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Comparative Immunomodulatory Activity of *Ascaris Suum* Eggs and *Dermatophagoides pteronyssinus* Extract in BALB/c Mice: Cytokine and Immunoglobulin Regulations

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Abstract

Allergy is highly prevalent in well-developed countries; hygiene hypothesis suggests that this is due to exposure to previous bacterial infection eliciting Th1-response that suppresses Th2-mediated responses. Aside from Th2 suppressing Th1-responses, Th2-mediated infections such as parasitism, appear to lower the risk of developing allergy. This study investigates the effects of *Ascaris suum* infection in *Dermatophagoides pteronyssinus*-induced allergic BALB/c mice. Ninety BALB/c mice divided into three trials were randomly grouped into: normal control (1), *A. suum* infected mice (2), *D. pteronyssinus* exposed mice (3), *A. suum* infected mice and then exposed to *D. pteronyssinus* (4), *D. pteronyssinus* exposed mice and then infected with *A. suum* (5), and mice exposed and infected with *D. pteronyssinus* and *A. suum*, simultaneously (6). Blood and fecal samples were collected at days 0, 36, and 72 for the measurement of cytokines and immunoglobulins through ELISA, and for fecal analysis. Results showed that *A. suum* infection suppressed *D. pteronyssinus*-induced allergy morphologically and histologically and downregulated the production of *D. pteronyssinus*-specific IgE. *A. suum* and *D. pteronyssinus* upregulated the production of IL-4 and IL-5, and only *A. suum* eggs enhanced the production of IL-10. *D. pteronyssinus* enhanced production of both *D. pteronyssinus*-specific and *A. suum*-specific IgE and IgG and may be accounted to cross-reacting antigens present in *D. pteronyssinus* and *A. suum*. IL-10 is upregulated in mice with *A. suum* infection and appears to protect mice against *D. pteronyssinus*-induced allergy. The possible role of IL-10 production in response to *A. suum* infection and against *D. pteronyssinus*-induced allergy needs further investigation.

Keywords: Hygiene; *Ascaris suum*; *Dermatophagoides pteronyssinus*; Allergy; Infection; Cytokines; Immunoglobulins

Abbreviations: *A. suum*: *Ascaris suum*; *D. pteronyssinus*: *Dermatophagoides pteronyssinus*

Introduction

Hygiene hypothesis is a proposed theoretical framework explaining the protection developed by an individual against certain diseases such as allergies as a result of previous exposure to infectious agents especially during childhood [1]. This hypothesis suggests that increase in the prevalence of chronic inflammatory disorders, such as allergies and autoimmunity in developed countries is attributable to diminishing exposure to the usual organisms in the environment, which have long been a part of the mammalian evolutionary history [2,3].

Most investigations on hygiene hypothesis focus on infections eliciting Th1 responses, like bacterial infections, to counteract certain immune reactions associated with Th2 response such as allergic reactions. Although a significant number of infectious agents that develop Th1 response have been thoroughly studied, the role of parasitic infections in allergies remains unresolved. Allergy and parasitism both induce Th2 immune response [4,5]. The shift from Th1 to Th2 or vice versa associated with the hygiene hypothesis is controversial because of contradicting results by a number of studies. On the one hand, some studies show that decrease in parasitic cases is associated with increasing cases of allergic diseases [6,8]. On the other hand, in a study conducted among preschool-aged Brazilian children, it showed that *Ascaris lumbricoides* and *Trichuris trichiura* infections are risk factors in the development of atopy. Thus children with parasitic infections are more prone to atopy and experience wheezing [9]. Still, another

study conducted in Rural China showed children with *A. lumbricoides* infection have increased risk of developing asthma. This manifestation is associated with sensitization to some aeroallergens [10].

The conflicting results on the role of helminth infections in the development of allergic diseases necessitate further studies. Helminth infections and allergy highly induce Th2 immune response [4,5]. Both allergy and helminth infections are associated with elevated levels of immunoglobulin E (IgE), tissue eosinophilia, mastocytosis, and Th2 cytokines IL-4 and IL-5. Because of these similarities in immune response, as well as the issue on hygiene hypothesis, it has been debated that Th2-response in helminth infection can protect an individual from allergic condition. IL-10 was also measured, since some studies have mentioned the inhibitory effect of this interleukin against other interleukins such as IL-4 and IL-5, thus, conferring resistance or protection for the host from developing lethal immunopathology [11]. IL-10 is usually induced in cases of chronic helminthiasis, together with TGF- β and IgG instead of IgE [3,12]. This present study examines

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the role of *A. suum* infection in *D. pteronyssinus*-induced allergy in BALB/c mice. Potential application of Hygiene hypothesis is indirectly investigated in this study by comparing the levels of interleukins and immunoglobulins involved in the immunology of helminth infection and allergy. Can *A. suum* infection protect BALB/c mice against *D. pteronyssinus*-induced allergy?

Materials and Methods

Study design

BALB/c mice (n=90), 6 to 8 weeks old, obtained from the University of the Philippines National Institute of Health, were used in this study. Permit to handle and use animals in research was secured from the University of Santo Tomas Institute of Animal Care and Use Committee (IACUC), with Permit No. AR-2011-2015. The mice were housed in the Thomas Aquinas Research Complex Animal House. They were acclimatized for two weeks, prior to the start of experimentation. Mice were treated and handled humanely following standard IACUC guidelines. Ninety BALB/c mice divided into three trials were randomly assigned into six groups labeled as normal control (1), *A. suum* infected mice (2), *D. pteronyssinus*-allergic mice (3), *A. suum* infected mice then exposed to *D. pteronyssinus* (4), *D. pteronyssinus*-allergic mice then infected with *A. suum* (5), and simultaneously infected with *A. suum* and exposed to *D. pteronyssinus* (6).

BALB/c mice in groups 2, 4, 5, and 6 received approximately

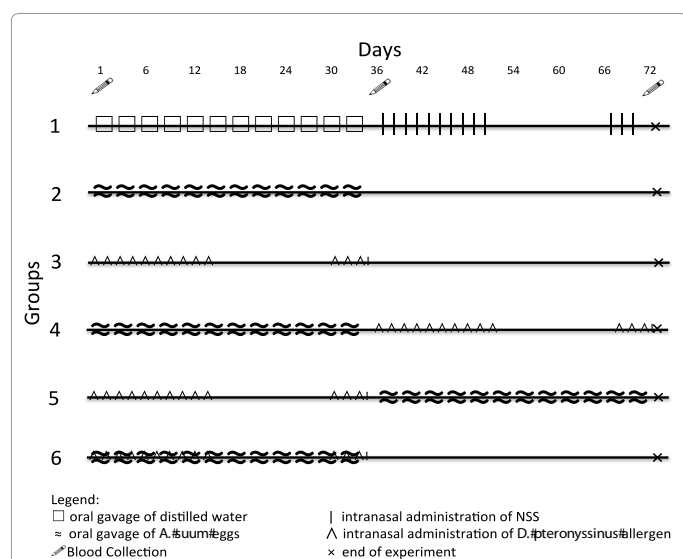


Figure 1: Infection/Induction Protocol. BALB/c mice were grouped into six groups.

Group 1 received only distilled water from days 1 to 36 and NSS from days 37 to 72 as placebo.

Group 2 received *A. suum* eggs every three days from days 1-36.

Group 3 received *D. pteronyssinus* extract from days 1-10 and booster from days 31-33.

Group 4 received *A. suum* eggs from days 1-36 every three days and *D. pteronyssinus* extract from days 37-46 and booster dose from days 66-69.

Group 5 received *D. pteronyssinus* extract from day 1-10 and from days 31-33 and received *A. suum* eggs every three days from days 37 to 72.

Group 6 received *A. suum* eggs every three days from days 1-36 and *D. pteronyssinus* extract from days 1-10 and from days 31-33.

Blood extraction and fecalysis were done at days 1, 36 and 72. At day 72, three mice per group were sacrificed for histopathologic analysis of the lungs, liver and intestines.

25,000 *A. suum* infective eggs suspended in 250 uL distilled water via oral gavage every three days. Groups 2, 4 and 6 received *A. suum* eggs starting from day 1 to day 36 while group 5 received *A. suum* eggs from day 37 to 72. This concentration mimicked chronic parasitism [13,14]. Via intranasal route, *D. pteronyssinus* extract was given to groups 3, 4, 5, and 6 groups of BALB/c mice at 2.5 mg/mL concentration suspended in 10 uL normal saline solution (NSS) to induce allergy [15]. Extract was administered for ten consecutive days dropwise, alternating between either nostril. Three weeks after the last dose, booster dose of *D. pteronyssinus* extract was given for three consecutive days. This procedure was given to groups 2, 5, and 6 starting on day 1 to 10, and booster dose from day 31 to 33, and to group 4 starting on Day 37 to 46 and booster dose from day 66 to 69 (Figure 1). NC group received only placebo, i.e., oral gavage of distilled water from day 1 to 36 and intranasal administration of NSS from day 37 to 46 and 66 to 69, respectively.

D. pteronyssinus extract

Ten grams of pure cultures of *D. pteronyssinus* were wrapped in aluminum foil and soaked in liquid nitrogen for twenty minutes. A total volume of 50 mL of 1X Tris-Buffered Saline (TBS) with 2 mM Phenyl Methyl Sulfonyl Fluoride (PMSF) (Sigma-Aldrich, Saint Louis, MO, USA) and 1 mM EDTA (BioRad, Hercules, CA, USA) were added slowly while grinding the mites for at least 30 minutes. After 16 hours of incubation at 4°C with constant shaking, the extract suspension was centrifuged at 10,000 rpm for 20 minutes (Hettich). Supernatant was collected and stored in -20°C freezer (Sanyo® Biomedical Freezer) until used. Protein content of the extract was quantified using the BioRad Protein Assay [16,17].

A. suum egg suspension

Adult *A. suum* worms were obtained from a slaughter house in La Loma, Quezon City, Philippines. Only female worms were used in this study. Worms were dissected longitudinally [18]. Uterus of each worm was isolated and placed in a mortar and pestle. Distilled water was added dropwise and the uterus was macerated into a paste-like consistency. *A. suum* egg suspension was examined under a compound microscope (low and high power objective). The suspension was then placed inside a petri dish with a moistened filter paper and stored under light protection at room temperature (25°C). The petri dishes were continuously moistened with distilled water to prevent dryness. Eggs were monitored everyday starting at day 8 until the infective stage of *A. suum* was observed (egg with larva inside). Culture of the infective egg stage was stored in an amber bottle with water until used. Culture is checked daily to ensure the eggs survival. Infective egg stage of *A. suum* will not hatch and will not undergo any developmental stage unless ingested by a host.

Blood collection

Blood samples were collected retro-orbitally from the mice at days 0, 36 and 72. Pasteur pipette was inserted in the medial portion of the eye. Approximately 500 uL of blood sample per mice was collected and placed in a yellow microtainer tube with gel separator and clot activator (Beckton Dickinson). The tube was then centrifuged at room temperature at 10,844 g for 5 minutes (Hettich). Sera collected were stored at -20°C until used (Sanyo® Biomedical Freezer).

Measurement of interleukins

Levels of interleukins IL-4, IL-5 and IL-10 were measured from blood sera using sandwich Enzyme Linked-Immunosorbent Assay

(Biolegend, Inc., USA) following the procedures provided by the manufacturer with slight modifications. In brief, 50 uL of diluted capture antibody (anti-mouse IL-4, anti-mouse IL-5, and anti-mouse IL-10) were incubated overnight at 4°C followed by one hour blocking step at room temperature with 100 uL 1X assay diluents. Samples and standards were added to appropriate wells and incubated for two hours at room temperature. Plates were then incubated with 50 uL of detection antibody for 1 hour followed by addition of 50 uL of diluted Avidin-Horse Radish Peroxidase (HRP) for 30 minutes at room temperature. Plates were washed with 200 uL of wash buffer between incubation steps. Detection was performed by incubation with 50 uL tetramethyl benzidine (TMB) substrate solution for 20 minutes. Colorimetric reaction was stopped by adding 50 uL of 2N sulfuric acid as stop solution to each well. Immediately within 30 minutes, absorbance was read at 450 nanometer (nm). The detection limits of the assays were 1 picogram/milliliter (pg/mL), 4 pg/mL, and 16 pg/mL for IL-4, IL-5, and IL-10, respectively.

Measurement of antigen-specific immunoglobulins

Serum levels of antigen specific IgE and IgG were measured through Indirect ELISA. Briefly, ELISA plate was coated with 50 uL of either 5 ug/mL *D. pteronyssinus* extract antigen or *A. suum* antigen in 0.1 M NaHCO₃ at pH 8.4 overnight. After washing, plate was blocked with 50 uL 1% BSA (bovine serum albumin) in PBS-T for two hours at RT. Sera was added at 1:10 dilution, followed by incubation with 50 uL of biotin anti-mouse IgE (or IgG) and 50 uL of ExtraAvidin Alkaline phosphatase. Reaction was developed in the dark at RT for 60 minutes by using 50 uL p-nitrophenyl phosphate (PNPP). Absorbance was read at 405 nm using an ELISA reader [13,16,19-21].

Histopathology

At day 72, mice were sacrificed and lungs, liver, and small intestines were harvested. Specimens were fixed in 10% formalin, and processed using routine histopathology procedures. Processed specimens were embedded in paraffin, sectioned and stained with hematoxylin and eosin solution [18]. Slides were examined by a pathologist blinded to the exposure status of the individual mice under the microscope using low and high power objective, with 100X and 400X magnification (Olympus). Scoring system was used to assess the pathology of the different organs. Lung sections were scored according to the following criteria: a. eosinophil infiltration 0=absent, 1=low grade cell influx, 2=moderate cell infiltration, low grade tissue damage, 3=moderate cell influx, marked tissue damage, 4=high cell influx and significant tissue pathology; b. mucosal edema 0=normal, 1=low grade diffuse edema, 2=moderate edema with moderately increased mucus production, 3=pronounced edema with excessive mucus production; and c. epithelial cell damage 0=normal, 1=low grade epithelial cell loss, 2=moderate grade epithelial cell loss, 3=high grade cell loss, epithelial metaplasia [22]. Liver was scored using the following criteria: a. gross 0=normal, 1=slight change in color, no visible necrosis, 2=change in color with moderate lesions, 3=change in color with severe necrosis; b. cellular infiltrate 0=absent, 1=one focal infiltrate, 2=multifocal infiltrate (<10 per field), 3=diffuse infiltrate (>10 per field); and c. necrosis 0=no visible necrosis, 1=small areas of necrosis, 2=moderate levels of necrosis (<50%), 3=marked necrosis (>50%) [23]. Sections of intestines were scored using the following criteria: 0=normal villi, 1=villi with tip distortion, 2=presence of Guggenheim's spaces, 3=villi with patchy disruption of the epithelial cells, 4=villi with exposed but intact lamina propria with sloughing, 5=exuded lamina propria, 6=villi with hemorrhage or denuded villi [24].

Statistical analyses

Mean and its standard errors (SEM) were used to summarize the data gathered. Mixed Model Analysis of Variance was used to determine if there is a significant difference among the groups (1-6), and trials (trial 1, trial 2 and trial 3). The groups were considered as fixed factors and the trials as random factors. In addition, Repeated Measures Analysis of Variance (RMANOVA) was used to determine if there is a significant change in the means from the baseline, with Bonferroni correction was used to compare the baseline with the third extraction. All statistical tests were performed at 5% level of significance using SPSS 17.0 for Windows.

Results

D. pteronyssinus extract and *A. suum* eggs upregulates IL-4 and IL-5 production

IL-4 and IL-5 levels increased significantly in BALB/c mice exposed to *D. pteronyssinus* extract and in mice infected with *A. suum* eggs. BALB/c mice in group 1 did not show any significant change in the level of IL-4 from day 0 to 36 ($p=0.266$) and from day 36 to 72 ($p=0.092$). Enhanced production of IL-4 was observed in groups 2, 3, 4, 5, and 6 (Figure 2A). In group 2, although *A. suum* eggs did not immediately increase the level of IL-4 at day 36 ($p=0.071$), increase was observed at day 72 ($p=0.010$). *A. suum* eggs in BALB/c mice

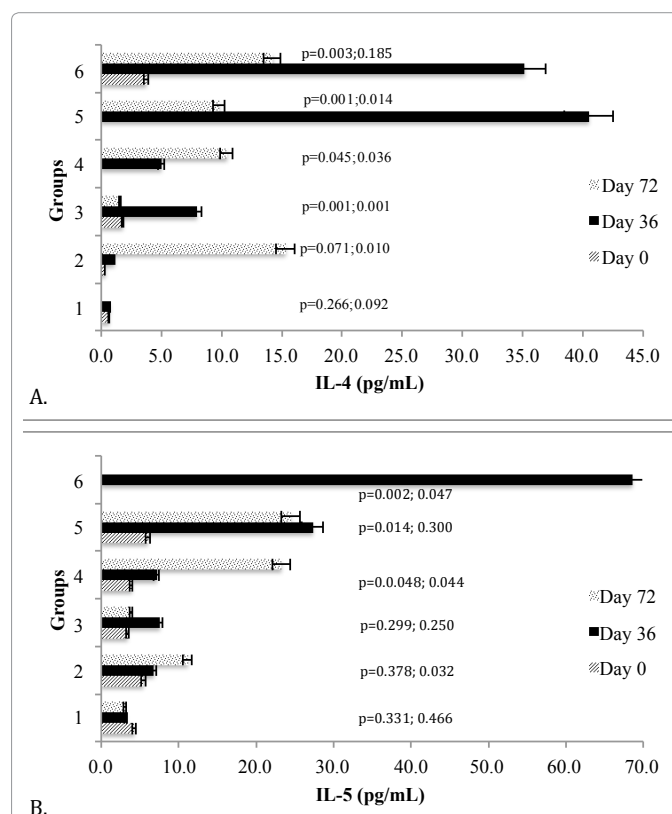


Figure 2: *A. suum* eggs and *D. pteronyssinus* extract upregulate production of IL-4 and IL-5. BALB/c mice were exposed to either *A. suum* eggs or *D. pteronyssinus* extract or both. Levels of IL-4 and IL-5 were measured at day 0, 36 and 72. P-values were determined using Repeated Measures Analysis of Variance (RMANOVA): $p=x;y$ where x =day 0 vs day 36 and y =day 36 vs day 72. Results were presented as mean of the three trials because Mixed Model Analysis of Variance indicates that the results per trial do not significantly differ ($p=0.132$). A. IL-4 levels. B. IL-5 levels.

upregulate the production of IL-4. While in group 3, marked increased in IL-4 production was observed immediately in mice exposed to *D. pteronyssinus* extract from day 0 to day 36 ($p=0.001$) and decreased significantly at day 72 ($p=0.001$). IL-4 levels also increased in group 4 after infection with *A. suum* from day 1 to 36 ($p=0.045$) and after exposure to *D. pteronyssinus* extract from day 37 to 72 ($p=0.036$). In groups 5 and 6, IL-4 level increased at day 36 ($p=0.001$; $p=0.003$) and decreased at day 72 ($p=0.014$; $p=0.185$) but still significantly higher than that of day 0 ($p=0.028$; $p=0.077$).

Similarly, in group 2, IL-5 increased slightly at day 36 ($p=0.378$) and upregulated at day 72 ($p=0.032$). IL-5 level in group 3 increased after administration of *D. pteronyssinus* extract, although not statistically significant ($p=0.299$) and decreased at day 72 ($p=0.250$). IL-5 also increased in group 4 after infection with *A. suum* at day 36 ($p=0.048$) and after exposure to *D. pteronyssinus* ($p=0.044$). Comparing the levels of IL-5 in group 3 at day 36 and group 4 at day 72, IL-5 in group 4 was markedly elevated ($p=0.044$). In group 5, IL-5 increased markedly at day 36 ($p=0.014$) and decreased slightly at day 72 after administration of *A. suum* eggs ($p=0.300$). In group 6, IL-5 level increased significantly at day 36 ($p=0.002$) and decreased at day 72 ($p=0.047$). Group 1 maintained a constant level of IL-5 throughout the experiment from day 0 to 36 and to 72 ($p=0.331$; 0.466) (Figure 2B).

A. suum upregulates and modulates IL-10 Production in *D. pteronyssinus*-induced allergy

Upregulation of IL-10 production was observed in BALB/c mice infected with *A. suum* eggs. Group 1 ($p=0.182$; 0.355) and group 3 ($p=0.247$; 0.0172) did not show significant increase in the level of IL-10 (Figure 3). In group 2, administration of *A. suum* eggs induced significant IL-10 production at day 36 ($p=0.036$) and increased further at day 72 ($p=0.255$). Significant IL-10 production was also observed in group 4 ($p=0.050$) at day 72. *A. suum* eggs induced a slight increase in IL-10 at day 36 and markedly increased at day 72 after administration of *D. pteronyssinus* extract. *D. pteronyssinus* extract alone did not induce significant IL-10 production as seen in group 3. *A. suum* eggs upregulated the levels of IL-10. Administration of *D. pteronyssinus* extract prior to and simultaneously with administration of *A. suum* eggs did not induce IL-10 production as seen in groups 5 and 6 ($p>0.05$).

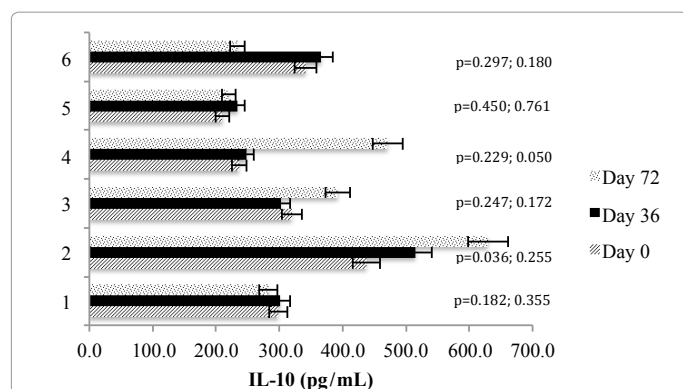


Figure 3: *A. suum* eggs upregulates the level of IL-10 in BALB/c mice. BALB/c mice were exposed to either *A. suum* eggs or *D. pteronyssinus* extract or both. Level of IL-10 was measured at day 0, 36 and 72. P-values were determined using RMANOVA: $p=x;y$ where x =day 0 vs day 36 and y =day 36 vs day 72. Results were presented as mean of the three trials because Mixed Model Analysis of Variance indicates that the results per trial do not significantly differ ($p=0.854$).

D. pteronyssinus extract caused allergic symptoms on BALB/c mice

Mice in groups 3 and 5 developed allergic symptoms. Allergic

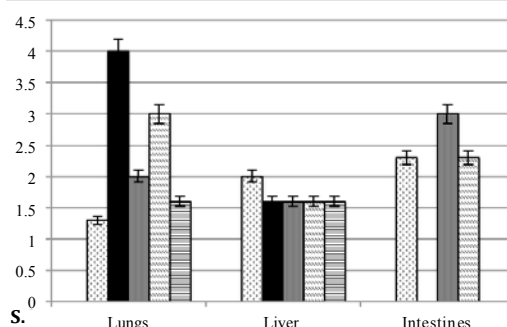
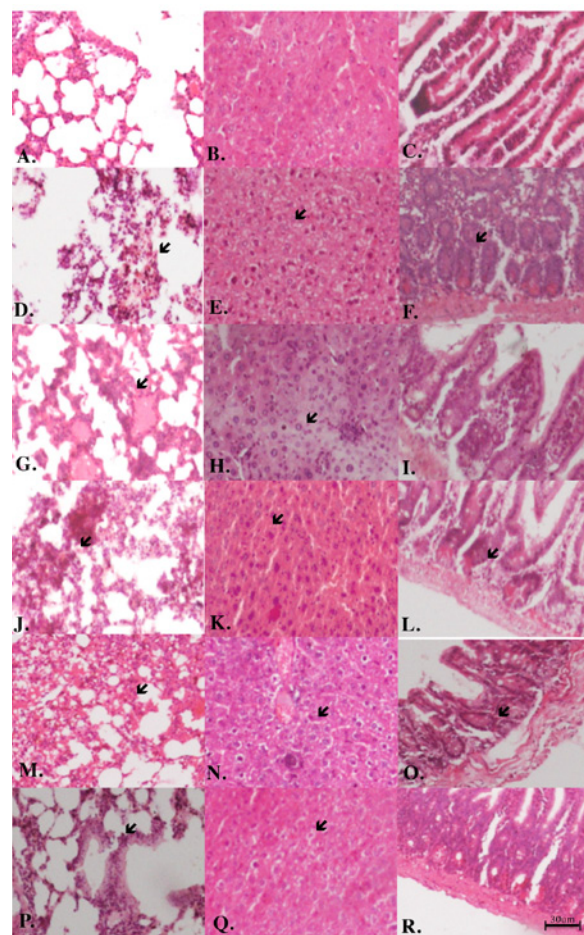


Figure 4: *A. suum* eggs and *D. pteronyssinus* extract affected the lungs, liver and intestines of BALB/c mice (400x magnification). **A-C:** Lungs, liver and small intestines of group 1 mice. **D-R:** Lungs, liver and intestines of group 2 (**D-F**), 3 (**G-I**), 4 (**J-L**), 5 (**M-O**), 6 (**P-R**). **S:** Levels of eosinophilia, edema and cell damage in the lungs; gross pathology, cellular infiltrate and necrosis in the liver; and morphological damage in the intestines were scored. Scores are presented as mean \pm SD, $n=3$. Pictures shown are from single mice per group and are representative of all mice examined in a group. Arrows indicate important pathological changes in the tissues such as congestion with neutrophilic infiltrates (**D, J, M, P**), focal hyperplastic changes (**F**), mild inflammation (**G**), atypia with lymphocytic infiltrates (**H**), atypia with lymphocytic infiltrates (**K**), atrophic villi (**L**), hyperplastic changes (**N**), hypertrophied villi (**O**), and focal microsteatosis (**Q**).

symptoms were observed after administering *D. pteronyssinus* extracts for 10 consecutive days and lasted until the end of the experiment. 8 out of 15 mice (3 in trial 1, 3 in trial 2, and 2 in trial 3) in group 3 and 7 out of 15 mice in group 5 (2 in trial 1, 3 in trial 2 and 2 in trial 3) experienced blepharitis (eyelid swelling and inflammation), nasal inflammation, inflammation of the neck and either the upper or lower limbs. These symptoms were not observed in groups 1, 2, 4, and 6.

Microscopic analysis showed that lung, liver, and intestinal tissues from groups 2, 3, 4, 5, and 6 differed histologically from those of uninfected BALB/c mice (group 1). Lungs, liver and small intestines of group 1 showed no pathological abnormality (Figures 4A-4C). Group 2 (Figure 4D) and group 6 (Figure 4P) mice showed moderate interstitial edema and congestion accompanied by moderate to dense interstitial neutrophilic infiltrates with sprinkling of lymphocytes. Peribronchial and moderate perivascular lymphocytic infiltrates are likewise appreciated. Group 3 mice (Figure 4G) showed mild inflammatory process. Group 4 mice (Figure 4J) showed moderate interstitial edema and congestion accompanied by moderate interstitial neutrophilic infiltrates. Group 5 mice (Figure 4M) showed mild to moderate interstitial edema and congestion accompanied by moderate interstitial neutrophilic infiltrates.

Liver tissues of mice infected with *A. suum* eggs (Figure 4E) showed mild perivascular lymphocytic infiltrates. Group 3 mice (Figure 4H) showed reactive atypia with mild perivascular lymphocytic infiltrates. Mild lymphocytic infiltrates often forming clusters were seen. Group 4 mice (Figure 4K) showed mild atypia with lymphocytic infiltrates. Group 5 mice (Figure 4N) showed hyperplastic changes with pleomorphism and hyperchromasia of hepatocytes. Group 6 mice (Figure 4Q) showed focal microsteatosis.

Normal appearance of the small intestines was observed from the tissues of mice in groups 1, 3, and 6 (Figures 4C, 4I, 4R). Group 2 mice (Figure 4F) showed focal hyperplastic changes. Group 4 mice (Figure 4L) showed atrophic villi. And group 5 mice (Figure 4O) showed hyperplastic changes and hypertrophied villi. All pathologic changes were scored following the criteria discussed above (Figure 4S). Slides of each organ per group of mice exhibited similar pathologic changes, except for liver specimen of mice 2 of group 2, with normal histological appearance (not shown).

***D. pteronyssinus* extract upregulates *D. pteronyssinus*-specific IgE and *A. suum*-specific IgE and IgG**

Anti-*D. pteronyssinus* IgE of group 3 mice increased significantly after administration of *D. pteronyssinus* extract and increased further at day 72 ($p=0.010$; $p=0.070$) as seen on Figure 5A. In group 5 mice, administration of *A. suum* eggs lowered anti-*D. pteronyssinus* IgE level ($p=0.159$). Comparing this with group 3 mice, instead of continuously increasing the level of anti-*D. pteronyssinus* IgE up to day 72, the level of anti-*D. pteronyssinus* IgE on allergic mice decreased upon administration of *A. suum* eggs. Anti-*D. pteronyssinus* IgE of group 2 mice after oral administration of *A. suum* eggs increased slightly but not significantly ($p=0.006$). Instead of increasing the level of anti-*D. pteronyssinus* IgE as seen in group 3 group, administration of *D. pteronyssinus* extract to parasitized mice (group 4) decreased the level of anti-*D. pteronyssinus* IgE ($p=0.001$). In group 6 mice, where both *A. suum* eggs and *D. pteronyssinus* extract were given simultaneously, anti-*D. pteronyssinus* IgE absorbance level did not change significantly from day 0 to day 36 and day 36 to day 72 ($p=0.378$; $p=0.490$).

Anti-*A. suum* IgE level of group 2 did not significantly change

after administration of *A. suum* eggs ($p=0.780$; $p=0.647$) (Figure 5B). On the other hand, group 3 showed an increased production of anti-*A. suum* IgE upon administration of *D. pteronyssinus* extract ($p=0.098$) and was further upregulated at day 72 ($p=0.000$). IgE specific for *A. suum* did not increase after infection with *A. suum* eggs but increased significantly upon administration of *D. pteronyssinus* extract. In group 4, level of anti-*A. suum* IgE decreased after administration of *D. pteronyssinus* extract but not significantly ($p=0.522$). As with group 5, administration of *A. suum* eggs lowered the absorbance level of anti-*A. suum* IgE ($p=0.172$). Comparing this with the response of mice in group 3, in group 3 anti-*A. suum* IgE continuously increased up to

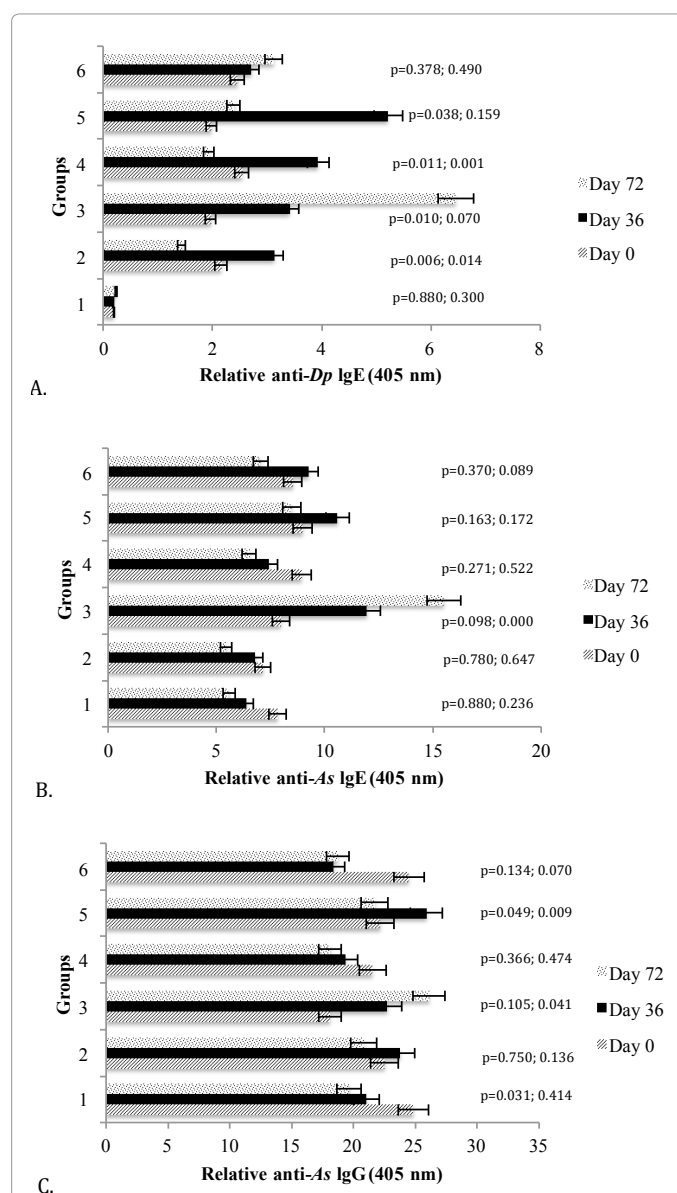


Figure 5: *D. pteronyssinus* extract enhances the production of *D. pteronyssinus*-specific IgE and *A. suum*-specific IgE and IgG. BALB/c mice were exposed to either *A. suum* eggs or *D. pteronyssinus* extract or both. Levels of antigen specific IgE and IgG were measured at day 0, 36 and 72. P-values were determined using RMANOVA: $p=x:y$ where x =day 0 vs day 36 and y =day 36 vs day 72. Results were presented as mean of the three trials because Mixed Model Analysis of Variance indicates that the results per trial do not significantly differ ($p>0.05$). A: Relative anti-*D. pteronyssinus* IgE B: Relative anti-*A. suum* IgE C: Relative anti-*A. suum* IgG.

day 72 but in group 5, the level of *A. suum*-specific antibody decreased upon administration of *A. suum* eggs on allergic mice. Instead of enhancing the production of anti-*A. suum* IgE, it has been observed that *A. suum* eggs decreased the level of *A. suum*-specific IgE. *D. pteronyssinus* extract, on the other hand, increased the level of anti-*A. suum* IgE. In group 6, level of anti-*A. suum* IgE did not significantly change ($p=0.370$; $p=0.089$).

Anti-*A. suum* IgG level of group 2 mice increased slightly but not significantly after oral administration of *A. suum* eggs ($p=0.750$; $p=0.136$) (Figure 5C). Unlike in group 2, group 3 showed a significant increase in the production of anti-*A. suum* IgG upon administration of *D. pteronyssinus* extract ($p=0.105$; $p=0.041$). IgG specific for *A. suum* did not increase after infection with *A. suum* eggs but increased after administration of *D. pteronyssinus* extract. In group 4, level of anti-*A. suum* IgG did not significantly change after administration of *A. suum* eggs at day 36 and after administration of *D. pteronyssinus* extract at day 72 ($p=0.366$; 0.474). As with group 5, administration of *A. suum* eggs lowered the level of anti-*A. suum* IgG ($p=0.009$). In group 6, anti-*A. suum* IgG did not change significantly ($p=0.134$; $p=0.070$).

Levels of anti-*D. pteronyssinus* IgE and anti-*A. suum* IgE and IgG of group 1 did not significantly change from day 0 to 36 to 72 ($p>0.05$).

Discussions

In this study, immunomodulatory activity of *A. suum* and *D. pteronyssinus* in BALB/c mice was investigated. Since both parasites and allergens are known Th2 cell inducers, three important Th2 cytokines were studied in this paper, namely, IL-4, IL-5, and IL-10. Other interleukins such as IL-13 may also be involved in Th2 immune response but their involvement is not as vital as those examined. Although IL-13 is also important, studies have shown the overlapping role of IL-4 and IL-13. IL-4 and IL-13 are not identical but their biological activity is the same. They are structurally similar and reside within a cluster of cytokine genes [25,26]. Oral administration of *A. suum* eggs (group 2) induced significant IL-4, IL-5, and IL-10 production in BALB/c mice. While intranasal administration of *D. pteronyssinus* in BALB/c mice (group 3) induced significant production of IL-4, and IL-5 and not of IL-10. In group 6, *A. suum* and *D. pteronyssinus* synergistically upregulated the levels of IL-4, and IL-5 but not of IL-10. Comparing the levels of these interleukins in groups 2, 3, and 6, it can be observed that administration of both *A. suum* and *D. pteronyssinus* upregulated the levels of IL-4 and IL-5 and only *A. suum* eggs enhanced the production of IL-10. IL-10 is a cytokine with anti-inflammatory function, thus only *A. suum* infected BALB/c mice have the capacity to modulate inflammatory response through production of this interleukin.

Immunomodulatory activity of *D. pteronyssinus* in parasitized BALB/c mice (group 4) and of *A. suum* in allergic BALB/c mice (group 5) were also monitored. Group 4 mice have increased levels of IL-4, IL-5, and IL-10 at day 72. While group 5 mice have decreased levels of IL-4, IL-5, and IL-10. IL-4 and IL-5 are interleukins with pro-inflammatory functions, while IL-10 has an anti-inflammatory function. Since activity of IL-10 counteracts the activity of IL-4 and IL-5, IL-10 in group 4 was able to block inflammatory actions of IL-4 and IL-5, and was exhibited by the absence of morphological inflammation in this group of mice as discussed above. Mice sensitized with *D. pteronyssinus* extract alone (group 3) and mice sensitized with *D. pteronyssinus* extract prior to administration of *A. suum* eggs (group 5) developed allergic inflammatory symptoms such as blepharitis, nasal inflammation, and/or neck and limb inflammation, while *A. suum* infected mice

(group 1) and mice infected with *A. suum* eggs prior to sensitization with *D. pteronyssinus* extract (group 4) and simultaneously with *D. pteronyssinus* extract (group 6) did not develop such allergic symptoms. In this study, we hypothesize that IL-10 production protected the mice against the damaging inflammatory responses due to sensitization to *D. pteronyssinus* extract. Enhanced IL-10 production in mice exposed to previous infection was observed as well in the results of other studies [27-30]. In the study of Mangan et al. instead of *Ascaris* infection, female BALB/c mice were exposed to *Schistosoma mansoni*, and results showed an increase allergen specific IL-4 and IL-10 [29]. Elevated IL-10 level protects the mice against allergic pulmonary inflammation. The use of *S. mansoni* infection to reduce airway inflammation was likewise observed in C57/B16 mice strain showing the effectiveness of chronic parasitic infection in inhibiting allergic airway inflammation via IL-10 production [30]. *S. japonicum* egg infection was also studied in BALB/c mice and results corroborated with other studies that IL-10 production inhibits antigen-induced airway inflammation and development of asthma [30]. In humans, the role of *S. haematobium* in atopy among children was studied, and found out that the parasite induced IL-10 is capable of suppressing atopy [31]. Another study used *Heligiosomoides polygyrus*, a gastrointestinal nematode, as the experimental parasite to induce helminth infection in female BALB/c mice. The study showed that *H. polygyrus* infection is capable of suppressing allergen-induced airway eosinophilia, bronchial hyperactivity and in-vitro allergen recall Th2 responses in an IL-10 dependent manner [27]. Protection against allergic airway inflammation is also observed in C57BL/6 mice infected with infectious muscle larvae of *Trichinella spiralis*. Reduction of allergic airway inflammation in this study is attributed to the increase of IL-10 and TGF. IL-10 inhibits expression of many pro-inflammatory cytokines, chemokines, and chemokine receptors, and mediates allergen tolerance after exposure to high doses of allergen in several mouse models such as BALB/c mice and C57BL/c mice [15,32,33].

Results of this study showed increased levels of IL-4 and IL-5 in BALB/c mice infected with *A. suum* and exposed to *D. pteronyssinus*. IL-4 and IL-5 productions are markers of immune response against chronic exposure to house dust mites and certain parasites [15,34,35]. In this present study, similar regulation of IL-4 and IL-5 was observed in both allergic mice and parasitized mice. IL-4 regulates allergic conditions and is also the protective immune response against helminthes and other extracellular parasites. IL-5 also contributes to induction of airway hyperreactivity in cases of allergy and asthma [33]. In previous studies, to regulate immune response against allergies, IL-4 production is a good target. Lowering IL-4 production balances immune response in terms of lowering IgE and eosinophil productions, and increasing interferon- γ , interferon- α and Th1 response [36]. However, in this present study, no lowering of IL-4 was observed to balance allergic response. Instead, there was enhanced production of IL-10. A study on the determination of the importance of IL-4 in airway inflammation was done to determine if absence of IL-4 would preclude immune response against chronic exposure to house dust mites (HDM). Results of this study showed that even in the absence of IL-4, allergic response against HDM is still possible through emergence of Th1 immunity. Thus, lowering IL-4 is not a potential candidate in preventing immune response against exposure to HDM. However, lack of IL-4 in mice causes defects in Th2 cell differentiation and reduces IgE and IgG1 [33,37]. Studies targeting IL-5 has been a topic of several researches to regulate allergic response, but is not yet proven because of conflicting results. However, in patients with eosinophilic asthma, reducing IL-5 is highly correlated with reduced exacerbations and eosinophils in blood and sputum [38]. Results of the previous studies

mentioned above indicate that downregulating the production of IL-4 and IL-5 does not protect the mice from allergic reactions. In group 4, *A. suum* infected mice might have protected the mice against *D. pteronyssinus*-induced allergy through production of IL-10 but not through downregulation of IL-4 and IL-5. While in group 5, *A. suum* infection in allergic BALB/c mice downregulated the levels of IL-4, and IL-5. Simultaneous infection with *A. suum* and allergic induction with *D. pteronyssinus* did not cause synergistic immune response in BALB/c mice but rather counteracts each other.

In terms of antibody productions, no significant increase in the relative level of IgE specific to *A. suum* was detected in *A. suum* infected mice (group 2). IgE, according to studies, is related to immunity rather than to exposure to infection and are usually produced only in cases of acute infection [39,40]. In acute infection, IgE is produced to combat the invading parasite together with eosinophils. Eosinophil degranulates against the parasite because parasites are too big to be phagocytized. Thus, IgE and eosinophil act as the host's protective mechanism against parasites [41,42]. Production of parasite-specific IgE is indicative of the host immunity against the parasite and is associated with resistance to infection or re-infection [12,41]. In this study, mice were infected with *A. suum* eggs to mimic chronic infection in humans following the protocol of Schopf et al. [14] with slight modification. The chronic parasitism was achieved and is indicated by the non-upregulation of As-specific IgE. As discussed in previous paragraphs, production of IL-4, IL-5, and IL-10 are enhanced in infected mice as compared to normal mice. Production of these interleukins may also have exerted an effect on the production of antibodies. IL-4 is a known cytokine that enhances antibody switching to IgE while IL-10 enhances antibody switching to IgG [43]. Since both IL-4 and IL-10 were markedly elevated in infected mice, effect of each cytokine counteracts each other, thus antibody switching is modulated. Presence of IL-10 can differentially regulate B-cells stimulated by IL-4 to produce IgG instead of IgE [44]. One study discussed three possible specific immune response against parasites: (1) host can be resistant to infection by balancing Th1 and Th2 cells controlled by T regulatory cells where both IgE and IgG4 are present but more IgE is produced; (2) host can have uncontrolled inflammatory Th1 disease where severe inflammation is observed and there is low IgG4 and significantly high IgE; and (3) host can be susceptible to infection and chronic infection is developed leading to a 'modified Th2 cell response' where more IgG and IL-10 are produced and less IgE [45]. The third type of immune response is exhibited in this study, that is, IL-10 is produced significantly, IgG is increasing and IgE production is not upregulated. Modified Th2 response is dominated by the production of IL-10. Although IL-4 with a known function in IgE and IgG switching, is also produced in this study, the dominating IL-10 inhibits B-cell switching to IgE while inducing production of IgG. Interestingly, a study conducted in an *A. lumbricoides* hyperendemic area, central province of Cameroon, measurable levels of IgG4 were detected in individuals infected and only 30% of them have detectable IgE levels. Individuals with detectable levels of IgE but not IgG4 had the lowest average levels of infection. Thus, IgG4 is positively correlated to the level of infection [12].

As seen on the results above, *D. pteronyssinus* extract was able to upregulate the production of *A. suum*-specific IgE and IgG. Based on previous studies, this upregulation can be accounted to cross-reacting antigens present in both *A. suum* and *D. pteronyssinus*. *Ascaris* and mites like *Dermatophagoides* spp. are closely related invertebrates belonging to the Infra-kingdom *Ecdysozoa*, thus it is expected that they share similar allergens [40]. According to one study using Western blot analysis, antigens like tropomyosin and paramyosin from *A.*

lumbricoides at approximately 35-37 kD level and at 100 kD level, respectively, match with the tropomyosin at approximately 37 kD of HDM group 10 and with the paramyosins at 98-102 kD level of HDM group 11 [46]. Further, another study identified high IgE reactivity with *Blomia tropicalis* and *D. pteronyssinus* at 40 kD allergen in *Ascaris*. This 40 kD allergen is confirmed as *A. lumbricoides* tropomyosin by Tandem LC-MS/MS and subsequent database search [47]. Tropomyosin and paramyosin are examples of cross-reactive antigens present in both *D. pteronyssinus* and *A. suum*. Tropomyosin is said to be highly conserved and antibodies against it may cross react to allergens from disparate species, thus termed as pan allergen. Paramyosin, on the other hand, is not as highly conserved and as cross-reactive as tropomyosin [48,49]. Tropomyosin found among invertebrates like house dust mites causes allergic reactions and is identified to have homology with the tropomyosins found in parasitic helminthes such as *A. suum*, *Anisakis*, *Onchocerca* and *T. spiralis* [50]. Paramyosin is found also in numerous invertebrates and in other parasites such as *Clonorchis*, *Schistosoma*, *Fasciola*, *Paragonimus*, *Onchocerca* and *Anisakis* and has been associated with the cross reactions observed between *A. lumbricoides* and house dust mites [46,51,52]. Another cross-reacting allergen found between *Ascaris* and dust mites (*D. pteronyssinus* and *B. tropicalis*) is glutathione-s-transferase at 23 kD level [41]. In this present study, *D. pteronyssinus* extract enhanced the production of *A. suum*-specific IgE and IgG. The capacity of *D. pteronyssinus* to upregulate *A. suum*-specific IgE and IgG is supported by a study conducted to evaluate cross reactivities between house dust mites, such as *B. tropicalis*, *D. pteronyssinus* and *D. farinae*, and *A. lumbricoides*, and the presence of high binding IgE antigens in *A. lumbricoides* against sera of allergic patients has been identified [46]. On the one hand, results of the present study showed that *A. suum* eggs did not induce significant production of *D. pteronyssinus*-specific IgE. This non-upregulation of *D. pteronyssinus*-specific IgE by *A. suum* eggs even in the presence of cross reacting antigens coincides with the result of Valmonte et al. [46] which states that subjects with ascariasis showed low reactivity against house dust mite allergens and that cross reactive antigens are present in higher amounts in *Ascaris* than among house dust mites. Also, in another study, ABA-1 (ASC S1), a body fluid allergen 1 specific to *A. suum*, has not been found to be cross-reactive with house dust mite allergens and that house dust mites were not found to have epitopes for ABA-1. ABA-1 is a nematode polypeptide allergen found at 15 kD level [41,47]. Mite allergens can cross-react with *Ascaris* epitopes and not vice versa [46,47]. *D. pteronyssinus* extract was able to upregulate the level of *A. suum*-specific IgE and IgG while *A. suum* eggs were not able to enhance the production of *D. pteronyssinus*-specific IgE and IgG. Upregulation of *A. suum*-specific IgE and IgG by *D. pteronyssinus* extract and non-upregulation of *D. pteronyssinus* specific IgE and IgG by *A. suum* eggs indirectly indicates and supports the idea that parasitism may protect an individual against allergic disorders but not vice versa. This is supported by the study of Valmonte et al. where ascariasis subjects showed low reactivity against HDM allergens and this low reactivity suggests that parasite products may be used as reagents to alleviate allergic disease [46]. Furthermore, another study by Flohr et al. in 2006 conform to this result that geohelminth infection lowers the risk of allergic sensitization thus components derived from parasites may be used as therapeutic regimens against allergy [53].

Histopathologic analysis of liver, lungs and small intestines were done to determine the pathological effect of *D. pteronyssinus* and *A. suum* in mice. Histological changes observed in the isolated tissue specimens of mice are due to either *A. suum* or *D. pteronyssinus* antigens. *D. pteronyssinus* extract caused edema, neutrophilic

infiltrates, congestion, and mild inflammatory process as observed in the lung tissues of mice in group 3. Group 3 mice manifested to have allergic symptoms and elevated levels of IL-4 and IL-5 and not IL-10. The levels of IL-4 and IL-5 were significantly elevated in allergic mice and have caused the inflammatory reactions of these mice as observed histologically and morphologically. Allergic symptoms and inflammation were not observed in group 2 group (parasitized mice) and group 4 (parasitized/allergic mice) mice histologically and morphologically. This was possibly due to the level of IL-10. Neutrophilic infiltrates were observed in groups 2, 3, 4, 5, and 6. Neutrophilic infiltrates are said to be common immunological responses against antigens [54,55]. Lungs, liver and small intestines from mice of groups 2 and 4 showed pathological abnormalities and are due to the migratory pattern of *A. suum*. Upon ingestion of *A. suum* eggs, eggs in the intestines hatched and released the larvae, which penetrated the intestinal wall to reach the venules and lymphatics. These larvae passed through the liver and lungs. Lungs, liver and small intestines of *A. suum* infected groups (2, 4 and 5) showed pathological conditions indicating that the ingested infective ova with larva inside may have hatched in the small intestines, larva migrated to the liver and to the lungs. *A. suum* infection caused hyperplastic changes and/or atrophic villi in the intestines of the mice. Cellular infiltration, for example, in the lungs of group 2 mice might have led to inflammation because of obstruction due to accumulation of the cells. Lymphocytic infiltration in group 2, as well, caused mild edema and congestion. In the liver tissues, lymphocytic infiltrates were also observed. Presence of these lymphocytes in the liver tissues is associated with persistent infection [54,55]. Histopathological changes in the different organs of the mice were results of the host's immunological responses against the antigens, either *A. suum* eggs or *D. pteronyssinus* extract. Both antigens were capable of eliciting the immune response of BALB/c mice [18].

Conclusions

A. suum infection lowers the risk of developing *D. pteronyssinus*-induced allergic reaction in BALB/c mice. *A. suum* infected BALB/c mice were protected from *D. pteronyssinus*-induced allergy. Mice infected with *A. suum* eggs and exposed to *D. pteronyssinus* extract did not show any allergy symptoms morphologically and histologically and has decreased levels of anti-*D. pteronyssinus* IgE. *A. suum* and *D. pteronyssinus* are capable of upregulating IL-4 and IL-5 while only *A. suum* upregulates IL-10. *D. pteronyssinus* extract enhanced the production of *A. suum*-specific IgE and IgG and is accounted to cross-reacting antigens present in *A. suum* and *D. pteronyssinus*. The possible role of IL-10 in the protection confers by *A. suum* infection against *D. pteronyssinus*-induced allergy needs further investigations. The use of IL-10 knock out mice may be useful. If proven, it will be the basis for the development of therapeutic regimens against allergy, which is a potential application of Hygiene Hypothesis. For future researches, analysis of bronchoalveolar lavage (BAL) is recommended, as well as other parameters such as neutrophil count and T cell recruitment to the lungs.

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