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Comparison of blood parameters according to fecal detection of *Mycobacterium avium* subspecies *paratuberculosis* in subclinically infected Holstein cattle

Seungmin Ha [1], **Seogjin Kang** [1], **Mooyoung Jung** [1], **Sang Bum Kim** [1], **Han Gyu Lee** [1], **Hong-Tae Park** [2], **Jun Ho Lee** [2], **Ki Choon Choi** [1], **Jinho Park** [3], **Ui-Hyung Kim** [1,*], **Han Sang Yoo** [2,*]

[1]National Institute of Animal Science, Rural Development Administration, Cheonan 31000, Korea
[2]Department of Infectious Diseases, College of Veterinary Medicine, Seoul National University, Seoul 08826, Korea
[3]Department of Veterinary Internal Medicine, College of Veterinary Medicine, Jeonbuk National University, Iksan 54596, Korea

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***Corresponding authors:**
Ui-Hyung Kim
National Institute of Animal Science, Rural Development Administration, 114 Sinbang 1-gil, Seonghwan-eup, Seobuk-gu, Cheonan 31000, Korea.
Email: uhkim@korea.kr
https://orcid.org/0000-0002-2197-5080

Hang Sang Yoo
Department of Infectious Diseases, College of Veterinary Medicine, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Korea.
Email: yoohs@snu.ac.kr
https://orcid.org/0000-0003-0548-0835

ABSTRACT

Background: *Mycobacterium avium* subspecies *paratuberculosis* (MAP) causes a chronic and progressive granulomatous enteritis and economic losses in dairy cattle in subclinical stages. Subclinical infection in cattle can be detected using serum MAP antibody enzyme-linked immunosorbent assay (ELISA) and fecal polymerase chain reaction (PCR) tests.
Objectives: To investigate the differences in blood parameters, according to the detection of MAP using serum antibody ELISA and fecal PCR tests.
Methods: We divided 33 subclinically infected adult cattle into three groups: seronegative and fecal-positive (SNFP, n = 5), seropositive and fecal-negative (SPFN, n = 10), and seropositive and fecal-positive (SPFP, n = 18). Hematological and serum biochemical analyses were performed.
Results: Although the cows were clinically healthy without any manifestations, the SNFP and SPFP groups had higher platelet counts, mean platelet volumes, plateletcrit, lactate dehydrogenase levels, lactate levels, and calcium levels but lower mean corpuscular volume concentration than the SPFN group ($p < 0.017$). The red blood cell count, hematocrit, monocyte count, glucose level, and calprotectin level were different according to the detection method ($p < 0.05$). The SNFP and SPFP groups had higher red blood cell counts, hematocrit and calprotectin levels, but lower monocyte counts and glucose levels than the SPFN group, although there were no significant differences ($p > 0.017$).
Conclusions: The cows with fecal-positive MAP status had different blood parameters from those with fecal-negative MAP status, although they were subclinically infected. These findings provide new insights into understanding the mechanism of MAP infection in subclinically infected cattle.

Keywords: Johne's disease; subclinical infection; blood cells; blood chemical analysis; Bos taurus

ORCID iDs

Seungmin Ha
https://orcid.org/0000-0002-5152-1979
Seogjin Kang
https://orcid.org/0000-0001-7838-9042
Mooyoung Jung
https://orcid.org/0000-0001-7396-6787
Sang Bum Kim
https://orcid.org/0000-0002-8187-4134
Han Gyu Lee
https://orcid.org/0000-0002-3531-1971
Hong-Tae Park
https://orcid.org/0000-0003-3415-2339
Jun Ho Lee
https://orcid.org/0009-0008-8953-6413
Ki Choon Choi
https://orcid.org/0000-0001-8071-5097
Jinho Park
https://orcid.org/0000-0001-5235-5717
Ui-Hyung Kim
https://orcid.org/0000-0002-2197-5080
Han Sang Yoo
https://orcid.org/0000-0003-0548-0835

Author Contributions

Conceptualization: Ha S, Kim UH, Yoo HS; Data curation: Ha S, Kang S, Jung M; Formal analysis: Ha S; Funding acquisition: Kim S, Kim UH; Investigation: Ha S, Kang S, Jung M, Lee HG, Park HT, Lee JH; Methodology: Ha S, Choi KC, Park J; Project administration: Kim SB, Kim UH; Resources: Kim SB; Software: Ha S; Supervision: Yoo HS; Validation: Ha S, Kim UH, Yoo HS; Visualization: Ha S; Writing - original draft: Ha S; Writing - review & editing: KIM UH, Yoo HS.

Conflict of Interest

The authors declare no conflicts of interest.

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INTRODUCTION

Mycobacterium avium subspecies *paratuberculosis* (MAP) is an obligate intracellular and gram-positive acid-fast bacterium that causes a chronic and progressive granulomatous enteritis known as Johne's disease (JD) or paratuberculosis in dairy cattle [1,2]. MAP has a thick lipid-rich wall that enables resistance to degradation by the host immune system and the environment outside of cattle [3,4]. The MAP shed via feces of the host can persist for 120 weeks in soil and water [5]. MAP can be transmitted via the fecal-oral route, in-utero, and via milk and colostrum [6]. Johne's disease is not inevitably caused by MAP infection; the clinical manifestation depends on other factors including immunity, parturition, lactation, and age [7]. Cattle show symptoms in 3–5 years after the infection of MAP given its long asymptomatic latency period [8]. Subclinically infected cows can also shed MAP through feces and milk [9]. Currently, there are no effective MAP vaccines or treatments for JD [7]. Therefore, it is difficult to accomplish disease control, management and eradication.

MAP causes considerable economic losses through decreased production and disease control. Subclinically infected cows show no other manifestations except for reduced milk yield. Hence, subclinical cases go unnoticed, resulting in a continuous economic impact [7]. Clinically infected cows develop many signs including reduced milk yield, diarrhea, edema, infertility, and weight loss [10]. Subclinically and clinically infected cows show over 15% reduction in milk yield [11]. The costs for monitoring, diagnosis, culling, prevention, and replacing cows also lead to economic losses [11].

Presently, MAP infection is detected using fecal culture, histopathological examination, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) [12]. Culturing MAP from infected tissues is considered the most accurate method of pathogen detection, but the process of culturing and isolating MAP is expensive and requires intensive labor, time, and multiple tissues samples [13]. Histopathological examination uses acid-fast staining; acid-fasting staining has the following drawbacks: non-specific staining of other acid-fast bacterial species and requirement of over 10^6 MAP organisms per gram of tissue [14,15]. ELISA and PCR are commonly used as alternative methods to detect MAP due to relative simplicity, rapidness, and cost-effectiveness [12,16].

Hematology and serum biochemistry analyses are used to assess the health and physiological status of cows, understand pathogenesis, confirm the diagnosis, and select treatments in bovine medicine. Significant differences in the hematological and serum biochemical parameters were observed in cases of clinical MAP infection in cattle presenting diarrhea [17-19]. Hematological and serum biochemical parameter levels in subclinical cases of MAP infection in cattle are similar to those in cattle without MAP infection, but different from those in clinical cases of MAP infection in cattle. However, subclinical cases of MAP infection in cattle show differences in blood protein levels according to results of serum MAP antibody ELISA and quantitative PCR tests [20] suggesting that there might be differences in hematological and serum biochemical parameters in subclinical cases of MAP infection in cattle according to the utilized detection methods.

To the best of our knowledge, the differences in the hematological and serum biochemical parameters have not been investigated in subclinical cases of MAP infection in Holstein cattle using serum MAP antibody ELISA and fecal PCR tests. We hypothesized that the differences in the detection of MAP infection may reflect the impact of the pathogen on the host. Thus,

the present study aimed to determine the differences in the hematological and serum biochemical parameters according to the detection method of MAP infection.

MATERIALS AND METHODS

Study design

The present study aimed to evaluate blood parameters according to the fecal detection of MAP in clinically healthy Holstein cattle. Once seropositive cattle were observed, we randomly chose cattle among seronegative cattle that had similar characteristics in the same barn as comparative and reference groups. Blood and fecal samples were collected on the day when we identified seropositive cattle. Then, the cattle were divided into three groups: seronegative and fecal-positive, seropositive and fecal-negative, and seropositive and fecal-positive group. We used seronegative and fecal-negative cattle as the reference group by excluding them in the comparison, since it was unclear whether the animals were uninfected with MAP or in the undetectable silent infection phase.

Animals and sample collection

The Holstein cattle, used in this study, were female and raised on a farm in the Republic of Korea. The farm had implemented a test-and-slaughter strategy to eradicate MAP. All cattle, older than 6 months, were tested with serological test using the *Mycobacterium paratuberculosis* antibody test kit (IDEXX, USA). We performed serological tests as the screening before the sample collection. We randomly selected seronegative cattle among cattle in similar circumstances to those of seropositive cattle, such as barn, age, parity, and pregnancy. Only non-pregnant animals were used in this study. They were clinically healthy without clinical signs of MAP, such as diarrhea, weight loss, pallor, lethargy, and emaciation. We obtained the blood and fecal samples from 59 clinically healthy cattle. Blood was drawn from the jugular vein and collected using ethylenediaminetetraacetic acid and serum-separating tubes. Fecal samples were collected from the rectum.

Blood analyses

The complete blood count (CBC) was analyzed using a hematology analyzer (Procyte Dx hematology analyzer, IDEXX Laboratories, USA). The CBC profile included three types of parameters: the erythrocyte parameters included red blood cell (RBC) count, hematocrit (HCT), hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width, and reticulocyte count. The leukocyte parameters included white blood cell, neutrophil, lymphocyte, monocyte, eosinophil, and basophil counts. The platelet parameters included platelet count, mean platelet volume (MPV), platelet distribution width, and plateletcrit (PCT).

The serum was separated by centrifuging the serum-separating tubes at 3,000 rpm (2,600g) for 10 min. The serum was frozen and stored at –70°C until analysis and the serum biochemical and mineral analyses were conducted on a single day using two biochemistry automatic analyzers (CatalystDx chemistry analyzer, IDEXX Laboratories and Hitachi Labospect 006, Hitachi Ltd., Japan). Sodium (Na), potassium (K), sodium/potassium (Na/K), Chloride (Cl), and lactate level were measured using a Catalyst Dx chemistry analyzer. Glucose, non-esterified fatty acids (NEFA), triglyceride, total cholesterol (TC), total protein (TP), albumin, blood urea nitrogen (BUN), creatinine, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT),

lactate dehydrogenase (LDH), creatine kinase (CK), calcium (Ca), magnesium (Mg), and inorganic phosphorus (P) levels were measured using Hitachi Labospect 006 after calibration and quality control assessments with commercial enzyme assay kits (Fujifilm Wako Pure Chemical Ltd., Japan; Shino-Test Corporation, Japan; Sekisui Medical Co. Ltd., Japan). The globulin level was calculated by subtracting the level of albumin from TP level.

The MAP test kit used was the *Mycobacterium paratuberculosis* antibody test kit (IDEXX). The levels of C-reactive protein, calprotectin, lactoferrin, and haptoglobin were determined using cattle C-reactive protein ELISA kit (Cat. No. MBS2702533, MyBioSource, USA), bovine calprotectin ELISA kit (Cat. No. MBS020907, MyBioSource), bovine lactoferrin ELISA kit (Cat. No. E11-126, Bethyl Laboratories, USA), and bovine haptoglobin ELISA kit (Cat. No. BCH61-K01, Eagle Biosciences, USA), respectively. The testing procedure was performed in accordance with the manufacturer's instructions.

The ELISA results were read from a microplate photometer, Tecan Nano Quant infinite M200 PRO Plate Reader (Tecan, Männedorf, Switzerland) that measured optical density at 450 nm wavelength. The presence or absence of the antibody to MAP was determined by the sample to positive (S/P) ratio. The results were interpreted in accordance with the manufacturer's test instructions as follows: S/P ratio ≤ 0.45 = negative, 0.45 < S/P ratio < 0.55 = suspect, and S/P ratio ≥ 0.55 = positive.

Fecal detection

On fecal testing for MAP, two MAP-specific targets, IS900 and ISMap02, were detected in the DNA extracted by real-time PCR, and the samples positive for both targets were considered positive for MAP infection. DNA was extracted from feces using a method described in a previous study [21]. Primers for IS900 and ISMap02 were used as previously described by Park et al. [21] and Sevilla et al. [22], respectively. Real-time PCR was performed in duplex using the TaqMan probe method, and the reaction mixture for the PCR contained 1 × Rotor-Gene Probe PCR master mix (Qiagen Inc., USA), 400 nM forward and reverse primers, 100 nM probe, and 4 μL of template solution. The final amount of 20 μL was prepared by adding distilled water. The PCR conditions were as follows: a total of 45 cycles of initial denaturation at 95°C for 5 min, annealing at 95°C for 15 sec, and 60°C for 1 min.

Experimental group definition

The Holstein cattle were classified according to the serological and fecal test results of MAP into the following group: seronegative and fecal-positive (SNFP, n = 5), seropositive and fecal-negative (SPFN, n = 10), and seropositive and fecal-positive (SPFP, n = 18). The parameters of the cattle in the seronegative and fecal-negative (SNFN, n = 26) group were used as the reference (**Table 1**).

Statistical analyses

Statistical analyses were performed using the SPSS software (version 27.0; IBM Corp., USA). The Kruskal–Wallis test and the Mann–Whitney U with Bonferroni's method were used. The chi-square test was used to determine the associations between the types of cattle (non-milking and milking cattle) and the classifications (SNFP, SPFN, and SPFP). Data were expressed as mean ± SD. Statistical significance was set at $p < 0.05$ for the Kruskal–Wallis test and $p < 0.017$ for the Mann–Whitney U with the Bonferroni's method.

Table 1. Descriptive statistics for Holstein cattle according to each group in the study

Variables	SNFP	SPFN	SPFP	*p* value	SNFN
Number	5	10	18		26
Non-lactating cattle	4 (80.0%)	7 (70.0%)	13 (72.2%)	0.917	17 (65.4%)
Lactating cattle	1 (20.0%)	3 (30.0%)	5 (27.8%)		9 (34.6%)
Age (yr)	5.7 ± 0.5	5.8 ± 1.6	5.3 ± 1.4	0.517	5.4 ± 1.7
Parity	0.2 ± 0.4	1.7 ± 1.1	1.1 ± 1.5	0.078	1.7 ± 1.2
S/P ratio	0.236 ± 0.160[a]	0.729 ± 0.200[b]	1.010 ± 0.508[b]	< 0.001	0.168 ± 0.108

The total of each level was 100% because the total number of each level was different. The parameters of SNFN were used as the reference.
Data obtained using the Kruskal–Wallis test are expressed as mean ± standard deviation.
Cross-tabulation analysis between each group and the type of cattle was performed using the χ^2 test.
SNFP, seronegative and fecal-positive for *Mycobacterium avium subspecies paratuberculosis*; SPFN, seropositive and fecal-negative for *Mycobacterium avium subspecies paratuberculosis*; SPFP, seropositive and fecal-positive for *Mycobacterium avium subspecies paratuberculosis*; SNFN, seronegative and fecal-negative for *Mycobacterium avium subspecies paratuberculosis*; S/P ratio; sample-to-positive ratio.
[a,b]Different letters in the same row indicate significant differences ($p < 0.017$, Mann-Whitney U test with Bonferroni's method).

Ethics approval

This research was approved by the Institutional Animal Care and Use Committee (IACUC) at the National Institute of Animal Science, the Republic of Korea (approved number: NIAS-2020127). All experimental procedures involving animals were conducted in strict accordance with relevant guidelines and regulations. All infected cattle had naturally occurring infections.

RESULTS

Descriptive statistics according to serological and fecal tests for of MAP detection

The cattle of each group (SNFP, SPFN, and SPFP) were not different without S/P ratio of MAP. The groups had similar ratios of non-lactating cattle to lactating cattle, with more non-lactating cattle than lactating cattle ($p > 0.05$). The age of each group was approximately 5 years. The parities between groups were not statistically different ($p > 0.05$). The S/P ratio of the SNFP group (0.236 ± 0.160) was lower than the ratios of the SPFN (0.729 ± 0.200) and SPFP (1.010 ± 0.508) groups ($p < 0.017$) (**Table 1**).

Association between fecal detection of MAP and hematological parameters

Regarding hematological parameters, the groups showed differences in RBC count, HCT, MCHC, monocyte count, platelet count, MPV, and PCT values ($p < 0.05$). The SNFP and the SPFP groups had higher hemoglobin than the SPFN group; however, these results were not significant ($p = 0.073$). The SNFP group had higher RBC count (7.0 ± 0.6 M/μL), HCT (35.1 ± 2.7%), platelet count (420.2 ± 29.7 K/μL), MPV (10.5 ± 0.6 fL), and PCT (0.4 ± 0.0%) and lower MCHC (33.5 ± 0.8 g/dL) values than the SPFN group (5.7 ± 0.7 M/μL, 29.4 ± 3.0%, 243.0 ± 72.0 K/μL, 7.5 ± 1.6 fL, 0.2 ± 0.1, and 35.1 ± 0.8 g/dL, respectively) ($p < 0.017$). The SPFP had lower MCHC (34.0 ± 0.6 g/dL) and monocyte (1.0 ± 0.4 K/μL) and higher platelet counts (385.0 ± 101.9 K/μL), MPV (10.1 ± 1.1 fL), and PCT (0.4 ± 0.1%) values than the SPFN group ($p < 0.017$). Although not at a significant level, the SNFP group had lower monocyte count (1.0 ± 0.1 K/μL) ($p = 0.04$) than the SPFN group (1.5 ± 0.7 K/μL) and the SPFP group had higher RBC count (6.4 ± 0.7 M/μL) ($p = 0.024$) than the SPFN group (**Fig. 1**).

Association between fecal detection of MAP and serum biochemical parameters

Regarding serum biochemical parameters, the levels of glucose, LDH, lactate, Ca, and calprotectin were different among the groups ($p < 0.05$). However, significant differences were not observed in NEFA, TG, TC, TP, albumin, globulin, BUN, creatinine, ALT, AST, GGT,

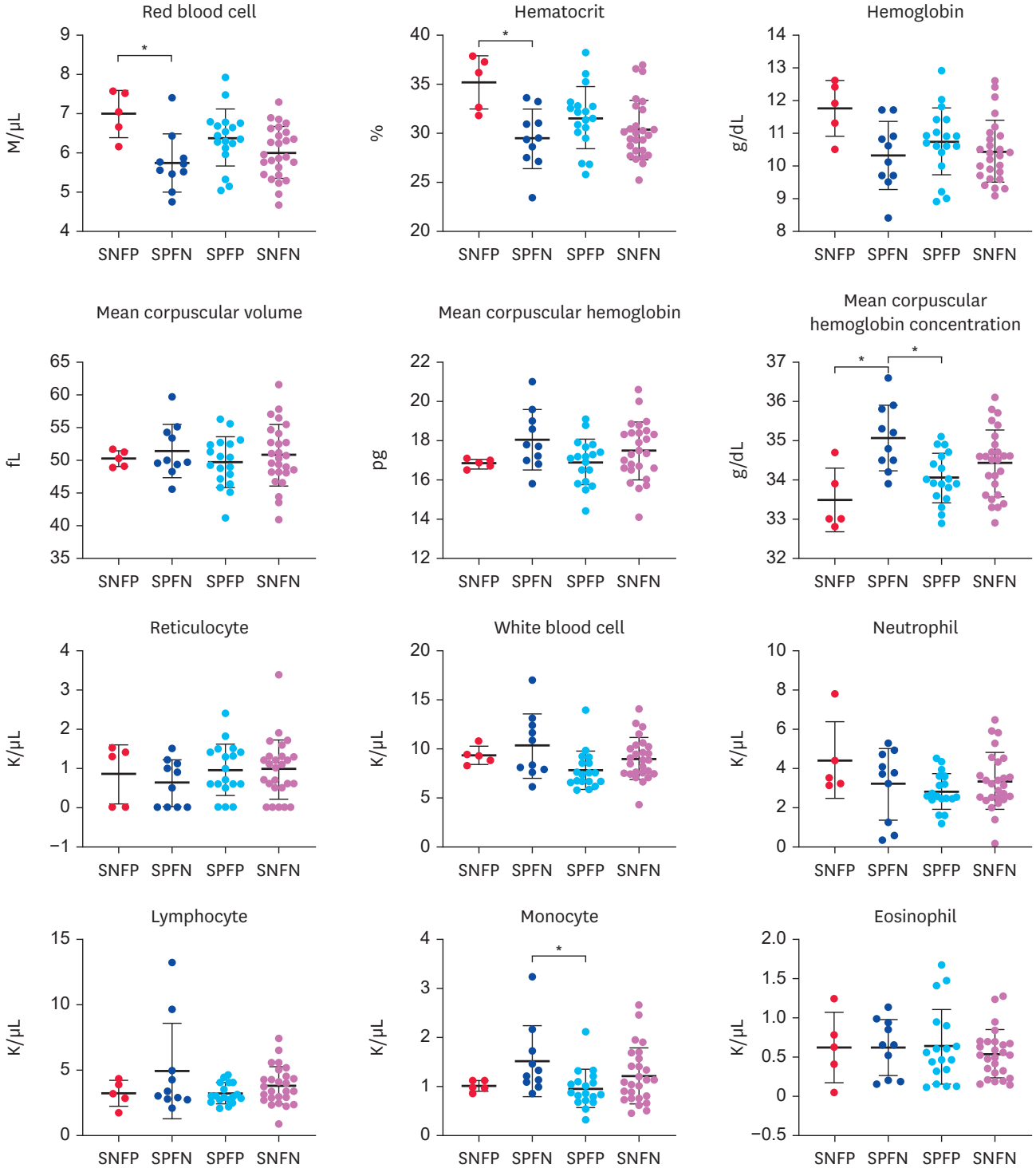

Fig. 1. Complete blood count results according to the detection methods of *Mycobacterium avium* subspecies *paratuberculosis* (n = 33: SNFP, 5; SPFN, 10; SPFP, 18). The parameters of SNFN were used as reference.
SNFP, seronegative and fecal-positive for *Mycobacterium avium subspecies paratuberculosis*; SPFN, seropositive and fecal-negative for *Mycobacterium avium subspecies paratuberculosis*; SPFP, seropositive and fecal-positive for *Mycobacterium avium subspecies paratuberculosis*; SNFN, seronegative and fecal-negative for *Mycobacterium avium subspecies paratuberculosis*; S/P ratio; sample-to-positive ratio.
*$p < 0.017$.

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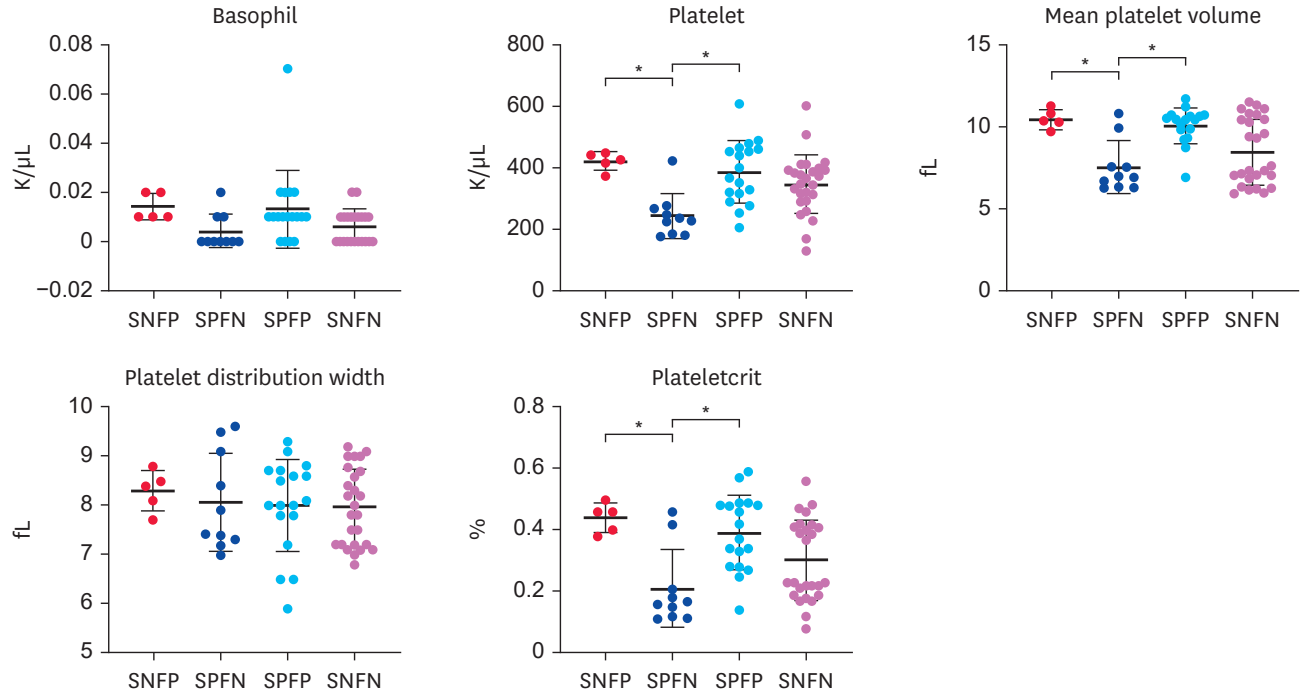

Fig. 1. (Continued) Complete blood count results according to the detection methods of *Mycobacterium avium* subspecies *paratuberculosis* (n = 33: SNFP, 5; SPFN, 10; SPFP, 18). The parameters of SNFN were used as reference.
SNFP, seronegative and fecal-positive for *Mycobacterium avium subspecies paratuberculosis*; SPFN, seropositive and fecal-negative for *Mycobacterium avium subspecies paratuberculosis*; SPFP, seropositive and fecal-positive for *Mycobacterium avium subspecies paratuberculosis*; SNFN, seronegative and fecal-negative for *Mycobacterium avium subspecies paratuberculosis*; S/P ratio; sample-to-positive ratio.
*$p < 0.017$.

CK, Na, K, Na/K, Cl, Mg, P, c-reactive protein, lactoferrin, and haptoglobin levels ($p > 0.05$). The SNFP and the SPFP groups had higher levels of LDH, lactate, and Ca than the SPFN group ($p < 0.017$). The SNFP group had lower level of glucose (51.2 ± 4.8 mg/dL, $p = 0.019$) and higher level of calprotectin (40.7 ± 4.9 ng/mL, $p = 0.019$) than the SPFN group (61.7 ± 6.2 mg/dL, 21.8 ± 13.2 ng/mL, respectively). The SPFP group also had lower level of glucose (56.0 ± 8.8 mg/dL, $p = 0.057$) and higher level of calprotectin (39.6 ± 22.5 ng/mL $p = 0.023$) than the SPFN group; however, these results were not significant (**Figs. 2**, **3**, and **4**).

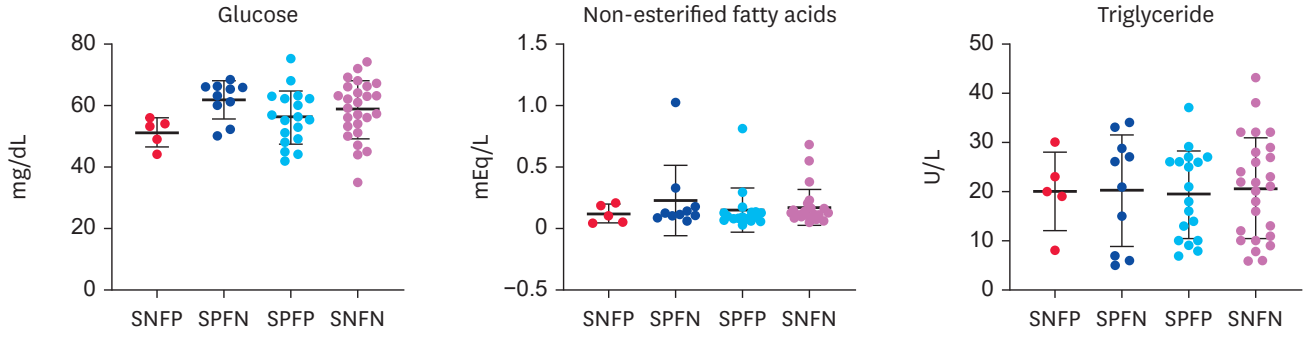

Fig. 2. Serum biochemical parameters results related to metabolism according to the detection methods of *Mycobacterium avium* subspecies *paratuberculosis* (n = 33: SNFP, 5; SPFN, 10; SPFP, 18). The parameters of SNFN were used as the reference.
SNFP, seronegative and fecal-positive for *Mycobacterium avium subspecies paratuberculosis*; SPFN, seropositive and fecal-negative for *Mycobacterium avium subspecies paratuberculosis*; SPFP, seropositive and fecal-positive for *Mycobacterium avium subspecies paratuberculosis*; SNFN, seronegative and fecal-negative for *Mycobacterium avium subspecies paratuberculosis*; S/P ratio; sample-to-positive ratio.
*$p < 0.017$; **$p < 0.003$.

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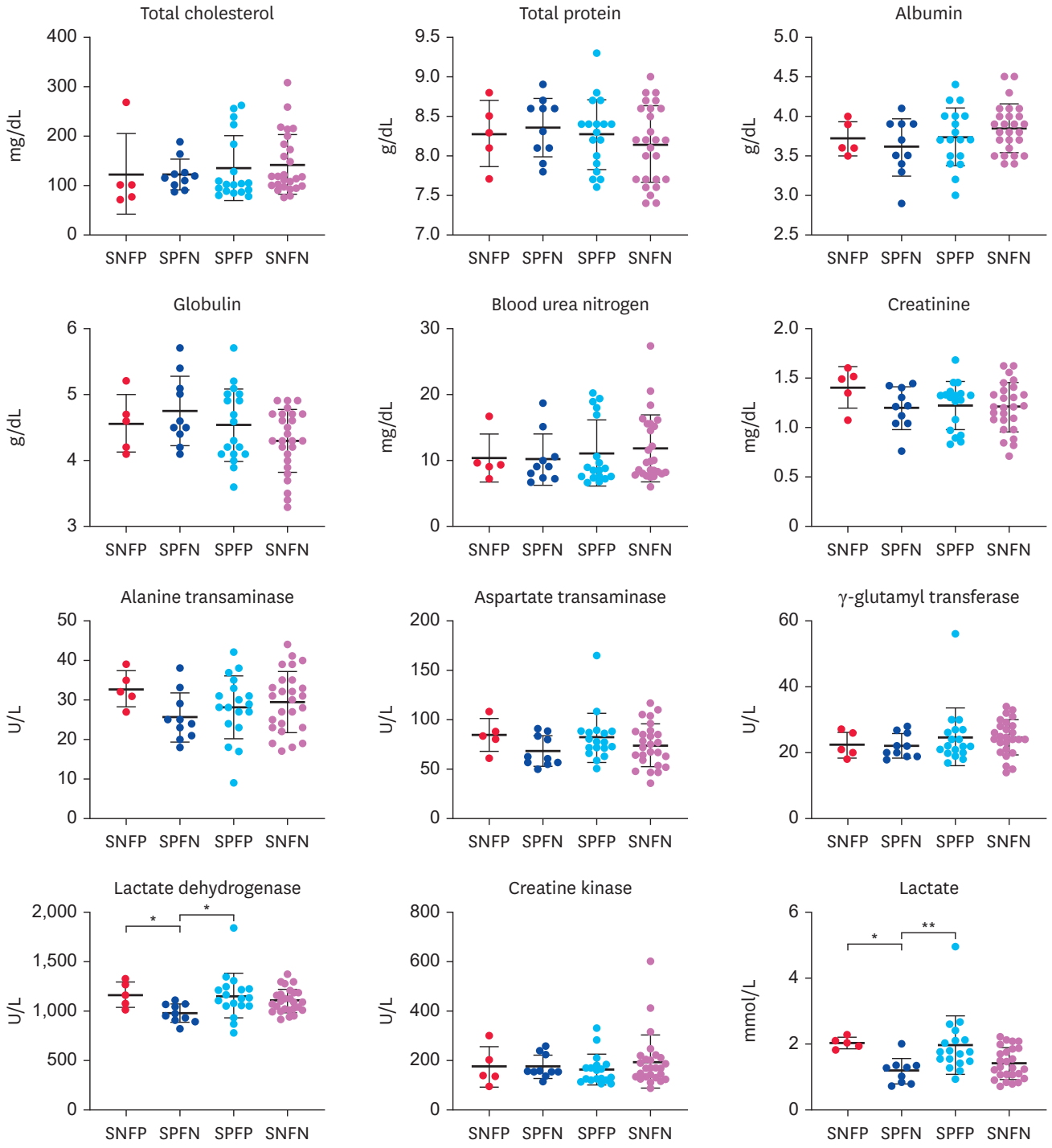

Fig. 2. (Continued) Serum biochemical parameters results related to metabolism according to the detection methods of *Mycobacterium avium* subspecies *paratuberculosis* (n = 33: SNFP, 5; SPFN, 10; SPFP, 18). The parameters of SNFN were used as the reference.
SNFP, seronegative and fecal-positive for *Mycobacterium avium subspecies paratuberculosis*; SPFN, seropositive and fecal-negative for *Mycobacterium avium subspecies paratuberculosis*; SPFP, seropositive and fecal-positive for *Mycobacterium avium subspecies paratuberculosis*; SNFN, seronegative and fecal-negative for *Mycobacterium avium subspecies paratuberculosis*; S/P ratio; sample-to-positive ratio.
*$p < 0.017$; **$p < 0.003$.

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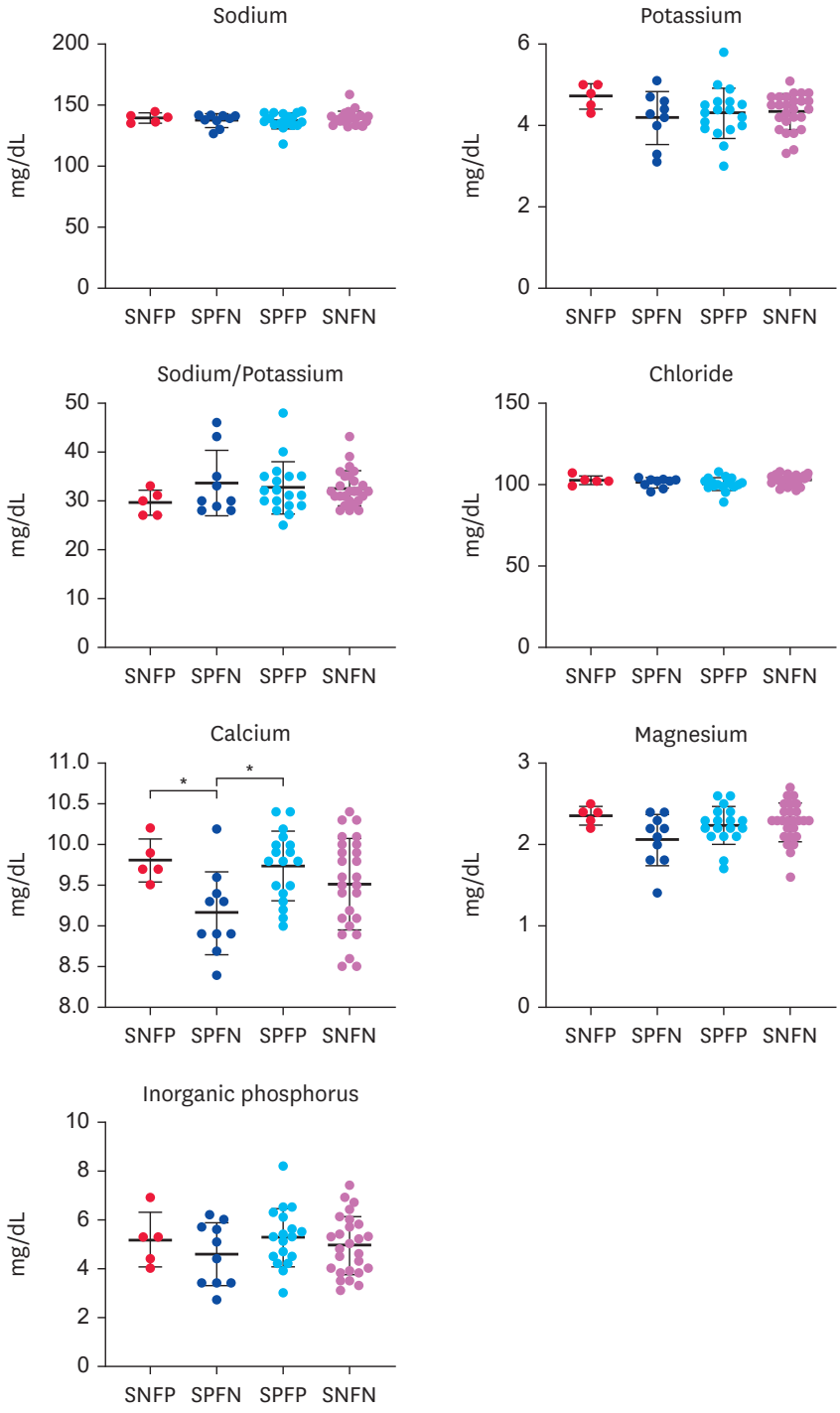

Fig. 3. Serum biochemical parameters results related to electrolytes according to the detection methods of *Mycobacterium avium* subspecies *paratuberculosis* (n = 33: SNFP, 5; SPFN, 10; SPFP, 18). The parameters of SNFN were used as the reference.
SNFP, seronegative and fecal-positive for *Mycobacterium avium subspecies paratuberculosis*; SPFN, seropositive and fecal-negative for *Mycobacterium avium subspecies paratuberculosis*; SPFP, seropositive and fecal-positive for *Mycobacterium avium subspecies paratuberculosis*; SNFN, seronegative and fecal-negative for *Mycobacterium avium subspecies paratuberculosis*; S/P ratio; sample-to-positive ratio.
*$p < 0.017$.

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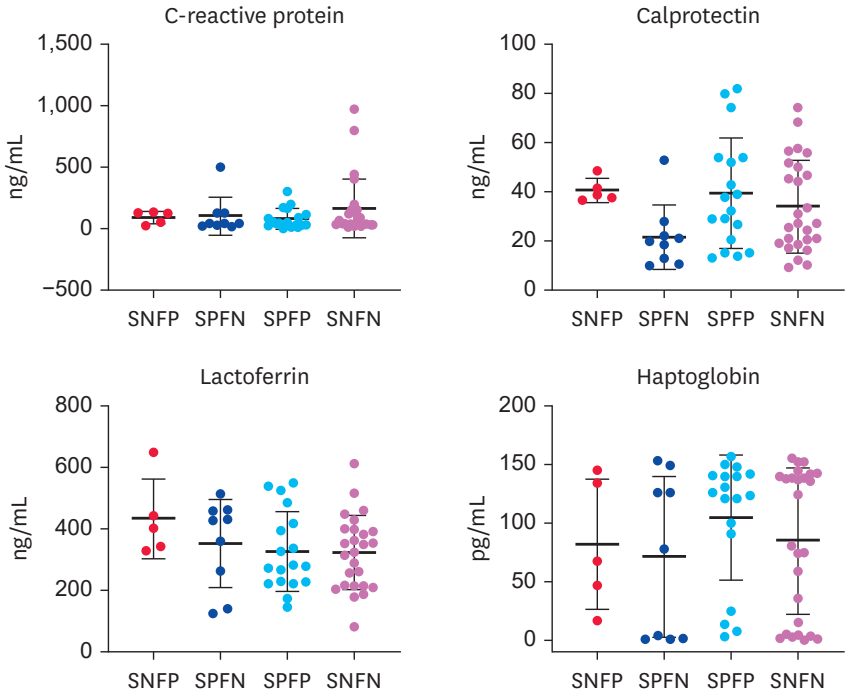

Fig. 4. Specific serum protein results according to the detection methods of *Mycobacterium avium* subspecies *paratuberculosis* (n = 33: SNFP, 5; SPFN, 10; SPFP, 18). The parameters of SNFN were used as the reference.
SNFP, seronegative and fecal-positive for *Mycobacterium avium subspecies paratuberculosis*; SPFN, seropositive and fecal-negative for *Mycobacterium avium subspecies paratuberculosis*; SPFP, seropositive and fecal-positive for *Mycobacterium avium subspecies paratuberculosis*; SNFN, seronegative and fecal-negative for *Mycobacterium avium subspecies paratuberculosis*; S/P ratio; sample-to-positive ratio.

DISCUSSION

In this study, we identified differences in the hematological and serum biochemical parameters of Holstein cows with subclinical MAP infection according to the detection of serum MAP antibody by ELISA and fecal PCR results. Although the cows were healthy with no clinical manifestations of MAP infection, the RBC count, HCT, MCHC, monocyte count, platelet count, MPV, PCT, glucose, LDH, lactate, Ca, and calprotectin values were significantly different according to the detection method. The cows with fecal-positive MAP status had higher RBC count, HCT, platelet count, MPV, PCT, LDH, lactate, calcium and calprotectin values but lower MCHC, monocyte count, and glucose values than those with seropositive and fecal-negative status. These results indicate that the cows shedding MAP in their feces may have more severe granulomatous lesions than those not shedding MAP in their feces, although they had subclinical MAP infection.

An interesting finding of this study is that the differences in hematological and serum biochemical parameters were related to iron disorders, according to shedding MAP in feces. MAP can survive and grow inside macrophages by acquiring iron. [23]. Paratuberculosis lesions that accumulate iron frequently shed MAP into feces [24]. The characteristics of MAP may affect the hematological indices. Iron deficiency and disorders are associated with increased erythropoiesis with hypochromasia and macrothrombocytosis [25]. Iron deficiency also results in abnormal erythrocyte morphology, such as anisocytosis, poikilocytosis, keratocytes, schistocytes, leptocytes, and dacrycytes [25]. High levels of erythropoietin in the plasma are speculated to stimulate megakaryopoiesis associated with increased platelets in patients with

iron deficiency [26]. Similarly, in this study, the cows shedding MAP in feces showed high RBC count with low MCHC and high platelet count with high MPV, compared to the cows that were not shedding MAP in feces. These findings suggest that cows shedding MAP in feces have greater iron accumulation in the lesions than those with fecal-negative MAP status. However, the MCV results did not support this supposition. The cows shedding MAP in feces had lower MCV than those not shedding MAP in feces, but, this difference was not significant. The reason for this observation remains unclear because the MCV value is affected by many factors, such as folate, cobalamin, pyridoxine, iron, and copper levels [25,27]. However, the reticulocyte result might offer a clue. Reticulocytes and young erythrocytes have higher MCV and lower MCHC values than mature erythrocytes [25,27]. The cows shedding MAP in feces had higher reticulocyte counts than those not shedding MAP in feces, although this difference was not significant. The difference in reticulocytes between cows shedding MAP and those not shedding MAP in feces might contribute to the MCV and MCHC results noted in this study.

Iron disorders may also influence the serum biochemical parameters. In this study, low levels of blood glucose were associated with shedding of MAP in feces. Mice with iron deficiency displayed impaired glucose production when gluconeogenic precursors were administered, resulting in hypoglycemia [28]. The low levels of blood glucose in the cattle shedding MAP in feces might indicate that iron accumulation in the lesions, resulting in reduced availability of circulating iron. The lactate and LDH levels were in accordance with the association between the level of blood glucose and shedding of MAP in feces. The abnormalities in the erythrocytes due to iron deficiency resulted in decreased oxygenation [29]. Tissue iron deficiency limits oxidative metabolism; iron-containing enzymes in the muscle and liver increase lactate production in the presence of iron deficiency in the tissues [30]. LDH functions as a catalyst to reversibly convert lactate to pyruvate and its level is increased to perform anaerobic metabolism in the case of iron deficiency [27,31]. In this study, cows with fecal-positive MAP status showed higher levels of lactate and LDH than those with fecal-negative status. Similar to the results of erythrocytes, platelets, and blood glucose, lactate and LDH levels also indicate that MAP shedding in feces is associated with iron disorders. However, since we did not measure the indicators related to iron disorders, such as serum iron level, total iron-binding capacity, and serum hepcidin and ferritin levels, further studies are required to elucidate the association between the detection of MAP in feces and iron availability.

Monocytes migrating into tissues differentiate into macrophages by exposure to the macrophage colony-stimulating factor, which is an inflammatory cytokine [25]. Monocytes store calprotectin as a major protein complex in the cytosol and tissue macrophages release calprotectin when recruited from the peripheral blood [32]. The type of paratuberculosis lesion that eventually develops is associated with the level of serum calprotectin [33]. These characteristics of monocytes and serum calprotectin may be implicated in the results of this study; hence, cattle shedding MAP in feces had low monocytes and high calprotectin; the transformation of monocytes in the blood to macrophages in the tissue may affect the levels of monocytes and calprotectin. Cattle shedding MAP in feces may have MAP lesions recruiting macrophages in the gut. The measurement of monocyte chemotactic proteins might be required to elucidate the reasons underlying the lower number of monocytes in subclinically infected cattle shedding MAP in feces than in those not shedding MAP in feces as well as those underlying the role of host–pathogen interaction in the development of MAP infection; monocyte chemotactic proteins that contribute to the migration of monocytes to lesions and granuloma formation are associated with MAP infection, and their levels increase and decline by time points [34-36].

Excessive production of 1,25 dihydroxycholecalciferol by macrophages and increased levels of parathyroid hormone-related protein in granulomatous inflammation are considered to cause increased serum calcium levels [27,37]. We found that serum calcium levels are associated with MAP shedding in feces. Our findings suggest that in the subclinical stage, cattle shedding MAP in feces may develop severe granulomatous lesions in the gut compared to cattle not shedding MAP in feces.

This study has a few limitations. We could not determine the mechanism underlying the shedding of MAP in feces and speculated that it influenced hematological and serum biochemical indices of the cattle. In addition, more cases should have been investigated for a rigorous statistical analysis. Further studies are required to elucidate how MAP affects the host in terms of serum iron and macrophages levels. To the best of our knowledge, this study is the first field study to compare the blood parameters of Holstein cattle subclinically infected with MAP using serum and fecal detection methods. Our findings shed light on the changes in the blood parameters in the host due to MAP infection.

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