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NOTE

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Cytokine levels of peripheral blood mononuclear cells in the clinical cases of Holstein calves infected with *Mycoplasma bovis*

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ABSTRACT. The immune related factors of peripheral blood mononuclear cells (PBMC) were analyzed in the clinical cases with *Mycoplasma* (*M.*) *bovis* infection. Seventy-eight Holstein calves in one farm were used. These calves were divided into three groups; the calves with *M. bovis* infection of poor outcome after treatment (Non-Recovery Group), the calves with *M. bovis* infection recovered (Recovery Group) and clinically healthy calves (Control Group). Blood samples were collected at days of the first medical treatment and the final treatment or euthanasia. IL-17A levels in the Non-Recovery Group were higher than those in the Recovery Group on both days. Our result suggested that the IL-17A of PBMC is an important factor to affect outcome of the calves with *M. bovis* infection.

KEY WORDS: calf, clinical case, immune factor, *Mycoplasma bovis*, peripheral blood mononuclear cell

Mycoplasma (*M.*) *bovis* is known to cause pneumonia, otitis media, arthritis and mastitis. *M. bovis* has been isolated frequently from lungs of affected animals during respiratory disease outbreaks [2]. Field cases of respiratory disease caused by *M. bovis* infection are sometimes accompanied by arthritis. Outbreaks of otitis media in dairy calves have been largely attributed to *M. bovis* infection [22], however animals with *M. bovis* may not develop clinical signs or show evidence of disease. Although the mechanisms of *M. bovis* pathogenesis remain to be elucidated, this pathogen can induce a broad range of immunomodulatory events by directly and indirectly affecting leukocytes through the induction of cytokine secretion from multiple cell types [6].

In the lung of calves experimentally infected with *M. bovis*, immune cell activation resulted in expression of cytokines such as interleukin (IL)-4, IL-10 and interferon (IFN)-γ and tumor necrosis factor (TNF)-α capable of inducing lung lesions and hyperplasia of the bronchus-associated lymphoid tissues [16]. Another previous report indicated that increased TNF-α, IL-12p40, and IFN-γ mRNA expression in bovine peripheral blood mononuclear cells (PBMC) was induced with *M. bovis* stimulation *in vitro* [6]. Although changes of serum T cell cytokines such as IL-4 and IFN-γ were observed in experimental intra-tracheal infection of *M. bovis* of calves [4], the relationship between the outcome and T cell cytokines levels in clinical cases of mycoplasma infection is not known. In this study, we investigated the expression of cytokines by PBMC in the field calves with *M. bovis* infection of different outcome.

Seventy-eight Holstein calves in one farm were used in this investigation, and these calves were divided into three groups; the calves with *M. bovis* infection of poor outcome after treatment (Non-Recover Group, N=13), the calves with *M. bovis* infection recovered after treatment (Recovery Group; N=51), and clinically healthy *M. bovis* undetected calves (Control Group, N=14). In the Non-Recovery Group, there were 8 cases of arthritis and 5 cases of otitis. In the Recovery Group, there were 11 cases of arthritis and 40 cases of otitis. The abscess samples were collected from the affected areas for detection of *M. bovis*. All calves with clinical symptoms were treated by repeated cleaning of the affected areas with sterile saline (lesion of ears or joints) and injection of antibiotic drugs (new quinolone or macrolide antibiotics) every day, as general treatment. The treatment period differed with each calf. Blood samples were collected from the jugular vein of calves with infection on the day of the first medical treatment (First day) and the day of final treatment or euthanasia (Last day). Age on the first day and on the last day were 56.80 ± 2.25 (mean ± standard error of the mean (SE)) and 70.48 ± 2.50 in the Non-Recovery Group, and 57.69 ± 6.35 and 91.46 ± 10.56 in the Recovery Group, respectively. In the calves of the Control Group, blood sample was collected once from the jugular vein at

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56.40 ± 7.15 days of age. Disappearance of symptoms such as fever, loss of appetite, ear drooping, or lameness was considered to be recovery from disease. Cases with exacerbation of symptoms were considered as having a poor outcome. The procedures used in the present study were performed in accordance with the principles and guideline for animal use set by the Animal Experiment and Care Committee of Rakuno Gakuen University, Ebetsu, Japan.

Blood samples were collected from the jugular vein into EDTA-2 potassium containing tubes for the analysis of cytokine mRNA expression of the peripheral blood mononuclear cells (PBMC), as well as counts of total white blood cells (WBC) and lymphocytes. Cell counts were determined by a blood cell counter (pocH-100iV Diff, Sysmex Co., Kobe, Japan).

In order for mRNA isolation and cDNA synthesis, SuperPrep cell lysis & RT kit for qPCR (TOYOBO, Ltd., Osaka, Japan) were used. 2×10^6 cells of each sample were mixed with 50 μl of cell lysis mixture (including gDNA remover) and reacted for 5 min before adding the stop solution. Then 8 μl each of lysis mixture was added into 32 μl of reaction solution. cDNA was synthesized by using PCR. Real-time PCR was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO, Ltd.) on an ABI prism 7300 Sequence Detector (Applied Biosystems, Foster City, CA, USA). The following genes were selected for analysis: β-actin, mRNA expression of IL-2, IL-4, IL-12p40, IL-17A, IL-21, IFN-γ, IL-17 receptor (R) A, CCL3 and CCR5 in PBMC. Primers were designed using the publicly available web based Primer3 program and are listed in Table 1. The Ct values define the threshold cycle of PCR, at which amplified products were detected. The final quantification of immune related molecule mRNA was carried out using the comparative CT (threshold cycle) method. This method was used after a validation experiment, which demonstrated that the efficiencies of the target and reference (β-actin) genes were approximately equal. The final quantification of immune related molecule mRNA was carried out using the comparative CT (threshold cycle) method as reported previously [13]. The calibrator samples used in this study were those with the lowest amount of mRNA for each gene of all the samples used for the respective mRNA.

The presence of *M. bovis* was detected as previously reported [8].

Statistical analysis for each parameter was performed using Tukey-Kramer test in order to determine the differences among the three groups for each parameter. The differences were considered significant at $P<0.05$. Data were expressed as mean ± standard error. For all immune factors, multiple regression analysis was performed using "no-effect of treatment" as an objective variable in the calves with Mycoplasma infection.

Table 2 shows the result of comparative levels of immune factors and counts of leukocytes among three groups. The mRNA levels of IL-17A, IFN-γ and CCR3 in the two groups with mycoplasma infection were significantly lower compared with the Control Group at both the First and Last days. Significantly higher mRNA levels of IL-17A were found in the Non-Recovery group compared with the Recovery Group at both days. The IL-17RA mRNA levels in the Recovery Group were significantly lower than those in the Control Group at both days. However, the differences in the other factors between the groups with mycoplasma infection were not significant, and neither group showed a significant difference compared with the Control Group ($P>0.05$) (Table 2). There were significant differences in leukocyte count among the Recovery Group (109.39 ± 7.00, 139.90 ± 9.16 × $10^2/\mu l$), the Non-Recovery Group (175.15 ± 34.26, 219.15 ± 51.36 × $10^2/\mu l$) and the Control Group (89.70 ± 8.34 × $10^2/\mu l$) at both the First and the Last days (respectively; $P<0.05$). Lymphocyte count in the Recovery Group (34.31 ± 1.53, 34.46 ± 1.05 × $10^2/\mu l$) and

Table 1. Primers used for real-time PCR expression analysis

Gene	Accession number	Product length	Primer designation	Sequence (5′-3′)
IL-2	NM_180997.2	113	Forward	AACCTCAACTCCTGCCACAAT
			Reverse	TCATTGTGTTCCCCGTAGAGC
IL-4	NM_173921	117	Forward	GCCCCAAAGAACACAACTGA
			Reverse	GAGATTCCTGTCAAGTCCGC
IL-12p40	NM_174356.1	101	Forward	CACCCCGCATTCCTACTTCT
			Reverse	TGACTTTGGCTGAGGTTTGG
IL-17A	NM_001008412.2	138	Forward	ATCTCACAGCGAGCACAAGT
			Reverse	GTGGGATGATGACTCCTGCC
IL-17RA	XM_010827963.1	97	Forward	TTCAGCAGCTGCCTCAACG
			Reverse	GGGGCGTATGGTCTTCATTG
IL-21	NM_198832.1	144	Forward	CAGCTCCAGAAGATGTAAAGAGAC
			Reverse	TGGCAGGTAGTTTCCTCTTCA
IFN-γ	NM_174086	108	Forward	TCAAATTCCGGTGGATGATCT
			Reverse	CTTCTCTTCCGCTTTCTGAGG
CCL3	NM_174511.2	117	Forward	CTGACGCTCAAGCCCACACT
			Reverse	CGCCAAATGGTGCCGAGAAG
CCR5	NM_001011672.2	90	Forward	GGCTCCCTACAACATCGTCC
			Reverse	GCTTGGTCCAGCCTGTTAGA
β-actin	NM_173979.3	76	Forward	CCCAGATCATGTTCGAGACC
			Reverse	GAGGCATACAGGGACAGCAC

Table 2. Cytokine mRNA expression of peripheral blood mononuclear cells in three groups

Particle	Sampling day	Unfavorable group (N=13)	Favorable group (N=51)	*P* value	Control group (N=14)
IL-2	First	3.20 ± 0.23	3.24 ± 0.18		4.54 ± 0.46
	Last	3.05 ± 0.31	2.86 ± 0.18		
IL-4	First	8.37 ± 0.48	8.24 ± 0.25		9.24 ± 0.57
	Last	7.20 ± 0.75	8.52 ± 0.22		
IL-12p40	First	5.25 ± 0.55	4.27 ± 0.26		5.21 ± 0.42
	Last	5.94 ± 0.59	4.60 ± 0.22		
IL-17A	First	8.86 ± 0.79 a)	7.52 ± 0.32 a)	*	11.17 ± 0.43 b)
	Last	8.67 ± 0.25 a)	7.49 ± 0.35 a)	*	
IL-17R	First	4.94 ± 0.32	4.37 ± 0.15 a)		6.63 ± 0.46 b)
	Last	5.11 ± 0.28	4.10 ± 0.13 a)		
IL-21	First	7.26 ± 0.78 a)	8.10 ± 0.24		9.53 ± 0.51 b)
	Last	8.41 ± 0.36	7.91 ± 0.21		
IFN-γ	First	10.32 ± 0.38 a)	9.93 ± 0.27 a)		13.15 ± 0.43 b)
	Last	10.56 ± 0.61 a)	9.80 ± 0.38 a)		
CCL3	First	8.13 ± 0.57	6.75 ± 0.31		8.23 ± 0.40
	Last	8.23 ± 0.53	6.52 ± 0.28		
CCR5	First	5.09 ± 0.43 a)	4.97 ± 0.21 a)		7.84 ± 0.65 b)
	Last	5.20 ± 0.49 a)	4.94 ± 0.22 a)		

Data are expressed as the mean ± SE (target gene mRNA/β-actin gene mRNA). Values denote relative expression of each parameters mRNA. Different letters indicate significant difference among groups ($P<0.05$). *P* value means significant difference between two infected groups.

the Non-Recovery Group (30.28 ± 2.68, 28.69 ± 1.92 × $10^2/\mu l$) were significantly lower compared with the Control Group (42.37 ± 3.01 × $10^2/\mu l$) at both sampling days ($P<0.05$ for both days). But a significant difference in the numbers of lymphocytes and granulocytes was not found between two infected groups (data not shown).

Mycoplasma induces a broad range of immunomodulatory events by directly affecting on macrophages, neutrophils, and lymphocytes, and by indirectly affecting through induction of cytokine secretion from immune cells [16]. In the calves with experimental *M. bovis* respiratory infection, markedly increase in the blood IL-17A level was detected at 14 days after infection, but no significant difference of IL-17A at 7 days [1]. It was reported that the expression levels of IL17A and IFN-β in bronchoalveolar lavage fluid decreased in the severe *M. pneumoniae* pneumonia children comparing to healthy children [23]. Since *M. bovis* causes the downregulation of lymphocyte proliferative responses to various mitogens *in vitro* [19], *M. bovis* appears to directly suppress lymphocyte function. Vanden Bush [20] indicated that *M. bovis* induces apoptotic death of bovine lymphocytes. In experimental sheep nasally infected with *M. agalactiae*, a prolonged depletion of peripheral T lymphocytes was induced [14]. We found lower mRNA levels of IL-17A, IFN-γ and CCR5 and counts of lymphocyte in the two groups with *M. bovis* infection compared to those in the Control Group. Suppression of lymphocyte function might have lowered several cytokines mRNA expression in the calves with *M. bovis* infection. Previous study indicated that experimental lung infection of cattle with *M. bovis* resulted in a Th2-skewed immune response [21]. Kurata *et al.* [12] showed that both Transforming Growth Factor (TGF)-β and IL-10 were involved in the suppression of IL-17A production in mouse with *M. pneumoniae* antigens stimulation both *in vivo* and *in vitro*. On the other hand, CCR5 is predominantly expressed on T cells, as a receptor to mediate chemotactic activity [17]. This study observed the lower mRNA level of CCR5 in the two groups with mycoplasma infection. Since CCR5 is required for the Th1 cell-selective transmigration [11], it is possible that lower CCR5 decreased IFN-γ production by T cells in the calves with *M. bovis* infection. Therefore, the imbalance among Th1 cells, Th2 cells and Th17 cells might influence pathophysiological mechanisms or disease outcomes in *M. bovis* infection of calves.

This study observed a significant difference in IL-17A at the First day using the multiple regression analysis with no-effect of treatment as the dependent variable. Previous study indicated higher frequencies of Th17 cell percentage in PBMC and higher levels of serum IL-17 were detected in patients with refractory *M. pneumoniae* infections [7]. This study indicated no increase of TGF-β level and peripheral lymphocyte count in the patients with *M. pneumoniae* pneumonia, and we speculated that in imbalance of circulating effector subset of $CD4^+$ T cells might be associated with the deterioration of pulmonary injury in patients with *M. pneumoniae* infections. Although IL-17A mRNA levels were lower in two infected groups compared with that in the Control Group in our present study, the imbalance among effector T cells might have influenced disease outcomes in mycoplasma infection of calves.

IL-17RA is expressed in leucocytes, epithelial cells or other cells [9]. Signaling downstream of IL-17RA mediates NF-κB activation, leading to the production of pro-inflammatory cytokines and chemokines [18]. It has been demonstrated in infectious models in which neutrophils are crucial for host defense, that IL-17RA deficiency results in reduced chemokine levels, reduced neutrophil numbers, and increased susceptibility to infection [3, 24]. Since a lack of IL-17RA expression was found in the affected

child with chronic infections of *Candida albicans* and *Staphylococcus aureus* [5], they suggested that decrease in IL-17A and IL-17RA might imply a severe malfunction in the IL-17 signaling pathway, conferring susceptibility to refractory infections. Since binding IL-17RA to IL-17A is critical for host defense against extracellular planktonic bacteria [10], decrease in IL-17A and IL-17RA might result in the reduction immune response to bacteria. But its function and regulation have not been clarified in bovine, further study needs to clarify the decreased levels of IL-17A and IL-17RA in the calves with *M. bovis* infections.

In conclusion, the alteration of IL-17A in PBMC might have biased function in host immune response in the calves with mycoplasma infection in the present study. Since neutralization of IL-17A after infiltration of T cells into lungs reduced disease severity in mice experiment, IL-17A is identified as a pivotal pathological element of murine respiratory mycoplasma infection [15]. More layers of investigation are necessary to uncover the ways the adaptive immune system of T cell effector responses in the calves with mycoplasma.

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REFERENCES

1. Chao, J., Han, X., Liu, K., Li, Q., Peng, Q., Lu, S., Zhu, X., Hu, G., Dong, Y., Hu, C., Chen, Y., Chen, J., Khan, F. A., Chen, H. and Guo, A. A. 2019. Calves infected with virulent and attenuated *Mycoplasma bovis* strains have upregulated Th17 inflammatory and Th1 protective responses, respectively. *Genes (Basel)* **10**: 10. [Medline] [CrossRef]
2. Castillo-Alcala, F., Bateman, K. G., Cai, H. Y., Schott, C. R., Parker, L., Clark, M. E., McRaild, P., McDowall, R. M., Foster, R. A., Archambault, M. and Caswell, J. L. 2012. Prevalence and genotype of *Mycoplasma bovis* in beef cattle after arrival at a feedlot. *Am. J. Vet. Res.* **73**: 1932–1943. [Medline] [CrossRef]
3. Crowe, C. R., Chen, K., Pociask, D. A., Alcorn, J. F., Krivich, C., Enelow, R. I., Ross, T. M., Witztum, J. L. and Kolls, J. K. 2009. Critical role of IL-17RA in immunopathology of influenza infection. *J. Immunol.* **183**: 5301–5310. [Medline] [CrossRef]
4. Dudek, K., Bednarek, D., Ayling, R. D. and Szacawa, E. 2013. Immunomodulatory effect of *Mycoplasma bovis* in experimentally infected calves. *Bull. Vet. Inst. Pulawy* **57**: 499–506. [CrossRef]
5. Fellmann, F., Angelini, F., Wassenberg, J., Perreau, M., Arenas Ramirez, N., Simon, G., Boyman, O., Demaria, O., Christen-Zaech, S., Hohl, D., Belfiore, M., von Scheven-Gete, A., Gilliet, M., Bochud, P. Y., Perrin, Y., Beck Popovic, M., Bart, P. A., Beckmann, J. S., Martinet, D. and Hofer, M. 2016. IL-17 receptor A and adenosine deaminase 2 deficiency in siblings with recurrent infections and chronic inflammation. *J. Allergy Clin. Immunol.* **137**: 1189–1196.e2. [Medline] [CrossRef]
6. Gondaira, S., Higuchi, H., Iwano, H., Nakajima, K., Kawai, K., Hashiguchi, S., Konnai, S. and Nagahata, H. 2015. Cytokine mRNA profiling and the proliferative response of bovine peripheral blood mononuclear cells to *Mycoplasma bovis*. *Vet. Immunol. Immunopathol.* **165**: 45–53. [Medline] [CrossRef]
7. Guo, H., He, Z., Li, M., Wang, T. and Zhang, L. 2016. Imbalance of peripheral blood Th17 and Treg responses in children with refractory *Mycoplasma pneumoniae* pneumonia. *J. Infect. Chemother.* **22**: 162–166. [Medline] [CrossRef]
8. Higuchi, H., Gondaira, S., Iwano, H., Hirose, K., Nakajima, K., Kawai, K., Hagiwara, K., Tamura, Y. and Nagahata, H. 2013. Mycoplasma species isolated from intramammary infection of Japanese dairy cows. *Vet. Rec.* **172**: 557. [Medline] [CrossRef]
9. Ho, A. W. and Gaffen, S. L. 2010. IL-17RC: a partner in IL-17 signaling and beyond. *Semin. Immunopathol.* **32**: 33–42. [Medline] [CrossRef]
10. Jin, W. and Dong, C. 2013. IL-17 cytokines in immunity and inflammation. *Emerg. Microbes Infect.* **2**: e60. [Medline] [CrossRef]
11. Kawai, T., Seki, M., Hiromatsu, K., Eastcott, J. W., Watts, G. F., Sugai, M., Smith, D. J., Porcelli, S. A. and Taubman, M. A. 1999. Selective diapedesis of Th1 cells induced by endothelial cell RANTES. *J. Immunol.* **163**: 3269–3278. [Medline]
12. Kurata, S., Osaki, T., Yonezawa, H., Arae, K., Taguchi, H. and Kamiya, S. 2014. Role of IL-17A and IL-10 in the antigen induced inflammation model by *Mycoplasma pneumoniae*. *BMC Microbiol.* **14**: 156. [Medline] [CrossRef]
13. Maeda, Y., Ohtsuka, H., Tomioka, M. and Oikawa, M. 2013. Effect of progesterone on Th1/Th2/Th17 and regulatory T cell-related genes in peripheral blood mononuclear cells during pregnancy in cows. *Vet. Res. Commun.* **37**: 43–49. [Medline] [CrossRef]
14. Marinaro, M., Greco, G., Tarsitano, E., Ventrella, G., Camero, M., Corrente, M., Rezza, G. and Buonavoglia, D. 2015. Changes in peripheral blood leucocytes of sheep experimentally infected with *Mycoplasma agalactiae*. *Vet. Microbiol.* **175**: 257–264. [Medline] [CrossRef]
15. Mize, M. T., Sun, X. L. and Simecka, J. W. 2018. Interleukin-17A exacerbates disease severity in BALB/c mice susceptible to lung infection with *Mycoplasma pulmonis*. *Infect. Immun.* **86**: 86. [Medline] [CrossRef]
16. Rodríguez, F., Castro, P., Poveda, J. B., Afonso, A. M. and Fernández, A. 2015. Immunohistochemical labelling of cytokines in calves infected experimentally with *Mycoplasma bovis*. *J. Comp. Pathol.* **152**: 243–247. [Medline] [CrossRef]
17. Samson, M., Labbe, O., Mollereau, C., Vassart, G. and Parmentier, M. 1996. Molecular cloning and functional expression of a new human CC-chemokine receptor gene. *Biochemistry* **35**: 3362–3367. [Medline] [CrossRef]
18. Shalom-Barak, T., Quach, J. and Lotz, M. 1998. Interleukin-17-induced gene expression in articular chondrocytes is associated with activation of mitogen-activated protein kinases and NF-kappaB. *J. Biol. Chem.* **273**: 27467–27473. [Medline] [CrossRef]
19. Thomas, C. B., Mettler, J., Sharp, P., Jensen-Kostenbader, J. and Schultz, R. D. 1990. *Mycoplasma bovis* suppression of bovine lymphocyte response to phytohemagglutinin. *Vet. Immunol. Immunopathol.* **26**: 143–155. [Medline] [CrossRef]
20. Vanden Bush, T. J. and Rosenbusch, R. F. 2002. *Mycoplasma bovis* induces apoptosis of bovine lymphocytes. *FEMS Immunol. Med. Microbiol.* **32**: 97–103. [Medline] [CrossRef]
21. Vanden Bush, T. J. and Rosenbusch, R. F. 2003. Characterization of the immune response to *Mycoplasma bovis* lung infection. *Vet. Immunol. Immunopathol.* **94**: 23–33. [Medline] [CrossRef]
22. Walz, P. H., Mullaney, T. P., Render, J. A., Walker, R. D., Mosser, T. and Baker, J. C. 1997. Otitis media in preweaned Holstein dairy calves in Michigan due to Mycoplasma bovis. *J. Vet. Diagn. Invest.* **9**: 250–254. [Medline] [CrossRef]
23. Yang, M., Meng, F., Wang, K., Gao, M., Lu, R., Li, M., Zhao, F., Huang, L., Zhang, Y., Cheng, G. and Wang, X. 2017. Interleukin 17A as a good predictor of the severity of *Mycoplasma pneumoniae* pneumonia in children. *Sci. Rep.* **7**: 12934. [Medline] [CrossRef]
24. Ye, P., Rodriguez, F. H., Kanaly, S., Stocking, K. L., Schurr, J., Schwarzenberger, P., Oliver, P., Huang, W., Zhang, P., Zhang, J., Shellito, J. E., Bagby, G. J., Nelson, S., Charrier, K., Peschon, J. J. and Kolls, J. K. 2001. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J. Exp. Med.* **194**: 519–527. [Medline] [CrossRef]