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Colostrum proinflammatory cytokines as biomarkers of bovine immune response to bovine tuberculosis (bTB)

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ABSTRACT

Bovine colostrum contains compounds, which provide passive immune protection from mother to newborn calves. Little is known about cytokine levels and their role in bovine colostrum. Moreover, the capacity of bovine colostrum cells to mount specific immune responses after natural exposure to bovine tuberculosis (bTB) antigens in dairy herds has not been studied, thus far. The purpose of this study was to identify biomarkers for bTB infection measurable in bovine colostrum. The present study reveals that isolated-immune colostrum cells can mount a specific immune response against bTB antigens, by measuring the novo IFN-γ release in cell culture. We found that IFN-γ levels in the responders (Bov$^+$) to bTB antigen were higher than in non-responders (Bov$^-$). On the other hand, proinflammatory cytokines contained in colostrum's whey were tested in Tuberculin Skin Test (TST) reactor (TST$^+$) and non-reactor (TST$^-$) animals to assess their potential role as biomarker. We observed that IFN-γ levels were lower or undetectable, as opposed to IL4 levels were measurable, the TNF-α level was higher in TST$^-$ than TST$^+$, while IL-6 levels showed the opposite reaction and with no statistical significance. Moreover, IL-1α mRNA expression levels were higher in colostrum mononuclear cells (CMC) in Bov$^+$ cattle. Collectively, these data suggest that the differential expression of pro and anti-inflammatory cytokines could have relevant value to diagnose bTB in cattle.

1. Introduction

The ruminant epitheliochorial placenta cannot transfer passive immunity (PI) from mother to fetus during gestation, thus resulting in a neonatal calf immune system that is often referred as immature [9,14]. Thus, PI in cattle is delivered through colostrum, which is the first milk produced after birth [47]. Colostrum is a complex fluid, rich in nutrients and characterized by its high level of bioactive components. Such as immunoglobulins [21] and growth factors, especially insulin-like growth factor-1 (IGF-1); transforming growth factor beta-2 (TGF-b2) [33], and growth hormone (GH). Notably, colostrum also contains antimicrobials [22], enzymes like lactoferrin, lysozyme and lacto peroxidase [34,39], as well as immune cells; and regulatory factors like cytokines [18,42].

It is known that the concentration of molecules and cellular components found in bovine colostrum, change significantly from the first three days post-partum throughout lactation [42].

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Therefore, it is imperative that colostrum is consumed by the calf, in a timely manner and in sufficient quantities during the 6–12 h after birth [29]. Since, maternal PI is essential to confer neonatal protection [5] and to reduce the failure of passive transfer (FTP) and its associated disorders. The FPT in calves has been associated with high neonatal morbidity and mortality, which decreases the dairy herd population and causes a long-term decline in milk production [4].

Although the concept of protective maternal immunity transfer by antibodies has been known for decades [7,47], only few studies have addressed the specific function of resident T cells in maternal colostrum [17] and their immunological impact in newborn calves. Once digested, colostrum immune cells migrate into the digestive tract, where they are transported through epithelium-associated Peyer's patches follicles of the neonate intestine [28]. These cells are important in promoting the development and maturation of the immune system in the newborn animals [26,37,38], and bovine colostral lymphocytes have been shown are able to response to concanavalin A [2].

T cells in human and bovine colostrum produce cytokines, which are soluble molecules [10], that mediate key cell signaling. However, little is known about the physiological significance of T cell presence in bovine colostrum or the specific profiles of Th1 or Th2 subsets [17].

While the intake of colostrum by the calves is essential, some studies have shown that it could become a vehicle for pathogens such as *Salmonella dublin*, *S. typhimurium* [35] and *Mycobacterium bovis* [20].

Thus, transmission of infectious diseases via colostrum ingestion represents a major health risk, since the intake of contaminated colostrum/milk is a natural means of bTB infection from cow to calf [20,54,55].

In developing countries like Mexico, the disease transmitted by *Mycobacterium bovis* causes both economic losses and health problems [12]. Despite the use of tuberculin skin test (TST) with PPD-B as antigen, to identify bTB reactor carriers for slaughter, according to NOM-031-ZOO-1995 in Mexico, the incidence and prevalence of bTB in cattle herds remains high. An alternative test to screen cattle is the BOVIGAM method, which is an *in vitro* assay based on the quantitation of cellular immune responses that detects IFN-γ production by T-lymphocytes from whole bovine blood, after stimulation with purified protein derivative (PPD-B) of the *M. bovis* [48,49].

The central aim of the present study was to identify potential markers of colostrum bTB transmission in reactor and non-reactor animals to bTB. To that end, the following studies were performed: 1) to evaluate the specific response of colostrum mononuclear cells (CMCs) against PPD-B antigen stimuli, using interferon gamma release assays (IGRA); 2) to assess the level of other cytokines in colostrum whey, as a means to compare their respective profile between T helper 1 (Th1) and Th2 cell subsets; and 3) to assess mRNA cytokine expression in CMCs.

We propose that the data resulting from this study could identify specific biomarkers of bTB infection, which could provide an effective means for early diagnosis of colostrum transmission in cattle and thus help to improve animal health and food safety.

2. Material & methods

2.1. Colostrum samples

Colostrum samples were collected by veterinary personnel according to approved guidelines, from eight different commercial herds dedicated to milk production in the State of Jalisco, México. Holstein–Friesian cattle herds lived in herds classified with high prevalence (>10%), which are naturally exposed to bTB. Samples were aseptically collected in flasks on the day of parturition (d0) or two days post-partum (d2). For cytokine assays in colostrum whey, all animals (n = 73) had previously undergone tuberculin skin testing (TST), according to NOM-031-ZOO-1995, followed by sample classification into TST^+ n = 31 or TST^- n = 42 groups. For assays using colostrum mononuclear cells (CMC), colostrum was collected the day of parturition (d0) and the criteria to set the study groups were based on the results of the Bovigam® IFN-γ assays (63320, Life Technologies) obtained from blood samples. The method is a laboratory-based test measuring *in vitro* in PBMC (peripheral blood mononuclear cells) responses, in which whole blood is incubated overnight in the absence or presence of PPD-B (purified protein derivative from bovine) and PPD-A (purified protein derivative from avian), at 37 °C. IFN-γ produced after antigen-specific stimulation and quantitatively measured by ELISA. Based on this assay, samples were classified as responders Bov^+ and non-responders Bov^-.

2.2. Colostrum whey sampling and colostrum mononuclear cells (CMC)

Colostrum whey: Seventy-three samples of colostrum whey were collected and frozen at −20 °C, until used. A volume of 50 ml of frozen colostrum was thawed on ice, then diluted (1 vol: 1 vol) with 1× sterile PBS (phosphate buffered saline), and centrifuged at 2500 rpm for 20 min at 4 °C. After centrifugation, the lipid layer was removed and discarded; 1 ml of supernatant was collected and aliquoted into 1.5 ml tubes and stored at −20 °C until ELISA assays were performed, to avoid repeated freeze-thaw cycles.

Thirty Colostrum Mononuclear Cells (CMC): Thirty samples were classified; 15 positive Bov^+ and 15 negative Bov^-, according to IFN-γ production assessed by Bovigam assay in blood samples. About 400 ml of fresh colostrum were collected in sterile plastic flasks after 12–24 h postpartum. Samples were diluted (1 vol: 1 vol) in 1× PBS and centrifuged at 2500 rpm, for 20 min at 4 °C. Total cells were washed three times with sterile PBS, suspended in RPMI-1640 media (R0883, Sigma), layered over a Histopaque (1077, SIGMA) gradient to enrich CMC, and centrifuged at 900 g by 30 min at 22 °C, following the protocol for blood samples adapted for bovine colostrum [8,18]. The interphase was removed and transferred to a new 15 ml tube, pelleted, and washed five times with sterile PBS. CMC cell viability was determined by trypan blue dye exclusion (15250-061, GIBCO). Cells were then suspended in RPMI-1640 media, supplemented with 10% heat-inactive fetal bovine serum (10082139, GIBCO), 100 U/l penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine (hereafter referred as media).

2.3. CMC interferon-gamma release assay after TBb antigen (PPD-B) stimulation

An Interferon-γ release assay (IGRA) from freshly isolated CMCs, was carried out after antigen stimulation and using ELISA. A total of 3×10^5 CMCs, isolated from Bov^+ n = 15 and Bov^- n = 15. Cells were seeded on 48-well flat-bottomed plate in 300 μl of total media: a) without stimuli (NIL); b) media plus bovine 100 μl PPD-B; c) media plus 100 μl of avian PPD-A; and d) media plus Concanavalin A (1.4 μg/ml, Con A) to provoke IFN-γ production. After adding all the stimuli, cells were incubated for 16 h in a humidified atmosphere, containing 5% CO_2. The plate was then centrifuged at 200 g for 20 min at room temperature; supernatant aliquots were collected into 1.5 ml tube and stored at −20 °C, until assays were carried out. The IGRA release by CMC was determined, using the kit for bovine IFN-γ (ESS0026B, Thermo Scientific), all assays were performed by triplicate, and the concentration of IFN-γ was

assessed by using a standard curve with dilutions of the recombinant IFN-γ standard with known concentration. The optimal incubation time was preemptively established after a series of tests (data not shown).

2.4. Cytokine measurements in colostrum whey

The bovine colostrum is liquid thick, sticky, yellowish with a high degree of fat. For this, in order to quantify the cytokines colostrum whey fraction was obtained; the whole colostrum was 1× PBS diluted and centrifuge, the fat was discarded and colostrum whey fraction was collected. The concentration of IL-6, TNF-α, and IFN-γ was measured, using commercially available ELISA kits: Bovine IL-6 (ESS0029, Thermo Scientific); bovine IFN-γ (MCA5638KZZ, RD System) and Bovine TNF-α (DY2279, AbD Serotec). All cytokine measurements were performed according to manufacturer's instructions. For IL-4 levels, the ELISA assay was optimized [3] with some modifications. Briefly, a 96 well plate was coated overnight with 50 μl of *anti*-IL4 antibody (MCA2371, AbD serotec) per well [2 μg/ml] in carbonate buffer (0.2 M sodium carbonate/bicarbonate, pH 9.4) at 4 °C. After incubation, the plate was washed three times with PBS/Tween 0.05% (washing buffer), and blocked with 2% low-fat milk (M7409 Sigma)/PBS (1×), the reason to use milk was as blocking reagent is it did not show un-specific binding, or high background in the control. Moreover, some ELISA assays using BSA (Bovine Serum Albumin) for blocking, have shown a non-specific binding interaction that occurred between an ELISA reactant and some preparations of BSA [50]. Subsequently, the plate was rinsed three times with washing buffer, followed by the addition of 100 μl of biotin-conjugated secondary mouse-anti bovine IL-4 antibody (MCA2372B, AbD serotec) at 2 μg/ml and incubated for 2 h at room temperature. The plate was washed five times and reacted with 100 μl R T.U. (SA5704, horseradish-Peroxidase streptavidin, vector Laboratories). Reaction was subsequently revealed after a 15 min incubation with 100 μl of the Ultra TMB substrate (34028, Thermo Scientific) and stopping the reaction with 100 μl of stop solution (2 N H_2SO_4). Bovine antigen IL-4 (PBP010, AbD Serotec) was used for standard curves. All samples were run in triplicates and results were calculated by interpolation of absorbance at OD 450 nm (spectrophotometer Xmark BIORAD 168-1150) of sample values against the standard curve. Results were presented as protein concentration in picograms per milliliters (pg/ml).

2.5. CMCs cytokine mRNA expression and semi-quantitative RT-PCR

mRNA levels of IL 1-α; NM_174092, IL-2; NM_180997, IL-4; NM_173921, IL-6; NM_173923.2, IL-10; NM_174088, TNF-α; BC134755, and IFN-γ; E01329.1 were assessed by RT-PCR, from freshly isolated CMC from Bov^+ n = 10 and Bov^- n = 10. Followed CMC isolation, cell lysis and RNA extraction were performed with 400 μl of TRIzol (15596026, Life Technologies), according to manufacturer's instructions. The concentration of RNA was measured by NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). 1 μg of total RNA was used to generate cDNA templates by reverse transcription, using RevertAid H cDNA Synthesis Kit (K1631, Thermo Scientific) and a thermal cycler (Biorad T100 186–1096), according to manufacturer's instructions. The resulting cDNA was used to amplify each cytokine (K0171, Thermo Scientific) by a standard PCR program: 95 °C for 5 min, followed by 35 cycles at 94 °C for 45s denaturation; annealing range between 53 °C and 65 °C for 45s (temperature for each cytokine is showing in Table 1); and amplification at 72 °C for 45s. The housekeeping β-actin; (NM_001033618.1) was used as an internal control to normalize for mRNA content The PCR products were run on a 1.5% agarose gel, using Tris-acetate-EDTA buffer and staining with Sybr Safe staining (S33102, Life Technologies) staining. Relative Gene expression changes were analyzed using Image J software with the β-actin. The primer cytokine set used for RT-PCR was designed with the Primer3 software and is summarized in Table 1.

2.6. Data analysis and statistics

Because cytokine concentration were not normally distributed, protein level results were analyzed by unpaired tests, using Student's *t*-test. For CMC IFN-γ release and cytokine mRNA expression data, paired sample Student's *t*-test analysis was applied. And in Figs. 2 and 3 the Bonferroni correction was applied to the p values. All statistical analysis were performed using the GraphPad Prism 6 version 6.0 b, Software (San Diego,CA, USA, www.graphpad.com). *p* values lower than 0.05 were considered significant.

3. Results

3.1. IFN-γ release by CMC, from responder Bov^+ and non-responder Bov^-

Immune cells from whole blood of bovine reactors to bTB are able to respond by producing and releasing IFN-γ after cell stimulation with bovine antigens such as PPD-B. We wanted to assess, whether isolated-CMC from colostrum would respond in a similar manner, which would allow us to classify herds into reactor and non-reactor cattle, using a non-invasive colostrum sample collection. To accomplish this, isolated-CMC from responders Bov^+ n = 15 and non-responder Bov^- n = 15 were incubated for 16 h with antigens PPD-B and PPD-A from the BOVIGAM kit. IFN-γ levels in the collected supernatants, were determined by ELISA assays. The results in Fig. 1 show that after PPD-B stimulation, the released IFN-γ levels were higher in Bov^+ than in Bov^- group, with a significant $p < 0.0001$ value. In contrast, no increase of IFN-γ release levels was observed in PPD-A stimulated CMC from either group. Consistent with the literature, the results with the positive control Con A were as expected, namely: the stimulated-CMC Bov^+ released more IFN-γ compared with Bov^- with a *p* value = < 0.001. Similarly, the non-stimulated CMC (NIL) negative controls did not produce IFN-γ. Remarkably, IFN-γ protein release levels from Bov^+ were higher with PPD-B stimulated and more specific than those treated with polyclonal Con A. Our findings are important because there was no previous evidence that CMC from responder cattle can mount a specific cell-mediated immune responses initiated by bTB signaling. Given the importance of the role of this molecule in response against *Mycobacterium bovis* [15,43,52], CMC might be a potential source of samples for classifying bovine as responders or non-responders and using a non-invasive colostrum sampling, which can be readily performed postpartum and be useful in dairy herds. It is also possible that this response will be a means to protect calves from imminent infection by *M. bovis* in colostrum that contain it. Although IFN-γ release has been shown in many studies using blood samples, the analysis in colostrum CMCs reported in this study, is the first non-invasive and simple approach that provides a quantitative tool for the dairy industry to carry out massive cattle screening tests in the herds.

3.2. IL-4, TNF-α, IL-6 and IFN-γ protein levels in colostrum whey

To evaluate the content of immune-modulatory factors in colostrum whey, which define the T helper (TH) cell profile between the experimental groups, cytokines IL4, IL-6, TNF-α and IFN-γ were analyzed from a large sample screen of defined experimental bovine groups using TST assay. In the 73 colostrum whey

Table 1
Primers used for mRNA cytokines expression by RT-PCR semi quantitative.

Primers	Sequence	Tm	Amplicon size (pb)	Accesion number
IL-1-α	F: ATGAGCCACTTCGTGAGGAC R: CTCCTTTAGCAAGACGGGTTC	57.6 °C	500 pb	NM_174092
IL-2	F: TTCAAGCTCTACGGGGAACAC R: TCAAGTCATTGTTGAGTAGATGCT	55 °C	400 pb	NM_180997
IL-4	F: GTCTCACCTACCAGCTGATCCG R: TTTCAGCGTATCTGTGCTCT	55 °C	350 pb	NM_173921
IL-6	F: TGACTTCTGCTTTCCCTACCC R: ATAGCTCTCAGGCTGAACTGC	57.6 °C	550 pb	NM_173923.2
IL-10	F: GCTCAGCACTACTCTGTTGC R: CCCTCTCTTGGAGCTCACTG	53.8 °C	450 pb	NM_174088
TNF-α	F: AGGAGGTGCTCTCCGAGAAA R: TGAGGGCATTGGCATACGAG	55.3 °C	300 pb	BC134755
IFN-γ	F: GTAGCTAAGGGTGGGCCTCT R: TCTGCAGATCATCCACCGGA	62.6 °C	250 pb	E01329.1
Bovine β-actin	F: TGCTGTCTCTGTACGCCTCT R: TGTAACCACGTTCCGTGAGG	57.6 °C	180 pb	NM_001033618.1

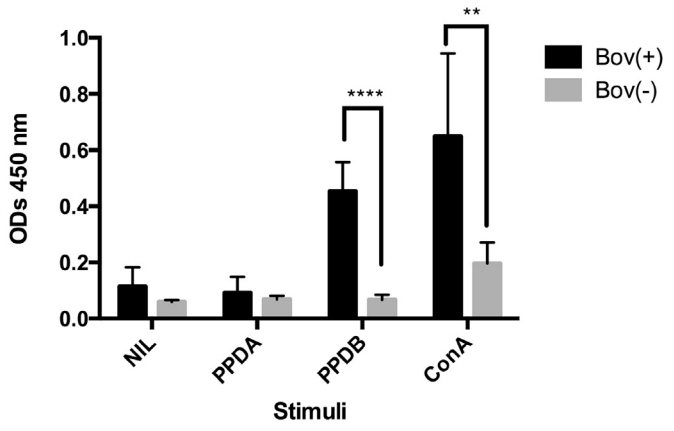

Fig. 1. IFN- γ *ex vivo* released by colostrum mononuclear cells.
Description: IFN-γ released assay (IGRA) by colostrum mononuclear cells (*ex vivo)* in Bov^+ n = 15 and Bov^- n = 15.3 × 10^5 CMCs were stimulated with PPD-A; PPD-B; with concanavalin A (1.5 μg/ml), and without stimuli (NIL). Comparison of IGRA levels was higher in Bov^+ than in Bov^- animals, with a value of $^{****}p < 0.0001$.

samples, comprising 31 TST^+ and 42 TST^- measurable immune-reactive levels of IL-4, TNF-α and IL-6 were observed in all colostrum whey, whereas only low or undetectable IFN-γ levels were registered in 58% of samples of the TST^+ and 54% of TST^-, as summarized in Table 2. On the other hand, the IL-4 median levels were 562.0 (range 842.1–329.6) pg/ml for TST^+ versus a median of 551.7 (range 724.2–328.8) pg/ml for TST^-. However, there was no significant difference between the groups studied. Similarly, IFN-γ levels did not show significant difference between the groups. For instance, the upper and lower quartiles results for TST^+ and TST^- were 88.33–0 pg/ml and 54.1–0 pg/ml respectively. While this result is at odds with the important role of IFN-γ in T cell mediated responses against bTB, it is conceivable that undetectable IFN- γ levels may be a reflection differential protein stability [30,53]. In contrast, the results of IL-6 levels showed a median of 287.5 (range 586.3–98.03) pg/ml for TST^+ versus median 171.5 (range 641.3–56.45) pg/ml for TST^- with a *p value of* < 0.0300 but when Bonferroni correction was applied, the p value showed that there was not significant difference. Moreover, a similar trend was seen for TNF-α levels, in which a median 82.58 (range 98.08–63.60) pg/ml for TST^+ and 116.3 (range 208.0–88.90) pg/ml for TST^- $p = 0.0043$ and when p value was corrected by Bonferroni the statistical significance remains $p = 0.0125$ was consistently noted. As such, Fig. 2 shows the same tendency in the levels (in pg/ml) of all the samples tested for IL-4, TNF-α, IL-6 and IFN-γ. Of note, the concentration of TNF-α concentration was higher in TST^- than TST^+, meanwhile IL-6 in colostrum whey was higher in TST^+ than TST^- although there was not significant relevant. These data collectively suggest a clear dichotomy between pro-inflammatory IL-6 and TNF-α protein, which could be a signature biomarker for cattle test screening of bovine colostrum whey. The pathophysiological meaning of such dichotomy remains to be defined.

3.3. Cytokine expression (mRNA) in responder Bov^+ and non-responder Bov^- cattle

To extend our expression analysis and investigate known whether other cytokines could be detected in the colostrum, mRNA levels were determined in CMCs. Thus, IL1-α, IL-2, IL-4, IL -6, IL-10, TNF-α and IFN-γ mRNA expression was assessed by semi-quantitative RT-PCR, using cDNA as template samples from CMC isolates from responders Bov^+ n = 10, and non-responder Bov^- n = 10, animals. The expression of each cytokine was obtained and normalized against an actin reference, which is then presented as relative expression. As shown in Fig. 3A, IL-6, IL-10, TNF-α, and IFN-γ were expressed at the same levels in both Bov^+ and Bov^- groups, whereas IL-1α mRNA levels were higher in Bov + when compared with Bov-with p = 0.044 by t-student and p = 0.01 after Bonferroni correction. On the other hand, IL-2 and IL-4 were not expressed in either group at any time. As a representative example Fig. 3B and C shows agarose gel images of amplified IL1-α, IL-6, IL-10, and TNF-α fragments from respective Bov^+ and Bov^- samples. Future studies should determine whether the correlation between protein and mRNA levels is the result of differential molecular stability, associated to downstream mRNA degradation and/or protein post-translational modifications. Nevertheless, the data presented in this study suggest that the major immune response between the Bov^+ and Bov-groups, resides in the expression of pro- IL-1α, and anti-inflammatory IL10 cytokines. Thus, the role of these cytokines as potential biomarkers of bTb transmission from bovine mothers to offsprings is a worthwhile concept.

4. Discussion

Although it is known that human and animal colostrum contains bioactive substances such as hormones, growth factors and cytokines, the overexpression or down-regulation effects of these immunomodulatory molecules in the calves are not completely understood. For instance, cell-depleted colostrum can decrease the ability of calf's lymphocytes to mount effective immune responses,

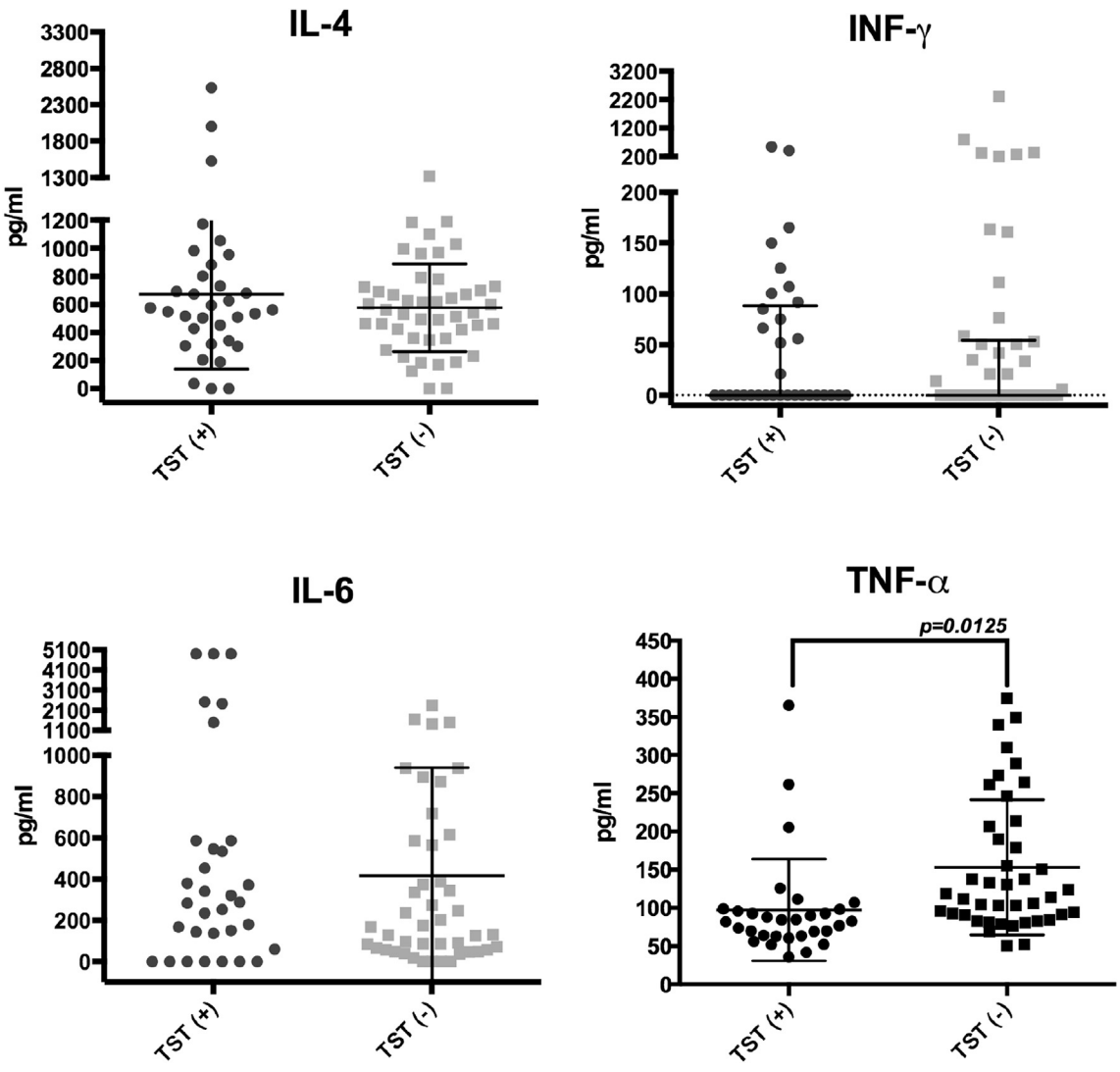

Fig. 2. IL-4, IFN-γ, IL-6 and TNF-α cytokines levels in all colostrum whey samples.
Description: Cytokines levels from reactors TST^{+} n = 31 and non-reactor TST^{-} n = 42. TNF-α level were higher in TST^{-} than TST^{+}; $p = 0.0125$ Bonferroni corrected and IL-6 levels were higher in TST^{+} than TST with not statistical significance according to Bonferroni correction.

including phagocytosis [40,41]. Such finding confirms the transfer of immune cells to the calves through colostrum. In keeping with this notion, the present study show that colostrum mononuclear cells (CMC) isolated from cattle classified as reactors by BOVIGAM blood tests, have an active cellular immune response against antigens (PPD-B). These include *de novo* production and release of IFN-γ protein in isolated cell supernatants, measured by ELISA. These results show a specific cellular immunity against *M. bovis* antigens in reactor cows, transferred by immune cells from the cow's colostrum to calves. Interestingly, the IGRA assays (Fig. 1) showed that the response of the Bov^{+} was higher against PPD-B than with ConA stimuli, a polyclonal mitogen widely used *in vitro* to induce cell-mediated immunity [2,24]. We suggest, that the cells responsible for the expression of IFN-γ might be T-CD8 (+) cells present in colostrum, this according to results of Katsuro [17]. Currently, there is no work that show T cells isolated from bovine colostrum such as T-CD4 or T-WC1 express high levels of IFN-γ.

In addition, it has been shown that T lymphocytes are the principal subpopulation in the cattle mammary secretions, which may represent an essential event in the adoptive transfer of cellular immunity via colostrum to the cattle [13].

Although, there are studies in isolated cells of bovine blood, in which have been conducted the quantification of IFN-gamma produced by mononuclear cells of peripheral blood (PBMC). In response to a vaccination in the effect against *Mycobacterium bovis* [27]; or in the modulation of response against *Mycobacterium bovis* [44–46]. Contrary, there are few studies that include the response of mononuclear cells from bovine colostrum [2,17,32].

Overall, these results show that the CMC are capable of producing IFN-γ in *ex vivo assays.*

It is equally known that soluble cytokines are transferred from the colostrum to the neonates [18,42] and play significant roles in offspring protection against pathogens and in priming the neonatal immune system. Proinflammatory cytokines in bovine colostrum potentiate the mitogenic response of peripheral blood mononuclear cells of newborn calves [51]. IL-18 can be detected in sera of pregnant cow and newborn calves [31] while IL-1 β is induced in healthy dairy cattle and transferred to calves via the colostrum [16]. However, the cytokine interactions among cells are multifaceted and can result in a complex network of activating and suppressive mechanisms.

Consistent with the studies, which report the amount of several

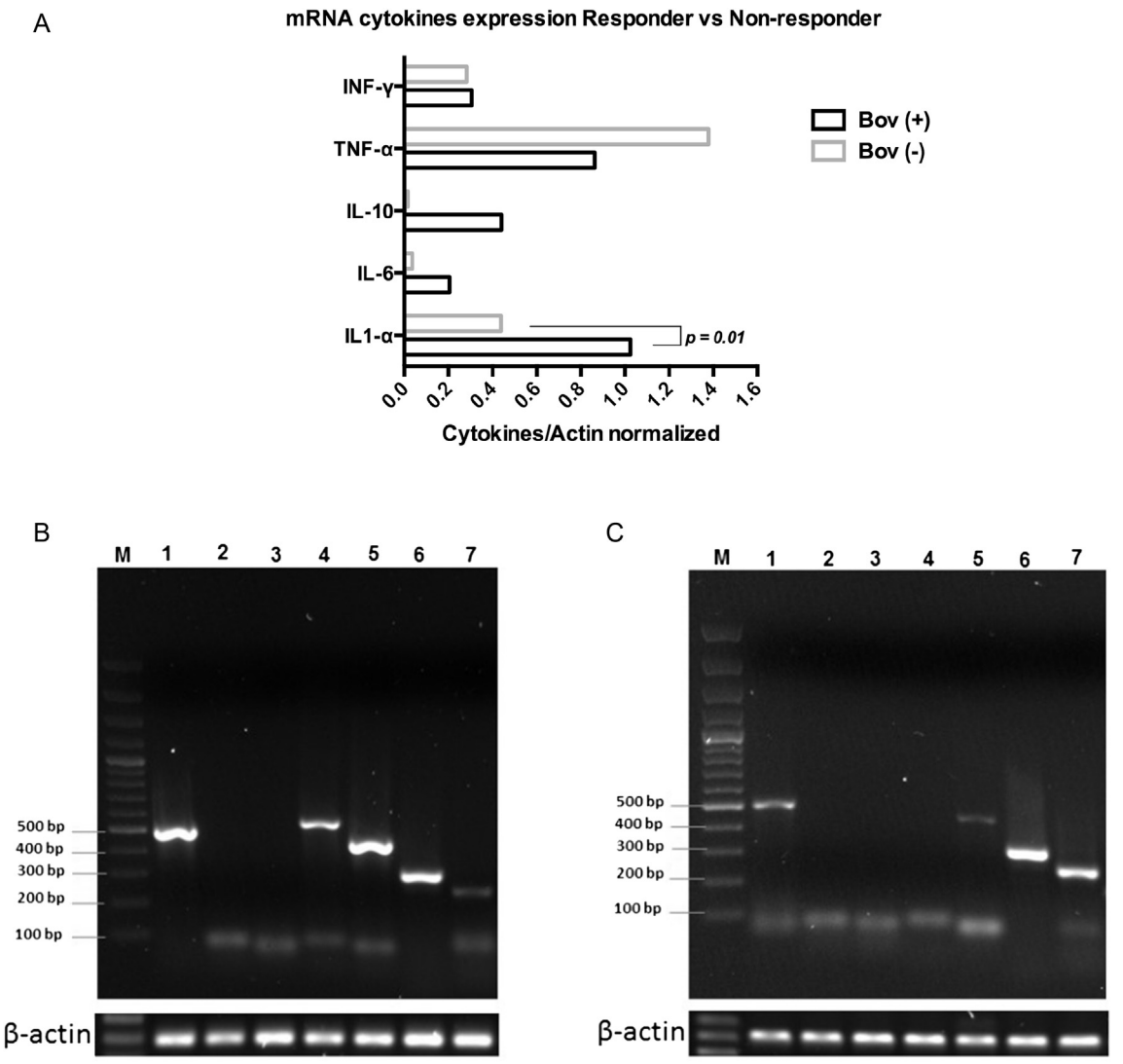

Fig. 3. Cytokines mRNA expression by colostrum mononuclear cells 3B and C.
Description: Cytokines mRNA expression of IFN-γ, TNF-α, IL-10, IL-6 and IL-1α in CMC. A) Graphic summarize expression mRNA of each cytokines, not statistically significant mRNA expression were in IFN-γ, TNF-α IL-6 or IL-10 in Bov^+ when compare with Bov^-, only IL-1-α shown statistical difference with value of p = 0.044 by t-student analysis and after of Bonferroni-corrected p-values with *p = 0.01*. B and C) PCR amplifies in agarose gels, lane: 1 to 7: IL1-α, IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ. M = DNA size marker. 3B) PCR fragments amplified and run in agarose from representative of Bov^+, 3C) PCR fragments amplified, run in agarose amplifies in agarose representative of Bov^-. Amplification of β-actin (control) is below gels.

Table 2
IL-4, INF-γ, IL-6 and TNF-α levels in colostrum whey samples of TST+ and TST−.

	TST^+	TST^-	Student test	Bonferroni correction
	n = 31	n = 42	*p-value*	*p = 0.05*
TNF-α (pg/ml)	82.58 (98.08–63.60)	116.3 (208.0–88.90)	***<0.0043***	***0.0125***
IL-6 (pg/ml)	287.5 (586.3–98.03)	171.5 (641.3–56.45)	*<0.0300*	*0.0166*
IL-4 (pg/ml)	562 (842.1–329.6)	551.7 (724.2–328.8)	*0.3094*	–
IFN-γ (pg/ml)	0 (88.33–0)	0 (54.1–0)	*0.4437*	–

Median (upper–lower quartiles).
Statistically significant *P-values* are in bold.

cytokines in colostrum, our present data show relevant IL-4 protein levels in colostrum whey, in both TST^+ (median 562 pg/ml) and TST^- samples (median 551 pg/ml), without significant difference between the two groups (Table 2). Although it is plausible that IL-4 expression may play an important role in colostrum T cell-mediated immune responses to other pathogens, its significance in the mechanisms implicated in cattle bTB transmission are yet to be established. On the other hand, it is noteworthy that while IFNγ mRNA levels were equal in cells isolated from Bov^+ and Bov(-), Fig. 3A–C, IFNγ protein levels in the colostrum whey were undetectable Fig. 2. However, IFNγ expression was readily inducible in *ex vivo assays* of isolated CMC from Bov^+ after *Mycobacterium*

stimuli (Fig. 1), thus confirming that immune cells from colostrum are able to respond specifically against *Mycobacterium bovis*.

In contrast, the pro-inflammatory cytokine TNF-α showed higher levels in TST^{-} (median of 82 pg/ml) than in TST^{+} (median 116 pg/ml). Similar results were obtained when TNF-α mRNA levels were measured, though the increase in the non-reactors showed p values that were not statistically significant (Fig. 3A–C). These results are in contrast with Hagiwara's findings, which show levels of TNF-alpha in healthy bovine colostrum whey, at levels 926 ng/ml [18]. Nevertheless, our results are consistent with levels found in human colostrum whey, around 65–183 pg/ml [1].

To date, the relationship between TNF-alpha and bovine tuberculosis levels are inconclusive. When considering IL-6, we found higher levels in TST^{+} (median 287 pg/ml) than in TST^{-} (median 171 pg/ml) with not statistically significant differences. IL-6 is a molecule related with inflammation, which is mainly increased in bovine mastitis [19]. Studies in a number of species have evaluated the role of IL-6 in tuberculosis, however conflicting data can be found when attempting to ascertain whether IL-6 exerts beneficial or detrimental effects in tuberculosis. For example, studies in IL-6 gene-deficient mice suggest a role in protection against tuberculosis [25]. In contrast, some human studies have shown an association between IL-6 and active pulmonary TB, compared to healthy subjects [11,36].

Consistent with this notion we propose that TNF- alpha and IL-6 could be a useful biomarker for the diagnosis and prognosis of bovine tuberculosis by diferent expression between and non reactors animals. Furthermore, our RT-PCR results show that IL-1-α, support the overall role of pro- and anti-inflammatory cytokines as biomarkers of dairy bTB transmission affecting calf health and food safety.

The only precedent related to the present work regarding serum cytokines present in colostrum is a study by Hagiwara et al. [17,18], in which they found cytokine expression at concentrations of ng/ml range. However a review in humans show, broad variations in cytokine concentrations in the milk, which makes cross-comparisons quite difficult [1].

Thus, reliable methods for the diagnosis of bTB infections are needed to be able to demonstrate the real state of the transmission to animals and now some cytokines have been studied for their potential as biomarkers of disease progression in bovine tuberculosis [6].

5. Conclusion

We propose IL-1-α and TNF-α as suitable means to identify reactors and non-reactor cattle to bovine tuberculosis and thus improve the current diagnostic screening routine that can be implemented in dairy herds. The methodology reialable now, is the diagnosis assays by ELISA to determine levels of this cytokines in reactors or responder in colostrum cattle. With the validation of many samples and the knowledge generated, we could be develop a test based on lateral flow strips with IL1-α and/or TNF-α antibodies, which could be useful directly in dairy herds.

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Conflicts of interest

None.

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