Intradermal Tuberculin Test in Water Buffalo (*Bubalus bubalis*): Experimental use of Mycobacterial Antigens for the Diagnosis of Bovine Tuberculosis

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Abstract: The study aims to evaluate the potential use of mycobacterial ESAT6 and CFP10 antigens, Early Secretory Proteins (ESP) in the Skin Test used for bovine tuberculosis (TB) diagnosis in Water Buffalo.

A pilot study was performed on 21 buffaloes from a TB outbreak and 11 buffaloes from a TB-free herd. Three concentrations of ESAT6-CFP10 (10, 20, and 30 μg) and two of ESP (50 and 100 μg) were inoculated in the Skin Test, along with PPDB, PPDA, and PBS as a negative control. Skin thickness was measured with calipers before the test and every 24 hours for 4 days. Then, to evaluate the specificity of the antigens, a field study was conducted, and 100 buffaloes from a TB-free herd were inoculated using the best antigens concentration derived from the pilot study.

In the positive buffaloes, the strongest skin response was to PPDB at 24h, with some subjects becoming inconclusive at 72 and 96 h. A peak response to PPDA at 48 hours was detected, followed by a slight decrease. The response to ESP-100 μg remained high at 24 and 48 h, then decreased, remaining positive at 72 h. In the 100 TB-free buffaloes, the best specificity was observed using ESAT6-CFP10 and ESP.

ESP yielded the best results, showing higher reactivity in infected animals and no reactivity in the healthy ones at 72 h. Therefore, ESP could be an excellent candidate for further extensive studies in the buffalo species to improve Skin Test performance.

Keywords: Water Buffalo, tuberculosis, diagnosis, tuberculin Skin Test, ESAT6-CFP10, *Mycobacterium bovis* antigens.

INTRODUCTION

Bovine tuberculosis (TB) is a chronic disease caused by mycobacteria of the *Mycobacterium tuberculosis* complex (MTC), specifically *Mycobacterium bovis*. Since TB is a zoonotic disease that represents a serious threat to human health [1], it is controlled through the application of eradication programs. In Italy, the control of TB in the water buffalo population is mainly carried out using the tuberculin Skin Test. The test measures the increase in skin thickness 72 hours (h) after the intradermal injection of mycobacterial purified protein derivatives (PPDs). The Skin Test can involve the single intradermal test with bovine PPD (PPDB) or the comparative test by adding a second injection with avian tuberculin (PPDA). PPD is a water-soluble crude protein extract of a heat-treated *M. bovis* culture (PPDB) or *M. avium* (PPDA) in a liquid synthetic medium [2].

The Skin Test exhibits cross-reactivity with environmental mycobacteria, *Mycobacterium avium* subsp. *avium* and *Mycobacterium avium* subsp. *paratuberculosis* [2]. Using a couple of PPDs, such as in the Comparative Skin Test, can reduce false positive results caused by non-tuberculosis mycobacteria (NTM) but hardly discriminate between infected animals and those exposed to NTM. Despite this, the Skin Test is an affordable method that has successfully eradicated tuberculosis in many areas. However,

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according to various studies, the test sensitivity and specificity can vary significantly in buffalo species [3-5].

In the gamma-interferon test (IFN-γ), an immunological *in vitro* assay developed to diagnose tuberculosis in cattle and buffaloes [2, 6], the use of more specific antigens such as 6kDa early secretory antigenic target (ESAT-6) and 10kDa culture filtrate protein (CFP-10) can improve specificity [7, 8]. Although it is an excellent and objective method that avoids many practical problems associated with the Skin Test (i.e., subjective interpretation), it is quite expensive, and there are logistical challenges linked to sensitizing and incubating blood samples with antigens within a specific time frame, 8 – 12 hours of collection. Using ESAT-6 and CFP-10, along with PPDB and PPDA in the Skin Test, has shown potential in improving TB diagnosis in healthy and naturally *M. bovis*-infected cattle or buffaloes [9, 10]. This method can identify more animals than the PPDB alone, especially in herds with a low TB prevalence. It has also been reported that ESAT6-CFP10 does not cause reactions in TB-free or Paratuberculosis-infected cattle [9].

In Italy, new *M. bovis* antigens, Early Secretory Proteins, have been produced at the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati" (IZSUM), used in cattle to improve IFN-γ test performance [11].

The present study aims to evaluate the efficacy of the ESAT-6/CFP-10 protein cocktail and Early Secretory Proteins, along with PPDB and PPDA in the Skin Test, in diagnosing TB in buffalo species.

MATERIALS AND METHODS

Antigens

The PPDs used in the study were those used in Italian territory for the National TB eradication program provided by IZSUM [12].

The ESAT6/CFP10 protein cocktail is produced and purified as described by Fontana *et al*. (2018) at Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Italy (IZSLER).

The Early Secretory Proteins (ESP) are produced according to the protocol previously described [11, 13] by IZSUM from an *M. bovis* AN5 liquid culture in a synthetic medium.

Pilot Study

To define the optimal concentration of the experimental antigens to be used in the Skin Test, twenty-one buffaloes from a TB-outbreak herd already resulted positive for the IFN-γ test for TB diagnosis, and eleven buffaloes from a TB-free herd were selected.

All animals were submitted to the IFN-γ test, using the same antigens (Ag) as the Skin Test, to evaluate the *in vitro* cellular response and to exclude anergic animals that did not respond to *in vitro* stimulation with the pokeweed mitogen (PWM). Eight intradermal injection sites were used on each animal's shoulder (4 on the right and 4 on the left side). Three concentrations of the ESAT6-CFP10 protein cocktail (10, 20, and 30 μg) and two concentrations of ESP (50 and 100 μg) were inoculated basing on previous studies [9, 11, 13]. PPDB (30,000 IU/mL) and PPDA (25,000 IU/mL) were always included in the Skin Test, along with PBS as a negative control. Skin reactions were measured with calipers before the test and every 24 hours for 4 days.

Field Study

A total of 100 buffaloes from 3 TB free Italian herds were enrolled. Five intradermal injection sites on animal shoulders (3 on the right and 2 on the left side) were used on each animal, with a volume of 0.1 mL for all antigens (ESAT-6 and CFP-10 at 20 μg/mL, ESP at 100 μg/mL, PPDA and PPDB), and PBS as a negative control. Skin reactions were measured with calipers before the test and at 72 hours from inoculation.

All 100 buffaloes were also submitted to the IFN-γ test to confirm TB negativity.

Skin Test Procedures

The Skin Test was performed according to the TB Italian National Eradication program and to Regulation (EU) 2016/429 and Regulation (EU) 2020/689. Skin reactions were measured using calipers; results were expressed in millimeters as the difference between the two measurements, i.e., before and 72h after the tuberculin inoculation. The reaction in the Single Intradermal Test (SIT), which provides the use of PPDB only, was considered positive if skin thickness increased by ≥ 4 mm, inconclusive if > 2 and < 4 mm, and negative if ≤ 2 mm.

In the Comparative Intradermal Test (SICT), which also uses the PPDA, the reaction was considered positive if the difference between the PPDB and PPDA measurements was ≥ 4mm, inconclusive if > 1 and < mm, and negative if ≤ 1 mm.

IFN-γ Test

The test was carried out using heparinized blood samples collected from each animal before the tuberculin inoculation. Six aliquots of 1 ml per animal were sensitised with PPDA and PPDB, supplied by Thermo-Fisher Scientific (Life Technologies, Thermo-Fisher Scientific, Schlieren, Switzerland), with a final concentration 10 µg/mL); early secretory antigens (ESP); ESAT6/CFP10 protein cocktail, the final concentration of each protein 4µg/mL; pokeweed mitogen (PWM), final concentration 1µg/mL, to control blood cells vitality; phosphate-buffered saline (PBS), as sample baseline. The samples were then incubated for 16-24 hours at 37 °C in a humidified atmosphere. IFN-γ levels were measured using a sandwich enzyme immunoassay (ELISA) following the manufacturer's instructions (Bovigam™, Life Technologies, Thermo-Fisher Scientific, Schlieren, Switzerland).

Samples with a plasma PPDB and ESP value ≥ 0.1 compared to PPDA and PBS values were considered positive, based on the interpretative criteria adopted by the Bovigam™ manufacturer. To define the positive sample to the antigen cocktail, the recommended cut-off was a net difference of ESAT6/CFP10 minus PBS ≥ 0.1 OD without taking into account PPDB and PPDA values.

Statistical Analysis

The data were analyzed using the nonparametric Kruskal-Wallis test by Proc NPAR1WAY procedure of SAS 9.4 (SAS Institute Inc., Cary, CA, USA). The statistical differences between responses were assessed at three levels of significance (*: P <0.05; **: P <0.01; ***: P < 0.001).

RESULTS AND DISCUSSION

In the pilot study, the ESAT6-CFP10 protein cocktail was inoculated at different dose concentrations (10, 20, and 30 µg of total protein). Additionally, two concentrations of ESP (50 and 100 µg) were also tested.

To evaluate the antigen's performance in the Skin Test, buffaloes naturally infected with *M. bovis* tested IFN-positive from a TB outbreak, and healthy buffaloes from TB-free herds were selected.

In the IFN-positive buffaloes, skin responses to all intradermally inoculated antigens were observed, especially at 24 h and 48 h (Figure **1A**); however, at 72h, the strongest response was observed to ESP (100 µg), followed by PPDA and then PPDB.

Skin response kinetics in infected subjects showed that the strongest response to PPDB was at 24 h, with some subjects becoming inconclusive at 72 and 96 h. A peak response was observed at 48 h to PPDA and 20 µg of the ESAT6-CFP10 protein cocktail, followed by a decrease.

The response to ESP-100 µg remained high at 24 and 48 h and then decreased, remaining positive at 72 h (Figure **1A**). The mean skin response at 72 h was 4.2 mm for ESP (100 µg) and 3.0 for ESAT6-CFP10 (20 µg), compared to 3.6 mm observed in reactions to PPDB. Unfortunately, for PPDA, mean skin response at 72 h was 3.8 mm (Figure **1A**), suggesting some sensitization to environmental mycobacteria that, in infected subjects, may lead to the attribution of a false-negative result with the application of the Comparative Skin Test (PPDB – PPDA).

During the pilot study, healthy subjects from TB-free herds exhibited a skin response to PPDB after 24 and 48 h, but by 72 h, the response had become completely negative.

This non-specific response observed in healthy buffalo in the first 48 hours after PPDB inoculation, as well as the greater response observed in *M. bovis* infected buffalo in the first 48 h but lesser at 72 h (Figure **1A** and **B**), is not observed in similar studies conducted in cattle [9].

Therefore, this matter should be further investigated since it is likely related to a different dermal immune response in buffalo compared to cattle [8].

On the other hand, ESP showed better performance as it had higher reactivity in *M. bovis*-infected animals and no reactivity in healthy animals at 72 h (Figure **1B**). The concentrations of 10 and 20 µg for the ESAT6-CFP10 protein cocktail were found to be the best dilutions to be used in the Skin Test. This was also reported in previous studies done on bovine and buffalo [9, 10], where, in particular, a concentration of 20 µg of the ESAT6-CFP10 protein cocktail elicited the best skin inflammatory response in naturally *M. bovis* infected cattle [9].

The best concentration to be used in the Skin Test for ESP was found to be 100 µg, as seen in Figure **1A**.

The pilot study showed ESP had higher specificity, as seen in Figures **2** and **3**.

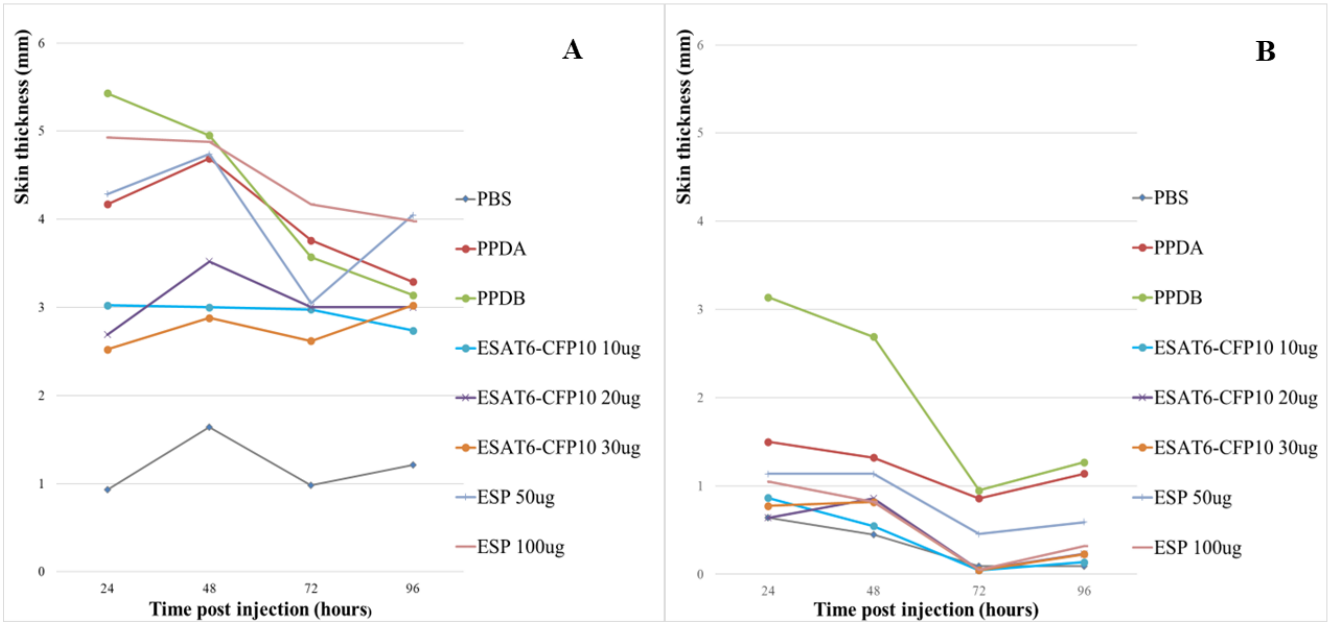

Figure 1: Kinetics of skin response to PBS; PPDB; PPDA; 10 µg, 20 µg and 30 µg of ESAT6-CEF10; 50 µg and 100 µg of ESP, measured every 24 hours on 21 TB-positive (**A**) and 11 TB-negative (**B**) buffaloes. Results indicate skin thickness difference between post and pre-skin test reading.

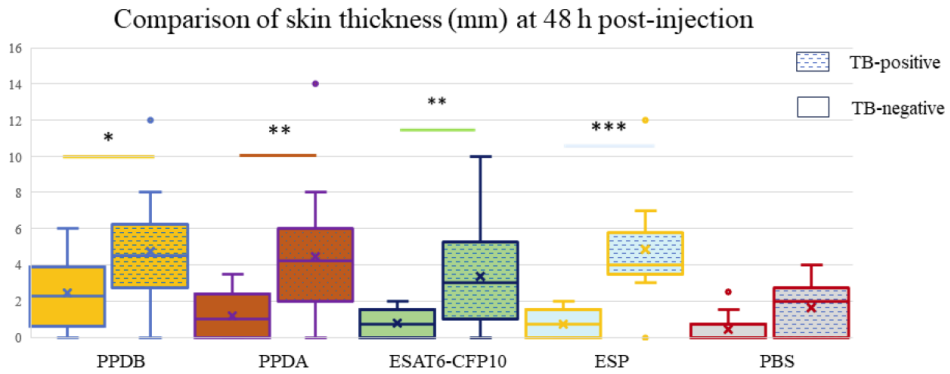

Figure 2: The skin responses (measured in nm) to the five antigens: PPDB; PPDA; ESAT6-CFP10 (20 µg); ESP (100 µg); and PBS as negative control, were compared at 48 h post injection between 21 TB-positive and 11 TB-negative buffaloes. Results are presented as mean (x), median (-) and rnages. Statistical differences between responses were analysed by the Kruskall-Wallis test (*, P<0.05; **, P<0.01; ***, P<0.001).

At 48 hours, the TB-negative group (healthy buffaloes from TB-free herds) and positive group (IFN-positive buffaloes from the TB outbreak) were clearly separated, with no overlapping between their confidence intervals (± 3 standard deviations). At 72 hours, ESAT6-CFP10 also shows a clear separation between the zero values found in the TB-negative subjects and the values of the TB-positive subjects (Figure **3**).

Concerning PPDB, only a few subjects in the TB outbreak had skin reaction values above the positive cut-off of 4 mm in the pilot study (Figure **3**). The other subjects had a reaction between 2 mm and 4 mm, which is in the range of interpretation of inconclusive outcomes. These subjects would have been considered inconclusive if only the interpretation of the Skin Test was taken into account. However, with the application of the IFN-γ test, they were correctly identified as positive.

The best concentrations of ESAT6-CFP10 (20 µg) and ESP (100 µg), found in the pilot study, also used in literature in previous studies [9, 10], were used in the field study on 100 buffaloes reared in TB-free farms, together with traditional PPDs. Skin responses to the protein cocktail, ESP, PPDA, and PPDB were evaluated and compared with responses to the IFN-γ test carried out on the same animals using the same antigens.

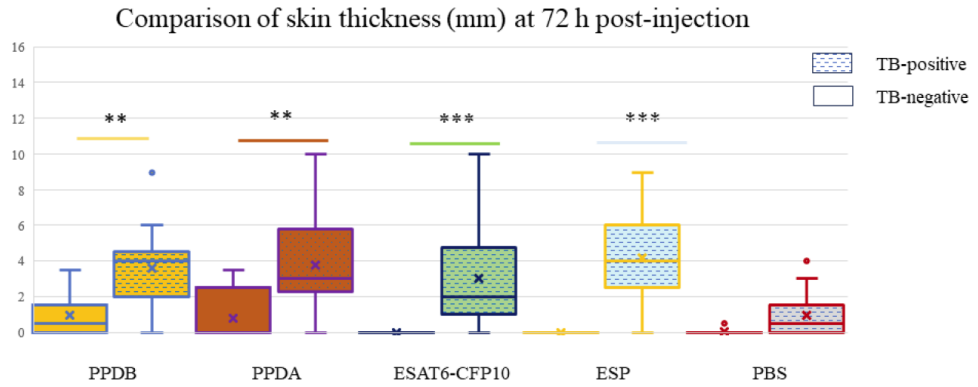

Figure 3: The skin responses (measured in nm) to the five antigens: PPDB; PPDA; ESAT6-CFP10 (20 µg); ESP (100 µg); and PBS as negative control, were compared at 72 h post injection between 21 TB-positive and 11 TB-negative buffaloes. Results are presented as mean (x), median (-) and rnages. Statistical differences between responses were analysed by the Kruskall-Wallis test (*, P<0.05; **, P<0.01; ***, P<0.001).

Of the 100 healthy subjects from TB-free herds, 12 showed inconclusive outcomes to SIT with PPDB, while the number of inconclusive subjects decreased to 3 with the SICT. The number of inconclusive results with ESP stimulation was 10, and with ESAT-6 and CFP-10 protein cocktail was 7. If we introduce the responses to stimulation with ESP and ESAT-6 and CFP-10 protein cocktail combined with the interpretation of SICT, the 3 inconclusive results would be reduced to 0.

The inconclusive outcomes of SIT and SICT found in healthy animals from TB-free herds with PPDB and PPDA stimulation could be due to non-specific responses. In buffaloes, this PPDA and PPDB non-specific reaction could be due to atypical mycobacterial infections as well as to the peculiarities of buffalo skin structure compared to bovine skin, *e.g.*, dermis thickness [8]. In fact, in the study conducted on cattle by Flores-Villalva *et al*. (2012), no marked reactions to PPDA and PPDB were reported in *M. bovis*-free cattle, in contrast to that observed in TB-negative buffaloes in the present study.

Regarding ESAT-6 and CFP-10 protein cocktails, they induced fewer non-specific reactions, as also reported in the literature [6, 7, 9]. Also, ESP, the experimental antigens used in this study, gave similar results, with low reactions in negative buffaloes, and thus ESAT6-CFP10 or ESP could be used in SIT or SICT in association with traditional PPDs to reduce false negative results in *M. bovis* free herds. Using the IFN-γ test, we excluded the presence of anergic animals since all 100 negative subjects reacted to the stimulus with the mitogen. Furthermore, the IFN-γ test in all the 100 healthy animals yielded negative results according to the interpretation criteria of the official EU IFN-γ test protocol published on the European Union Reference Laboratory (EU-RL) website, even if with slight non-specific reactions to PPDs, but excluding false-positive results.

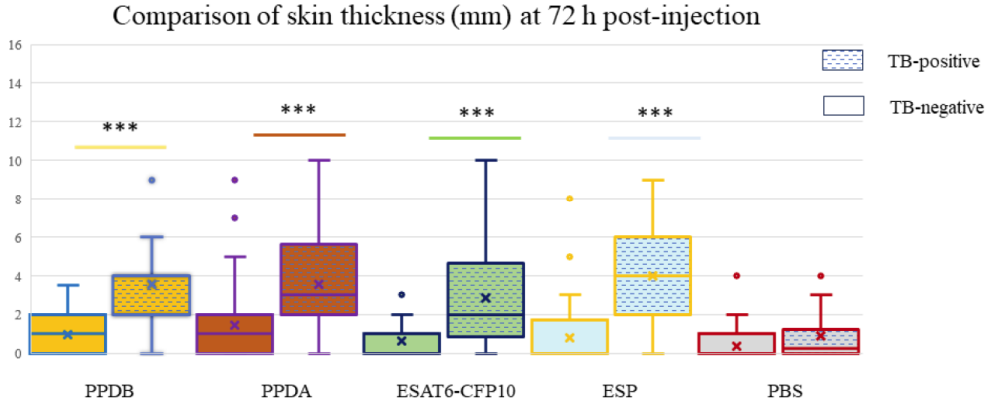

Figure 4: The skin responses (measured in nm) to the five antigens: PPDB; PPDA; ESAT6-CFP10 (20 µg); ESP (100 µg); and PBS as negative control, were compared at 72 h post injection between 21 TB-positive and 111 TB-negative buffaloes. Results are presented as mean (x), median (-) and rnages. Statistical differences between responses were analysed by the Kruskall-Wallis test (*, P<0.05; **, P<0.01; ***, P<0.001).

Comparing the results obtained in the two studies, 21 positive subjects and 111 negative subjects, we observed a significant difference in the skin response to all antigens at 72 hours (*P<0.001*) between negative and infected subjects (Figure **4**). However, it should be noted that the difference was more marked when ESP was used.

Therefore, ESP could be an excellent candidate for further extensive studies in the buffalo species to improve Skin Test Sensitivity and Specificity.

CONCLUSION

Based on our results, using the ESAT6-CFP10 cocktail and Early Secretory Proteins in the Skin Test could greatly improve the performance of TB diagnosis in both *M. bovis*-infected and *M. bovis*-free buffalo herds. In particular, the best performance was obtained with Early Secretory Proteins, used for the first time in buffalo in this study. Although our results are promising, further validation through post-mortem tests for TB is necessary to ensure their reliability.

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ETHICS APPROVAL

The experimental protocol for animal care, handling, and sampling defined in the present study was approved by the Italian Ministry of Health under authorization number 2/2021-PR. Furthermore, the authors complied with the European legislation on protecting animals used for scientific purposes, maintenance, and experimental protocols [14].

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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