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## THE DYNAMICS OF ANTIGEN SPECIFIC PROLIFERATIVE RESPONSES OF LYMPHOCYTES AT EARLY STAGES OF BOVINE PARATUBERCULOSIS INFECTION

### *Studi Longitudinal Terhadap Respon Proliferasi Antigen Spesifik dari Limfosit pada Tingkat Awal Infeksi Bovine Paratuberculosis*

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### ABSTRACT

The present study was aimed to quantify the dynamics of early antigen specific proliferative responses of lymphocytes to (protein) antigens associated with experimental *Mycobacterium avium* subsp. *paratuberculosis* (Mp) infection cattle. The data were collected from 20 experimentally infected calves, and 10 uninfected control animals, during the first 2 years of their lives. Several purified protein derivative antigens (Ppdp, Ppda, and Ppdb), two recombinant Mp heatshock proteins (Hsp65 and Hsp70) and whole bacteria (sonicated Mp strain 316F) were used to measure lymphocyte proliferation in a lymphocyte proliferation assay. Data were analyzed using a linear mixed effect (LME) model. The results showed significant group and timed effects for all antigens tested. At several time points, the responses in the infected group were found significantly higher as compared to control group. The Ppd antigens induced similar lymphocyte proliferation patterns, as compared to whole bacteria antigen and Hsp70. These results indicated that the antigen specific proliferative responses of lymphocytes differs for different antigens, probably related to differences in their availability during different stages of infection. The application of LME model is a useful tool for analyzing the quantitative longitudinal datasets.

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Keywords: dynamics, Mp, antigen, LME

### ABSTRAK

Studi longitudinal ini ditujukan mengukur dinamika respon tingkat awal proliferasi antigen spesifik dari limfosit terhadap antigen (protein) yang berhubungan dengan infeksi *Mycobacterium avium* subsp. *paratuberculosis* (Mp) pada sapi. Data dikumpulkan dari 20 ekor pedet yang diinfeksi sebagai perlakuan, dan 10 ekor pedet yang tidak diinfeksi sebagai kontrol, dalam usia 2 tahun pertama. Sejumlah antigen purified protein derivative (Ppdp, Ppda, dan Ppdb), dua rekombinan Mp heatshock proteins (Hsp65 dan Hsp70) dan whole bacteria (hasil sonikasi dari Mp strain 316F) digunakan untuk mengukur proliferasi limfosit dengan assay. Data dianalisis menggunakan metode linear mixed effect (LME) model. Hasil pengujian memperlihatkan pengaruh yang signifikan dari kelompok perlakuan dan waktu pada semua antigen yang diuji. Respon dari kelompok perlakuan secara signifikan lebih tinggi dibandingkan dengan kelompok kontrol yang diamati pada sejumlah titik waktu. Seluruh antigen Ppd menghasilkan pola proliferasi limfosit yang serupa, ketika dibandingkan dengan whole bacteria dan Hsp70. Hasil tersebut menunjukkan bahwa respon proliferasi antigen spesifik dari limfosit berbeda antar antigen, kemungkinan dikarenakan perbedaan dari ketersediaan mereka pada tingkat infeksi berbeda. Penggunaan metode LME merupakan cara yang tepat untuk menganalisis dataset longitudinal kuantitatif.

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Kata kunci: dinamika, Mp, antigen, LME

## INTRODUCTION

Paratuberculosis (John's disease) is a chronic, infectious intestinal disease of cattle and other ruminants, and causes substantial economic losses, mainly affecting the dairy industry (Rebhun, 1995; and Hutchinson, 1996). The disease is caused by *Mycobacterium avium* subsp. *paratuberculosis* (Mp) (Chiodini *et al.*, 1984; Chiodini, 1996; Mainar-Jaime and Vazquez-Boland, 1998; and Jakobsen *et al.*, 2000).

In cattle, the infection of paratuberculosis could occur at a very early age (Roermund *et al.*, 2000), and followed by an incubation period of two to six years, which is typical before the onset of clinical stage (Jakobsen *et al.*, 2000). After ingestion, the bacteria are translocated in the terminal ileum and taken up by macrophages underlying the M-cells in the dome epithelium of Peyers patches (Chiodini, 1996). One of the crucial pathogenic event is the fact that Mp is able to survive inside the macrophages and avoid bactericidal mechanisms to a large extent (Bermudez *et al.*, 1997). Bacterial antigens (either secreted or available from dead bacteria) will be degraded, and afterward presented on the cell surface of the macrophage. This will subsequently lead to activation of so called helper T cells, which play a crucial task in control (and elimination) of infectious agents (Andersen, 1994; and Waters, 1999). Two general types of antigens have been reported to play an important role in animals with regard to the Mp infection. Those are the purified protein derivative (Ppd) and to a lesser extent the heatshock proteins (Hsp) to which, upon Mp infection, antigen specific proliferative responses of lymphocytes have been measured in various studies (Koets *et al.*, 1999; Reichel *et al.*, 1999; Moseley, 2000; and Stabel, 2000). Purified protein derivative is prepared from

excreted Mp culture filtrate antigens, whereas Hsp is a non-excreted intracellular antigen of Mp (Koets *et al.*, 1999). Because those antigens are inherently different, the immunologic responses are likely to be different as well.

Several different methods have been used in studies of the proliferative responses of lymphocytes to Ppd and/or Hsp antigens related to Mp infection in animals, for example, the lymphocyte stimulation test (Koets *et al.*, 1999), migration inhibition factor (Kreeger and Snider-III, 1991), interferon gamma assay and interleukin-2 assay (Rebhun, 1995; and Reichel *et al.*, 1999). Both cross-sectional and longitudinal studies have been done on adult and young animals. However, there is still not enough quantitative information available on the dynamics of the immunological events in Mp infection. In the present study we quantified longitudinal data of responses of lymphocytes to antigens of Mp in young cattle, which were observed through the lymphocyte stimulation test.

The aim of this study was to quantify the dynamics of the early antigen specific responses of lymphocytes to (protein) antigens associated with Mp infection. Ultimately, this approach may be helpful as a tool for solving diagnostic and pathogenesis related problems of bovine paratuberculosis infection.

## MATERIALS AND METHODS

### Data

The data originated from an ongoing experiment of Mp infection in cattle. The study used 20 animals from an experimentally infected group (infected with Mp in the first month of life) and 10 animals in a control (non-infected) group.

Heparinized blood was collected from all animals once a month for the duration of experiment. The blood was used to isolate peripheral blood mononuclear cells (PBMC) using density gradient centrifugation (Koets *et al.*, 1999). The PBMC were used in a lymphocyte proliferation assay using  $2.10^5$  PBMC per well, (in 100  $\mu$ l), triplicate of which were stimulated with 100  $\mu$ l of antigen solution in 96 well microtiter plates. The antigens used in this assay were the purified protein derivative of Mp strain 3+5/c (10  $\mu$ g/ml) (Ppdp), *Mycobacterium bovis* strain AN5 (10  $\mu$ g/ml) (Ppdb), and *Mycobacterium avium* strain D4 (10  $\mu$ g/ml) (Ppda). In addition, recombinant Hsp of 65 kD from Mp (10  $\mu$ g/ml) (Hsp 65), recombinant Hsp of 70 kD from Mp (10  $\mu$ g/ml) (Hsp 70), and sonicated Mp strain 316F in a concentration of  $1.10^7$  CFU/ml (10 per APC) (Wb10:1) were also used as antigens. The mitogen Concanavalin A (ConA) was used as a positive control (2.5  $\mu$ g/ml) and the medium alone as a negative control.

### Statistical Analysis

Lymphocyte stimulation test results were expressed as the stimulation index (SI), calculated as geometric mean count per minute (cpm) antigen divided by geometric mean cpm medium. Responses were considered positive when the  $SI \geq 2$ .

To better approximate a normal distribution of the data, all SI responses variables (Ppdp, Ppda, Ppdb, Hsp65, Hsp70 and Wb10:1) were transformed by using a logarithmic transformation. Subsequently, a linear mixed effect (LME) model was used to analyze the time effect, the group effect and their interaction (Verbeke and Molenberghs, 2000). To take into account the dependence of data, a random calf effect was included. Besides the time of sample collection was taken as a fixed effect.

Because the residuals were heterogeneous we included a variance of structure into the model, which allowed different standard deviations for each time point. Then, to model any remaining dependence in the data we introduced an autoregressive order 1 (AR (1)) correlation structure, which assumes the current residual to be only dependent on the residual at the previous time point (Pinheiro and Bates, 2000). In particular, the restricted maximum likelihood (REML) method was used, since in general it gives less biased results than the maximum likelihood (ML) method for comparing different random parts (Pinheiro and Bates, 2000; Verbeke and Molenberghs, 2000). For computation, we used S-PLUS 2000 statistical software (Pinheiro and Bates, 2000).

To determine at which time points the infected and the control groups differed significantly, pooled T-tests on the logarithmically transformed SI values were performed. Bonferroni correction was applied to account for multiple comparisons (P value specified as  $P_B$ ) (Armitage and Berry, 2000). Significant differences are indicated in the graphs, which depict the geometric means of the SI of both groups.

## RESULTS AND DISCUSSION

### Linear Mixed Effect Model

The LME test showed that all antigens had a significant time and group-effect. The Ppdp and whole bacteria antigens gave the most significant group effect ( $P < 0.0001$ ). Then, five antigens gave significant outcome when tested for interaction, but one (Ppdb) did not (Table 1).

Table 1. Results of the linear mixed effect model for SI of all antigens specific proliferation responses of lymphocytes to Ppd, Hsp and whole bacteria antigens (time and group are both corrected for each other)

Response Variable	Group (p-value)	Time (p-value)	Interaction (p-value)
Ppdp	< 0.0001	< 0.0001	< 0.0001
Ppda	< 0.005	< 0.0001	< 0.0001
Ppdb	< 0.05	< 0.0001	NS
Hsp65	< 0.05	< 0.0001	< 0.0001
Hsp70	< 0.05	< 0.0001	< 0.0001
Wb10:1	< 0.0001	< 0.0001	< 0.0001

Ppd : Purified protein derivative  
 Hsp : Heatshock protein  
 Wb10:1 : Whole bacteria  
 NS : Not significant

**LST to Antigens Specific Proliferative Responses of PbmC**

The SI proliferative responses of Ppd antigens in the infected group reached values larger than 2 at multiple time points (Figure 1). The lymphocyte proliferation was declined from time point 1 to time point 2, and then sharply increased to time point 3, and next showed a sudden drop to time point 4. During these time points only Ppdp at time point 3, when comparing the infected and the control group, was reached a significant difference. The antigens responses were declined from time point 4

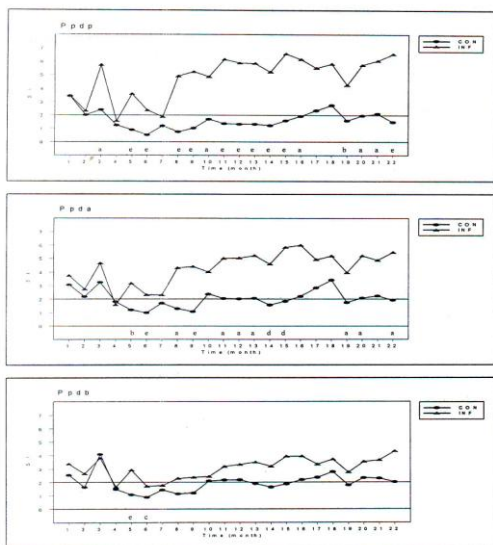


Figure 1. Proliferative responses of lymphocytes to Ppdp, Ppda and Ppdb at 22 time points, expressed as Stimulation Index (S.I.) for the infected group and the control group. The horizontal line above the horizontal axis indicates a SI of 2. Letters indicate statistical difference (a :  $P_B < 0.05$ ; b :  $P_B < 0.01$  ; c :  $P_B < 0.005$  ; d :  $P_B < 0.001$  ; e :  $P_B < 0.0001$ ) according to pooled T-tests with Bonferroni correction.

to time point 7, and proved to be significantly higher to the infected group than that to the control group at time points 5 and 6. Next, the responses were expressed an upward trend to time point 8, and remained steady until the end of experiment. For Ppdp and Ppda, from time point 7 to time point 22, almost at all time points were showed significantly higher responses in the infected group as compared to the control group. But, this was not the case for Ppdb.

Proliferative responses of lymphocytes to Hsp65 were not observed in either group ( $SI < 2$ ) and at nearly all time points the differences between the infected and control group were non-significant ( $P_B > 0.05$ ) (Figure 2a). From time point 1 to time point 6, the responses were flow smoothly. After that, rose to time point 7 and showed a plateau pattern until time point 22. The proliferative responses in the control group seem also followed a similar pattern as in the infected group. On the other hand, the SI of Hsp70 was exceeded 2 at three time points in the infected group (Figure 2b). Lymphocyte proliferation also showed a smooth response from time point 10 to time point 11, and then remained steady until the end of time point. In the control group, the responses were expressed leveling off until Time point 22. Compared both groups, the proliferative responses to Hsp70 were

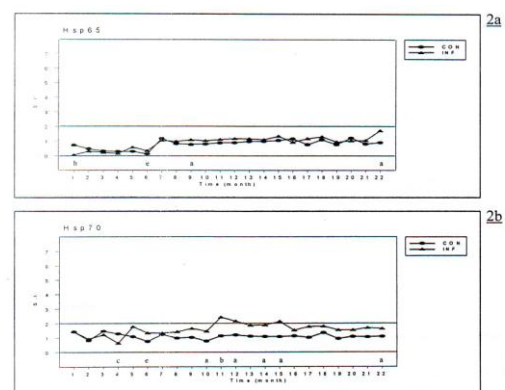


Figure 2. Proliferative responses of lymphocytes to Hsp65 and Hsp70 at 22 time points, expressed as Stimulation Index (S.I.) for the infected group and the control group. The horizontal line above the horizontal axis indicates a SI of 2. Letters indicate statistical difference (a :  $P_B < 0.05$ ; b :  $P_B < 0.01$  ; c :  $P_B < 0.005$  ; d :  $P_B < 0.001$  ; e :  $P_B < 0.0001$ ) according to pooled T-tests with Bonferroni correction.

significantly higher for the infected group at several more time points ( $P_B < 0.05$ ).

The geometric mean SI of lymphocyte proliferation to whole bacteria in the infected group proved to be both larger than that in the control group ( $P_B < 0.05$ ) at the majority of time points (Figure 3). The proliferative responses in the infected group rose from time point 1 to time point 2, then smoothly to time point 3 continued fell off to time point 4. After that, increased sharply to time point 5 and decreased to time point 6. Next, lymphocyte proliferation remained steady until time point 10 before showed an upward trend to time point 22. On the other hand, in the control group the proliferative responses expressed a downward trend from time point 1 until time point 4, and then remained steady until the end of experiment.

The present study quantified the dynamics of the early antigen specific proliferative responses of lymphocytes to a panel of mycobacterium antigens during the early stages of experimental Mp infection in cattle.

The data in this longitudinal study was modeled using LME model, which to our knowledge, has not been done previously. Linear mixed effects is very versatile in coping with dependent observations. By including a random calf effect, dependence between observations on the same animal was partly corrected for. Any remaining dependence was modeled using an autoregressive correlation structure of order 1, which assumes residuals to only depend on the residual of the previous observation. Inclusion of an extra variation structure solved the problem of heterogeneity of the variances of the residuals at the various time points (Pinheiro and Bates, 2000). In this way it was possible to analyze the group and time

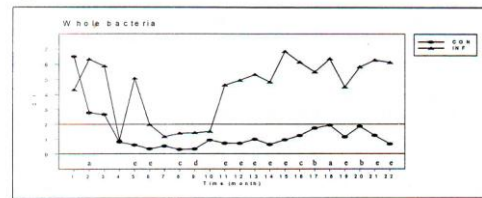


Figure 3. Proliferative responses of lymphocytes to whole bacteria antigen at 22 time points, expressed as Stimulation Index (S.I.) for the infected group and the control group. The horizontal line above the horizontal axis indicates a SI of 2. Letters indicate statistical difference (a :  $P_B < 0.05$ ; b :  $P_B < 0.01$ ; c :  $P_B < 0.005$ ; d :  $P_B < 0.001$ ; e :  $P_B < 0.0001$ ) according to pooled T-test with Bonferroni correction.

main effects plus their interaction parametrically, resulting in a more powerful analysis.

The antigens that were used during this study can be separated in two different types. That are the Ppd-type antigens representing secreted antigens and the Hsps and the whole bacterial antigen representing structural antigens. The latter group can be subdivided to the extent that recombinant Hsp65 and Hsp70 are single protein antigens and whole bacteria is inherently a much more complex mix of structural antigens. In general, the two types tested antigens induced proliferative responses of lymphocytes in Mp infected calves at one or more time points which were significantly higher when compared to controls. However, not all of them induced a biologically relevant reaction ( $SI \geq 2$ ) with regard to the immunologic pathogenic process.

Minor proliferative responses of lymphocytes to Ppd, Hsp, and whole bacteria antigens were observed in uninfected cattle consistent with those reported in previous studies (Koets *et al.*, 1999; Koets, 2000; Whist *et al.*, 2000; and Stabel and Whitlock, 2001). Probably, here they found their origin in sensitization of these animals to antigen determinants between Mp, *M. avium* and *M. bovis* (Koets *et al.*, 1999), indicating the presence of antigenic cross reactivity (Pillai *et al.*, 2001) between those different mycobacterial species (Whist *et al.*, 2000; and Stabel and Whitlock, 2001).

Focusing on the experimentally infected population of animals a general pattern can be observed, indicating early proliferative responses during the first 5-6 months following infection. Then, a relatively 'silent' phase for a period up to 10 months and a subsequent exponential increase leading to a prolonged plateau phase which is sustained to the end of the second year of life. The results indicated a temporal difference between responses to the Ppd type (excreted) antigens and the structural antigens which may be related to the difference in availability of these different antigens during infections as has been previously observed by several other investigators ( Koets *et al.*, 1999; Koets, 2000; Whist *et al.*, 2000; and Stabel and Whitlock, 2001). Most likely, the secreted antigens are available in earlier stages of infection, when bacteria replicating in macrophages are metabolically active, as compared to the structural antigens which will be available only when bacteria are degraded or appear free in circulation following the death of their host cell. The observed period of relative quiescence may be related to a decrease in bacterial activity following the initial responses, indicating a successful immune response. However, extreme multiplication of the bacteria may also lead to a temporary state of peripheral energy induced by the abundance of antigens and the redistribution of T cells to infected intestinal tissue. Analysis of fecal culture data indicative of the bacterial load in this period may provide more insight into the observed phenomena. In the third phase, the response return and lead to a rather stable level of immune reactivity. Taken together with absence of any clinical signs of infection during this period this may be regarded as a sign of competent immune responses controlling the infection.

When comparing the responses between the complex antigens (Ppd and whole bacteria) to the lymphocyte proliferation induced by Hsp antigens, the responses to these antigens are of a lower magnitude as can be expected for simple protein antigens. However, the Hsp70 clearly induced more pronounced responses as compared to Hsp65. This result is similar to previous cross-sectional studies, indicating that Hsp65 is not a dominant T cell antigen in *Mp* infected cattle (Koets *et al.*, 1999).

## CONCLUSION

The antigens specific proliferative responses of lymphocytes to Ppd antigens increased stronger and reached a plateau stage earlier in subclinical *Mp* infected cattle as compared to other *Mp* antigens. Thus, the results of present study supported the further analysis of the constituents of Ppd antigens with regard to their potential diagnostic value and pathogenesis approach in bovine paratuberculosis infection.

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