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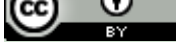


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## Comparison of Cytokine Profile between Indonesian Thin-Tailed and Merino Sheep during A Primary Infection with *Fasciola gigantica*

(PERBANDINGAN PROFIL SITOKIN ANTARA DOMBA EKOR TIPIS DAN DOMBA MERINO  
YANG DIINFEKSI PERTAMA DENGAN *FASCIOLA GIGANTICA*)

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### ABSTRACT

The aim of this study was to investigate the expression of cytokines profiles interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-5 (IL-5) and IL-10 in *Rasciola gigantica* resistant Indonesian thin-tailed (ITT) sheep compared to susceptible merino sheep infected with *Fasciola gigantica*. A total of ten ITT and merino sheep were randomly allocated into infected (n=5) and control (n=5) groups, sheep were infected with 250 viable metacercariae of *F. gigantica*. The cytokines were determined by isolated mRNA from hepatic lymph node by semi-quantitative RT-PCR (Reverse Transcriptase -Polymerase Chain Reaction). The result showed ITT sheep produced significantly higher IL-5 and 10 (  $P<0,05$  ) than merino sheep, while ITT sheep produced less IFN- $\gamma$  (  $P<0,05$  ) than merino sheep at 10 weeks post infection. It could be concluded that merino sheep tend to develop T type 1 cells, while the ITT sheep tend to develop T type 2 cells which effectively killed *F. gigantica*.

Key Words: *Fasciola gigantica*; Indonesian thin-tailed sheep; merino sheep; IL-5; IL-10; IFN- $\gamma$

### ABSTRAK

Tujuan dari penelitian ini adalah untuk mengetahui perbedaan profil sitokin  $\gamma$ -interferon ( $\gamma$ -IFN), interlukin-5 (IL-5) and IL-10 antara domba ekor tipis (ET) yang resisten dengan domba merino yang peka terhadap infeksi *Fasciola gigantica*. Masing-masing 10 ekor bangsa domba dibagi menjadi dua, kelompok kontrol (tidak diinfeksi) dan kelompok diinfeksi dengan 250 metaserkaria *F. gigantica*. Sitokin diidentifikasi dengan cara ekstraksi mRNA dari sel yang berasal dari kelenjar getah bening hepatis dengan cara semi-kuantitatif RT-PCR (*Reverse Transcriptase -Polymerase Chain Reaction*). Hasil menunjukkan bahwa domba ET memproduksi IL-5 dan IL-10 lebih banyak secara signifikan (  $P<0.05$  ) dibandingkan domba merino, tetapi produksi  $\gamma$ -IFN lebih banyak secara signifikan (  $P<0.05$  ) pada domba merino daripada domba ET pada 10 minggu setelah infeksi. Disimpulkan bahwa domba merino cenderung ke arah perkembangan sel T tipe 1, sedangkan domba ET cenderung ke arah sel T tipe 2 yang efektif membunuh cacing *F. gigantica*.

Kata kunci: *Fasciola gigantica*; domba ekor tipis; domba merino ; IL-5; IL-10;  $\gamma$ -IFN

### INTRODUCTION

Tropical fasciolosis is a significant disease in ruminants causing losses to agricultural and livestock productions. It is caused by the tropical liver fluke *F. gigantica*. *F. gigantica* is a digenetic leaf-like trematode that requires a lymnaeid snail, *Lymnae rubiginosa auricularia*, as an

intermediate host (Torgeson and Claxton, 1999). The parasite invades the liver and maturity occurs in the bile ducts. *F. gigantica* is generally restricted to the tropics and having been recorded in Africa, the Middle East, eastern Europe, and south and eastern Asia (Mas-Coma *et al.*, 2005). *F. gigantica* causes a significant reduction in meat and milk production, growth rate,

fertility and draught power in infected animals. Other causes of economic losses are condemnation of infected livers at slaughter and the cost of control measures (Spithill *et al.*, 1999). Recent reports indicate that fasciolosis is also an important emerging pathogen in humans, with approximately 2.5 million people infected worldwide (WHO, 1995).

Immune responses to parasitic infections are regulated by T helper cells via their production of cytokines. Development of protective immunity to infections is influenced by these cells and their cytokines (Abbas *et al.*, 1996). It is well accepted that CD4<sup>+</sup>Th (helper) cells can be separated into two major subsets, Th1 and Th2, on the basis of their cytokine production patterns and function (Fitch *et al.*, 1993). T-helper1 cells produce interferon (IFN)- $\gamma$ , interleukin (IL)-2 and IL-12 and promote the activation of macrophages and mediate delayed type hypersensitivity. In contrast, Th2 cells produce IL-4, IL-5, IL-6, and IL-10 and promote immediate type hypersensitivity reactions involving IgE, eosinophils and mast cells. The cytokines of each T cell subtypes are mutually inhibitory for the differentiation and effector functions of the reciprocal subset resulting in the polarization of the immune response to either type 1 or type 2.

The previous studies had shown that ITT sheep are more resistant to infection with *F. gigantica* than the merino sheep (Wiedosari and Copeman, 1990). The innate cytokine response occurred during infection is a critical component which can control the production of cytokines in the microenvironment of the responding lymph node (Abbas *et al.*, 1996). This study was conducted by measuring IFN- $\gamma$ , IL-5, and IL-10 which were determined by isolated mRNA from hepatic lymph node following *F. gigantica* infection by semi-quantitative RT-PCR. The purpose of this study is to investigate the cytokine expression in tissues of ITT and merino sheep during *F. gigantica* infection which will show critical differences between the T cells from the two sheep breeds allowing to understand why ITT sheep are better at killing this parasite.

## RESEARCH METHODS

### Experimental Animals and Design

Ten of each ITT and merino sheep, approximately nine months old at the time of infection with no detectable eggs of *F. gigantica* in faeces, were used in this experiment. Animals

in each breed were allocated at random into infected (n=5) and control (n=5) groups

The trial was carried out at Research Centre for Biotechnology Indonesian Institute of Sciences (LIPI), Bogor, West Java, Indonesia where sheep were maintained in open pens on a diet of freshly cut *Pennisetum purpureum* and concentrate. Metacercariae for infections were obtained from infected *L. rubiginosa* snails collected at Surade, West Java, Indonesia. Sheep were infected with 250 viable metacercariae of *F. gigantica* by loading the required metacercariae on filter paper which was placed inside gelatin capsules and delivered orally using a dosing gun (Wiedosari and Copeman, 1990).

### Necropsy and Lymph Node Collection

Sheep were sacrificed at 10 weeks postinfection by severing the jugular vein. Immediately following slaughter, the hepatic lymph node was dissected from each animal and rinsed in 100% ethanol to remove excess blood and tissue. Approximately 0.5 - 1 g of tissue was cut into slices of 0.5 cm diameter, rinsed in cold PBS and immersed in 5 ml of RNA later (Ambion). Samples were kept at 4°C for 12 hours before long term storage at -20°C.

### Homogenisation, RNA Extraction and cDNA Synthesis (Clery and Mulcahy, 1998) :

Samples were thawed by gentle rotation at 4 °C and approximately 400-500mg of tissue was removed, rinsed in cold PBS and homogenised in 5 ml of QIAzol (Qiagen) for 30 seconds using a sterilized polytron homogenizer. Samples were stored in 1ml aliquots at -70 °C until RNA was extracted.

RNA was extracted using the Lipid RNeasy© Mini Kit (Qiagen) according to manufacturer's guidelines. Genomic DNA contamination was eliminated by treating each sample with 27 Kunitz of RNase free DNase I (Qiagen) during the extraction procedure as recommended by the manufacturer. High quality RNA (OD 260nm/280nm > 1.9) was eluted from the columns using RNase-free water and quantitated using a Biophotometer (Eppendorf).

Total RNA (2  $\mu$ g) was reversed transcribed using Omniscript® Reverse Transcription kit (Qiagen) according to manufacturer's guidelines in a total volume of 20  $\mu$ l containing 2  $\mu$ l of 10 x Reverse Transcriptase Buffer (Qiagen), 2  $\mu$ l dNTP mix (Qiagen), 0.5  $\mu$ g Oligo-dT primer (Invitrogen), 10 units RNase Inhibitor (Promega),

4 Units of Omniscript Reverse Transcriptase (Qiagen), Rnase free water, and 2 µg RNA. The final reaction mixture was incubated at 37 °C for 70 minutes and aliquots stored at -20 °C or use directly in PCR reaction. A 10 µl of the RT reaction was used in a 100 µl PCR reaction which contained 0.5 µl of Taq DNA polymerase (Qiagen), 10 µl of 10 x PCR buffer, 0.2 µl of 10 mM dNTP, 4 µl of 50 mM MgCl, 1 µl of each appropriate forward and reverse primers. Oligonucleotides were used to amplify ovine IL-5, IL-10 and IFN-γ and the reference gene, GAPDH. Oligonucleotides were synthesised by GeneWorks Pty Ltd (Adelaide, Australia) and were used at a concentration of 10µM. Prior to PCR amplification a denaturation step (95°C for 15 minutes) was used followed by 34 cycles of denaturation (95°C for 0.5 min); annealing (53°C for 1 min); and elongation (72°C for 1 min). A final extension step was performed by heating to 72°C for 8 min. The number of PCR cycles and the total RNA input in the RT reaction were determined in a preliminary experiment to ensure the amplification was well below the saturation under this assay condition.

Primer sequence IFN- g:

**IFN- g (F):** AGGGTGGGCTCTTTTCTC (262bp)

**IFN- g (R):** ATTGATGGCTTTGCGCTG

Primer sequence IL-10:

**IL-10 (F):** AGCTGTACCCACTTCCCA (305bp)

**IL-10 (R):** GAAAACGATGACAGCGCC

Primer sequence IL-5:

**IL-5 (F):** CCTTGACACTGCTCTCCACGC (303bp)

**IL-5 (R):** CTTTCCATCGTCCACTCTGTC

Primer sequence GAPDH:

**GAPDH (F):** ATCACTGCCACCCAGAAGACT (200bp)

**GAPDH (R):** CATGCCAGTGAGCTTCCCGTT

After PCR amplification, a 10µl aliquot was mixed with 2 µl loading buffer and electrophoresed on a 1.2 % agarose gel containing ethidium bromide (0.25 µg/ml). The results of DNA bands in the gel were photographed (Figure 1, 2 and 3) while the quantitation of cytokine mRNA was accomplished by 1 D Image Analysis Software. The mRNA level of each cytokine was normalized to GAPDH (control) and expressed as ratio to GAPDH mRNA for each sample.

### Statistical Analysis

Statistical analysis was performed using SAS system (1998), the ANOVA and the Duncan

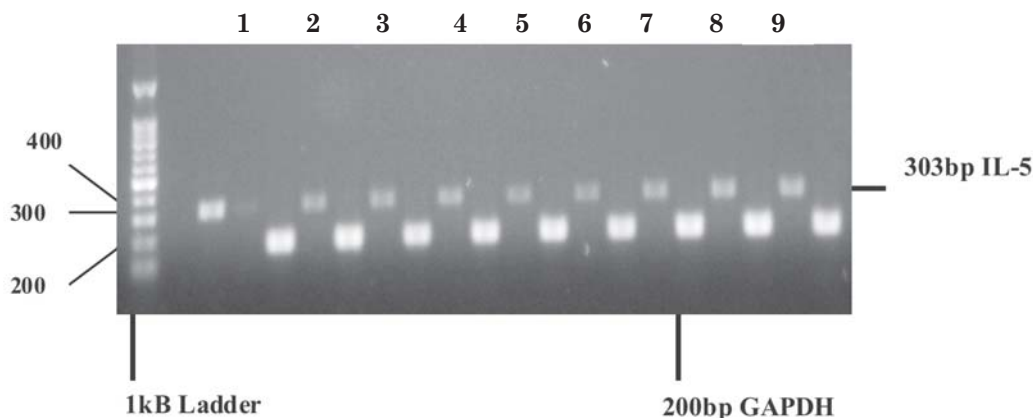
test were used to evaluate the result. Differences at the  $P < 0,05$  level were considered significant.

## RESULTS AND DISCUSSION

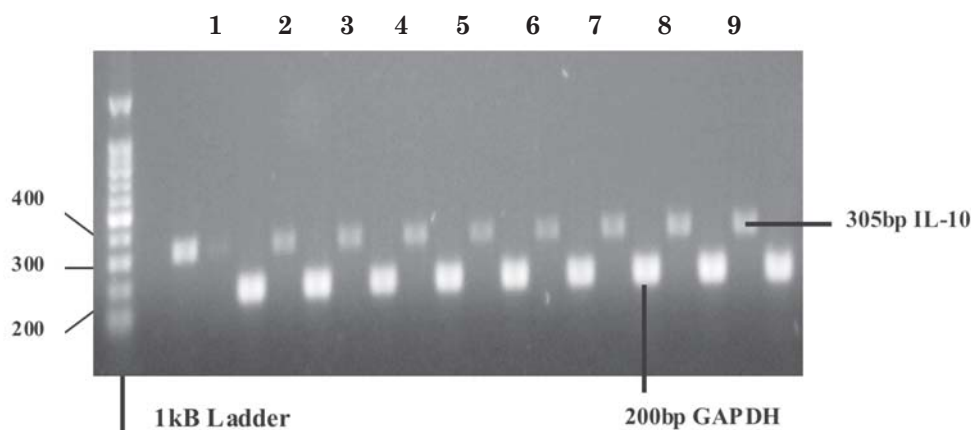
The result showed that hepatic lymph node cells from ITT sheep produced significantly more IL-5 ( $P < 0,05$ ) than cells from merino sheep at 10 weeks post infections with *F. gigantica* (Figure 4). The presence of IL-5 indicates a stimulation of a Th2-type of response and it participates in the polarization of the immune response towards a Th2 response. The main activities of IL-5 are to promote the growth and terminal differentiation of eosinophils, to attract eosinophils to sites of inflammation, and to enhance IgG1 induced eosinophil degranulation (Abbas *et al.*, 1996). Interleukin-5 has also been implicated in eosinophil activation and shown to enhance the killing of nematode larval in sheep (Rainbird *et al.*, 1998).

The role of eosinophils during infection with *F. gigantica* has been suggested as one of the main effector cells involved in the killing of young flukes. Van Milligen *et al.*, (1998) reported that eosinophils may be associated with the expression of resistance to *F. hepatica* as these cells infiltrate the intestine and selectively adhere to newly-excysted juveniles, where their degranulation causing severe damage and perhaps death to the young flukes (Burden *et al.*, 1983). Immunological analysis of response in ITT and merino sheep following a primary infection showed ITT sheep exhibited a significantly higher eosinophilia than merino sheep (Wiedosari, 2005).

A study by Piedrafita (2001) revealed that eosinophils were identified as the major immune cell-types within the peritoneum during the early migration of *F. gigantica* parasites in ITT sheep. These immune cells from the ITT sheep could indeed effectively kill immature *F. gigantica* but this ability was critically dependent on the presence of sera from *F. gigantica*-infected sheep (Piedrafita, 2001) By using a series of inhibitors, the author was further able to determine that the major cytotoxic molecules mediating parasite killing, which were produced by these cells, were superoxide radicals. Consequently, the enhanced resistance of ITT sheep to *F. gigantica* is probably associated with IL-5, as indicated in these observations that IL-5 of ITT sheep was higher compared to merino sheep.



**Figure 1.** Lane 1-9 were an example of PCR products using specific primers for sheep IL-5 and the housekeeping gene GAPDH from all of 20 sheep, were visualised by electrophoresis using a 1,2% agarose gel containing ethidium bromida.



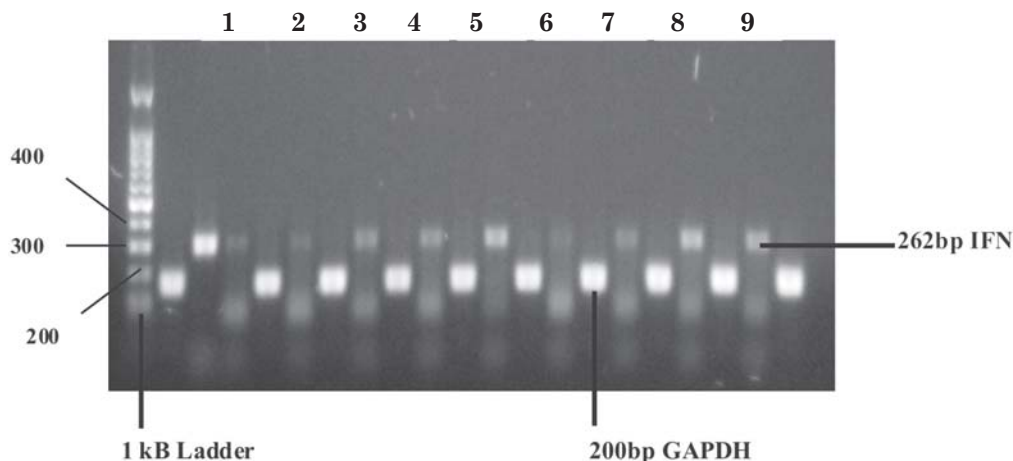
**Figure 2.** Lane 1-9 were an example of PCR products using specific primers for sheep IL-10 and the housekeeping gene GAPDH from all of 20 sheep, were visualised by electrophoresis using a 1,2% agarose gel containing ethidium bromida.

A Th2 response was also indicated by IL-10 which was observed at 10 weeks postinfection and the production was higher ( $P < 0,05$ ) in ITT sheep than merino sheep (Figure 5). A recognized role of IL-10 in mediating Th2 responses is its inhibition of Th1 cell differentiation via suppression of IFN- $\gamma$  production by Th1-committed cells. Interleukin-10 does not act directly on Th1 cells but impairs the capacity of splenic and peritoneal macrophages to stimulate Th1 cytokine synthesis by Th1 cells. This effect is partly due to the ability of IL-10 to down-regulated the expression of class II MHC on APCs that interact with antigen-activated Th1 cells to induce cytokine synthesis (Tizard, 2000). Schopf (2002) demonstrated that a deficiency in IL-10 can lead to increased susceptibility to *Trichuris muris* and even death. Interestingly, this lethal outcome is not only to *Trichuris muris* infections, because

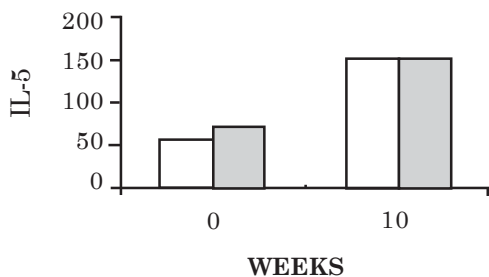
mortality of IL-10 deficient mice has also been observed during *Toxoplasma gondii*, *Trypanosoma cruzi*, *Plasmodium chabaudi-chabaudi*, and *Schistosoma mansoni* infection. It is, therefore, likely that the increased production of IL-10 in the ITT sheep have an enhanced capacity for the sheep to kill *F. gigantica* in vivo.

In present study, the production of IFN- $\gamma$  was higher ( $P < 0,05$ ) in merino sheep than ITT sheep (Figure 6). Interferon- $\gamma$  is an important Th1 cytokine that serves primarily to activate natural killer cells and mononuclear phagocytes (e.g. macrophages) which are responsible for cytotoxic activity against tumor cells and microbes (Abbas *et al.*, 1996). Research suggests that IFN- $\gamma$  inhibits or delays protective immunity to nematodes by suppressing the growth and expansion of Th2 cells via a down-

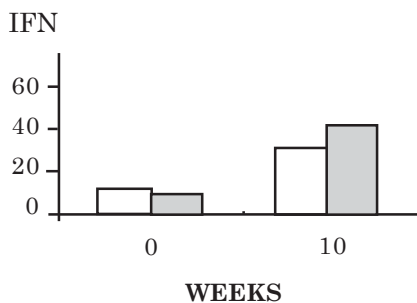




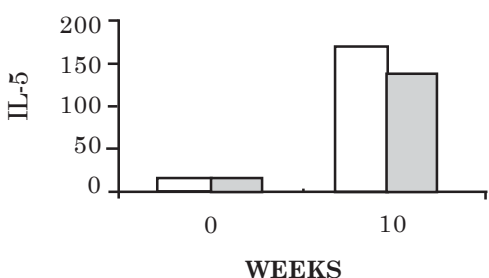
**Figure 3.** Lane 1-9 were an example of PCR products using specific primers for sheep IFN- $\gamma$  and the housekeeping gene GAPDH from all of 20 sheep, were visualised by electrophoresis using a 1,2% agarose gel containing ethidium bromida.



**Figure 4.** IL-5 profile in Indonesian Thin-tailed and Merino sheep at 10 weeks post infection with *Fasciola gigantica* □ ITT █ Merino



**Figure 6.** IFN $\gamma$  profile in Indonesian Thin-tailed and Merino sheep at 10 weeks post infection with *Fasciola gigantica* □ ITT █ Merino



**Figure 5.** IL-10 profile in Indonesian Thin-tailed and Merino sheep at 10 weeks post infection with *Fasciola gigantica* □ ITT █ Merino

regulation of IL-5, inhibiting production of IgG1, blocking eosinophilia (Urban *et al.*, 1993). These inhibitory effects of IFN- $\gamma$  on Th2 effector responses have been associated with prolonged survival of intestinal nematode parasites, such

as *Trichuris muris* in rodents (Schopf, 2002)). Wiedosari (2005) reported that ITT sheep produced significantly lower levels of IgG2 antibody relatives to the high level detected in merino sheep. The production of IgG2 has been shown to be strongly up-regulated by IFN- $\gamma$  in mice, humans and cattle (Abbas *et al.*, 1996). It is therefore likely that elevated IFN-g levels are related to elevated IgG2 levels in the susceptible merino sheep. This observation is intriguing given by Bielefeldt and Babiuk (1985) that IFN-g, a typical Th1-type cytokine, decreases superoxide radicals production by sheep immune cells.

Thus, the immunological pathways which could inhibit an effector mechanism in the resistant ITT sheep, appear to be up-regulated in the susceptible merino host. Fitch *et al.*, (1993) reported that characteristic cytokine products of Th1 and Th2 cells are mutually antagonistic for the differentiation and activity of effectors

belonging to the reciprocal phenotype. Hence, IL-10 inhibits cytokine synthesis by Th1 cells and macrophage function, whereas IFN- $\gamma$  suppresses the production of Th2-type effectors and cytokines. These results strongly support that the protective immune response in ITT sheep to *F. gigantica* infection involves the selective expansion of Th2-type subset cells with strong IL-5 and IL-10-secreting activities, with minimal activation (and possibly active down-regulation) of Th1-type IFN- $\gamma$ -secreting activity.

### CONCLUSION

The resistant ITT sheep tends to develop Th2-cells, while susceptible merino sheep develop Th1-cells. *Fasciola gigantica* infection in ITT sheep could be prevented by producing IL-10, IL-5 and activating eosinophils expressed by Th2-cells that effectively killed *F. gigantica*. The development of significant IFN- $\gamma$  response by Th1 which inhibited superoxide production may be the primary explanation for the increased susceptibility of merino sheep.

### ACKNOWLEDGEMENTS

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