# Factors affecting the performance of P22 ELISA for the diagnosis of caprine tuberculosis in milk samples

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## ARTICLE INFO

## ABSTRACT

Caprine tuberculosis (TB) is a zoonosis caused by members of the *Mycobacterium tuberculosis* complex (MTBC). Caprine TB eradication programmes are based mainly on intradermal tuberculin tests and slaughterhouse surveillance. However, the use of serological test has been extended as a potential diagnostic tool in goats through the use of serum, plasma, or even milk samples. Milk production and the antibodies (Ab) present in milk can vary depending on several circumstances. In the present study, different factors that may affect the performance of humoral TB diagnosis were analysed using goat milk samples: 1) lactation stage, 2) a recent previous skin test (booster effect) and 3) the effect of freeze-thaw cycles on milk samples preserved with azidiol. TB-infected animals ($n = 44$) were selected to evaluate the evolution of the Ab levels during the 6-month lactation period, along with its potential effect on the P22 ELISA results. In general, no significant changes ($p = 0.079$) were observed throughout the study as regards Ab levels in milk samples between consecutive analysis although the reactivity to P22 ELISA decreased when samplings were performed at the last two months of the lactation. Regarding the booster effect, the quantitative results showed a significant variation ($p < 0.001$) for both milk and serum samples when serological tests were carried out 15 days after the skin test. Finally, there were no significant differences ($p = 0.99$) in the P22 ELISA results when using milk samples preserved with azidiol that had undergone freeze-thaw cycles.

## 1. Introduction

Caprine tuberculosis (TB) is a chronic infectious disease caused mainly by *Mycobacterium bovis* and *M. caprae*, with *M. caprae* being the principal cause of TB in goats in Spain (Rodríguez et al., 2011). Unlike TB in cattle, the application of eradication programmes in goats is not generalised in certain countries, one of which is Spain, and is rather focused on caprine herds that are epidemiologically linked to bovine herds or located in specific areas (MAPA, 2021). The diagnosis of TB in domestic ruminants is based on test and cull strategies, using the single and comparative intradermal tuberculin tests (SITT and CITT, respectively) and, in certain cases, using the interferon-gamma release assay (IGRA) as an ancillary diagnostic tool (Bezos et al., 2014). However, these diagnostic techniques, which are based on cellular immune response, are not able to detect all TB-infected animals (Schiller et al., 2010).

It is for this reason that humoral diagnostic tools have, in recent years, been considered to detect TB-infected animals that do not react to cellular immune response tests and thus maximize the detection of TB-infected ruminants and herds using serum and plasma (Casal et al., 2017; O'Brien et al., 2017; Bezos et al., 2018; Infantes-Lorenzo et al., 2019; Infantes-Lorenzo et al., 2020). Moreover, milk has been proposed as an alternative sample to measure specific antibodies (Ab) against MTBC in ruminants (Jeon et al., 2010; Waters et al., 2011; Buddle et al., 2013; O'Brien et al., 2017; Roy et al., 2020). The usefulness of P22 ELISA, an immunoassay that detects specific Ab against a protein complex purified from bovine PPD (Purified Protein Derivatives from *M. bovis*) (Infantes-Lorenzo et al., 2017), has been demonstrated as regards TB diagnosis (Roy et al., 2020). Milk samples could, therefore, be a valuable means to diagnose caprine TB, as occurs with other

diseases such as bovine virus diarrhoea virus (BVD) and *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Lanyon et al., 2014; Pesqueira et al., 2017; Bauman et al., 2019). However, the levels of Ab in milk may fluctuate during the lactation period. This has been observed in previous studies in which levels of immunoglobulins (Ig) against BVD and MAP have been found to be higher in the early and/or late stages of lactation in ruminants (Niskanen et al., 1989; Nielsen et al., 2002; Angelidou et al., 2014; Ngu Ngwa et al., 2016). Nevertheless, a previous study carried out in order to detect Ab against TB in cattle showed small variations in Ab levels in milk samples obtained from cattle during different stages of lactation (Buddle et al., 2013). However, no similar studies have been carried out in goats.

Several studies have demonstrated that Ab levels increase in serum after recent intradermal tuberculin tests in ruminants (Casal et al., 2014; Waters et al., 2015; Casal et al., 2017). This booster effect has also been observed in milk samples taken from cattle (Buddle et al., 2013). With regard to milk samples obtained from goats, a previous study showed higher levels of Ab 8 days after PPD inoculation (O'Brien et al., 2017), although the booster effect has not been studied in depth in this species when employing milk samples.

Antibody-based diagnosis in milk samples is not affected by the freezing process (Roy et al., 2020). However, the effect of freeze-thaw cycles on P22 ELISA results has not been evaluated in goats milk samples. Milk samples can be preserved by means of other procedures such as azidiol, which is used in the Public Health programmes of several countries, including Spain, in order to analyse the composition of milk and the presence/absence of antibiotics (Beltrán et al., 2014). In this context, the same milk samples preserved with azidiol could be used to detect specific TB Ab if azidiol does not affect ELISA results. Moreover, these samples could undergo freeze-thaw cycles, thus making them adequate for the daily work schedule of the laboratory. The effect of freeze-thaw cycles on milk samples preserved with azidiol should, therefore, be evaluated.

The main objectives of the present study were: i) to evaluate antibody detection in milk samples obtained from lactating TB-infected goats; ii) to evaluate the booster effect in milk samples, and iii) to evaluate the effect of freeze-thaw cycles on milk samples preserved with azidiol.

## 2. Material and methods

### 2.1. Study design

The study was performed in a dairy herd of Guadarrama breed goats located in the central region of Spain. The herd ($n = 163$) was previously confirmed as TB-infected by bacteriological culture (*M. bovis* SB0121). Moreover, this herd had undergone a vaccination programme against MAP, using the Gudair vaccine (CZ Vaccines, Spain) in 6 month–old animals, and was dewormed with Panacur (MSD Animal Health, Spain) previously to this study. The animals were subjected to SITT, CITT, IGRA in plasma samples and P22 ELISA in serum samples, with an apparent prevalence of 60.7% (95% CI 53.1–67.9), 37.4% (95% CI 30.4–45.1), 55.8% (95% CI 48.2–63.2) and 53.4% (95% CI 45.7–60.9), respectively.

First, forty-four goats from this TB-infected herd were randomly selected, and individual milk samples taken from this group were assayed using P22 ELISA in order to evaluate the Ab levels in milk during the lactation period and determine whether the time of sampling can affect the results. Milk samples were, therefore, collected monthly for 6 months in order to evaluate the evolution of the Ab levels continuously, resulting in a total of six samplings (T1-T6) from April (month of kidding) to September. Moreover, serum samples were collected in T1 and T5 to observe the relationship between the optical densities (ODs) in the milk and serum samples.

In a second study, thirty-seven animals from the same herd ($n = 163$) were randomly selected in order to evaluate the booster effect on serum and milk samples. These goats were subjected to SITT, CITT and IGRA at

day 0. The animals were also tested by means of P22 ELISA in serum and milk samples at days 0, 15, 30 and 60 after PPD inoculation. All milk and serum samples from day 0 were obtained before the intradermal testing.

Finally, milk samples from goats ($n = 20$) were randomly selected and preserved with azidiol according to Spanish Royal Decree 1728/2007 (133 μl in 40 ml of milk). They were then stored at −20 °C. In order to assess the effect of freeze-thaw cycles on the detection of specific antibodies in P22 ELISA, samples were defrosted and analysed by employing P22 ELISA at days 1, 8, 30, 45, 75 and 105. A total of 6 freeze-thaw cycles were carried out.

The animals included in this study were not experimental animals. All samplings and other procedures were carried out in accordance with Spanish legislation (Royal Decree 727/2011). In order to homogenize parameters as the milk production or the lactation persistency all goats included in the present study were multiparous. The milk yield mean per goat was 1.5 l/day. A mixture of cereals (corn, barley and oats) as well as correctors (copper, zinc, phosphate and magnesium) composed the feeding of the animals. In addition, the animals went out to graze every day. No animal showed TB compatible clinical signs (cough, dyspnea, loss of body condition, reduced milk yield, anorexia or lethargy) or died throughout the study.

### 2.2. Milk and serum sample collection

Individual milk samples (30 ml) were collected during the milking session, after which 1 ml was centrifuged at 13,000*g* for 5 min, the superficial cream and the pellet were removed and an aliquot of whey was obtained and stored at −20 °C until further testing. Serum samples were obtained from the jugular vein, collected in plastic serum tubes, centrifuged (2500 *g* for 15 min) and stored at −20 °C until the ELISA assay.

### 2.3. Indirect ELISA

Serum and milk samples were analysed by employing an in-house indirect ELISA that detects antibodies against the P22 protein complex (Infantes-Lorenzo et al., 2017). The ELISA was performed as described elsewhere (Roy et al., 2020). Briefly, a 5% skimmed milk powder solution in PBS was used to block the plates coated with P22. The protocol was adapted to the type of sample using the optimal dilution of serum and milk samples (Bezos et al., 2018; Roy et al., 2020) in skimmed milk solution and incubated for 60 min at 37 °C. The secondary antibody anti-sheep IgG-HRP conjugated (South-ern Biotech, USA) was then added and the plates were agitated for 30 s and incubated for 30 min at room temperature (RT). One hundred microliters of *o*-phenylenediamine dihydrochloride substrate (FAST OPD, Sigma–Aldrich, USA) was subsequently added in order to develop the reaction, and the plates were agitated and incubated for 15 min in darkness and under RT conditions, after which fifty microliters of $H_2SO_4$ (3 N) were added in order to stop the reaction. Finally, the optical density (OD) was measured at 492 nm with an ELISA reader. The serum and milk sample results were expressed as an ELISA percentage (E%), calculated by employing the following formula: sample E% = [mean sample OD / (2 x mean of negative control OD)] x 100. Serum and milk samples with E% values greater than 150 were considered positive.

### 2.4. Intradermal tuberculin tests

The animals were tested by means of SITT and CITT, which were carried out by the intradermic inoculation of 0.1 ml of bovine and avian PPDs (CZ Vaccines, Porriño Spain) simultaneously in the left and right sides of the neck, respectively, using a Dermojet syringe (Akra Dermojet, France). The SITT and CITT were performed according to Regulation EU 2016/429, Commission Delegated Regulation EU 2020/688 and Spanish Royal Decree 2611/1996 (standard interpretation). The results were interpreted as described elsewhere (Bezos et al., 2015). Briefly, a goat

was considered as positive reactor to the SITT when there was an increase ≥4 mm in the skinfold thickness measures and/or the presence of clinical signs (oedema, exudation or necrosis) were observed on the bovine PPD inoculation site. Regarding to the CITT, a goat was considered as positive reactor when the bovine reaction was greater than the avian reaction by more than 4 mm and/or there were clinical signs previously mentioned on the bovine PPD inoculation site.

### 2.5. Interferon-Gamma Release Assay (IGRA)

Blood samples were collected from the jugular vein by employing venepuncture, using evacuated tubes (BD Vacutainer Becton, Dickinson and Company, Franklin Lakes, USA) with heparin, and stimulated with bovine and avian PPDs (CZ Vaccines, Lugo, Porriño, Spain) at a final concentration of 20 μg/ml for the detection of IFN-γ production. The blood samples were then processed as described previously (Bezos et al., 2011). IFN-γ production was measured using a commercial IGRA (Bovigam TB kit, Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions, and the results were interpreted as described elsewhere (Roy et al., 2019).

### 2.6. Statistical analysis

Data were analysed using the SPSS Statistics 25 commercial statistical software (IBM, New York, NY, USA), and interpreted by considering a *p*-value of 0.05 in order to determine statistical significance. Quantitative differences in the ELISA percentage (E%) during the lactation period and different preservation procedures were evaluated using the Kruskal-Wallis test. The relationship between ODs in the milk and serum samples was evaluated by using the Spearman's rank correlation coefficient (rs). Quantitative differences in the E% during the study concerning the booster effect were analysed using the Wilcoxon signed-rank test. The proportion of animals in the group belonging to the lactation period and booster effect studies that were positive to P22 ELISA when using milk and serum samples was compared using McNemar's test. Wilson's 95% confidence intervals (95% CI) were calculated for the percentage of animals that were positive to the different tests.

## 3. Results

### 3.1. Effect of sampling time

Of the 44 animals selected for the study, 24 (54.5%), 35 (79.5%) and 32 (72.7%) goats were positive to IGRA and P22 ELISA in serum and milk samples, respectively, in the first testing event carried out 2 weeks after the beginning of lactation. Quantitative values (E% in P22 ELISA) were also analysed in order to assess the effect of the changes observed during lactation. With regard to E%, there were no significant differences between the different sampling events of the milk samples (*p* = 0.079) (Fig. 1). However, small variations in the E% values obtained during the different samplings led to a change in the number of positives to P22 ELISA in milk samples between the different testing events (Fig. 2). In spite of these variations, the differences in the percentage of positives to P22 ELISA only were significant between two consecutive samplings (T4 and T5) when using milk samples (*p* = 0.039) (Fig. 2). Nevertheless, the number of positives to P22 ELISA in milk samples decrease significantly in the last stages of the lactation compared to previous samplings (*p* = 0.019) (Fig. 2). The correlation observed in the quantitative results (ODs) obtained in serum and milk samples was very strong in those samplings in which both samples were collected: T1 (rs = 0.803) and T5 (rs = 0.931).

### 3.2. Effect of a recent previous intradermal test (booster effect)

Twenty-nine (78.4%) and four (10.8%) out of thirty-seven goats were reactors to the SITT and CITT respectively, at day 0 of the study. The mean (and standard deviation) increases in skin fold thickness observed after the PPDs injection on the bovine PPD and avian PPD sites were 5.27 mm (± 2.18) and 4.29 mm (± 2.54), respectively. In this group, 18 (48.6%) animals were positive to IGRA and 25 (67.6%) and 29 (78.4%) goats were positive to P22 ELISA (E% 150) when using serum and milk samples, respectively, at day 0. The number of reactors was higher for the serum and milk samples collected 15 and 30 days post-intradermal test, 100% (37/37), when compared to the serum [67.6% (25/37)] and milk samples [78.4% (29/37)] collected prior to PPD inoculation. With regard to sampling at day 60, all the animals were positive to P22 ELISA when using milk samples, and only one goat was negative when using serum samples (Fig. 3). Significant differences were observed as regards the number of positives to P22 ELISA between day
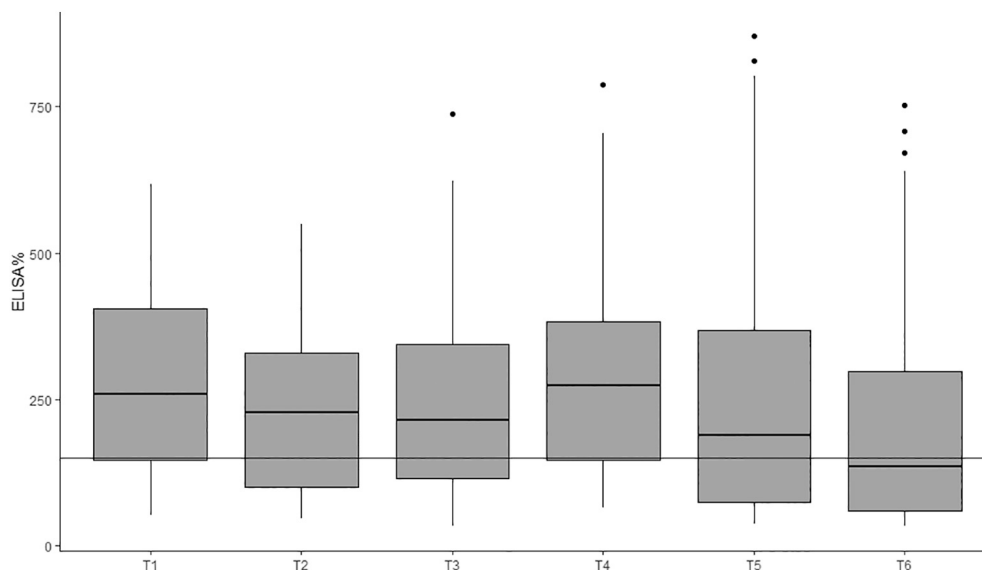


**Fig. 1.** ELISA % in milk samples during different stages of lactation in TB-infected goats with a cut-off point of 150 E%. No significant differences (*p* = 0.079) in the E % between the different sampling events carried out throughout the lactation period.
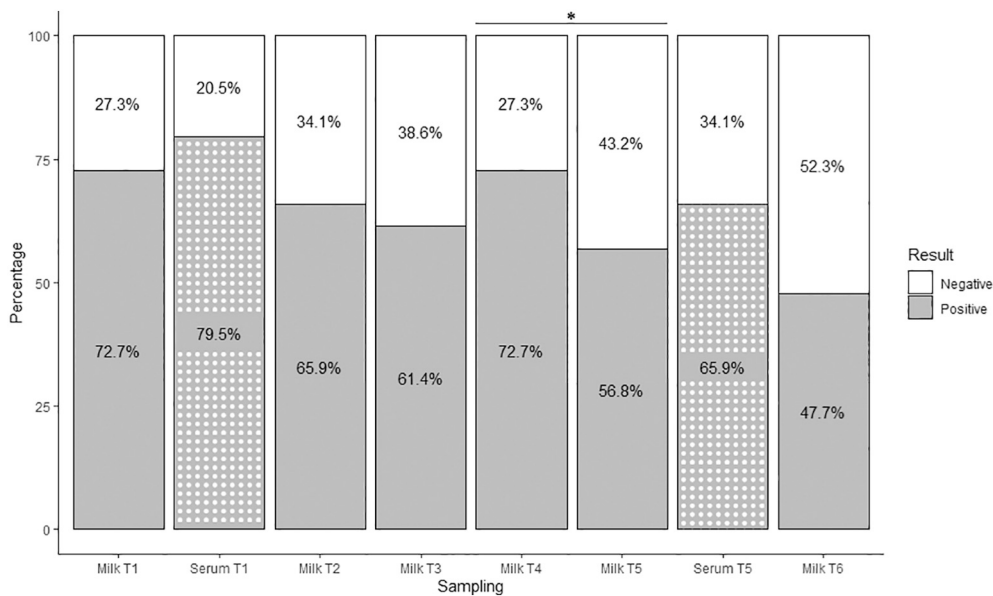
**Fig. 2.** Percentage of animals that tested positive (grey) and negative (white) to P22 ELISA in milk and serum out of the 44 animals analysed during the different sampling times of the lactation period evaluated. The differences with regard to the percentage of positives to P22 ELISA were significant between samplings T4 and T5 when using milk samples *$p < 0.05$.
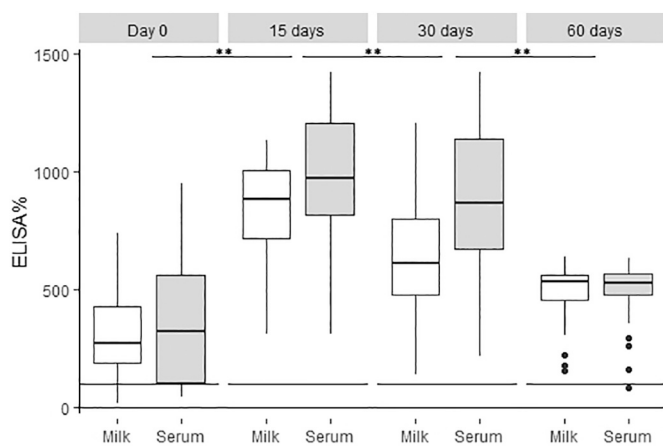


**Fig. 3.** ELISA % in milk (white) and serum (grey) at days 0, 15, 30 and 60 of boosting effect study in TB-infected goats with a cut-off point of 150 E%. The differences between the E% values obtained in all four samplings were significant, regardless of the type of samples (serum or milk) **$p < 0.001$.

0 and the rest of the sampling times when using milk ($p = 0.008$) and serum ($p < 0.0001$). The booster effect was similarly performed using milk and serum samples, and the Ab levels were significantly higher in both types of samples at 15 days following the PPD inoculation. A progressive decrease in the E% was subsequently observed when using both types of samples at days 30 and 60, up to levels similar to those observed before the tuberculin test (Fig. 3). The differences between the E% values obtained in all four samplings were significant, regardless of the type of samples (serum or milk) ($p < 0.001$).

### 3.3. Effect of storage and azidiol preservation

Of the 20 samples analysed, thirteen were positive to P22 ELISA in all the analyses (T1-T6), while six were negative. The qualitative result of only one sample changed from negative to positive. This sample was negative in the first three analyses and positive from fourth to the sixth. No significant differences ($p = 0.99$) were observed in either the positive or the negative goats as regards the E% values of the milk samples

preserved with azidiol that were subjected to six freeze-thaw cycles (Fig. 4).

## 4. Discussion

In the present study, the Ab response in milk samples was higher at the beginning than at the end of lactation. Moreover, the Ab levels in milk increased significantly 15 days after PPD inoculation, similarly to that which occurred when using serum samples. The preservation of frozen milk samples with azidiol did not significantly affect the P22 ELISA results.

The results from the present study showed that there were no differences in the P22 ELISA quantitative results obtained for the different sampling events during the course of lactation. However, the number of positive reactors to the P22 ELISA using milk samples was higher when using samples collected between the beginning and the fourth month of lactation compared to samples collected at the two las months of lactation due to small variations in the Ab response. Therefore, the performance of the P22 ELISA as regards the diagnosis of caprine TB using milk samples collected during the lactation period was optimal at
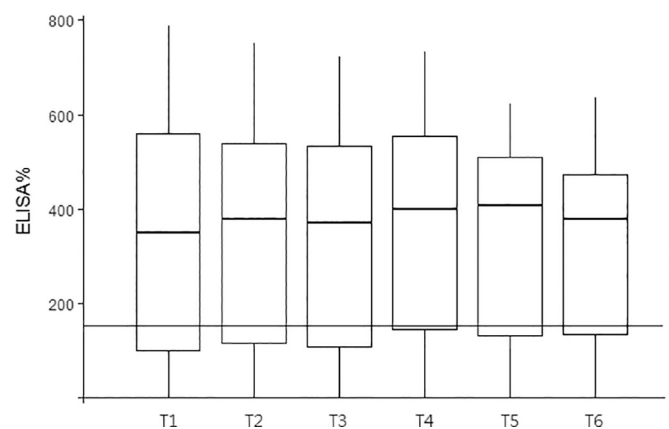


**Fig. 4.** ELISA % in milk samples preserved with azidiol at six different times over four months. No significant differences ($p = 0.99$) in the P22 results were observed between analysis.

the first stages of lactation. In this respect, several previous studies in cattle and goats have reported that the levels of Ab present in milk were higher in the early stages of lactation. This occurred mainly in the first days owing to the formation of colostrum and/or at the end of lactation, and it was used for the diagnosis of other diseases such as MAP and BVD (Niskanen et al., 1989; Nielsen et al., 2002; Angelidou et al., 2014; Ngu Ngwa et al., 2016). These findings were attributed to the increases in the concentration of IgG at the beginning and at the end of lactation, and decreases in the IgG concentration at the peak of milk production owing to the dilution effect caused by increases in milk production (Nielsen et al., 2002; Ngu Ngwa et al., 2016). However, a previous study in goats did not observe high Ab levels at the beginning of lactation, but rather at a late stage, and these were related to differences in milking frequency and husbandry practices (Angelidou et al., 2014). In this respect, the concentration of IgG in milk was negatively correlated with milking frequency and the stage of lactation in goats, leading to a high Ab concentration in milk in late lactation (Hernández-Castellano et al., 2011; Moreno-Indias et al., 2012).

Although no significant differences in the E% were observed during the different lactation stages, there were small variations among the different sampling times which could explain the changes in the individual qualitative results obtained for some animals, especially as regards those values that were close to the cut-off point. However, the small variations in Ig levels observed during lactation are supported by a previous study, in which slight differences with respect to the levels of Ab against TB in cattle at different times during lactation were observed in the ELISA response in milk samples (Buddle et al., 2013). The main difference between the study in question and our experiment was the sampling times, since in the study of Buddle and collaborators, samples were collected at three different stages (early, mid and late), whereas in our study, the evolution of Ab levels during the lactation period was evaluated more frequently. Our results suggest that the detection of reactors to P22 ELISA decrease when samples are collected at the last two month of the lactation period. Therefore, in order to minimize the risk of getting false negatives this factor should be taken into account. In our study, there was a very strong correlation between serum and milk sample results. These results were in line with those of a previous study in goats whose results for a high prevalence herd showed that the parallel use of milk and serum P22 ELISA tests, or either of these two tests with the SIT test, was similar in terms of sensitivity (Se) (Roy et al., 2020). In this sense, milk could be a valuable sample to the diagnosis of caprine TB since early Ab response (4–5 weeks after *M. bovis* infection) has been previously detected in cattle (Waters et al., 2006) and goats (Arrieta-Villegas et al., 2018).

The increase in the Ab response in serum following bovine PPD inoculation (booster effect) has been reported in several studies carried out on cattle (Waters et al., 2015; Casal et al., 2017) and, in a lesser extent, in other species such as alpaca, red deer and goats (Gutiérrez et al., 1998; Bezos et al., 2013; Che-Amat et al., 2016; Bezos et al., 2018). Moreover, this booster effect has been observed in milk samples obtained from cattle (Buddle et al., 2013) but not in other species. In this previous study, the proportion of positive reactors obtained when using milk samples from TB-infected animals was significantly higher when the samples were collected between 2 and 4 weeks after the PPD inoculation (Buddle et al., 2013). This was the reason that the present study evaluated the performance of the P22 ELISA as regards the detection of specific Ab against MTBC members in goat milk samples 15 days after bovine PPD inoculation, and evaluated the evolution at 30 and 60 days. Our results showed that the antibody levels were significantly higher 15 days after tuberculin injection than those observed 30 and 60 days after skin tests, suggesting a better performance of the test when milk samples were collected 15 days after tuberculin administration. Similar results have been observed in other studies in ruminants using serum samples (Gutiérrez et al., 1998; Casal et al., 2014). In cattle, a low Se (23.9–32.6%) was observed on the day of the PPD inoculation, which increased significantly, up to 66.7–85.2%, 15 days later (Casal et al.,

2014). In goats, the Se increased from 54.9% on the day of the PPD inoculation to 88.6% 15 days later (Gutiérrez et al., 1998). In this respect, the booster effect observed in milk samples in the present study was similar to that observed in cattle when using milk samples (Buddle et al., 2013). Our results suggest that the booster effect using milk samples could be a valuable strategy for caprine TB immunological diagnosis in order to maximize the detection of TB-infected animals in herds of dairy goats.

Finally, a recent study demonstrated that, at least under the conditions evaluated, no significant differences were observed in the P22 ELISA results when using different preservation procedures (freeze-thaw cycles and preservation with azidiol) or samples collected from different mammary glands (Roy et al., 2020). This is consistent with the results of our study, since, the P22 ELISA results were not affected after several freeze-thaw cycles when using milk samples preserved with azidiol. These results suggest that milk samples could be preserved and stored with azidiol and subsequently be analysed according to the daily work schedule of the laboratory. In fact, azidiol is used in several countries (including Spain) in order to preserve milk samples employed to detect antibiotics and to analyse milk composition in goats (Beltrán et al., 2014).

In conclusion, the use of milk samples collected in the fifth and sixth month of lactation led to a decrease of the number of positives reactors to P22 ELISA compared to the milk samples collected at first stages of the lactation period. With regard to the booster effect, the collection of milk samples after a recent PPD inoculation could be a valuable strategy for TB diagnosis since it maximized the number of reactors detected when samples were collected 15–30 days after the PPD injection. Furthermore, several freeze-thaw cycles of milk samples preserved with azidiol did not significantly affect antibody detection. All of these results will be useful for the potential implementation of antibody-based platforms for caprine TB diagnosis using milk samples.

## Author contributions

JO, JB and JAI were involved in the conceptualization, investigation, methodology, data analysis and writing of the manuscript. JB and LdJ were in charge of funding acquisition and project administration. AR, BR, IM, LD and made supervision and collaborated in the investigation and formal analysis. All authors reviewed and approved the manuscript.

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## Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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