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Research paper

Identification of potential biomarkers of disease progression in bovine tuberculosis

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ABSTRACT

Bovine tuberculosis (bTB) remains an important animal and zoonotic disease in many countries. The diagnosis of bTB is based on tuberculin skin test and IFN-γ release assays (IGRA). Positive animals are separated from the herd and sacrificed. The cost of this procedure is difficult to afford for developing countries with high prevalence of bTB; therefore, the improvement of diagnostic methods and the identification of animals in different stages of the disease will be helpful to control the infection. To identify biomarkers that can discriminate between tuberculin positive cattle with and without tuberculosis lesions (ML+ and ML−, respectively), we assessed a group of immunological parameters with three different classification methods: lineal discriminant analysis (LDA), quadratic discriminant analysis (QDA) and K nearest neighbors (k-nn). For this purpose, we used data from 30 experimentally infected cattle. All the classifiers (LDA, QDA and k-nn) selected IL-2 and IL-17 as the most discriminatory variables. The best classification method was LDA using IL-17 and IL-2 as predictors. The addition of IL-10 to LDA improves the performance of the classifier to discriminate ML-individuals (93.3% vs. 86.7%). Thus, the expression of IL-17, IL-2 and, in some cases, IL-10 would serve as an additional tool to study disease progression in herds with a history of bTB.

1. Introduction

Bovine tuberculosis (bTB) is an important animal and zoonotic disease that causes significant financial loss and is a public health hazard. *Mycobacterium bovis*, the causative agent of bTB, is closely related to *Mycobacterium tuberculosis* and both species are included in the *M. tuberculosis* complex. In humans and cattle the disease is primarily an infection of the respiratory system with similar characteristics. The pathogen is transmitted from cattle to humans via aerosol or ingestion of contaminated dairy products. The presence of bTB hinders the development of the dairy and meat industry and is an impediment to international trade. Therefore, the control and eventually the eradication of this disease are essential.

In many of the developed countries, the control of bTB is based on "test and slaughter" programs. Field and/or laboratory diagnostic tests are used to identify potentially infected herds for quarantine, which may be followed by additional diagnostic testing and slaughter of all cattle that show positive test reactions. The economical limitations of developing countries with high prevalence of bTB turn this test and slaughter policy in a non-viable option. In this particular situation, the use of complementary strategies such

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as vaccination and diagnostic improvements will be useful for controlling the infection. Blood- or serum-based methods with the potential to differentiate animals in different stages of the disease could help to isolate spreader-animals and limit the dissemination of the pathogen. Biomarkers of disease could improve the ante-mortem diagnosis, which is currently based only on the tuberculin skin test (the official diagnostic test) and IFN-γ determination, as an alternative/complementary method. IFN-γ release assay (IGRA) in whole blood upon specific antigen stimulation has been a useful tool for the detection of infected cattle (Wood and Jones, 2001; Gormley et al., 2006). However, this method is not suitable to differentiate animals with active disease from those infected but with no visible lesions at *post-mortem* examination (Lim et al., 2012; Vordermeier et al., 2002).

To identify biomarkers that can discriminate between tuberculin-positive cattle with and without tuberculosis lesions, we assess in this study three different classification methods: lineal discriminant analysis (LDA), quadratic discriminant analysis (QDA) and K nearest neighbors (k-nn). For this purpose, we used a database of immunological parameters determined in a group of experimentally infected cattle.

2. Materials and methods

2.1. Cattle infections, sample collection and necropsy

Thirty Holstein-Fresian calves (3–6 months old) from three independent experimental infections were inoculated intratracheally with 10^6 to 10^7 colony forming units (CFU) of *M. bovis* NCTC 10772 or *M. bovis* 04-303, as described previously (Blanco et al., 2011). All experiments conformed to local and national guidelines on the use of experimental animals and category III infectious organisms. The thirty animals used in this study were negative for IFN-γ by ELISA assay (Bovigam, Zurich, Switzerland) and tuberculin skin test at the beginning of the experiments but positive by both assays at the end of the experiments (data not shown). Blood samples were taken at the beginning of the experiment, for evaluation of preimmune status, and at several times after infection. For this particular study, all the analyzed samples corresponded to 60 days post infection (dpi). Heparinized blood (10 mL) from each animal was used for Bovigam and peripheral blood mononuclear cells (PBMC) isolation by gradient centrifugation over Histopaque 1077 (Sigma–Aldrich, St. Louis, USA) following the manufacturer's protocol. PBMCs were incubated at 37 °C in RPMI complete medium supplemented with 10% of bovine fetal serum (Internegocios, Mercedes, Argentina) and 20 μg/mL final concentration of purified protein derivative from *M. bovis* (PPDB) (Biocor, Zurich, Switzerland). The incubations were performed on 12-well tissue culture plates for 16 h for RNA extraction and 48 h for flow cytometry determinations. At the end of each experiment, the calves were euthanized and thin slices of lungs and lymph nodes of the head and pulmonary region were analyzed looking for granuloma formations. The animals were classified in two groups ($n = 15$) based on the presence or absence of macroscopic lesions. Representative images of granulomas are shown in supplementary Fig. 1.

Supplementary Figure 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2014.04.008.

2.2. Interferon gamma release assay

Heparinized blood samples were dispensed in 200 μl aliquots into individual wells of a 96-well plate. The wells contained whole blood plus 20 μg/ml *M. bovis* PPD (Prionics, Zurich, Switzerland) or PBS. The blood cultures were incubated for 18 h and plasma was harvested and stored at −80 °C. Interferon gamma concentrations in stimulated plasma were determined using a commercial ELISA based kit (Bovigam; Prionics, Zurich, Switzerland). Absorbance of standards and test samples were read at 450 nm. The optical density (OD) for the PBS controls, which was usually approximately 0.1 OD units, was used to normalize individual readouts and to calculate optical density indexes (ODIs), where the results obtained by antigen stimulation were divided by the results for the PBS-stimulated cultures.

2.3. Flow cytometry

For flow cytometry determinations, 2×10^6 PBMCs were incubated with PPDB. To evaluate the expression of CD4 (MCA1653A647, IgG2a), CD8 (MCA837PE, IgG2a) and CD25 (MCA2430F and MCA2430PE) surface markers, cells were stained with fluorescent conjugated monoclonal antibodies (AdDSerotec, Oxford, UK). The stained cells were analyzed in a FACSCalibur cytometer (BD, Franklin Lakes, NJ, USA) using Cell Quest software. Analysis gates were set on lymphocytes according to forward and side scatter. IL-2R expression was analyzed in CD4+ and CD8+ populations. Percentages of IL-2R-expressing cells were calculated as the ratio of CD4+ or CD8+ cells expressing CD25 and total CD4+ or CD8+ cells. Representative dot-plots are included in supplementary Fig. 2.

Supplementary Figure 2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2014.04.008.

2.4. Expression of cytokines in peripheral blood mononuclear cells stimulated with purified protein derivative from M. bovis

Cytokine mRNA expression analyses were performed in peripheral mononuclear blood cells. Briefly, PBMCs were isolated from heparinized blood by gradient centrifugation over histopaque 1077 (Sigma–Aldrich, St. Louis, USA), following the manufacturer's protocol, and subsequently incubated at 37 °C with PPDB for 16 hs (Blanco et al., 2009b). Immediately after incubation, cells were resuspended in RNAeasy RLT stabilization buffer and total RNA was extracted using the rest of the commercial kit and following the manufacturer's protocol (RNAeasy, QIAGEN, Hilden, Germany). RNA quality and quantity as well as cDNA synthesis were assayed as described previously (Blanco et al., 2009b).

Table 1
Dataset used in the classification studies.

Animal no.	Class	IFN-γ ELISA (ODI)	IL-17 (FC)	IL-2 (FC)	IL-10 (FC)	IL-4 (FC)	CD4+CD25+ (%)	CD8+CD25+ (%)
371-2011	ML+	8.16	−1.52	0.68	−0.61	−2.80	20.17	7.03
352-2011	ML+	18.08	3.68	4.07	−0.52	−0.87	40.60	36.29
365-2011	ML+	21.35	3.04	4.30	1.18	0.06	33.83	16.00
363-2011	ML−	12.43	−8.59	−2.01	−11.29	−5.77	19.35	8.57
362-2011	ML+	8.14	1.92	2.68	−1.58	−0.62	25.48	18.28
355-2011	ML+	11.52	4.94	4.38	−1.18	2.61	32.26	24.79
377-2011	ML−	12.14	0.48	1.65	−0.50	−3.03	15.79	15.96
350-2011	ML−	1.57	3.03	8.15	2.90	2.40	45.31	22.25
373-2011	ML−	2.09	0.19	6.95	−0.11	−1.63	13.37	9.16
358-2011	ML−	20.12	1.50	6.36	−0.78	3.20	23.89	15.00
368-2011	ML−	23.84	−1.46	2.00	−0.87	−0.22	47.35	49.61
366-2011	ML−	27.28	0.81	3.23	1.68	0.98	54.70	45.52
361-2011	ML−	3.14	0.11	5.47	−0.33	2.46	19.42	36.00
364-2011	ML−	2.77	3.27	7.79	−0.48	1.16	39.65	41.89
370-2011	ML−	1.60	5.81	1.77	−0.24	−1.59	22.30	8.53
248-2009	ML−	11.63	−0.57	5.04	−3.06	5.29	50.70	49.10
251-2009	ML−	5.54	1.68	5.00	−2.04	4.22	45.50	61.40
74-2009	ML−	11.86	−0.08	4.44	−1.24	4.75	26.60	39.10
244-2009	ML−	15.81	−0.35	2.93	−2.95	3.62	29.60	34.30
246-2009	ML+	16.19	1.29	4.50	−2.89	4.56	30.00	35.00
63-2009	ML−	12.65	−0.19	3.25	−1.32	3.57	42.80	43.00
66-2009	ML+	7.66	3.26	7.08	−2.15	5.78	54.10	44.60
318-2010	ML+	22.64	5.35	3.12	−4.06	1.39	17.87	7.91
325-2010	ML+	12.16	6.10	5.99	−3.84	3.39	28.84	21.42
418-2010	ML+	16.34	5.04	3.62	−2.40	2.62	69.60	41.52
424-2010	ML+	17.43	4.98	5.77	−2.12	6.07	84.40	76.60
000-2010	ML+	16.14	7.03	3.32	2.94	4.02	54.72	43.79
008-2010	ML+	13.86	7.12	4.01	1.76	5.64	ND	ND
276-2010	ML+	16.64	8.99	4.80	2.61	4.26	28.79	46.57
307-2010	ML+	17.92	7.52	4.79	2.54	4.45	59.12	59.36

Macroscopic lesions and immunological parameters observed/determined in the 30 experimentally infected cattle. ML+ and ML−: animals with and without macroscopic lesions; ODI: optical density index; FC: fold change (log 2); ND: not determined.

Cytokine mRNAs (IL-2, IL-4, IL-10, IL-17, and IFN-γ) were quantified by reverse transcription and quantitative polymerase chain reaction (RT-qPCR) using specific primers (Blanco et al., 2009a). The amplification program consisted of an initial denaturation step of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplification efficiencies for every pair of primers were determined using calibration dilution curves (Pfaffl, 2001) and increase of absolute fluorescence method (Ramakers et al., 2003): *gapdh*: 1.95; IL-2: 1.91; IL-4: 2.00; IL-10: 1.72; IL-17: 1.86 and IFN-γ: 1.96. The results of qPCR were analyzed using the REST software as described previously (Blanco et al., 2009b). The pre-immune condition was used as the calibrator and *gadph* was used as a reference gene. Glyceraldehyde 3-phosphate dehydrogenase expression was stable between the different conditions and was employed as a reference gene in our previous studies (Blanco et al., 2011, 2012) and others publications (Widdison et al., 2006; Rhodes et al., 2007).

2.5. Skin test

All animals were tested for skin tuberculin test reactivity before and after *M. bovis* inoculation. Animals were intradermally injected with 0.1 ml of PPDB and the thickness of the caudal fold tuberculin skin test was measured using calipers (Stanley, New Britain, USA) immediately before the injection and again 72 h later. PPDB (32,500 IU/ml) was obtained from the National Service of Agricultural and Food Health and Quality (SENASA, Buenos Aires, Argentina).

2.6. Data analysis

All the classifiers and receiver operating characteristic (ROC) curves were performed using R version 3.0.2 (R Core Team (2013)). The procedures implemented in the MASS library were used for the discriminant analyses. The k-nn was carried out with the class library and the ROC curves were analyzed with the ROCR library (Sing et al., 2005).

3. Results

Animals were divided in two groups according to *post-mortem* pathology scores. Cattle with macroscopic lesions in lungs and lung lymph nodes conformed the macroscopic lesions (ML) positive group ($N = 15$) and animals without lesions the ML negative group ($N = 15$). A dataset of immunological variables were used to discriminate between both groups of animals (Table 1). The classifiers tested for discrimination were lineal discriminant analysis (LDA), quadratic discriminant analysis (QDA) and K nearest neighbors (k-nn). The initial dataset had seven putative predictive variables: IFN-γ (ELISA), expression of IL-2, IL-4, IL-10 and IL-17 by RT-qPCR as well as activation of CD4 and CD8 lymphocyte subsets determined by FACS. In a

Table 2
Classification of cattle using lineal discriminant analysis (LDA).

Observed	Predicted	
	ML+	ML−
A		
ML+	10 (71.4%)	4
ML−	3	12 (80%)
B		
ML+	12 (80%)	3
ML−	2	13 (86.7%)
C		
ML+	11 (73.3%)	4
ML−	1	14 (93.3%)

A: including all the immunological parameters studied. B: With IL-17 and IL-2. C: using IL-2, IL-10 and IL-17. The tables show the number of animals with or without TB lesions confirmed at necropsy (observed) and predicted by the classifier (predicted). (ML+ and ML−: animals with and without macroscopic lesions respectively).

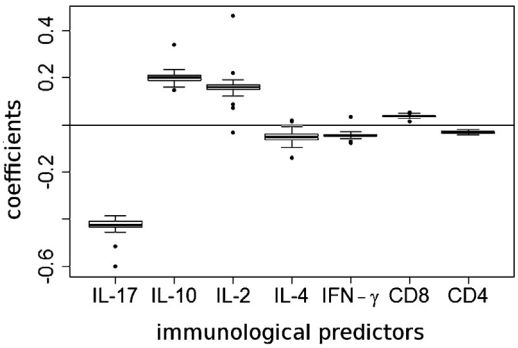

Fig. 1. Distribution of LDA coefficients. The boxplots show the distribution of the coefficients for each predictor produced by the leave-one-out process applied to the LDA. The largest the absolute value of the coefficient, the largest its predicting value.

previous analysis, the ranges of the predictors were normalized between 0 and 1 to discard any spurious effect caused by variables with large ranges but with little or none discriminating power. However, the performances of the range-normalized classifiers were consistently lower than those observed with the untransformed dataset (data not shown). This effect could be due to that several variables were already expressed as ratios between the infected and pre-infection stages, which would be enough to render comparable variables. To assess the performance of classifiers, and at the same time take into account the relatively small size of the dataset, we performed a leave-one-out validation strategy. Briefly, a classifier was trained with all records minus one, which was reserved for class prediction. This process is repeated iteratively leaving a different record out in each loop until all of them were used for prediction. Each predicting variable will contribute differentially to the discriminant power of the classifier. The sign and magnitude of their coefficients in the lineal discriminant function is a quantitative measure of their importance for classification.

3.1. Classification with lineal discriminant analysis

First, we built classifiers using lineal discriminant analysis as implemented in the *lda* function of the MASS package of R. The MASS package covers different types of supplementary methods that enhance R core (Venables and Ripley, 2002). The rationale of this method is to generate, for each class, multivariate distributions, whose parameters are used to derive lineal discriminant functions. The output of these functions is the class prediction. Applying the leave-one-out-strategy outlined before, we generated 29 discriminant functions (one record had missing data for activation of CD4 and CD8 lymphocyte subsets) and made predictions for the 29 subjects that were left out. The classifier correctly predicted the infection stage in 75.9% of the cases and its performance was slightly better for the prediction of ML− animals than for ML+ animals (80% vs. 71.4%). Table 2A summarizes the results.

Not all immunological variables were good predictors; furthermore, the presence of some of them could actually reduce the overall predicting ability of a classifier. To determine if this is so, we pooled the coefficients for each variable for the 29 discriminant functions and plotted them in a boxplot (Fig. 1). This plot indicates that IL-17 is the most influential variable for classification, with minor influences from IL-2 and IL-10.

To further clarify this point, we built a classifier that included all predicting variables except IL-17. This new classifier could only predict the correct class for only 51.7% of the test records and a multivariate analysis of variance (manova) showed that this classifier had no significant explanatory power over class (Pillai's statistics: 0.23637; *P*: 0.375). These results indicate that a classifier without IL-17 would basically behave as a random classifier.

A lineal discriminant classifier with IL-17 as the only variable showed an overall success rate of 80.0% and both class levels (ML+ and ML−) were also correctly predicted in 80% of the cases. Although we demonstrated that a classifier without IL-17 was no better than making random predictions, a classifier with IL-17 and some other variable(s) could still make better predictions than IL-17 alone. To investigate this possibility, we built lineal discriminant classifiers with IL-17 and each other variable. The best results were obtained with a classifier with IL-2 and IL-17 as predictors: the overall success rate was 83.3%, in which 80% and 86.7% were correctly predicted in the ML+ group and in ML− group, respectively (Table 2B). We kept these classifiers for further comparisons.

Alternatively, an LDA including IL-2, IL-10 and IL-17 also showed an overall success rate of 83.3%, but with an increased range between both groups: 73.3% and 93.3% correctly classified into ML+ and ML− animals respectively (Table 2C). This last LDA is more efficient to discriminate animals without lesions.

3.2. Classification with quadratic discriminant analysis

Quadratic discriminant analysis is a technique that relaxes the equal variance assumption of the lineal discriminant analysis, but requires more calculation power. However, with a modern computer its calculation, even for a larger dataset, should not be a problem. We tested all the variants described before (with and without normalization, IL-17 alone and IL-17 combined with other variables).

We observed the same pattern as before; the best classifier included only IL-2 and IL-17 as predictors, without normalization, which presented identical performance rates as the lineal discriminant classifier.

3.3. Classification with K-nearest neighbors

The third classification method tested here was the k-nearest neightbors (k-nn) algorithm. This method has its root in machine learning rather than in statistics, as is the case for discriminant analysis. With k-nn the class of a test individual is determined according to the known classes of the majority of its k nearest points. For this reason, the number of neighbors (k) is usually an odd number. The neighborhood criterion is the distance between points using the euclidean distance in a multidimensional space where each dimension corresponds to a predicting variable.

We built k-nn classifiers using all the predictors and two values of k (3 and 5). In all cases, the performance was very low and comparable to a random classifier. When we repeated these analyses using only IL-2 and IL-17 as predictors, the performance of the classifiers improved. The overall success rate was 80.0%, while the per-class success rate was 73.3% and 93.3%. Compared to the lineal and quadratic discriminant analyses with IL-2 and IL-17, the overall success rates for k-nn were lower but the spreading of class predictions was greater. When we used k-nn, the percent of correct predictions was lower for ML+ (73.3% vs. 80.0%) but higher for the ML− class (93.3% vs. 86.7%), as compared to LDA analysis. Identical correct predictions were obtained in LDA analysis (using the three cytokines IL-2, IL-10 and IL-17) and in k-nn method (using IL-2 and IL-17). However, in LDA analysis, the overall success rate was higher (83.3% vs 80%).

3.4. Receiver operating characteristic curves

To examine the trade-offs between the detection of true and false positives we plotted ROC curves for the ML+ class (Fig. 2). These curves combine the ML+ class predictions obtained during the cross-validation process and the actual class of the animals. The ROC curve for the LDA with IL-2, IL-10 and IL-17 as predictors presented an area under the curve (AUC) of 0.83 (dotted line, Fig. 2). When IL-2 and IL-17 were used as predictors, the ROC curves were the same for LDA and QDA classifiers, with an AUC of 0.79 (dashed line, Fig. 2), while the best k-nn classifier presented an AUC of 0.81 with the same predictors (continuous line, Fig. 2). Although the AUC for the LDA with three predictors is higher than the AUC of the other classifiers, the reverse happens with the ROC curves for the ML− class (data not shown). Thus, the classifiers with IL-2 and IL-17 are more stable, with a slightly higher AUC for the k-nn. However, a closer inspection of the ROC curves suggests that the improvement in k-nn occurs at the extremes of the sensitivity and false positive rate ranges; in contrast, both the LDA and QDA had better performance at the more useful mid-range values.

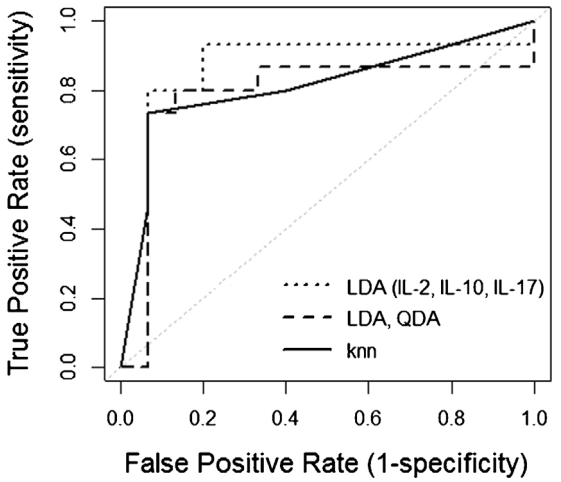

Fig. 2. Receiver operating characteristic (ROC) curve for the different classifiers: LDA using IL-2, IL-10 and IL-17 as predictors (dotted line); LDA and QDA (dashed line) and best k-nearest neighbors (k-nn) classifier (continuous line) using IL-2 and IL-17 as predictors. The thin dotted diagonal corresponds to a random classifier. The areas under the ROC curves (AUC) were 0.85 for the LDA with three predictors, 0.79 and 0.81 for the discriminant and k-nn classifiers with two predictors, respectively. All the ROC curves shown correspond to the detection of infected animals with lesions (ML+).

4. Discussion

Several studies have been conducted to find novel TB biomarkers, many of them using a multivariate statistical analysis. Discriminant analyses were applied in clinical studies for the differentiation of healthy-tuberculin skin test positive patients and TB patients to improve human TB diagnostic (Sánchez et al., 1994; Jacobsen et al., 2007).

This is the first report analyzing bTB biomarkers of active disease using discriminant analysis and k-nn as classifiers. Although the analysis will need validation in a larger cohort, in this preliminary study we identified which immunological parameters correlates with advanced disease in our experimental infected heard. The addition of new datasets to this database could improve the test and lead to a better understanding of immune parameters involved in the pathological progression of bTB.

Alternatively, immunological datasets from natural infected herd might be used for evaluating the group of biomarkers but a *post-mortem* examination is essential to confirm TB lesions. This last issue is particular difficult because of the complexity of tracing cattle to the slaughter facilities.

The cytokines selected as the most discriminant variables in the analysis have been subject of several studies conducted in natural and experimentally infected cattle (Widdison et al., 2006; Rhodes et al., 2000; Thacker et al., 2007). In general, in infected animals a typical Th1 response is characterized by high levels of IL-2 and IFN-γ (S G Rhodes et al., 2000). However, IFN-γ transcripts and proteins were not useful for separating ML+ from ML− cattle in the current study because the levels of IFN-γ detected using the whole blood IFN-γ ELISA assay or RT-qPCR varied considerably among animals. Similar findings were described by Lim et al. (2012).

Although interleukin 10 has shown to be downregulated in animals with exacerbated pathology in several experimental infections (Thacker et al., 2007; Welsh et al., 2005), in the present study we did not detect downregulation of IL-10 in ML+ and the expression of this anti-inflammatory cytokine was similar in both ML− and ML+ groups. However, when we analyzed the coefficients for the discriminant functions in the LDA (Fig. 1), IL-10, together with IL-2, contributed to enhance the discriminant power of a classifier with the variable IL-17 alone, and improved the classification of ML− cattle.

In a previous report, we had found a positive correlation between IL-17 expression and pathology (Blanco et al., 2011). This observation was consistent with other report where IL-17 has been suggested as a biomarker of disease (Aranday-Cortes et al., 2012). This particular cytokine is the most important variable selected by the LDA to identify animals with granuloma formations in lungs. Moreover, the expression of this cytokine between both groups was statistically different and this difference was the largest observed among all the variables studied in the experimental infections.

Although the mRNA quantification is a validated method to determine the production of cytokines in bovine PBMC samples (Lim et al., 2012; Kim et al., 2013), this quantification requires specific laboratory facilities, such as real time thermo-cyclers, that would limit its use on field. Fortunately, ELISA commercial kits for quantification of bovine cytokines IL-2 and IL-17 have been very recently developed (Kingfisher biotech, Saint Paul, USA and Uscnk Life Science, Wuhan, China). The use of these ELISA systems for the quantification of the biomarkers here identified is crucial to validate them.

In this study we evaluated three different statistical approaches and found that all the classifiers identified IL-2 and IL-17 as the most discriminatory variables. This observation supports the importance of IL-2 and IL-17 as biomarkers of disease in bTB. The inclusion of IL-10 improves the identification of ML− animals but it is important to evaluate the cost and benefit balance of adding another transcript expression assessment. For example, the addition of IL-10 into the expression analysis could be useful to monitor the progression of the disease in herds with no clinical symptoms of bTB but with history of positive reactor to tuberculin test.

The advantage of multivariate analysis is the overall evaluation of a set of immune mediators instead of the particular function of every parameter in the disease. The results of these preliminary studies are useful to identify a signature of advanced disease in cattle.

Conflict of interest

The authors state that they do not have conflict of interests regarding the publication of this article.

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