Establishment a real-time reverse transcription PCR based on host biomarkers for the detection of the subclinical cases of Mycobacterium avium subsp. paratuberculosis

Hyun-Eui Park¹, Hong-Tae Park¹, Young Hoon Jung², Han Sang Yoo¹,³*

¹ Department of Infectious Disease, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea, ² Department of Animal Resources Development, National Institute of Animal Science, Rural Development Administration, Cheonan, Republic of Korea, ³ Institute of Green Bio Science and Technology, Seoul National University, Pyeongchang, Republic of Korea

* yoohs@snu.ac.kr

Abstract

Bovine paratuberculosis (PTB) is a chronic enteric inflammatory disease of ruminants caused by Mycobacterium avium subsp. paratuberculosis (MAP) that causes large economic losses in the dairy industry. Spread of PTB is mainly provoked by a long subclinical stage during which MAP is shed into the environment with feces; accordingly, detection of subclinical animals is very important to its control. However, current diagnostic methods are not suitable for detection of subclinical animals. Therefore, the current study was conducted to develop a diagnostic method for analysis of the expression of genes of prognostic potential biomarker candidates in the whole blood of cattle naturally infected with MAP. Real-time PCR with nine potential biomarker candidates was developed for the diagnosis of MAP subclinical infection. Animals were divided into four groups based on fecal MAP PCR and serum ELISA. Eight genes (Timp1, Hp, Serpine1, Tfrc, Mmp9, Delfb1, Delfb10, and S100a8) were up-regulated in MAP-infected cattle (p < 0.05). Moreover, ROC analysis revealed that eight genes (Timp1, Hp, Serpine1, Tfrc, Mmp9, Delfb1, Delfb10, and S100a8) showed fair diagnostic performance (AUC ≥ 0.8). Four biomarkers (Timp1, S100a8, Delfb1, and Delfb10) showed the highest diagnostic accuracy in the PCR positive and ELISA negative group (PN group) and three biomarkers (Tfrc, Hp, and Serpine1) showed the highest diagnostic accuracy in the PCR negative and ELISA positive group (NP group). Moreover, three biomarkers (S100a8, Hp, and Delfb10) were considered the most reliable for the PCR positive and ELISA positive group (PP group). Taken together, our data suggest that real-time PCR based on eight biomarkers (Timp1, Hp, Serpine1, Tfrc, Mmp9, Delfb1, Delfb10, and S100a8) might be useful for diagnosis of JD, including subclinical stage cases.
Introduction

Johne’s disease (JD) is a chronic inflammatory disease of the gastrointestinal tract of ruminants with granulomatous lesions that is caused by Mycobacterium avium subsp. paratuberculosis (MAP) (Whitlock et al., 1996). Johne’s disease can be divided into four stages depending on the clinical signs and MAP shedding levels including the silent, subclinical, clinical, and advanced clinical stage [1]. In the silent stage, the infected animals do not show any clinical sign or excrete MAP into the environment [2]. During the subclinical stage, animals still do not have clinical symptoms; however, they shed low numbers of MAP into the environment, which can be circulated in the herd and infect other animals [2]. After the subclinical stage, animals enter clinical stage and start to show clinical signs such as gradual weight loss, diarrhea, and decreased milk production [2]. Finally, animals become cachectic and lethargic in advanced clinical stage [2]. Accordingly, it is very important to remove animals in the subclinical stage to control the disease. However, current diagnostic methods are insufficient for diagnosis of subclinical stages of disease [3]. Although fecal culture has been considered a gold standard for the diagnosis of MAP [4], this method is time-consuming and shows low sensitivity, especially in subclinical stages of the disease [5, 6]. PCR allows rapid detection of MAP in clinical samples such as feces, milk and blood [7]; however, PCR-based methods are also limited in their usefulness for diagnosis of subclinical stages of disease because of the low sensitivity [8] and low specificity caused by genetic similarities with other mycobacteria [9, 10]. Although ELISA has been used for detection of antibodies to MAP in clinical samples such as serum and milk, this method is also inadequate for diagnosis of fecal shedders in the subclinical stage, especially in 1–2 year old cattle [11]. Therefore, new diagnostic tools have been requested to detect MAP-infected animals at early stage of infection.

Biomarkers, which are considered indicators of specific pathogenic conditions or therapeutic responses to treatment [12], are commonly used as diagnostic tools for various diseases [13–16]. Recently, host biomarkers discovered using transcriptomics, metabolomics, and proteomics have been proposed as alternative diagnostic methods for paratuberculosis [17–20]. Biomarkers indicating early stages of MAP-infection were proposed by analyzing gene expression profiles of blood in cattle with experimental MAP infection [17, 18]. A metabolic profiling in cattle with experimental infection of MAP revealed that four metabolites (iso-butyrate, branched chain amino acids, leucine, and isoleucine) were increased in serum of the MAP-infected cattle while citrate was decreased [19]. Moreover, six proteins (transferrin, gelsolin isoforms α & β, complement subcomponent C1r, complement component C3, amine oxidase-copper containing 3, and coagulation factor II) were proposed as biomarkers after they were found to increase by at least 2-fold in MAP-infected cattle, as were two proteins (coagulation factor XIII-B polypeptide, and fibrinogen γ chain and its precursor) that were reduced by nearly two-fold in MAP-infected cattle [20]. Our previous studies also proposed several biomarkers that were up-regulated in MAP infected macrophages, mice, and cattle [21–23]. Transcriptional profiles of MAP-infected macrophage RAW 264.7 cells and a mouse model suggested five and three genes as prognostic biomarkers, respectively [21, 22]. β-defensins were also suggested as prognostic biomarkers in subclinical animals of MAP-naturally infected cattle [23]. However, application of those biomarkers for diagnosis of JD has yet to be investigated. Therefore, we developed a real-time PCR method using the biomarkers for diagnosis of bovine paratuberculosis by measuring the gene expression level of several biomarkers in whole blood.
Materials and methods

Experimental design and animals

About 300 Holstein cattle were raised on the national farm in Cheonan city which located in mid-west region of the South Korea. The cattle were regularly tested for absence of JD two times per year using fecal PCR and serum ELISA. A total of three to eight year old forty-four cows were selected for further analysis after detection of MAP-specific antibodies using a commercial ELISA kit (IDEXX Laboratories, Inc., Westbrook, ME, USA) and MAP in the feces by PCR [24]. The detection was performed four times with a 6-month interval to enable accurate classification of infection status. The animals were divided into the following groups based on the results of PCR and ELISA: NN, ELISA and PCR negative; PN, ELISA negative and PCR positive; NP, ELISA positive and PCR negative; PP, ELISA positive and PCR positive. All animal procedures were approved by the National Institute of Animal Science (2013–046). Detailed characteristics of study subjects are shown in Table 1.

Selection of biomarker candidates

Nine genes that were significantly up-regulated in MAP infected macrophages, mice, and cattle were selected for use as diagnostic biomarkers based on our previous studies (Table 2) [21–23, 25]. All datasets used in selection of the biomarkers are available at Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo website) under accession number GSE62836, http://dx.doi.org/10.4014/jmb.1302.02021, and http://dx.doi.org/10.4014/jmb.1408.08059.

Extraction of total RNA from blood

Peripheral blood was collected from the tail vein of cattle using a BD Vacutainer® Plus Plastic K$_2$EDTA Tubes. A total of 125 μl of whole blood was then mixed with 125 μl of RNase-free water and 750 μl of Trizol LS reagent (Ambion) and incubated at room temperature for 5 min. Next, 200 μl of chloroform was added to the mixture and it was centrifuged at 13,523 g and 4°C for 15 min. The supernatant was subsequently transferred to an RNAeasy column (Qiagen, Hilden, Germany) and centrifuged at 8,500 g for 15 sec. After washing, RNA was eluted in 30 μl of RNase-free water and immediately stored at -80°C until use.

Optimization of primer and probe concentrations

The optimal concentration of primer and probe concentration was determined with an identical cDNA template for each biomarker gene. Three concentrations (0.5μM, 0.75μM, 1μM) of both forward and reverse primers with a constant probe concentration were tested. The combination showing the highest fluorescence value was tested at three different concentrations of the probe (0.1μM, 0.2μM, 0.3μM). For further experiment, primer and probe concentration that showing the highest fluorescence value was selected.

Real-time PCR

Total RNA was employed to prepare cDNA with random primers using a QuantiTect® Reverse Transcription Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s instructions. The expression of nine biomarker genes was measured by quantitative real time RT-PCR, which was conducted using a Rotor-Gene multiplex PCR kit (Qiagen Inc). In brief, total of 18μl reaction mixture was prepared consists of 10μl Master mix, RNase-free water, 0.5μM forward and reverse primers, and 0.1μM probe for each of the biomarker genes. After that, 2μl of cDNA template was added to a final volume of 20μl. The specificity of the primers
and probes for each biomarker genes was confirmed by homology search (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and agarose gel electrophoresis. The primers and probe used in this study are shown in Table 3. Sensitivity of real-time PCR reactions was confirmed by real-time PCR reaction using the known copy numbers calculated from purified PCR products which serially diluted from $10^9$ to $10^2$ copies of the templates. The real-time PCR was conducted for 45 cycles and $C_T$ values were obtained. Negative control was included with no template. Real-time PCR was conducted by subjecting the samples to 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 45 s. The expression level was determined by the $2^{-\Delta\Delta C_T}$ method using the housekeeping gene, $\beta$-actin, as a reference.

### Statistical analysis

Data are reported as the means ± the standard error of the mean (S.E.M.) of three independent experiments. Statistical significance was determined by ANOVA ($p \leq 0.05$) with Dunnett’s post hoc test using the GraphPad Prism software version 7.00 (GraphPad Software, Inc., La Jolla, CA, USA). Receiver operator characteristics (ROC) curve analysis was conducted using the statistical package for social science (SPSS) software version 21.0 (SPSS Inc., Chicago, IL, USA) and the MedCalc Statistical Software version 13.3.3 (MedCalc Software, Ostend, Belgium). Higher AUC scores were considered to show better discriminatory powers as follows: excellent discriminatory power, $AUC \geq 0.9$; good discriminatory power, $0.8 < AUC < 0.9$; fair

### Table 1. Characteristics of study subjects.

| Number of subjects | All (n = 44) | NN group (n = 11) | PN group (n = 12) | NP group (n = 14) | PP group (n = 7) |
|--------------------|-------------|------------------|------------------|------------------|------------------|
| Heifers, n (%)     | 44 (100)    | 11 (100)         | 12 (100)         | 14 (100)         | 7 (100)          |
| Median age (Years) | 6 (4 to 9)  | 4 (4 to 7)       | 6 (4 to 9)       | 6.5 (4 to 8)     | 6 (5 to 8)       |
| Serum ELISA        |             |                  |                  |                  |                  |
| Positive, n (%)    | 21 (47.7)   | 0 (0)            | 0 (0)            | 14 (100)         | 7 (100)          |
| Negative, n (%)    | 23 (52.3)   | 11 (100)         | 12 (100)         | 0 (0)            | 0 (0)            |
| Fecal PCR          |             |                  |                  |                  |                  |
| Positive, n (%)    | 19 (43.2)   | 0 (0)            | 12 (100)         | 0 (0)            | 7 (100)          |
| Negative, n (%)    | 25 (56.8)   | 11 (100)         | 0 (0)            | 14 (100)         | 0 (0)            |

### Table 2. Mean fold change of selected biomarker genes between infected animals and non-infected animals.

| Accession No. | Gene symbol | Gene name | Location | Mean fold change (log2 value) | PN vs. NN P value | NP vs. NN P value | PP vs. NN P value | P value |
|---------------|-------------|-----------|----------|-------------------------------|-------------------|-------------------|-------------------|--------|
| NM_003234.2   | Tfrc        | Transferrin receptor (p90, CD71) | Plasma membrane | 1.6                           | 0.0005            | 1.3               | 0.0021            | 1.9     | 0.0004 |
| NM_174744     | Mmp9        | Matrix metallopeptidase 9 | Extracellular space | 2.9                           | 0.008             | 2.4               | 0.0342            | 3.6     | 0.0052 |
| NM_002964.4   | S100a8      | S100 calcium binding protein A8 | Cytoplasm | 1.6                           | 0.0039            | 0.9               | 0.146             | 1.7     | 0.0069 |
| NM_002965.3   | S100a9      | S100 calcium binding protein A9 | Cytoplasm | 0.4                           | 0.6228            | 0.6               | 0.2596            | 1.1     | 0.0548 |
| NM_174137     | Serpine1    | Serpin peptidase inhibitor | Extracellular space | 1.9                           | 0.0041            | 1.6               | 0.0183            | 2.6     | 0.0009 |
| NM_005143.3   | Hp          | Haptoglobin | Extracellular space | 2.3                           | 0.0031            | 3.2               | <0.0001           | 3.3     | 0.0003 |
| NM_174471.3   | Timp1       | Tissue inhibitor of metallopeptidase 1 | Extracellular space | 1.7                           | <0.0001           | 1.4               | 0.0002            | 0.5     | 0.415  |
| NM_001324544.1| Defb1       | Defensin beta 1 | Extracellular space | 5.2                           | 0.0009            | 3.3               | 0.039             | 1.8     | 0.5842 |
| NM_001115084.1| Defb10      | Defensin beta 10 | Extracellular space | 2.3                           | 0.0017            | 1.6               | 0.0313            | 1.6     | 0.1009 |
discriminatory power, $0.7 \leq \text{AUC} < 0.8$; poor discriminatory power, $\text{AUC} < 0.7$ [26]. The optimal cutoff values were calculated for each ROC curve while maximizing the Youden Index. Sensitivity and specificity were calculated based on cut-off value which showed highest AUC value in the ROC curve for each biomarker gene. A $p < 0.05$ was considered to indicate statistical significance.

### Results

#### Specificity of probe and primers

Specificity of primers and probes were confirmed by homology search. Also, to confirm the specificity for each biomarker genes, RT-PCR and agarose gel electrophoresis was performed. Single PCR band were confirmed for each biomarker gene and the $\beta$-actin gene and non-specific PCR product was not observed confirmed in the negative control with no cDNA sample (Fig 1).

#### Table 3. Oligonucleotide sequence of primer and probe used for real-time PCR in this study.

| Target gene | Primer sequence (5' to 3') | PCR product size (base pair) | Reference |
|-------------|---------------------------|-----------------------------|-----------|
| $\beta$-actin | F                         | GCAAGCAGGACTACGATGAG         | 134       |
|             | R                         | GCCATGCAACTTCCTACCTG        | In this study |
| Probe       | FAM-CTTCAGGCGACTTGGCTTGAC-BHQ1 |                            |           |
| Mmp9        | F                         | CCCCAGTCCTTGAGCTAGGCC      | 177       |
|             | R                         | GGCGGAGGACACATACAGTG       | [25]      |
| Probe       | HEX-AGTGTGGCCACGGCTGTTTACGAGBHQ1 |                       |           |
| Serpine1    | F                         | CTGGAAATCAGGCTGCGG         | 191       |
|             | R                         | GGATGAGAAAACCACGTGCG       | [25]      |
| Probe       | FAM-AGAAGTGAGGAGGCCAGACACTGTCAGGG-BHQ1 |                  |           |
| Timp1       | F                         | TCTGCAACTCCGGATGTTG        | 125       |
|             | R                         | CTCAGGCTGAGGACCATCTCTC     | In this study |
| Probe       | HEX-GTTGGGAGCGGCAGCTCAATG-BHQ1 |                        |           |
| Hp          | F                         | CCAQTACGAGCGAGCCACC        | 131       |
|             | R                         | ACCATCTAGCCACACAGCC        | In this study |
| Probe       | FAM-AACGACAGGAGGAGCCAGCAGACCTGTCG-BHQ1 |                |           |
| S100a8      | F                         | ATTTTGAGGAGACCTTGGG        | 124       |
|             | R                         | ACGCGGCTGTATTCCCCTTT       | [25]      |
| Probe       | FAM-TAAGTCCCCATGGAGCTCACCACAG-BHQ1 |                     |           |
| S100a9      | F                         | AGGTCTAGGGAGACCCAGG        | 134       |
|             | R                         | GCTGCCCCCTGCTTAGGTGG       | [25]      |
| Probe       | HEX-AGTGGAGGCTGACCGGCAACGCTACCCAG-BHQ1 |              |           |
| Tfrc        | F                         | CAAAGTTCTGCGACCCACC        | 188       |
|             | R                         | AACGAAAGAGGACCGCTGTGG       | [25]      |
| Probe       | HEX-TACGAGAACCGCATGCTGGACCAAG-BHQ1 |                |           |
| Defb1       | F                         | CGAAGGAGGCATCTGTTG         | 110       |
|             | R                         | CTTGGCTCTTCTCTACCAAGGA       | In this study |
| Probe       | FAM-TGCCCTGAGCATACTGAGGTTGCA-BHQ1 |                     |           |
| Defb10      | F                         | ATCTAGGCGCTGCGGGGAAAT      | 97        |
|             | R                         | CATTTTAACCTGGGGCCCTGTA       | In this study |
| Probe       | HEX-GTTTGCTTAAACAGGCTGTCGCCAC-BHQ1 |                  |           |

https://doi.org/10.1371/journal.pone.0178336.t003
Sensitivity of real-time PCR reactions

Real-time PCR for the each biomarker gene was performed using the specific primers, probes and the purified PCR products. Amplification plots were presented for biomarker genes with increased template copy numbers from $10^2$ to $10^9$. Amplification plot shows that fluorescence increase with increased template copy numbers (Fig 2). Also, real-time PCR was highly sensitive to detect low level of gene expression of biomarker genes (about $10^2$ copies of the template cDNA) and negative control sample with no template DNA showed no increasing of fluorescence (Fig 2).

Optimization of primer and probe concentrations

The optimal concentration of primer and probe concentration was determined by conducting real-time PCR with three primer and probe concentrations. The combination of forward and reverse primer at 0.5 $\mu$M for biomarker genes and $\beta$-actin gene revealed highest florescence and lowest $C_T$ value. With this primer concentration, 0.1 $\mu$M of probe showed highest florescence and lowest $C_T$ value. Combination of 0.5 $\mu$M forward and reverse primers and 0.1 $\mu$M probe concentration was used in further analysis.

Gene expression level of biomarkers in MAP infected cattle

Experimental animals were divided into four groups based on the results of fecal PCR and serum ELISA conducted three times with a 6 month interval (Table 1). When compared with the non-infected NN group, expression of eight genes ($\beta$-actin, Defb1, Defb10, Mmp9, Timp1, Hp, Serpine1, and Tfrc) showed higher expression in the PN group ($p < 0.05$), while higher expression of seven other genes ($Timp1$, $Hp$, $Serpine1$, $Tfrc$, $Defb1$, $Defb10$, and $Mmp9$) was observed in the NP group ($p < 0.05$). Moreover, in the PP group, five genes ($S100a8$, $Mmp9$, $Hp$, $Serpine1$, and $Tfrc$) showed significantly higher expression in the PP group ($p < 0.05$). Four genes ($Tfrc$, $Hp$, $Serpine1$, and $Mmp9$) were up-regulated in all infected groups, while three genes ($Timp1$, $Defb1$, and $Defb10$) were up-regulated in the PN group and the NP group, and $S100a8$ was up-regulated in the PN group and the PP group (Fig 3). The mean fold changes of each biomarker are shown in Table 2.

Discrimination between infected and non-infected animals

The AUC score of biomarkers was calculated during ROC analysis. In the PN group, the AUC scores of eight genes ($Timp1$, $Defb1$, $Tfrc$, $Defb10$, $S100a8$, $Serpine1$, $Mmp9$, and $Hp$) were $\geq 0.8$. In the NP group, four genes ($Hp$, $Timp1$, $Tfrc$, and $Serpine1$) had AUC scores $\geq 0.8$, while six genes ($S100a8$, $Hp$, $Serpine1$, $Tfrc$, $Mmp9$, and $Defb10$) in the PP group had AUC scores $\geq 0.8$ (Fig 4). When the diagnostic accuracies of individual biomarkers were calculated by ROC
curve analysis, the most accurate biomarker in the PN group was *Timp1*, with an AUC value of 0.985, while the most accurate biomarker in the NP group was *Hp*, with an AUC value of 0.942. Additionally, the most accurate biomarker in the PP group was *S100a8*, with an AUC value of 0.896. Similarly, in the PN group, *Timp1* showed the most accurate diagnostic performance, with a sensitivity of 100% and a specificity of 90.9%. In the NP group, *Hp* showed the most accurate diagnostic performance, with a sensitivity of 92.9% and a specificity of 90.9%. Moreover, *S100a8* showed the most accurate diagnostic performance in the PP group, with a sensitivity of 85.7% and a specificity of 90%. Other details pertaining to the diagnostic performance of biomarkers are shown in Table 4.

**Discussion**

Early diagnosis of JD is the most important requirement to eradicate it from MAP-infected herds. However, current diagnostic methods are not sufficient for the diagnosis of subclinical stage animals that are actively dispersing MAP into the environment via fecal shedding [2]. Recently, several studies have attempted to diagnose subclinical stages of JD by analyzing host-pathogen interactions, including gene expression, miRNA, protein, and metabolites to MAP infection [17–20, 27]. Some of the studies have been conducted to identify prognostic biomarkers of JD by understanding host response to infection during the progression of JD [21, 28–31]. However, no attempt has been made to apply biomarkers as diagnostic tools. Therefore, the present study was conducted to diagnose MAP infection using a real-time PCR method based on potential prognostic biomarkers.

In the present study, several biomarkers showed good discriminatory ability (AUC>0.8) between MAP-infected cattle and non-infected cattle. Three genes (*Hp*, *Serpin1*, and *Tfrc*) showed good discriminatory ability (AUC≥0.8) in fecal PCR-positive and/or serum ELISA-positive groups (PN, NP, PP). Acute phase proteins are blood proteins that respond to infection and inflammation and have been used as diagnostic and prognostic biomarkers in veterinary medicine [32]. *Hp* is the major acute phase protein of cattle that responds to infection.
Moreover, Hp is known to exert anti-inflammatory activity by down-regulating neutrophil activity via inhibition of both lipoxygenase and cycloxygenase [35] and to inhibit bacterial growth by interfering with iron acquisition by the host cell [36]. Moreover, Hp inhibits phagocytosis and intracellular killing of pathogens [37]. This anti-inflammatory response induced by Hp might reduce the harmful aspects of inflammation that could be destructive to the host itself. In that regard, up-regulation of Hp in MAP-infected animals might be a host response to early infection of MAP. Hp showed highest diagnostic accuracy for the NP group and whole infected animals, with AUC values of 0.942 and 0.901, respectively.

The initial response to MAP infection is dominant cell-mediated immunity, which is characterized by increasing interferon gamma release [38]. Serpine1 is known to be an essential element of the fibrinolytic system that is related to blood coagulation [39]. Serpine1 also acts as an inflammatory mediator by increasing the level of interferon gamma in blood to eliminate the pathogen in the early phase of an infectious disease [40, 41]. Therefore, increasing gene expression levels of Serpine1 might be related to interferon gamma release due to MAP infection. In addition, expression of MAP0403 in MAP was increased in infected macrophages and MAC-T cells in recent study [42]. MAP0403 is kind of serine protease which served as a key element of
the stress response network in intraphagosomal survival of MAP [42]. Up-regulation of Serpine1 might be a counter response to intraphagosomal survival of MAP in host cells. The diagnostic accuracy of Serpine1 was good (AUC≥0.8) in all infected animals (PN, NP, PP group).

Iron is an important nutrient in innate immune response to bacterial pathogen [43]. Tfrc, which is one of the key elements of iron metabolism, transfers iron to cells from transferrin protein [43]. Tfrc is known to down-regulated in response to intracellular pathogen infection; however, its expression was significantly increased in all infected animals in the present study. This phenomenon might be related to the alternative iron acquisition system of MAP, which acts in a host-independent manner using mycobactin [44]; however, further studies are needed to confirm this.

Mmp9 is a matrix metalloproteinase related to leukocyte migration to infection sites and tissue destruction if it is secreted in excess amounts [45]. The level of Mmp9 was regulated by Timp1, which inhibits the activity of MMP9 [45]. Mmp9 and Timp1 are known to be up-

Fig 4. Discriminatory ability of biomarkers between infected animals and control animals. Receiver operator characteristics curves of biomarker genes in cattle infected with Mycobacterium avium subspecies paratuberculosis compared to non-infected cattle. (A) Hp; (B) Timp1; (C) Serpine1; (D) TFRC; (E) Defb10; (F) Mmp9; (G) Defb1; (H) S100a8; (I) S100a9

https://doi.org/10.1371/journal.pone.0178336.g004
Table 4. Diagnostic performance of biomarkers for diagnosis of JD.

| Biomarker | AUC  | P value  | Cut-off (fold change) | Sensitivity (%) | Specificity (%) |
|-----------|------|----------|-----------------------|----------------|-----------------|
|           | PN vs. NN | NP vs. NN | PP vs. NN | PN vs. NN | NP vs. NN | PP vs. NN | PN vs. NN | NP vs. NN | PP vs. NN | PN vs. NN | NP vs. NN | PP vs. NN |
| Tfrc      | 0.909 | 0.89 | 0.857  | <0.0001 | <0.0001 | 0.0012 | >0.94 | >0.47 | >0.94 | 75 | 92.9 | 71.4 | 100 | 72.7 | 100 |
| Mmp9      | 0.871 | 0.76 | 0.857  | <0.0001 | 0.0102 | 0.0012 | >0.455 | >2.73 | >2.73 | 91.7 | 57.1 | 71.1 | 72.7 | 100 | 100 |
| S100a8    | 0.894 | 0.721 | 0.896  | <0.0001 | 0.045 | <0.0001 | >0.829 | >0.095 | >0.829 | 91.7 | 92.9 | 85.7 | 90.9 | 54.5 | 90 |
| S100a9    | 0.693 | 0.669 | 0.727  | 0.11 | 0.1315 | 0.1365 | >0.07 | >0.511 | >0.856 | 91.7 | 57.1 | 57.1 | 54.5 | 81.8 | 100 |
| Serpine1  | 0.875 | 0.864 | 0.883  | <0.0001 | <0.0001 | 0.0001 | >0.463 | >1.022 | >0.372 | 91.7 | 78.6 | 100 | 72.7 | 100 | 72.7 |
| Hp        | 0.864 | 0.942 | 0.883  | <0.0001 | <0.0001 | 0.0001 | >1.806 | >1.806 | >1.806 | 75 | 92.9 | 85.7 | 90.9 | 90.9 | 90.9 |
| Timp1     | 0.985 | 0.929 | 0.701  | <0.0001 | <0.0001 | 0.1815 | >0.682 | >0.682 | >1.022 | 100 | 78.6 | 42.9 | 90.9 | 90.9 | 100 |
| Defb1     | 0.955 | 0.786 | 0.558  | <0.0001 | 0.0024 | 0.7681 | >0.951 | >0.325 | >0.325 | 83.3 | 64.3 | 57.1 | 100 | 90.9 | 90.9 |
| Defb10    | 0.905 | 0.773 | 0.831  | <0.0001 | 0.0052 | 0.0118 | >1.154 | >1.815 | >1.154 | 83.3 | 57.1 | 85.7 | 90.9 | 100 | 90.9 |

In the present study, innate and adaptive immune responses by down-regulating pro-inflammatory cytokines [48]. The simultaneous up-regulation of -defensins exhibit antimicrobial functions, providing first protection against pathogens while playing an immune-modulation role [47]. Moreover, -defensins interplay between innate and adaptive immune responses by down-regulating pro-inflammatory cytokines [48]. In the present study, Defb1 and Defb10 were significantly up-regulated in both the PN group and the NP group. Moreover, Defb1 and Defb10 showed excellent discriminatory ability (AUC≥0.9) in the PN group.

S100a8 and S100a9 are members of a calcium-binding cytosolic protein family that are located in the cytoplasm [49]. S100a8 and S100a9 form a heterodimer known as calprotectin that induces an inflammatory response via activation of TLR4 signaling [50]. Moreover, calprotectin is known to induce leukocyte migration in the early phase of bacterial infection [51]. In previous studies, serum S100A8/A9 have been proposed as prognostic biomarkers for disease progression and therapeutic response in inflammatory bowel diseases (IBD)[52, 53]. In the present study, S100a8 showed good discriminatory ability (AUC≥0.8) in the PN and PP groups. However, gene expression of S100a9 was not significant in all infected animals. Generally, S100a8 and S100a9 exist as heterodimers, but they also exist as homodimers [54]. The inconsistent gene expression levels between S100a8 and S100a9 might be related to the presence of the homodimer form.

An ideal biomarker for diagnosis of JD should be able to discriminate between infected and non-infected animals with high sensitivity and specificity. Our data showed that the response of eight biomarkers (Hp, Timp1, Mmp9, Serpine1, Tfrc, S100a8, Defb1, and Defb10) significantly discriminated MAP-infected and non-infected animals. Moreover, eight biomarkers (Hp, Timp1, Mmp9, Serpine1, Tfrc, S100a8, Defb1, and Defb10) showed good accuracy (AUC≥0.7) for diagnosis of subclinical animals. Additionally, four genes (Timp1, S100a8, Defb1, and Defb10) showed sensitivity over 80% and specificity over 90%. It is generally very difficult to detect subclinical stages of JD using currently available diagnostic methods such as bacterial culture, fecal PCR and serum ELISA [3]. Fecal PCR is a reliable method for diagnosis of MAP infection; however, intermittent shedding of MAP into feces because of immunological changes during the progress of disease can interfere with accurate diagnosis [55]. Moreover, although serum ELISA is a simple, fast and cost-effective method for diagnosis of JD, it is
known to have low sensitivity for MAP-infected animals that do not show clinical signs [56]. However, our real-time PCR method based on biomarkers showed relatively precise diagnostic results. In that regard, combination of eight biomarker genes (Hp, Timp1, Mmp9, Serpine1, Tfrc, S100a8, Defb1, and Defb10) might be used for diagnosis of JD, including in subclinical stage animals.

In conclusion, a real-time PCR method was developed based on eight biomarkers that can be used as a new diagnostic tool for JD with good diagnostic performance. Moreover, this real-time PCR based on biomarkers might be used for diagnosis of JD, especially in subclinical stage animals that cannot be detected by current diagnostic methods. Although our developed diagnostic method might be applied to field test, this method will be more concreted if possible limitations in our study such as the low number of samples and sampling times would be addressed in future studies by including large scale field investigations.

Acknowledgments
We are grateful to all our research participants, and staff of the National Institute of Animal Science, who contributed to this study.

Author Contributions
Conceptualization: HEP HSY.
Data curation: HEP.
Formal analysis: HEP.
Funding acquisition: HSY.
Investigation: HEP HTP YHJ HSY.
Methodology: HEP HTP.
Project administration: YHJ HSY.
Resources: YHJ HSY.
Software: HEP.
Supervision: HSY.
Validation: HEP HTP.
Visualization: HEP.
Writing – original draft: HEP HSY.
Writing – review & editing: HEP HSY.

References
1. Whitlock RH, Buergeilt C. Preclinical and clinical manifestations of paratuberculosis (including pathology). Vet Clin North Am Food Anim Pract. 1996; 12: 345–3 PMID: 8828109
2. Tiwari A, VanLeeuwen JA, McKenna SL, Keefe GP, Barkema HW. Johne's disease in Canada Part I: clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. Can Vet J. 2006; 47: 874–882. PMID: 17017652
3. Mortier RA, Barkema HW, De Buck J. Susceptibility to and diagnosis of Mycobacterium avium subspecies paratuberculosis infection in dairy calves: A review. Prev Vet Med. 2015; 121(3–4): 189–98. https://doi.org/10.1016/j.prevetmed.2015.08.011 PMID: 26321657
4. Whittington RJ, Whittington AM, Waldron A, Begg DJ, de Silva K, Purdie AC, et al. Development and validation of a liquid medium (M7HC) for routine culture of Mycobacterium avium subsp. paratuberculosis to replace modified Bactec 12B medium. J Clin Microbiol. 2013; 51(12): 3993–4000. https://doi.org/10.1128/JCM.01373-13 PMID: 24048541

5. Bögli-Stuber K, Kohler C, Seitter G, Glanemann B, Antognozzi MC, Salman MD, et al. Detection of Mycobacterium avium subspecies paratuberculosis in Swiss dairy cattle by real-time PCR and culture. A comparison of the two assays. J Appl Microbiol. 2005; 99: 587–597. https://doi.org/10.1111/j.1365-2672.2005.02645.x PMID: 16108801

6. Sackett DC, Carr DJ, Collins MT. Evaluation of conventional and radiometric fecal culture and a commercial DNA probe for diagnosis of Mycobacterium paratuberculosis infections in cattle. Can J Vet Res. 1992; 56(2): 148–53. PMID: 1591658

7. Sevilla IA, Garrido JM, Molina E, Geijo MV, Elguézabal N, Vázquez P, et al. Development and evaluation of a novel multicopy-element-targeting triplex PCR for detection of Mycobacterium avium subsp. paratuberculosis in feces. Appl Environ Microbiol. 2014; 80(12): 3757–68. https://doi.org/10.1128/AEM.01026-14 PMID: 24727272

8. Wells SJ, Collins MT, Faaberg KS, Wees C, Tavornpanich S, Petrini KR, et al. Evaluation of a rapid fecal PCR test for detection of Mycobacterium avium subsp. paratuberculosis in dairy cattle. Clin Vaccine Immunol. 2006; 13(10): 1125–30. https://doi.org/10.1128/CVI.00236-06 PMID: 16929864

9. Cousins DV, Whittington R, Marsh I, Masters A, Evans RJ, Klver P. Mycobacterium avium from feces of ruminants possess IS900-like sequences detectable IS900 polymerase chain reaction: implications for diagnosis. Mol Cell Probes. 1999; 13(6): 431–42. https://doi.org/10.1006/mcpp.1999.0275 PMID: 10657148

10. Englund S, Böliske G, Johansson KE, An IS900-like sequence found in a Mycobacterium sp. other than Mycobacterium avium subsp. paratuberculosis. FEMS Microbiol Lett. 2002; 209: 267–271. PMID: 12007816

11. Al Hajri SM, Alluwaimi AM. ELISA and PCR for evaluation of subclinical paratuberculosis in the Saudi dairy herds. Vet Microbiol. 2007; 121: 384–385. https://doi.org/10.1016/j.vetmic.2007.01.025 PMID: 17339085

12. Strimbu K, Tavel JA. What are biomarkers? Curr Opin HIV AIDS. 2010; 5(6): 463–6. https://doi.org/10.1097/COH.0b013e32833ed177 PMID: 20978388

13. Dehnad A, Ravindran R, Subbian S, Khan IH. Development of immune-biomarkers of pulmonary tuberculosis in a rabbit model. Tuberculosis (Edinb). 2016; 101: 1–7.

14. Goyal N, Kashyap B, Singh NP, Kaur IR. Neopterin and oxidative stress markers in the diagnosis of extrapulmonary tuberculosis. Biomarkers. 2016; 8: 1–6.

15. Walker SJ, Beavers DP, Fortunato J, Krigsman A. A Putative Blood-Based Biomarker for Autism Spectrum Disorder-Associated Ileoclitis. Sci Rep. 2016; 6: 35820. https://doi.org/10.1038/srep35820 PMID: 27767057

16. Waters WR, Maggioli MF, Palmer MV, McGill JL, Vordenmeier HM, et al. Interleukin-17A as a Biomarker for Bovine Tuberculosis. Clin Vaccine Immunol. 2015; 23: 168–80. https://doi.org/10.1128/CVI.00637-15 PMID: 26677202

17. David J, Barkema HW, Mortier R, Ghosh S, le Guan L, De Buck J. Gene expression profiling and putative biomarkers of calves 3 months after infection with Mycobacterium avium subsp. paratuberculosis. Vet Immunol Immunopathol. 2014; 160: 107–117. https://doi.org/10.1016/j.vetimm.2014.04.006 PMID: 24841484

18. David J, Barkema HW, le Guan L, De Buck J. Gene-expression profiling of calves 6 and 9 months after inoculation with Mycobacterium avium subsp. paratuberculosis. Vet Res. 2014; 45: 96. https://doi.org/10.1186/s13571-014-0096-5 PMID: 25294045

19. De Buck J, Shakhutdinov R, Barkema HW, Vogel HJ. Metabolic profiling in cattle experimentally infected with Mycobacterium avium subsp. paratuberculosis. PLoS One. 2014; 9(11): e111872. https://doi.org/10.1371/journal.pone.0111872 PMID: 25372282

20. You Q, Verschoor CP, Pant SD, Macri J, Kirby GM, Karrow NA. Proteomic analysis of plasma from Holstein cows testing positive for Mycobacterium avium subsp. paratuberculosis (MAP). Vet Immunol Immunopathol. 2012; 148(3–4): 243–51. https://doi.org/10.1016/j.vetimm.2012.05.002 PMID: 22633222

21. Cha SB, Yoo A, Park HT, Sung KY, Shin MK, Yoo HS. Analysis of transcriptional profiles to discover biomarker candidates in Mycobacterium avium subsp. paratuberculosis-infected macrophages, RAW 264.7. J Microbiol Biotechnol. 2013; 23: 1167–1175. PMID: 23727801

22. Shin MK, Park HT, Shin SW, Jung M, Im YB, Park HE, et al. Whole-blood gene-expression profiles of cows infected with Mycobacterium avium subsp. paratuberculosis reveal changes in immune response and lipid metabolism. J Microbiol Biotechnol. 2015; 25: 255–267. PMID: 25248984
23. Shin MK, Park H, Shin SW, Jung M, Lee SH, Kim DY, et al. Transcriptional Profiles and Immuno-pathologic Response following Mycobacterium avium subsp. paratuberculosis Infection in Mice. PLoS One. 2015; 10(10): e0138770. https://doi.org/10.1371/journal.pone.0138770 PMID: 26439498

24. Park HT, Shin MK, Park HE, Cho YI, Yoo HS. PCR-based detection of Mycobacterium avium subsp. paratuberculosis infection in cattle in South Korea using fecal samples. J Vet Med Sci. 2016; 78(9): 1537–1540. https://doi.org/10.1292/jvms.15-0271 PMID: 27301582

25. Park HE, Shin MK, Park HT, Jung M, Cho YI, Yoo HS. Gene expression profiles of putative biomarker candidates in Mycobacterium avium subsp. paratuberculosis-infected cattle. Pathog Dis. 2016; 74(4): ftw022. https://doi.org/10.1093/femspd/ftw022 PMID: 27029383

26. Muller MP, Tomlinson G, Marrie TJ, Tang P, McGeer A, Low DE, et al. Can routine laboratory tests discriminate between severe acute respiratory syndrome and other causes of community-acquired pneumonia? Clin Infect Dis. 2005; 40(8): 1079–86. https://doi.org/10.1086/428577 PMID: 15791504

27. Malvisi M, Palazzo F, Morandi N, Lazzari B, Williams JL, Pagnacco G, et al. Responses of Bovine Innate Immunity to Mycobacterium avium subsp. paratuberculosis Infection Revealed by Changes in Gene Expression and Levels of MicroRNA. PLoS One. 2016; 11(10): e0164461. https://doi.org/10.1371/journal.pone.0164461 PMID: 27760169

28. Plain KM, de Silva K, Earl J, Begg DJ, Purdie AC, Whittington RJ. Indoleamine 2,3-dioxygenase, tryptophan catabolism, and Mycobacterium avium subsp. paratuberculosis: a model for chronic mycobacterial infections. Infect Immun. 2011; 79(9): 3821–32. https://doi.org/10.1128/IAI.05204-11 PMID: 21730087

29. Seth M, Lamont EA, Janagama HK, Widdel A, Vuchanova L, Stabel JR, et al. Biomarker discovery in subclinical mycobacterial infections of cattle. PLoS One. 2009; 4(5): e5478. https://doi.org/10.1371/journal.pone.0005478 PMID: 19244922

30. Verschoor CP, Pant SD, You Q, Kelton DF, Karrow NA. Gene expression profiling of PBMCs from Holstein and Jersey cows sub-clinically infected with Mycobacterium avium ssp. paratuberculosis. Vet Immunol Immunopathol. 2010; 137: 1–11. https://doi.org/10.1016/j.vetimm.2010.03.026 PMID: 20447668

31. Wang X, Wang H, Aodon-geril, Shu Y, Momotani Y, Nagata R, et al. Decreased expression of matrix metalloproteinase-9 and increased expression of tissue inhibitors of matrix metalloproteinase-1 in paratuberculosis-infected cattle in the ELISA-negative subclinical stage. Anim Biotechnol. 2011; 22(1): 44–9. https://doi.org/10.1080/10495398.2010.536096 PMID: 21328105

32. Eckerseid PB, Bell R. Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine. Vet J. 2010; 185(1): 23–7. https://doi.org/10.1016/j.tvjl.2010.04.009 PMID: 20621712

33. Seppä-Lassila L, Orto T, Lassen B, Lasonen R, Pelkonen S, et al. Intestinal pathogenes, diarrhea and acute phase proteins in naturally infected dairy calves. Comp Immunol Microbiol Infect Dis. 2015; 41: 10–6. https://doi.org/10.1016/j.cimid.2015.05.004 PMID: 26264522

34. El-Deeb WM, Elmoslem any AM. Acute phase proteins as biomarkers of urinary tract infection in dairy cows: diagnostic and prognostic accuracy. Jpn J Vet Res. 2016; 64(1): 57–66. PMID: 27348889

35. Saeed SA, Ahmad N, Ahmed S. Dual inhibition of cyclooxygenase and lipoxygenase by human haptoglobin: its polymorphism and relation to hemoglobin binding. Biochem Biophys Res Commun. 2007; 353(4): 915–20. https://doi.org/10.1016/j.bbrc.2006.12.092 PMID: 17198677

36. Eaton JW, Brandt P, Mahoney JR, Lee JT Jr. Haptoglobin: a natural bacteriostat. Science. 1982; 215(4533): 691–3. PMID: 7036344

37. Rossbach er J, Wagner L, Pasternack MS. Inhibitory effect of haptoglobin on granulocyte chemotaxis, phagocytosis and bactericidal activity. Scand J Immunol 1999; 50(4): 399–404. PMID: 10520180

38. Stabel JR. Transitions in immune responses to Mycobacterium paratuberculosis. Vet Microbiol. 2000; 77(3–4): 465–73. PMID: 11118731

39. Furie B, Furie BC. The molecular basis of blood coagulation. Cell. 1988; 53(4): 505–18. PMID: 3286010

40. Lim JH, Woo CH, Li JD. Critical role of type 1 plasminogen activator inhibitor (PAI-1) in early host defense against nontypeable Haemophilus influenzae (NTHi) infection. Biochem Biophys Res Commun. 2011; 414(1): 67–72. https://doi.org/10.1016/j.bbrc.2011.09.023 PMID: 21945446

41. Wang Z, Zhao Q, Han Y, Zhang D, Zhang L, Luo D. PAI-1 and IFN-γ in the regulation of innate immune homeostasis during sublethal yersiniosis. Blood Cells Mol Dis. 2013; 50(3): 196–201. https://doi.org/10.1016/j.bcmd.2012.11.005 PMID: 23218129

42. Kugadas A, Lamont EA, Bannantine JP, Shoyama FM, Brenner E, Janagama HK, et al. A Mycobacterium avium subsp. paratuberculosis Predicted Serine Protease Is Associated with Acid Stress and Infragasomal Survival. Front Cell Infect Microbiol. 2016; 6: 85. https://doi.org/10.3389/fcimb.2016.00085 PMID: 27597934
43. Johnson EE, Wessling-Resnick M. Iron metabolism and the innate immune response to infection. Microbes Infect. 2012; 14(3): 207–16. https://doi.org/10.1016/j.micinf.2011.10.001 PMID: 22033148

44. Wang J, Moolji J, Dufort A, Staffa A, Domenech P, Reed MB, et al. Iron Acquisition in Mycobacterium avium subsp. paratuberculosis. J Bacteriol. 2015; 198(5): 857–66. https://doi.org/10.1128/JB.00922-15 PMID: 26712939

45. Goetzl EJ, Banda MJ, Leppert D. Matrix metalloproteinases in immunity. J Immunol. 1996; 156(1): 1–4. PMID: 8598448

46. Chen Y, Wang J, Ge P, Cao D, Miao B, Robertson I, et al. Iron Acquisition in Mycobacterium avium subsp. paratuberculosis. Mol Med Rep. 2017; 15(1): 483–487. https://doi.org/10.3892/mmr.2016.5998 PMID: 27959391

47. Meade KG, Cormican P, Narciandi F, Lloyd A, O’Farrelly C. Bovine β-defensin gene family: opportunities to improve animal health? Physiol Genomics. 2014; 46(1): 17–28. https://doi.org/10.1152/physiolgenomics.00085.2013 PMID: 24220329

48. Allaker RP. Host defence peptides—a bridge between the innate and adaptive immune responses. Trans R Soc Trop Med Hyg. 2008; 102(1): 3–4. https://doi.org/10.1016/j.trstmh.2007.07.005 PMID: 17727907

49. Schiopu A, Cotoi OS. S100A8 and S100A9: DAMPs at the crossroads between innate immunity, traditional risk factors, and cardiovascular disease. Mediators Inflamm. 2013; 2013: 828354. https://doi.org/10.1155/2013/828354 PMID: 24453429

50. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat Med. 2007; 13(9): 1042–9. https://doi.org/10.1038/nm1638 PMID: 17767165

51. Achouiti A, Vogl T, Urban CF, Röhm M, Hommes TJ, van Zoelen MA, et al. Myeloid-related protein-14 contributes to protective immunity in gram-negative pneumonia derived sepsis. PLoS Pathog. 2012; 8(10): e1002987. https://doi.org/10.1371/journal.ppat.1002987 PMID: 23133376

52. Leach ST, Yang Z, Messina I, Song C, Geczy CL, Cunningham AM, et al. Serum and mucosal S100 proteins, calprotectin (S100A8/S100A9) and S100A12, are elevated at diagnosis in children with inflammatory bowel disease. Scand J Gastroenterol. 2007; 42(11): 1321–31. https://doi.org/10.1080/00365520701416709 PMID: 17852869

53. Cayatte C, Joyce-Shaikh B, Vega F, Boniface K, Grein J, Murphy E, et al. Biomarkers of Therapeutic Response in the IL-23 Pathway in Inflammatory Bowel Disease. Clin Transl Gastroenterol. 2012; 3: e10. https://doi.org/10.1038/ctg.2012.2 PMID: 23238132

54. Vogl T, Ludwig S, Goebeler M, Strey A, Thorey IS, Reichelt R, et al. MRP8 and MRP14 control microtubule reorganization during transendothelial migration of phagocytes. Blood. 2004; 104: 4260–8. https://doi.org/10.1182/blood-2004-02-0446 PMID: 15331440

55. Mitchell RM, Schukken Y, Koets A, Weber M, Bakker D, Stabel J, et al. Differences in intermittent and continuous fecal shedding patterns between natural and experimental Mycobacterium avium subspecies paratuberculosis infections in cattle. Vet Res. 2015; 19: 46–66.

56. Nielsen SS, Toft N. Ante mortem diagnosis of paratuberculosis: a review of accuracies of ELISA, interferon-gamma assay and faecal culture techniques. Vet Microbiol. 2008; 129: 217–35. https://doi.org/10.1016/j.vetmic.2007.12.011 PMID: 18255239