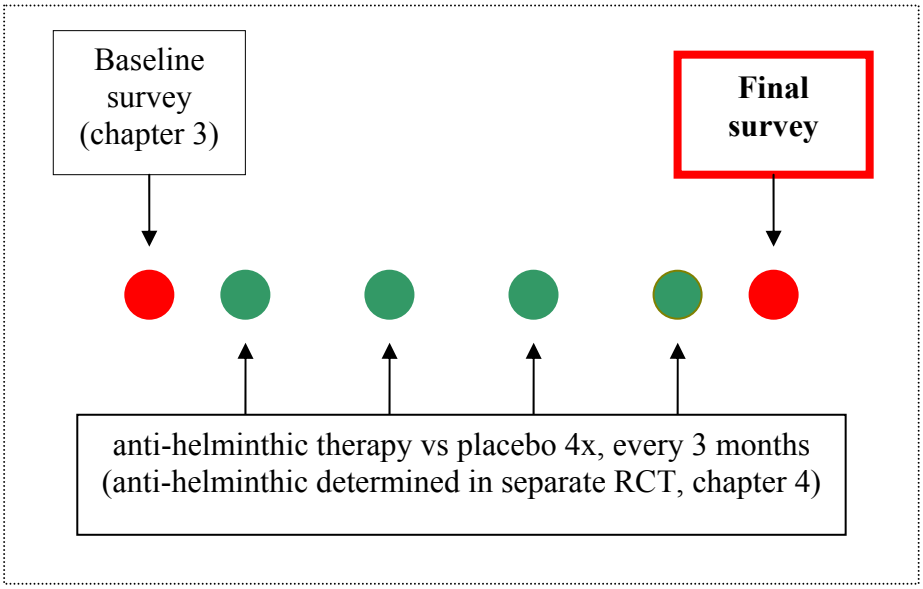
In summary, single dose generic mebendazole (Phardazone®) was not significantly superior to placebo against hookworm, with an estimated reduction in mean epg of 25%. This was particularly surprising, since single dose Phardazone® is currently being used by the World Health Organization-led national gut worm control campaign in Vietnam (Flohr et al., 2007a).

There are a number of factors that may have contributed to the low efficacy of single dose mebendazole. Faecal helminth egg counts are highly variable, reflected here in the relatively wide confidence interval for mebendazole efficacy (Hall, 1981). Variability can be reduced by performing repeated egg counts over a number of days, but such variation should not lead to bias, and the upper 95% confidence interval for mebendazole efficacy in our sample was still only 52%. Egg counts may also vary over time in untreated individuals, as shown here by a decline in egg counts post-treatment in the placebo group.

Similar observations have been made in other studies, and this emphasises the importance of comparing efficacy to a control group (De Clercq et al., 1997). Drug quality has been reported to affect mebendazole efficacy in some studies (Wesche and Barnish, 1994). However, the Phardazone® tablets we used were independently quality tested by the Bureau of Food and Drugs, Department of Health, Republic of the Philippines in August 2005 (independent WHO-accredited reference laboratory for mebendazole) and met international standards.

Whatever the reasons for low treatment efficacy were, our findings made it necessary to perform a second study to seek a more effective anti-helminthic regimen. As a recent systematic review suggested that albendazole has a higher efficacy against hookworm infection than mebendazole, we compared single and triple dose albendazole with triple dose mebendazole in our second study (Bennett and Guyatt, 2000, Horton, 2000). The results show that all treatments significantly reduced epg with the highest egg count reductions seen with triple dose albendazole, and we therefore continued the main study with triple dose albendazole, a total of three times, three months apart.

CHAPTER 5: THE INTERVENTION STUDY



As described in chapter 3, initially all primary and secondary schoolchildren in grades 1 to 9 in Khanh Son district were invited to take part in the baseline survey. Since children in grade 9 were about to leave school (n=35), only children in grades 1 to 8, who had taken part in the baseline survey in April/May 2005, were eligible to be randomised into the intervention study (n=1566). The final survey was conducted in April/May 2006.

5.1 METHODS

The methodology has already been described in detail in chapter 2. In brief, after the initial baseline survey (chapter 3), consenting children were randomised to receive either anti-helminthic therapy immediately and then at 3, 6 and 9 months, or an indentical looking placebo control

(chapter 4). At 12 months, the initial baseline measures were repeated in all children. The primary study endpoint was within-person mean % fall in peak flow from baseline after anti-helminthic treatment at 12 months. We determined *a priori* from an available estimate of the standard deviation for fall in PEFR of 6% from the study by Burr (Burr et al., 1989) that 1341 children (670 per group) would provide 86% power to detect a difference of 1% in the fall in PEFR between intervention and control group, and over 99% power to detect a difference of 2% between groups (alpha=5%). For our secondary outcomes of prevalence change in skin prick test positivity, flexural eczema, and questionnaire-derived wheeze and rhinitis at 12 months, using the same assumptions, for an outcome with a prevalence of around 10% (such as sensitisation to *D. pteronyssinus*), this sample size would provide 90% power to detect a change to 16% prevalence. For outcomes with a prevalence of around 5%, such as "wheeze", the study would have 92% power to look for a change to a prevalence of 10%.

All trial data were double entered by two data entry clerks, using SPSS Data Entry Station 4.0 and then analysed in SPSS version 14.0. The data were analysed on an intention-to-treat basis. The change in exercise-induced bronchospasm (% fall in PEFR after exercise) between baseline and 12 months was normally distributed, and compared between the intervention and control group by analysis of covariance, adjusting for a chance baseline difference between groups in allergen sensitisation. Those with missing data on the primary

outcome were excluded from this analysis, but we performed a sensitivity analysis, using an imputation method assuming no change in PEFR (percent reduction after exercise) between baseline and end of study in those with missing data on the primary outcome.

All binary outcomes were analysed by logistic regression, fitting the effect of treatment group, and adjusting for baseline difference in allergen sensitisation. The randomisation sequence was not broken until after completion of the primary analysis. Treatment efficacy was calculated as loss of any helminth infection (hookworm, *A. lumbricoides*, or *T. trichiura*) at 12 months follow-up in the treatment compared to the placebo group.

The cross-sectional survey suggested a stronger protective effect for *A. lumbricoides* on allergen skin sensitisation than for hookworm. In addition, the relationship between hookworm and allergen skin sensitisation was infection intensity related. In a post-hoc analysis, we therefore tested for interaction between type of helminth infection as well as infection intensity (epg) and treatment allocation (anti-helminthic therapy vs placebo) on all study outcomes. Where significant interaction was present, a subgroup analysis was conducted within those with specific infection at baseline.

5.2 RESULTS

The participant flow is shown in Figure 5.1 .

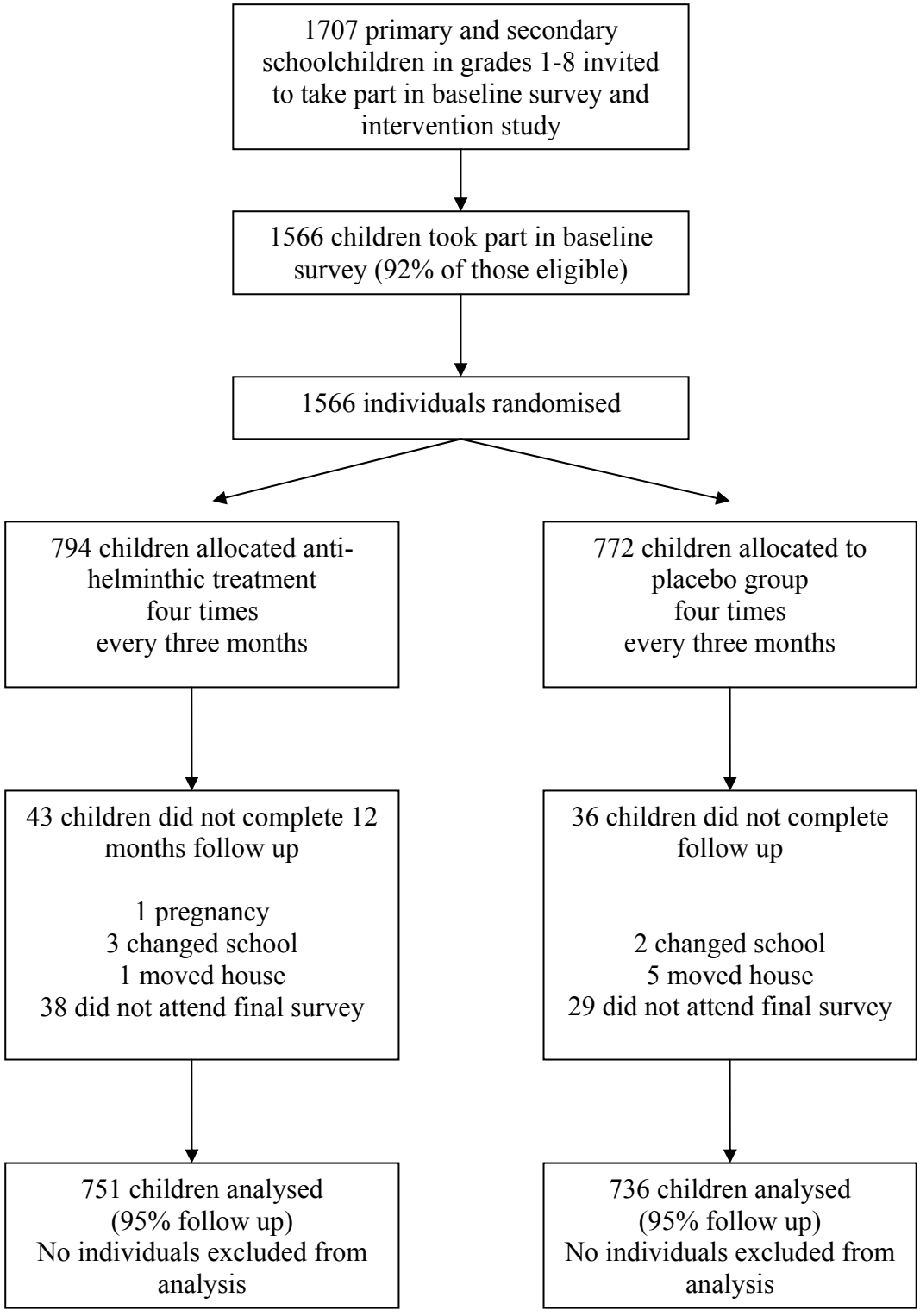


Figure 5.1 Flow diagram intervention study

A total of 1566 primary and secondary schoolchildren in grades 1 to 8 (age range 6 to 17, mean age 8.7) took part in the baseline survey. 64.8% (1015/1566) of children were infected with hookworm (mean epg 286, range 0-5400). *A. lumbricoides* was present in 7% (109/1566) of children (mean epg 63, range 0-13850). All children and their parents consented to be randomised to one of the two treatment groups, 794 to active treatment and 772 to placebo. A total of 1487 children (95% of those randomised) completed the study. In the active group there were 43 who did not complete, including one girl who became pregnant, three who changed school, one who moved house and 38 who did not attend the final survey without giving further reasons. In the control group, 36 children did not complete the study, including two who changed school, five who moved house, and 29 who failed to attend the final survey for unknown reasons (Fig. 5.1). The baseline characteristics of the children lost to follow-up were not different from those who took part and did not differ between groups (data not shown). The baseline characteristics of the two study groups were similar except for the prevalence of allergen skin sensitisation, which was slightly higher in the active treatment group (41.9%) than the placebo group (38.6%, Table 5.1).

Table 5.1 Baseline characteristics

Characteristic	Anti-helminthic treatment N (%)	Placebo N (%)
Total	794	772
Sex		
Male	375 (47.2)	372 (48.2)
Female	419 (52.8)	400 (51.8)
Age group		
6-8	466 (58.7)	449 (58.2)
9-11	250 (31.5)	250 (32.4)
12+	78 (9.8)	73 (9.5)
Area		
Thanh Son	185 (23.3)	185 (24.0)
Son Lam	291 (36.6)	278 (36.0)
Son Binh	214 (27.0)	204 (26.4)
Ba Cum Nam	104 (13.1)	105 (13.6)
Ethnic group		
Raclay	623 (78.5)	635 (82.5)
Kinh	159 (20.0)	126 (16.4)
Other	12 (1.5)	9 (1.2)
Parental education		
Illiterate	196 (24.7)	193 (25.0)
Primary	406 (51.1)	395 (51.2)
Secondary or higher	192 (24.2)	184 (23.8)
Hookworm		
Yes	515 (64.9)	500 (64.8)
No	279 (35.1)	272 (35.2)
Ascaris		
Yes	55 (6.9)	54 (7.0)
No	739 (93.1)	718 (93.0)
Trichuris		
Yes	6 (0.8)	6 (0.8)
No	788 (98.2)	766 (99.2)
Hookworm epg		
Mean (range)	100 (0 – 5400)	100 (0 – 7300)
Ascaris epg		
Mean (range)	39 (0-10150)	88 (0-13850)
Malaria		
Yes	9 (1.1)	7 (0.9)
No	785 (98.9)	765 (99.1)
Exercise-induced bronchspasm (% fall in PEFR after exercise)	-2.4 (SD 7.1)	-2.3 (SD 7.6)
Wheeze (questionnaire)	40 (5.0)	36 (4.7)
Rhintis (questionnaire)	146 (18.4)	138 (17.9)
Flexural eczema (skin examination)	4 (0.5)	3 (0.4)
Atopy		
Any allergen	273 (34.4)	248 (32.1)
D. pteronyssinus	65 (8.2)	43 (5.6)
D. farinae	95 (12.0)	78 (10.1)
Cockroach	232 (29.2)	197 (25.5)

The supervising fieldworkers reported that 96% of children received all doses of their allocated treatment under direct observation at 0, 3, 6, and 9 months. Adherence to the treatment protocol was similar in both study groups. While availability of over-the-counter antihelminthics is extremely limited in Khanh Son, we recognised that some parents might have treated their children for gut worms at their own initiative, and therefore asked parents at follow up whether any extra anti-helminthics had been given during the study period. The parents of two children, both in the active treatment group, said that they had initiated treatment. It is unlikely that treatment led to unblinding of participants and fieldworkers, since reported sighting of passed adult worms was equally frequent in the treatment (6.8%, 51/751) and placebo groups (6.5%, 48/736) at 12 months follow up. There was no significant difference in the frequency of reported side effects between study groups, the commonest being headache (9.3% active treatment vs 7.5% placebo), abdominal pain (7.6% active treatment vs 8.4% placebo), and a skin rash (0.9% active treatment vs 1.0% placebo). At 12 months follow up, there was a 90% reduction in hookworm infection prevalence in the active treatment group compared to placebo (8.9% vs 49.6%, OR=0.10, 95% CI 0.08-0.13).

At 12 months follow up there was a marked fall in helminth prevalence in the treatment group (any helminth from 67.4% at baseline to 9.2% after treatment; hookworm from 64.9% to 8.9%), compared to a slight change in the placebo group (any helminth from 66.4% to 50.4%; hookworm from 64.8% to 49.6%), and a highly significant effect of

treatment vs placebo on prevalence at 12 months of any helminth (OR=0.10, 0.08-0.13, p<0.001) or hookworm (OR=0.10, 0.08-0.13, p<0.001). Effects of treatment on *A. lumbricoides* prevalence at 12 months were less marked (OR=0.49, 0.12-1.96, p=0.30), as *A. lumbricoides* prevalence declined in both treatment (from 6.9% to 0.4%) and placebo (from 7.0% to 0.8%) groups. There was an 86% reduction in mean hookworm epg at 12 months in the treatment compared to placebo group, and a smaller reduction of 32% in mean *A. lumbricoides* epg.

The mean percent fall in peak expiratory flow in the active and placebo groups increased by 2.25% (SD 7.3) and 2.19% (SD 7.8) respectively between the baseline and 12 month assessments. After adjustment for the difference in prevalence of sensitisation between treatment groups at baseline, the difference between them was 0.06% (95% CI -0.71 to 0.83, Table 5.2). The results of the sensitivity analysis were very similar (mean change (SD) in the intervention group 2.13 (7.13), control group 2.07 (7.64), adjusted mean difference 0.06, 95% CI -0.68 to 0.79), indicating that missing values did not appreciably affect our results.

There was a significant increase in the prevalence of allergen skin sensitisation in those in the active compared to the placebo group at 12 months (OR=1.28, 1.03-1.60, p=0.03), and this difference remained significant after adjustment for baseline prevalences (adjusted OR=1.31, 1.02-1.67, p=0.03, Table 5.2). Similar effects were seen for all individual allergen responses, but these were only of borderline

statistical significance after adjustment for baseline prevalences (*D. pteronyssinus* adjusted OR=1.26, 0.81-1.95, p=0.31; *D. farinae* adjusted OR=1.45, 0.98-2.15, p=0.06; Cockroach adjusted OR=1.26, 0.96-1.66, p=0.09). There were no significant risk increases in the treatment group for the other secondary outcomes, questionnaire-derived wheeze (adjusted OR=1.16, 0.35-3.82, p=0.8) and rhinitis (adjusted OR=1.39, 0.89-2.15, p=0.1), and flexural eczema on physical examination (adjusted OR=1.17, 0.39-3.49, p=0.8). None of these effect estimates were altered by additional adjustment for ethnic group.

Table 5.2 Effect of anti-helminthic treatment on study outcomes at 12 months follow up

	Anti-helminthic treatment N (%)	**Placebo N (%)**	**Size of effect* (95% CI)**	**P value**
			Mean difference (95% CI)	
Change in exercise-induced bronchospasm (%) mean (SD)	2.25 (7.3)	2.19 (7.8)	0.06 (-0.71,0.83)	0.9
			Odds ratio (95% CI)	
Wheeze since start of treatment	6 (0.8)	5 (0.7)	1.16 (0.35-3.82)	0.8
Rhinitis since start of treatment	50 (6.7)	36 (4.9)	1.39 (0.89-2.15)	0.1
Flexural eczema (skin examination)	7 (0.9)	6 (0.8)	1.17 (0.39-3.49)	0.8
Skin sensitisation				
D.pteronyssinus	59 (7.9)	42 (5.7)	1.26 (0.81-1.95)†	0.31
D. farinae	76 (10.1)	51 (6.9)	1.45 (0.98-2.15)†	0.06
Cockroach	215 (28.6)	175 (23.8)	1.26 (0.96-1.66)†	0.09
Any of the above	251 (33.4)	207 (28.1)	1.31 (1.02-1.67)*	0.03

* all adjusted for the observed difference between groups in atopy (any sensitisation) at baseline,
† adjusted for difference between groups in specific sensitisation at baseline

There was no significant interaction between hookworm infection status or infection intensity at baseline and treatment effects on any of the study outcomes. In post-hoc analysis, however, there was a significant interaction between *A. lumbricoides* infection at baseline and the effect of treatment on allergen skin sensitisation (p=0.04), such that the risk of sensitisation to any allergen at 12 months in those who had received anti-helminthic treatment compared to placebo was more marked in those with *Ascaris* infection at baseline (adjusted OR=4.90, 1.48-16.19, p=0.009). Numbers were too small to calculate risk estimates for individual allergens. No significant risk increases were seen for any of the other primary and secondary outcomes, and there were no interactions with *A. lumbricoides* baseline infection intensity.

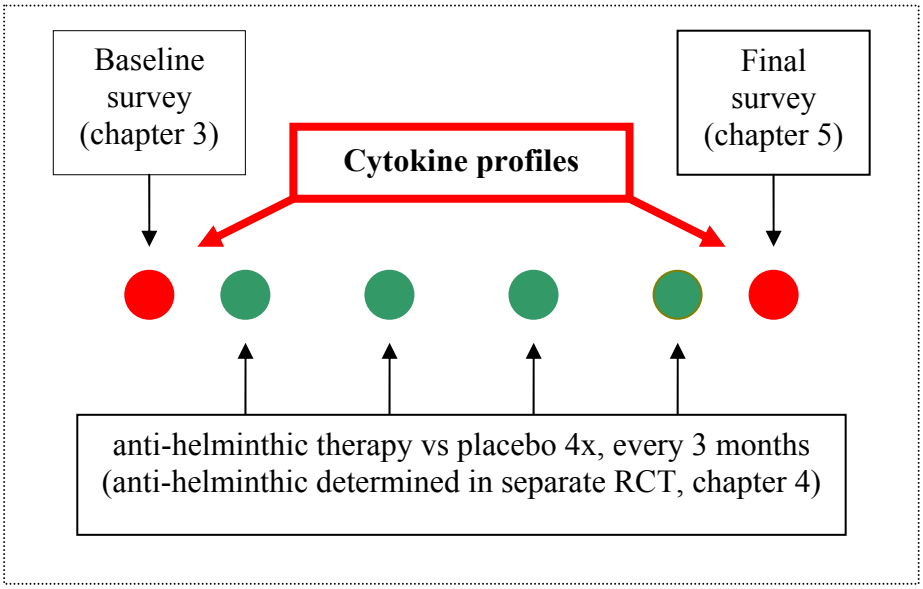
5.3 SUMMARY

The intervention study shows that three-monthly anti-helminthic therapy over a 12 month period had no effect on the primary endpoint of exercise-induced bronchospasm in Vietnamese children from an area with a high prevalence of hookworm infection, but significantly increased the risk of allergen skin sensitisation. This is in keeping with the cross-sectional baseline survey results (chapter 3) and suggests a direct immuno-modulatory effect of helminth infection. This effect was particularly marked for children with *Ascaris* infection, the majority of whom had dual helminth infection.

The still remaining question was whether the observed inverse relationship between skin sensitisation and helminth infection could at

least in part be explained by differences in cytokine profiles, especially helminth-induced anti-inflammatory IL-10. The cytokine analysis is described in the next chapter.

CHAPTER 6: CYTOKINE PROFILES



Hookworm-specific host cytokine responses were measured for IL-10 (anti-inflammatory cytokine), IL-5 (Th2 cytokine), IL-13 (Th2 cytokine), and IFN-γ (Th1 cytokine). The main hypothesis was that allergen sensitisation was inversely related to hookworm-specific IL-10 cross-sectionally at baseline, and that anti-helminthic treatment would lead to a reduction in hookworm-induced IL-10 compared to placebo at 12 months follow up. Results are presented separately for the cross-sectional analysis at baseline and the randomised controlled trial. Please refer to chapter 1 for more background on the cytokines implicated in helminth-allergy links.

6.1 METHODS

7.5mls of venous blood was taken from 244 secondary schoolchildren at baseline and from a further 144 after anti-helminthic treatment at 12 months (only those who were hookworm infected at baseline). The blood was transported directly to the Pasteur Institute Nha Trang at ambient temperature. Samples were kept out of direct sun exposure and care was taken to avoid shaking. All samples reached the laboratory within 3 hours post venesection. After arrival, samples were gently mixed and 1.5ml of blood was removed into Falcon tubes and diluted 1:4 with 6ml of RPMI 1640 culture medium (Sigma), supplemented with penicillin (100U/mL), streptomycin (100microgr/mL), and 2m*M* L-glutamine (all Sigma). 1mL of diluted blood was stimulated with 50 microlitres of either *Necator americanus* excretory antigen (500micrograms/ml, provided by Prof David Pritchard's laboratory, University of Nottingham) or phytohemagglutinin (200micrograms/ml, PHA, Sigma; positive control) were added to sterile 48-well culture plates. A third well was left unstimulated (negative control). All plates were incubated at 37^{o}C under 5% CO_2. Following incubation, 400 microlitres of supernatant was removed into 2mL cryotubes at 48 hours and 5 days and immediately frozen at –80C. All samples were sent to the Oxford University Clinical Research Unit in Ho Chi Minh City on dry ice for cytokine ELISA testing.

Cytokine ELISAs. We used paired monoclonal antibodies (MAbs) to detect IL-5 (purified anti-human MAb, 500pg/mL, Becton-Dickson

PharMingen), IL-10 (purified anti-human MAb, 500pg/mL, Becton-Dickson PharMingen), IFN-γ (purified anti-human MAb, 300pg/mL, Becton-Dickson PharMingen), and IL-13 (purified anti-human MAb, 360microgr/mL, R&D Systems). The results presented in chapter 6 are from the 48 hour time point, since the later time point proved not to be optimal for the detection of the above cytokines. Quantification of cytokines was determined by reference to commercial recombinant human standards expressed in pg/mL (Faulkner et al., 2002).

Cytokine raw data were entered and analysed in SPSS 14.0. Since cytokine responses were not normally distributed, medians and interquartile ranges (IQRs) are presented and log-transformed data was used where possible. Cross-sectionally, we examined whether any cytokine response was associated with skin prick test positivity, using the Mann-Whitney U test to compare cytokine responses between sensitised children and children with negative skin prick tests. In addition, all exposures found to be significant in cross-sectional analysis of the main baseline survey (chapter 3) were assessed for confounding by calculating univariate odds ratio estimates with corresponding 95% confidence intervals, using any skin prick test as outcome. For the intervention study, changes in cytokine responses were calculated as pre-treatment minus post-treatment responses, and these were compared between the anti-helminthic and the placebo group by Mann-Whitney U test. An association was considered significant when p was less than 0.05.

6.2 CYTOKINE RESULTS BASELINE SURVEY

The 244 schoolchildren that donated venous blood at baseline had a mean age of 11.7 (range 9-16). 102 (42%) were boys and 142 (58%) girls. As with the whole study sample, the majority of children belonged to the Raclay ethnic minority (173/244, 71%) with the remainder being either ethnic Vietnamese or (very few) of mixed ethnic origin (71/244, 29%). While hookworm was the main helminth infection with a prevalence of 63%, only 3% of children were infected with *Ascaris lumbricoides*. No other parasites were found. Consequently, we measured only hookworm-specific cytokine responses. Table 6.2 shows the baseline characteristics of children who donated blood.

Hookworm-specific cytokine responses were relatively low for all cytokines (IL-10 median 7.7 pg/mL (IQR 1.0-28.1), IFN-γ median 0 pg/mL (0-5.3), IL-5 median 1.1 pg/mL (0-0.6). IL-13 responses were mostly undetectable and therefore not analysed further. In contrast, PHA-induced responses were much stronger (PHA IL-10 median 834.9 pg/mL (449.1-1243.8), PHA IFN-γ median 8826.9 pg/mL (6868.9-11807.4), PHA IL-5 median 168.4 pg/mL (32.3-379.7), Figure 6.1).

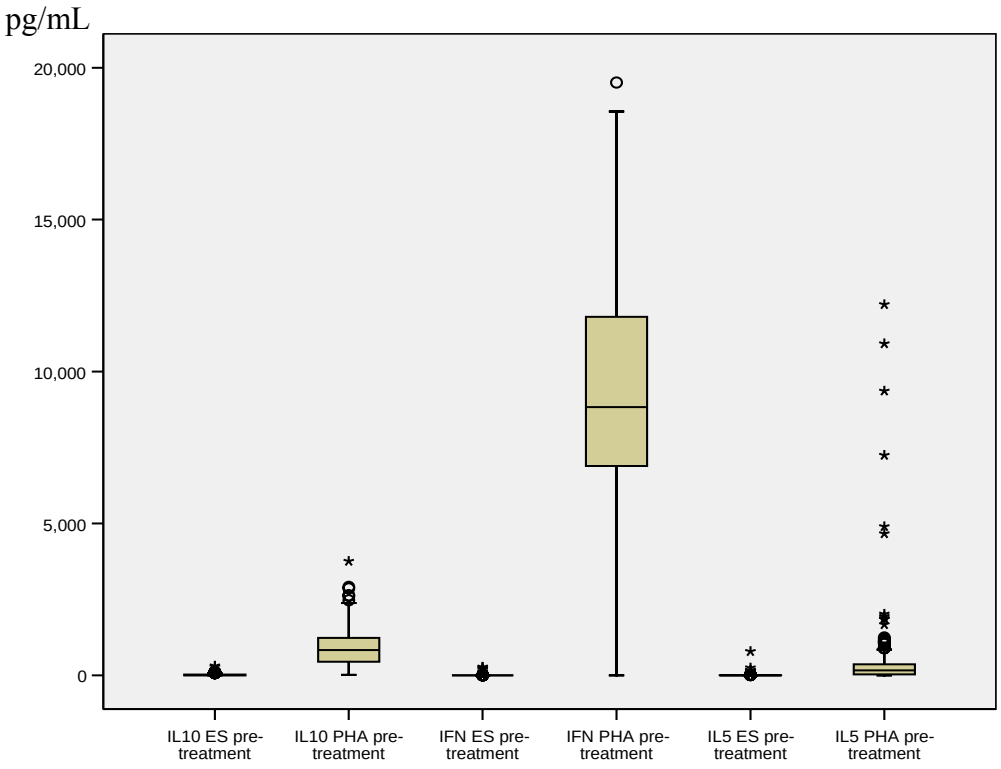


Fig. 6.1 Boxplots of cytokine responses (pg/mL) at baseline. Hookworm-specific IL-10, IFN-γ, and IL-5, as well as PHA-induced IL-10, IFN-γ, and IL-5.

ES=hookworm antigen

Hookworm-specific IL-10 responses were lower in atopic compared to non-atopic children, but this result was not statistically significant (median atopics=4.9 pg/mL (IQR 0.8-16.5) vs median non-atopics=8.8 pg/mL (1.2-32.8), p=0.07). There was also no significant difference in hookworm-specific IFN-γ (p=0.5) and IL-5 responses (p=0.6) between atopics and non-atopics. The same was true for PHA-induced cytokine levels (Table 6.1).

Table 6.1 Cytokine responses at baseline, using any positive skin prick test as outcome (Mann-Whitney U test)

Cytokine	Atopy	Median (IQR)	P value
IL-10 Hookworm (pg/mL)			
	Yes	4.9 (0.8-16.5)	0.07
	No	8.8 (1.2-32.8)	
IFN-γ Hookworm (pg/mL)			
	Yes	0 (0-1.0)	0.5
	No	0 (0-0.3)	
IL-5 Hookworm (pg/mL)			
	Yes	0.8 (0-5.1)	0.6
	No	1.1 (0.5.5)	
IL-10 PHA (pg/mL)			
	Yes	904.4 (370.7-1344.3)	0.9
	No	819.5 (473.9-1221.8)	
IFN-γ PHA (pg/mL)			
	Yes	8798.5 (6457.6-11849.7)	0.9
	No	8829.7 (6997.8-11816.1)	
Il-5 PHA (pg/mL)			
	Yes	165.9 (35.5-405.4)	1.0
	No	172.9 (19.5-376.3)	

The crude odds ratio for hookworm-specific IL-10 in atopics compared to non-atopics was 0.70 (95% CI 0.48-1.03, p=0.07; Tab. 6.2), 1.15 (0.71-1.85, p=0.6) for hookworm-specific IFN-γ and 0.84 (0.53-1.33, p=0.5) for IL-5.

Table 6.2 Baseline characteristics of children who donated venous blood for cytokine analysis with univariate OR estimates for any positive skin prick test as outcome.

	N (%)	At least one pos SPT [N (%)]	Crude OR (95% CI)	P value
IL-10 hookworm* (per pg/mL increase)	-	-	0.70 (0.48-1.03)	0.07
IFN-γ hookworm* (per pg/mL increase)	-	-	1.15 (0.71-1.85)	0.6
Il-5 hookworm* (per pg/mL increase)	-	-	0.84 (0.53-1.33)	0.5
Gender				
Male	102 (41.8)	37 (36.3)	1	0.9
Female	142 (58.2)	50 (35.2)	0.96 (0.56-1.62)	
Age			1	0.06
Per year increase	-	-	1.15 (1.00-1.32)	
Ethnic group				
Raclay	173 (70.9)	53 (30.6)	1	0.01*
Kinh or other	71 (29.9)	34 (47.9)	2.08 (1.18-3.67)	
Area				
Son Lam	169 (69.3)	70 (41.4)	1	0.005*
Ba Cum Nam	75 (30.7)	17 (22.7)	0.42 (0.22-0.77)	
Hookworm				
No	91 (37.8)	33 (36.3)	1	0.9
Yes	153 (62.7)	54 (35.3)	1.04 (0.61-1.79)	
Hookworm				
None	91 (37.2)	33 (36.3)	1	0.4 (P_{trend})
1-199	61 (25.0)	23 (37.7)	1.06 (0.54-2.08)	
200-349	39 (16.0)	17 (43.6)	1.36 (0.63-2.92)	
350+	53 (21.7)	14 (26.4)	0.63 (0.30-1.33)	
Ascaris				
No	238 (97.5)	86 (36.1)	1	0.3
Yes	6 (2.5)	1 (16.7)	0.35 (0.04-3.08)	
Toilet facilities				
None/bush/pit	244 (100.0)	87 (35.7)	-	-
Flush toilet	0 (0.0)	-	-	
Drinking water source				
Stream	65 (26.6)	24 (36.9)	1	0.8
Well or piped	179 (73.4)	63 (35.2)	0.93 (0.51-1.67)	

*Continuous variable, SPT=skin prick test

As for potential confounders of cytokine responses, out of the environmental exposures found to be associated with skin prick test positivity at baseline (see chapter 3) only ethnic group (OR=2.08, 1.18-3.67, p=0.01) and area (OR=0.42, 0.22-0.77, p=0.005) were significantly associated with atopic status in this subgroup of children (Table 6.2). Adjustment of hookworm-specific IL-10 responses for ethnic group and area rendered results even less significant (adjusted OR=0.72, 0.44-1.18, p=0.2).

6.3 CYTOKINE RESULTS INTERVENTION STUDY

144 schoolchildren were infected with hookworm at baseline and therefore re-bled after 12 months of anti-helminthic treatment to study the effect of loss of hookworm infection on cytokine responses. 82 children were randomised to receive anti-helminthic treatment and 71 children were in the placebo group. Baseline characteristics were grossly comparable between groups (Table 6.3).

Table 6.3 Baseline characteristics in those infected with hookworm

Characteristic	Anti-helminthic N (%)	Placebo N (%)
Total	82	71
Mean age (range)	11.84 (9-16)	11.85 (9-15)
Sex		
Male	42 (60.0)	28 (40.0)
Female	40 (48.2)	43 (51.8)
Ethnic group		
Raclay	67 (53.6)	58 (46.4)
Kinh or other	15 (53.6)	13 (46.4)
Area		
Ba Cum Nam	23 (52.3)	21 (47.7)
Son Lam	59 (54.1)	50 (45.9)
Drinking water source		
Well or piped	56 (53.3)	49 (46.7)
Stream	26 (54.2)	22 (45.8)
Toilet facilities		
None/bush/pit	82 (53.6)	71 (46.4)
Flush toilet	0	0
Hookworm		
Yes	82 (53.6)	71 (46.4)
No	0	0
Hookworm epg		
Median (IQR)	100 (0-300)	100 (0-325)
Ascaris		
Yes	3 (3.7)	2 (2.8)
No	79 (96.3)	69 (97.2)
CYTOKINES	**Median (IQR)**	**Median (IQR)**
IL-10 hookworm pg/mL	6.5 (1.3-21.5)	8.9 (1.3-29.2)
IFN hookworm pg/mL	0 (0-0.01)	0 (0-0.2)
IL-5 hookworm pg/mL	0.9 (0-4.0)	1.0 (0-6.5)
IL-10 PHA pg/mL	875.9 (403.3-1230.1)	833.9 (427.0-1185.4)
IFN PHA pg/mL	8136.9 (5966.7-11661.2)	8922.6 (6362.1-11824.8)
IL-5 PHA pg/mL	212.7 (7.7-348.2)	127.2 (42.7-319.1)

After treatment at 12 months follow up, hookworm-specific IL-10 levels were lower in the treatment compared to the placebo group (p=0.09). However, this trend was already seen at baseline, and there

was no significant difference in the change in pre-post treatment cytokine responses for hookworm-specific IL-10, IFN-γ, and IL-5 between groups (p=0.3 for all three, Table 6.4).

Table 6.4 Cytokine responses pre- and post-treatment, and pre minus post treatment change (Mann-Whitney U test)

Cytokine	Treatment group	Median pre-treatment (IQR)	Median post-treatment (IQR)	P value	Median pre minus post treatment (IQR)	P value
IL-10 Hookworm (pg/mL)						
	Anti-helminthic	6.5 (1.3-21.5)	2.7 (0-8.79)	0.09	0.4 (-1.8-11.2)	0.3
	Placebo	9.3 (2.4-29.3)	5.6 (0.25-20.51)		0 (-5.7-9.3)	
IFN-γ Hookworm (pg/mL)						
	Anti-helminthic	0 (0-0.01)	0 (0-5.44)	0.4	0 (-2.9-0)	0.3
	Placebo	0 (0-0.2)	0 (0-8.63)		0 (-2.0-0)	
IL-5 Hookworm (pg/mL)						
	Anti-helminthic	0.9 (0-4.0)	1.2 (0-4.52)	0.3	0 (-1.0-0.8)	0.3
	Placebo	1.0 (0-6.5)	2.1 (0-6.15)		0 (-2.6-0.4)	
IL-10 PHA (pg/mL)						
	Anti-helminthic	875.9 (403.3-1230.1)	1244.6 (394.4-1854.8)	0.7	-201.0 (-660.5-61.3)	0.2
	Placebo	833.9 (427.0-1185.4)	1359.3 (762.4-1770.91		-401.0 (-667.9-0)	
IFN-γ PHA (pg/mL)						
	Anti-helminthic	8136.9 (5966.7-11661.2)	8579.7 (5452.8-12699.1)	0.9	63.5 (-2056.9-1660.8)	0.3
	Placebo	8922.6 (6362.1-11824.8)	7840.2 (5319.3-12699.1)		437.2 (-1784.1-2630.5)	
IL-5 PHA (pg/mL)						
	Anti-helminthic	212.7 (7.7-348.2)	223.9 (60.7-408.0)	0.6	-2.0 (-129.0-126.5)	0.7
	Placebo	127.2 (42.7-319.1)	185.6 (67.9-374.9)		0 (-183.3-63.1)	

While PHA-induced IL-10 responses were stronger at follow up, especially in the placebo group, this pre-post treatment change was statistically not significant between groups (p=0.2). PHA-induced IFN-γ levels were both weaker after treatment than at baseline, more so in the placebo than in the active treatment group. As for PHA-induced IL-10, this change was statistically not significant (p=0.3). There was virtually no change in PHA-induced IL-5 responses between baseline and follow up in both study arms (p=0.7).

6.4 SUMMARY

Above results provide very little support for a direct role of helminth-induced IL-10-mediated suppression of allergen skin sensitisation. There were also no significant correlation between IL-5 or IFN-γ responses and skin prick test positivity. Anti-helminthic treatment did not significantly alter cytokine responses compared to placebo.

CHAPTER 7: DISCUSSION AND CONCLUSIONS

7.1 PRINCIPAL FINDINGS

The initial cross-sectional study suggested that poor hygiene and hookworm as well as *Ascaris lumbricoides* infection independently protect against allergen skin sensitisation. This protective effect of helminth infection on allergen skin sensitisation but not the primary outcome of exercise-induced bronchospasm or other secondary clinical outcomes (flexural eczema and questionnaire-derived wheeze and rhinitis) was consequently confirmed in a substantial individually randomised, double blind, placebo-controlled trial with an anti-helminthic agent. Post-hoc analysis indicated that this effect was particularly strong in children with *Ascaris lumbricoides* infection, most of whom also had infection with hookworm. An additive effect of dual infection is therefore likely. However, in our population this effect did not appear to be meadiated by IL-10 or any other cytokines that we measured.

7.2 STUDY STRENGTHS AND WEAKNESSES

The studies presented here are the first epidemiological studies on allergy-helminth links in East Asia and the first such studies conducted in a geographical area where hookworm predominates. With 92% of those eligible taking part in the baseline survey, initial participation was high, and we succeeded in following up 95% of randomised study participants.

With regard to the cross-sectional study, it is possible that some of the observed helminth effects were reduced by misclassification of infection, because large participant numbers only allowed collection of one stool sample, and a few low worm egg counts may have been missed. However, we confirmed in a separate validation study that the sensitivity of McMaster salt flotation in the field had a sensitivity comparable to the gold standard laboratory method, formol-ether sedimentation (Flohr et al., 2007a). It may also be that low study power obscured some effects seen at baseline, since our power calculation was aimed at the intervention study and because the estimates of effect of hookworm and Ascaris infection, toilet facilities, and drinking water were broadly similar for both house dust mite and cockroach, but more significant for the former. At the same time, it is unlikely that other unmeasured infectious or parasitic diseases confounded study results. Helminths and malaria, which we tested for, are the prevalent endoparasite infections in our study area. There is no schistosomiasis in Vietnam, and recent surveys conducted by the World Health Organization in our study area did not find any cases of lymphatic filariasis (unpublished internal report National Institute of Malariology, Parasitology, and Entomology (NIMPE), Hanoi, March 2006). To date, no case of HIV has been detected in Khanh Son, despite routine screening of all people who seek medical treatment in the district's only hospital.

As for our intervention study, it was individually randomized and double blind and therefore more robust to bias and confounding than

other study designs. Blinding is likely to have been successful for the majority of children, since hookworm was the main gut worm infection and in contrast to *Ascaris lumbricoides*, adult hookworms passed in the faeces after therapy are small and unlikely to be seen. There was no difference between the treatment and placebo groups in the proportion of children reporting sightings of worms after treatment. A further strength of our studies is that we used validated and objective markers of allergic disease outcomes assessed by fieldworkers blinded to intervention status. Since we used a population-based sample of all schoolchildren enrolled in grades 1 to 8 in one well-defined geographical area, and since over 95% of children there are known to attend school, our findings are likely to be generally representative of the target population.

Although mebendazole is being used for the national helminth control programme in Vietnam, we found to our surprise that single dose mebendazole 500mg was not superior to placebo, and we therefore had to change the trial regimen to albendazole 400mg daily for three consecutive days. As a result, those in the active treatment group received efficacious anti-helminthic therapy for only 9 rather than the intended 12 months. We were unable, for logistic and funding reasons, to extend the follow up to compensate for this. The increase in skin prick test positivity in the treatment group might therefore have been greater had we used albendazole from the start or, indeed, given anti-helminthic treatment for longer than 12 months. On the other hand, our study protocol ensured that treatment efficacy was assessed after the

first treatment round, and the change from mebendazole to albendazole is likely to have contributed to the validity of our findings.

7.3 ALLERGIC DISEASE AND HELMINTHS

Cooper et al suggested in a recent cluster randomised trial in Ecuadorian children that regular anti-helminthic treatment does not increase clinical allergic disease, namely exercise-induced bronchospasm, wheeze, rhinitis, and flexural eczema (Cooper et al., 2006). This study in Vietnam confirmed these results with regard to clinical allergy symptoms. In both studies, the lack of an association between loss of exposure to helminths and clinical allergic disease may be due to a lack of causality, or alternatively because helminth eradication was incomplete and/or short-lived in an environment where most children's immune systems are repeatedly exposed to gut worm infection from early in life. On the other hand, the available cross-sectional evidence summarised in a recent systematic review and meta-analysis suggests that hookworm is protective against asthma, while *A. lumbricoides* infection may be associated with an increase in asthma risk (Leonardi-Bee et al., 2006). In addition, better control of asthma symptoms has been reported after anti-helminthic therapy in a study among asthmatics in Venezuela, who were infected with *A. lumbricoides* and *T. trichiura* (Lynch et al., 1997). However, no objective change in pulmonary function was demonstrated.

Early priming of the infant's immune system both *in utero* and postnatally may also be important for the protection from clinical

allergic disease, as suggested by a small randomised, double blind, placebo-controlled trial comparing the risk of eczema development in 103 Ugandan infants whose mothers had been allocated to either single dose anti-helminthic treatment or placebo during the second or third trimester (Elliott et al., 2005). Children whose mothers had received anti-helminthic treatment rather than placebo had a more than two-fold increase in cumulative eczema risk up to age 15 months, though this effect was not statistically significant, possibly because of small sample size. In view of this partly conflicting evidence, it will be important to explore the effect of early loss of helminth infection on clinical allergic disease further through carefully conducted birth cohort and intervention studies.

7.4 ALLERGIC SKIN SENSITISATION AND HELMINTHS

Like us, others have found an inverse relationship between helminth infections and allergen skin sensitisation in cross-sectional analyses (Cooper et al., 2003b, Schafer et al., 2005, Hagel et al., 1993b, Cooper et al., 2004, Cooper et al., 2003a, Nyan et al., 2001, van den Biggelaar et al., 2000, Araujo et al., 2000). Where a distinction between types of helminths was made in the analysis, the effect sizes for *Ascaris lumbricoides* and hookworm infection on atopy were in keeping with the ones demonstrated here.

The evidence from intervention studies has been conflicting though, posssbily resulting from differences in the species, timing, chronicity and intensity of helminth infection. In the large study by Cooper et al,

there was no increase in skin prick test responses after anti-helminthic therapy, while skin prick test positivity did increase in two smaller intervention studies in Gabon and Venezuela following anti-helminthic treatment, and this is in keeping with the results presented here (Lynch et al., 1993b, van den Biggelaar et al., 2004, Cooper et al., 2006). Our expectation when designing the study was that either hookworm or *A. lumbricoides*, both of which have a systemic phase in their life cycle, would protect against allergic sensitisation. In the event, the protective effect in our study was particularly strong for children habouring infection with *A. lumbricoides*. Since 81.7% (89/109) of these children had dual infection with hookworm (89/109), it is likely that the increase in atopy risk we observed was at least partly caused by an additive effect of these infections on host skin prick test responses.

On the basis of data from a series of studies conducted in Venezuela it has long been argued that low intensity chronic helminth infection might increase the risk of allergic disease, while high intensity infection acts protectively (Cooper, 2002, Lynch et al., 1984, Lynch et al., 1987, Yazdanbakhsh et al., 2002). However, we found a significant increase in skin prick test positivity following loss of gut worm infection in a population where the majority of children has, according to WHO criteria, low hookworm infection intensity [mean epg 100 (Montresor et al., 2002)]. This suggests that loss of exposure to helminths can have a direct positive effect on allergen skin prick test reactivity even when infection intensity is low.

7.5 IL-10, HELMINTHS AND ALLERGIC DISEASE

IL-10 has been suggested by a number of authors to play a key role in the helminth-induced down-regulation of allergic host immune responses (Yazdanbakhsh et al., 2002). For instance, a cross-sectional study in Gabonese children demonstrated an inverse relationship between skin prick test positivity to house dust mite and schistosomiasis-specific IL-10 (van den Biggelaar et al., 2000). In addition, a study in children and adults with asthma in Brazil suggested that house dust mite-induced IL-10 was down-regulated after anti-helminthic treatment in asthmatics infected with schistosomiasis (Araujo et al., 2004). While the exact mechanisms of IL-10-induced anti-inflammatory action are currently uncertain, parasite-induced regulatory T cells have been argued to play a pivotal role in the increased expression of IL-10 and seem to be part of the regulatory network that provides a subtle immunological balance between host and parasite (Chatila, 2005, Maizels and Yazdanbakhsh, 2003). An up-regulation of IL-10 is likely to have anti-inflammatory action but may also affect mast cells in the skin, which would influence allergen skin reactivity. For instance, IL-10 is known to inhibit the expression of high affinity FcεRI receptors on mast cells and mast cell activation, which could lead to reduced mast cell degranulation and histamine release (Royer et al., 2001, Gillespie et al., 2004). However, we could not find much evidence to support an immuno-modulatory role for parasite-induced IL-10 in our study population. This may be because cytokine responses were measured by whole blood stimulation rather

than in peripheral blood mononuclear cells, as used by Biggelaar *et al*. and Araujo *et al*. (van den Biggelaar et al., 2000, Araujo et al., 2004). Cytokine responses were relatively low in our study population, which may have led to low power to detect differences in cytokine profiles between atopics and non-atopics at baseline as well as before and after treatment. Alternatively, the previously observed inverse association between schistosomiasis-induced IL-10 and skin prick test positivity (van den Biggelaar et al., 2000) may either be specific to this parasite rather than helminth infection *per se* or an epiphenomenon of other immunological mechanisms underlying the observed reduction in skin prick test responses.

7.6 CONCLUSIONS

This study provides independent evidence of a protective effect of hookworm and *Ascaris lumbricoides* infection on allergen skin prick test responses not only in cross-sectional analysis, but also from a large individually randomised, double blind, placebo-controlled trial with an anti-helminthic agent. However, while the reduction in skin prick test positivity was marked in cross-sectional analysis at baseline (adjusted OR *A. lumbricoides*=0.28, 0.10-0.78 and adjusted OR hookworm 350+ epg vs 0 epg=0.61, 0.39-0.96) the overall increase in skin prick test positivity risk following anti-helminthic therapy was only 31% compared to placebo (adjusted OR=1.31, 1.02-1.67). Even the stronger effect seen for children infected with *Ascaris lumbricoides* at baseline (univariate OR=2.92, 1.17-7.26, adjusted OR=4.90, 1.48-16.19), albeit

being statistically highly significant, represents only an absolute difference of 11 sensitised children in the treatment compared to the placebo group (8 more sensitised in the treatment and 3 less in the placebo group at 12 months follow up, compared to at baseline).

7.6.1 Implications for clinical practice

Given this rather small effect in absolute terms, it is difficult to extrapolate the clinical significance of the study findings. Clearly, had we started our study with albendazole rather than mebendazole and if anti-helminthic treatment had continued beyond the study period of 12 months, the effect seen might have been stronger, but not necessarily so. In addition, since we did not see an equal increase in clinical allergy, we cannot conclude that loss of exposure to endoparasites, for example as a consequence of mass-treatment campaigns with anti-helminthic agents, irrevocably leads to an increase in asthma and flexural eczema in the long-term, in particular, since a dissociation of skin prick test positivity and clinical allergic disease has been noted in developing country settings before (Faniran et al., 1999, Sunyer et al., 2000, Scrivener et al., 2001, Flohr et al., 2007b).

Despite such uncertainties, there are already a number of therapeutic clinical trials in progress that are assessing the therapeutic effects of helminth infection on established asthma and rhinitis, and the results are being eagerly awaited.

7.6.2 Suggestions for future research

Even if such trials were to produce no therapeutic benefits, the relation between helminth infection and allergy remains worthy of further investigation. One area that should receive particular attention is the role of helminth infection around the time of birth, the question being whether *in utero* or early post-natal exposure to helminth infection protects against the clinical expression of allergic disease.

In addition to the protective effect of *A. lumbricoides* and hookworm infections on skin prick test responses, we also saw a strong and uniform effect of ethnicity on allergen skin sensitisation in the cross-sectional baseline survey (chapter 3). It is possible, that ethnic group operated as a surrogate marker of socioeconomic status, reflecting subtle differences in lifestyle between ethnic groups not captured by our measure of ownership of goods. Alternatively, such effects may be a result of genetic traits, which contribute to strong Th2 responses to helminths and/or environmental allergens, and such genetic traits have been described before in other ethnic groups (Blumenthal et al., 2004). Future studies should therefore also make use of genetic tools.

Finally, the cross-sectional baseline study suggested a significant independent protective effect of markers of poor hygiene (ie drinking water source: well/piped water vs stream water adjusted OR=1.33, 1.02-1.75) and poor sanitation (toilet facilities: flush toilet vs none/bush/pit adjusted OR=2.51, 1.00-6.28) on skin prick test responses. These results would be in keeping with a protective effect of gastrointestinal infections other than helminths, and future studies

would benefit from measuring exposure for instance to hepatitis A virus (Matricardi et al., 1997, Matricardi et al., 2000, Matricardi et al., 2002).

Indeed, helminth infections are likely to be just one little piece of a complex jigsaw of gene-environment interactions that make up the increased risk of allergic disease seen in urban compared to rural populations in developing countries, such as Vietnam. For instance, one further contributing factor may be differences in the gut microflora (Bjorksten, 2006). Evidence comes from studies comparing the gut microflora of children from Estonia and Sweden, suggesting that early physiological gut colonization with enterococci and bifidobacteria is crucial in driving the immune system away from the type 2 T helper cell dominance found at birth into a type 1 T helper cell-dominanted direction, protecting the individual from allergic predisposition and sensitisation (Bjorksten et al., 1999, Bjorksten et al., 2001). However, very little work has been done on bacterial gut colonisation in developing countries and this, together with other recognised risk factors on incidence of allergic disease and allergic sensitisation, such as dietary factors, obesity, antibiotic prescribing, smoking and indoor fuel use, may play its part in enhancing allergy risk and should be incorporated in future studies.

Only with such a comprehensive approach will we be able to fully answer P.J. Preston's original question dating back to 1970, which marked the beginning of the research on helminth-allergy links: "…Is the atopic syndrome a consequence of good hygiene? … Could it then

be that the biological advantage associated with an efficient IgE producing mechanism is related to the maintenance of the balance between host and parasite in worm infestations, …?" (Preston, 1970)

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APPENDIX

THE BASELINE STUDY QUESTIONNAIRE

I. THE CHILD AND THE CHILD's FAMILY	
1. Is the child a boy or a girl? 1. Boy 2.Girl	/__/
2. In which year was the child born? _____	/__/__/__/__/
3. Where was the child born? 1. Khanh Son 2. Elsewhere	/__/
4. What ethnic group does the child belong to? 1. Raclay 2. Kinh 3. Mixed 4. Nùng 5. Tay 6. Other	/__/
5. How many <u>older</u> brothers and sisters does the child have? _____	/__/
6. How many <u>younger</u> brothers and sisters does the child have?_____	/__/
7. Was the child breast fed? 1. Yes 2. No <u>If yes</u>, was this 1. Less than 6 months 2. More than 6 months	/__/ /__/
8. Father's educational status 1. Illiterate 2. Primary school 3. Secondary school 4. Higher than secondary school	/__/
9. Mother's educational status 1. Illiterate 2. Primary school 3. Secondary school 4. Higher than secondary school	/__/
10. Does anybody in the house smoke? 1. Yes 2. No <u>If yes</u>, how many people in the house smoke? _____	/__/ /__/

II. THE HOUSE

11. How many people live in the house? _____ /__/

12. How many rooms are there in the house? _____ /__/

13. Has the child ever lived anywhere else? 1. Yes 2. No /__/

14. What type of roof does the house have? /__/
    1. Corrugated iron 2. Thatched 3. Tiles 4. Other

15. What are the walls of the house made of? /__/
    1. Mud 2. Bamboo 3. Wood 4. Bricks 5. Other

16. What type of floor does the house have? /__/
    1. Mud 2. Wood 3. Cement 4. Tiles 5. Other

17. Where does the child ususally sleep? /__/
    1. Floor 2. Hammock 3. Mattress (and no bed) 4. Bed 5. Other

18. Where is most of the cooking done? (tick only one) /__/
    1. Inside the house 2. Outside the house

19. How often are the following used for cooking? (for each fuel tick one box)

a) Charcoal	1. Never	2. Sometimes	3. Every day	/__/
b) Wood	1. Never	2. Sometimes	3. Every day	/__/
c) Leaves	1. Never	2. Sometimes	3. Every day	/__/
d) Kerosene	1. Never	2. Sometimes	3. Every day	/__/
e) Gas	1. Never	2. Sometimes	3. Every day	/__/
f) Electricity	1. Never	2. Sometimes	3. Every day	/__/

20. Which of the following animals does the household keep? (tick all applicable) 1. Cat 2. Dog 3. Chicken/Duck 4. Cow/Waterbuffalo 5. Pig 6. Goat 7. None	/__/__/__/__/__/__/__/
21. Are any insecticides used in the house? 1. Yes 2. No	/__/
22. Which of the following does the child's family own? 1. Car 2. Tractor 3. Motorbike 4. Bicycle 5. Refridgerator 6. Gas stove 7. Mobile phone 8. Telephone 9. Computer 10. TV and Video/VCD 11. TV 12. Radio 13. None of these	/__/__/__/__/__/__/ /__/__/__/__/__/ /__/__/__/__/
23. What is your main source of drinking water? 1. Piped 2. Well 3. Stream (nước suối) 4. Rain water	/__/
24. Do you usually boil water before you drink it? 1. Yes 2. No	/__/
25. What main type of toilet facility does the child use? 1. Flush toilet 2. Pit toilet (hố xí) 3. None/bush/field	/__/

III. THE CHILD's HEALTH

26. Does the child have an EPI card? 1. Yes 2. No /__/

[Interviewer: Politely ask for the EPI card.]

27. Has the child received the following vaccinations?

Lao (BCG) 1. Yes 2. No /__/

If yes, scar present? 1. Yes 2. No /__/

Bai Liet (OPV) 1. Yes 2. No /__/

If yes, confirmed through record (such as EPI card)? 1. Yes 2. No /__/

BH-HG-UV (DPT) 1. Yes 2. No /__/

If yes, confirmed through record (such as EPI card)? 1. Yes 2. No /__/

Soi (Measles) 1.Yes 2. No /__/

If yes, confirmed through record (such as EPI card)? 1. Yes 2. No /__/

Viem Gan B (HBV) 1. Yes 2. No /__/

If yes, confirmed through record (such as EPI card)? 1. Yes 2. No /__/

28. Has your child ever been diagnosed by a health professional (doctor or doctor assistant) to have any of the following diseases?

Tuberculosis 1. Yes 2. No /__/

Measles 1. Yes 2. No /__/

Malaria 1. Yes 2. No /__/

Gut worms 1. Yes 2. No /__/

29. Has the child ever taken any medicines against gut parasites? 1. Yes 2. No /__/

If yes, was this within the past 6 months? 1. Yes 2. No /__/

30. Has the child ever taken any antibiotics? 1. Yes 2. No /__/

IV. LUNG QUESTIONS

31. Has your child ever had wheezing or whistling in the chest at any time in the past? 1. Yes 2. No /__/

IF THE ANSWER IS "NO" PLEASE SKIP AND GO TO QUESTION 36.

32. Has your child had wheezing or whistling in the chest in the last 12 months?
1. Yes 2. No /__/

IF THE ANSWER IS "NO" PLEASE SKIP AND GO TO QUESTION 36.

33. How many attacks of wheezing has your child had in the last 12 months?
1. None 2. One to three 3. Four to twelve 4. More than twelve /__/

34. In the last 12 months, how often, on average, has your child's sleep been disturbed due to wheezing?
1. Never woke with wheezing 2. Less than one night per week /__/
3. One or more nights per week

35. In the last 12 months, has wheezing ever been severe enough to limit your child's speech to only one or two words at a time between breaths? 1. Yes 2. No /__/

36. Has your child ever had asthma? 1. Yes 2. No /__/
If yes, was this confirmed by a doctor or physician assistant? 1. Yes 2. No /__/

37. In the last 12 months, has your child had a dry cough at night, apart from a cough associated with a cold or chest infection? 1. Yes 2. No /__/

38. In the last 12 months, has your child taken any treatment (medicines, tablets, inhalers) for wheezing or asthma? 1. Yes 2. No /__/
If yes, what? 1. Inhaler (Name, frequency) _____
_____
2. Tablets/other medicine (Name, frequency)_____
_____

V. NOSE QUESTIONS

The following questions are about nose problems, when the child DOES NOT have a cold or flu.

39. Has your child ever had a problem with sneezing or a runny nose or a blocked nose when he/she did NOT have a cold or flu? 1. Yes 2. No /__/

IF THE ANSWER IS "NO" PLEASE SKIP AND GO TO QUESTION 42.

40. In the last 12 months, has your child had a problem with sneezing or a runny nose or a blocked nose when he/she did NOT have a cold or flu? 1. Yes 2. No /__/

IF THE ANSWER IS "NO" PLEASE SKIP AND GO TO QUESTION 42.

41. In the past 12 months, has this nose problem been accompanied by itchy-watery eyes? 1. Yes 2. No /__/

42. Has your child ever had allergic rhinitis? 1. Yes 2. No /__/

If yes, was this confirmed by a doctor or physician assistant? 1. Yes 2. No /__/

VI. SKIN QUESTIONS

43. In the last 12 months, has your child had an ITCHY skin condition? (By 'itchy' we mean scratching or rubbing the skin.) 1. Yes 2. No /__/

IF THE ANSWER IS "NO" THIS IS THE END OF THE QUESTIONNAIRE.

44. Has your child had this ITCHY skin condition in the last week? 1. Yes 2. No /__/

45. At what age did this itchy skin condition first occur?
1. < 2 years 2. Age 2-4 years 3. Age 5 or more /__/

46. Has this skin condition *ever* affected the skin creases? (By *skin creases* we mean the fronts of the elbows, behind the knees, the front of the ankles, around the neck, or around the eyes.) 1. Yes 2. No /__/

47. In the last 12 months, has your child *ever* suffered from generally dry skin?
1. Yes 2. No /__/

48. Has your child ever had eczema? 1. Yes 2. No /__/

If yes, was this confirmed by a doctor or doctor assistant? 1. Yes 2. No /__/

THE INTERVENTION STUDY QUESTIONNAIRE, USED AT 12 MONTHS FOLLOW UP (AFTER FOUR ROUNDS OF TREATMENT)

I. LUNG QUESTIONS As you know, we gave your child tablets over the past 12 months. The following questions refer to the time period since the first treatment was given. 1. Since we gave your child the first treatment 12 months ago, has he/she had any wheezing or whistling in the chest ? 1. Yes 2. No	/__/
IF THE ANSWER IS "NO" PLEASE GO TO QUESTION 7. --- 2. Since we gave your child the first treatment 12 months ago, how many attacks of wheezing has he/she had? 1. None 2. One to three 3. Four to twelve 4. More than twelve	/__/
3. Since we gave your child the first treatment 12 months ago, how often, on average, has your child's sleep been disturbed due to wheezing? 1. Never woke with wheezing 2. Less than one night per week 3. One or more nights per week	/__/
4. Since we gave your child the first treatment 12 months ago, has wheezing ever been severe enough to limit your child's speech to only one or two words at a time between breaths? 1. Yes 2. No	/__/
5. Since we gave your child the first treatment 12 months ago, has your child had a dry cough at night, apart from a cough associated with a cold or chest infection? 1. Yes 2. No	/__/
6. Since we gave your child the first treatment 12 months ago, has your child taken any treatment (medicines, tablets, inhalers) for wheezing or asthma? 1. Yes 2. No If yes, what? 1. Inhaler (Name, frequency) _____ 2. Tablets/other medicine (Name, frequency)_____	/__/

II. NOSE QUESTIONS The following questions are about nose problems, when the child DOES NOT have a cold or flu. 7. Since we gave your child the first treatment 12 months ago, has he/she had a problem with sneezing or a runny nose or a blocked nose when he/she did NOT have a cold or flu? 1. Yes 2. No *IF THE ANSWER IS "NO" PLEASE GO TO QUESTION 9.* --- 8. Since we gave your child the first treatment 12 months ago, has this nose problem been accompanied by itchy-watery eyes? 1. Yes 2. No	/__/ /__/

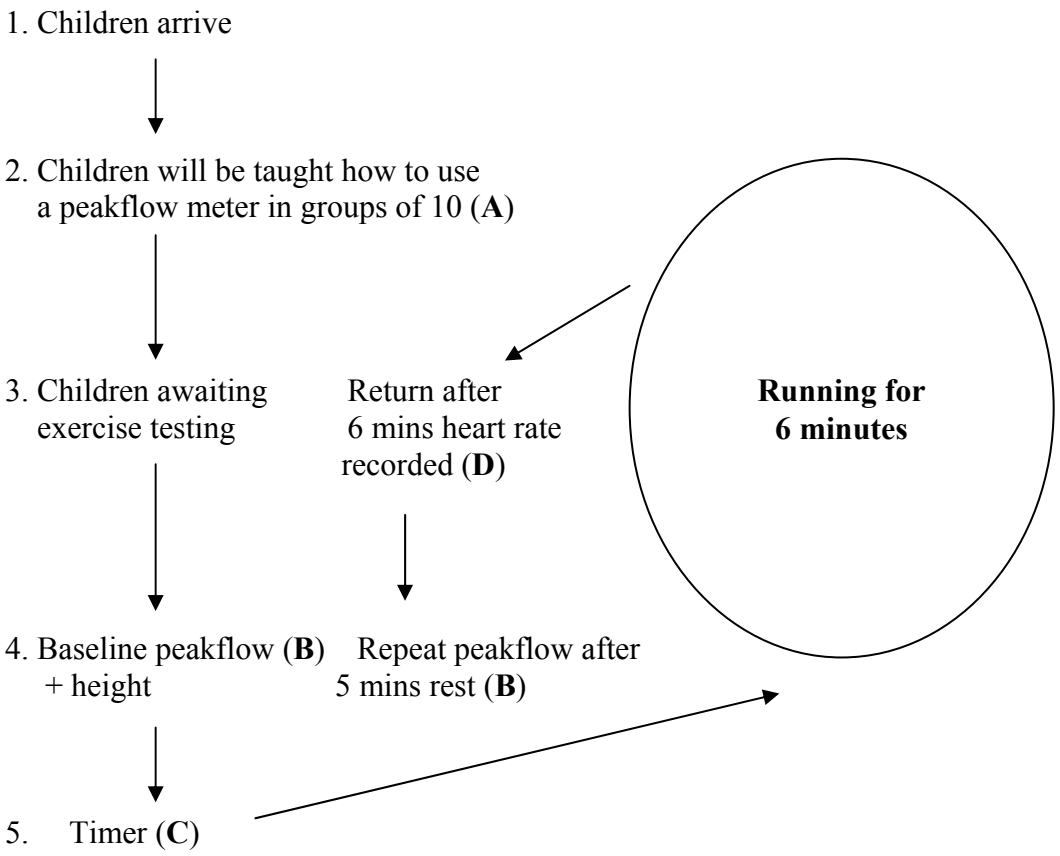
III. SKIN QUESTIONS 9. Since we gave your child the first treatment 12 months ago, has your child had an ITCHY skin condition? (By 'itchy' we mean scratching or rubbing the skin) 1. Yes 2. No *IF THE ANSWER IS "NO" THIS IS THE END OF THE QUESTIONNAIRE.* --- 10. Has your child had this ITCHY skin condition in the last week? 1. Yes 2. No 11. Has this skin condition affected the skin creases? (By *skin creases* we mean the fronts of the elbows, behind the knees, the front of the ankles, around the neck, or around the eyes.) 1. Yes 2. No 12. Since we gave your child the first treatment 12 months ago, has your child suffered from generally dry skin? 1. Yes 2. No	/__/ /__/ /__/ /__/

EXERCISE TESTING (FIELDWORKER INSTRUCTIONS)

Children should be seen in groups of 10, but exact numbers will depend on how many children will be tested on the day (around 50). Four fieldworkers are needed.

One fieldworker (A) performs the peakflow training;
One fieldworker (B) performs the peakflow testing;
One fieldworker (C) monitors the timing;
One fieldworker (D) takes heart rates after exercise.

<u>Diagram of the testing station:</u>



The layout of the testing area is shown in the diagram. A table and two benches or rows of chairs (for children awaiting peakflow testing) are essential.
When the group of children has arrived, one fieldworker will ask them to stand in a circle. Each child will then be given their own peak flow meter. After intitial instructions have been given to the whole group, children will be allowed to practice in groups of 5, until good technique has been observed: blowing through the peakflow meter from the peak of a deep breath giving a sharp blast and not a prolonged blow without any air leaks from around the mouth piece, until three readings less than 5% apart are achieved.

Following these instructions and practice, children's height will be measured by B. Peakflow readings are then performed as described above. B has a list of minimum acceptable peakflow readings for children of given heights. Those with lower values are not exercised on that occasion and will have to receive more training in the blowing technique.

Peak flow rates will be measured in each child three times immediately before, and 5 minutes after, a 6 minute period of free outdoor running at jogging pace, aiming at a heart rate of 170 beats/min or 85% maximum for age, whichever is greater. The maximum of each set of readings will be used to calculate the percentage fall in peak flow after exercise. Do not record readings where there has been an obvious air leak between lips and mouthpiece.

The pulse rate will be measured for 15 seconds immediately after completion of the run, as an indicator of the intensity of the exercise. Any children using inhaled bronchodilators for known asthma (very unlikely in this rural Vietnamese setting) will be tested at least 6 hours after their last inhaler dose.

C should have a digital clock and sheets of paper on a clipboard. It is best to start the children off at minute intervals, recording serially:

14:21 Bo Bo Thien
14:22 Cao Bo
14:23 Mau Thi Hoa

The children can chose what footwear (if any) to wear. Socks without shoes may cause falls. Jumpers, pullovers etc make children very hot and are best removed at the start.

When the seventh child starts running, the first is called back to sit down, he/she having exercised for 6 minutes. **D** records the heart rate immediately after completion of the run for 15 seconds and multiplies by 4 to give the rate per minute. It is remarkable how quickly the heart rate slows down in some children. It can be recorded either by palpation at the wrist or by auscultation (light clothing does not need to be removed). The latter is usually easier.

Insist that the children remain seated and in the right order (moving up as necessary) while awaiting the repeat peakflow measurement. **C** alerts **B** when the first child is due for repeat peakflow testing, and checks that successive children are tested at 1 minute intervals. This is usually easy, since each re-testing coincides with a child being called back to sit down, so that there are never more than 5 children waiting.

Children who show a substantial drop in peakflow should be given inhaled Salbutamol and asked to sit down for a few more minutes and then re-tested. This is the advantage of placing children with known

breathing problems last: the Salbutamol can be administered by **D**, who is no longer recording heart rates. Keep children with very low peakflow readings under observation, until it has returned back to normal. The final value will not be used in any analysis but should be entered on the form.

For primary school children the low reading peakflow meter will be used, whereas it may be necessary to give children in secondary school an adult peakflow meter if they can blow beyond the range of the children peakflow meter.

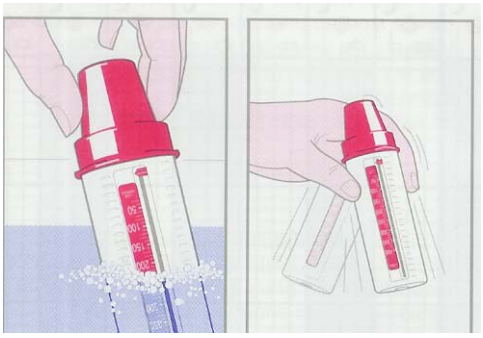
Always ensure that each child is tested using the same peakflow meter before and after exercise. Sometimes peakflow meters become very damp after continued use and may temporarily fail to work. If this happens, it is not a problem, since cleaning them usually puts them back into working order (for details see below).

The white mouthpieces should be cleaned between each child with an antiseptic solution.

Children who cannot run for any reason should be interviewed by **A** and only perform a baseline peakflow test. It is best to ask children with known breathing problems to run last. For asthmatic children a salbutamol inhaler will be available if he/she becomes wheezy.

Try to get absentees on another visit to the school.

Please ensure that all peakflow meters are working at the end of each survey day. For cleaning, immerse the peakflow meter in warm (but not hot) mild detergent solution for 2-3 minutes (maximum 5 minutes). Shake the instrument gently to ensure thorough cleaning. Then rinse in clean warm water and shake gently to remove any excess water. Allow to dry thoroughly before using again.



If this does not put the instrument back into working order, the team leader needs to inform the study supervisor to get a replacement.

SKIN PRICK TEST PROTOCOL

It is very important that the skin prick test procedure is explained to the child in very simple non-threatening language. If the child is anxious, it may be helpful to demonstrate the procedure on the fieldwoker's forearm, especially to show that the test is not painful.

Allergen solutions

1. Histamine (positive control)
2. House dust mite 1 (*D. pteronyssinus*)
3. House dust mite 2 (*D. farinae*)
4. Cockroach
5. Saline (negative control)

Perform these 5 tests on the left forearm. Place the allergens on the tray in the same order as they are put on the forearm. Store allergen solutions in a refrigerator between test sessions.

Applying the solutions

Check that the skin of the forearm is free of eczema. The test should not be performed on inflamed or broken skin. Place the left arm palm upwards on the table in front of the examiner.

Using a ball point pen, mark and code the inside of the forearm onto Scotch Magic tape (with + and – for the controls). The test site should be >5cm above the wrist to >3cm from the cubital fossa, with the tests 3cm apart.

Open the packaging of the lancets before doing the test. They should be placed ready to be taken out of the package with one hand.

Open the bottles with the allergen solutions, one at a time.

Put one drop of each allergen on the left forearm in the above sequence. Do this always in the same sequence. Do not use too much allergen and take care that the different allergens do not run together or run off the arm.

Put the bottle back to its position on the tray. Do not change the order of the bottles!

Performing the prick test

Always use a new lancet for each allergen.

Prick the lancet for 2 seconds vertically through the drop into the skin using firm pressure.

Put the used lancets into the disposable container.

After pricking blot the forearm dry. Do not wipe dry, since this might cause cross-contamination between the allergens.

Set the alarm clock for 15 minutes.

Close the allergen bottles with their own coloured caps.

Reading the reaction

After 15 minutes outline the contours of the wheal with a thin felt-tip pen, again on Scotch Magic Tape. Do not spread the skin. Hold the pen vertically. Ensure adequate lighting.

The contour should be drawn at the outside of the wheal. If there is no reaction mark that non-reactive position with a little dot.

Remove the prenumbered tape.

Paste a transparent tape onto the wheals to transfer the contours.

Press the tape onto the skin to make sure that the whole contour is transferred to the sticky side.

Remove the tape from the skin and paste it into the record sheet.

Measurement of each weal

Record measurements in millimetres, rounded to the next higher integer, using a flexible plastic ruler.

Always measure the inside of the felt-tip pen contour.

Identify and measure the longest diameter first.

Then drop a perpendicular line through the middle of the longest diameter and measure the length of this line.

Calculate the mean of the two diameters.

A measurement ≥3mm than the saline control is considered a positive test.

There should be no reaction to the saline control. The histamine positive control should yield a weal size of 3-4mm.

SKIN PRICK TEST RECORD SHEET

ID number: _____ School code: _____

Child's name: _____

Date: _____/_____/_____ Field worker name: _____

LEFT FOREARM	**Tape ID#**	**Tape Test Result**	**Wheal size (mm)**
Test allergen			
1. Histamine (positive control)			
2. House dust mite 1 (Dp)			
3. House dust mite 2 (Df)			
4. Cockroach			
5. Saline (negative control)			

DIAMETERS MEASURED TO THE NEAREST WHOLE MILLIMETRE.

a+b/2 = weal size

FLEXURAL ECZEMA PROTOCOL

STOOL ANALYSIS PROTOCOL (FOR LABORATORY TECHNICIANS)

We will perform both qualitative and quantitative stool sample analysis, using salt flotation and McMaster counting chambers.

The McMaster counting technique is a quantitative technique to determine the number of eggs present per gram of faeces (epg). A flotation fluid is used to separate eggs from faecal material in a counting chamber (McMaster) with two compartments. The technique described below will detect 50 or more epg.

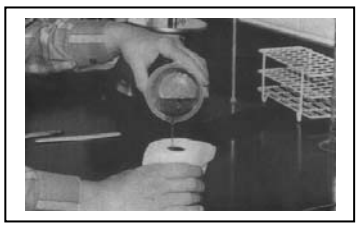
Equipment
1. Beakers or plastic containers
2. Balance
3. A tea strainer or cheesecloth
4. Measuring cylinder
5. Stirring device (fork or tongue depressor)
6. Pasteur pipettes and (rubber) teats
7. Flotation fluid (see below for formulation)
8. McMaster counting chamber
9. Microscope

Procedure
1. Weigh 4 g of faeces and place into Container 1.

2. Add 56ml of flotation fluid.

3. Stir the contents thoroughly with the stirring device.

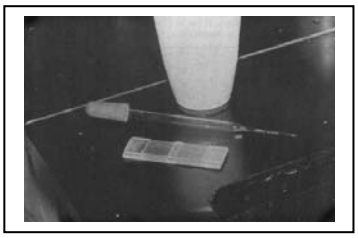
4. Filter the faecal suspension through a tea strainer into container 2.



5. While stirring the filtrate in container 2, take a sub-sample with a Pasteur pipette.

6. Fill both sides of the McMaster counting chambers with the subsample.

7. Allow the counting chamber to stand for 5 minutes (THIS IS IMPORTANT).



8. Examine the sub-sample of the filtrate under a microscope at 10x10 magnification.

9. Count all eggs within the engraved area of both chambers.

10. The number of epg can be calculated as follows: Add the egg counts of the two chambers together. Multiply the total by 50. This gives the epg.
(Example: 12 eggs seen in chamber 1 and 15 eggs seen in chamber 2 = (12+15)x50 = 1350 epg)