Field evaluation of Specific Mycobacterial Proteins-Based Skin Test for the Differentiation of *Mycobacterium bovis*-Infected and Bacillus Calmette Guerin-Vaccinated Crossbred Cattle in Ethiopia

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Summary

Bovine tuberculosis (bTB) challenges intensive dairy production in Ethiopia and implementation of the test and slaughter control strategy is not economically acceptable in the country. Vaccination of cattle with Bacillus Calmette-Guerin (BCG) could be an important adjunct to control, which would require a diagnostic test to differentiate *Mycobacterium bovis* (*M. bovis*)-infected and BCG-vaccinated animals (DIVA role). This study describes evaluation of a DIVA skin test (DST) that is based on a cocktail (DSTc) or fusion (DSTf) of specific (ESAT-6, CFP-10 and Rv3615c) M. *bovis* proteins in Zebu-Holstein crossbred cattle in Ethiopia. The study animals used were 74 calves (35 BCG-vaccinated and 39 unvaccinated) aged less than three weeks at the start and 68 known bTB positive cows. Six weeks after vaccination, the 74 calves were tested with DSTc and the single intradermal cervical comparative tuberculin (SICCT) test. The cows were tested with DSTc and SICCT test. Reactions to DSTc were not observed in BCG-vaccinated and unvaccinated calves while SICCT test reactions were detected in vaccinated calves. DSTc reactions were detected in 95.6% of the cows and single intradermal tuberculin (SIT) positive reactions were found in 98.2% (95% confidence interval, CI, 92.1–100%). The sensitivity of DSTc was 95.6% (95% CI, 87.6–99.1%), and significantly ($P<0.001$) higher than the sensitivity (75%, 95% CI, 63.0-84.7%) of the SICCT test at 4mm cutoff. DSTf and DSTc reactions were correlated ($r = 0.75$; 95% CI =0.53–0.88). In conclusion, DSTc could differentiate *M. bovis*-infected from BCG-vaccinated cattle in Ethiopia. DST had higher sensitivity than the SICCT test. Hence, DSTc could be used as a diagnostic tool for bTB if BCG vaccination is implemented for the control of bTB in Ethiopia and other countries.

Key words: Bovine tuberculosis, BCG vaccination, Crossbred cattle, DIVA skin test, Specific mycobacterial proteins

1. INTRODUCTION

In Ethiopia, intensive dairy farms that raise genetically improved dairy cows have been established around cities and towns (Ameni et al., 2018; Mekonnen et al., 2019) to address national nutritional needs. The development of the dairy sector has been constrained by the emergence of diseases associated with intensivisation, including bovine tuberculosis (bTB) (Ameni et al., 2007; Berg et al., 2009; Firedissa et al., 2012). bTB is an endemic disease in Ethiopia (reviewed by Sibhat et al., 2017) and the disease is affecting livestock production through reduction of productivity and trade restrictions (Tschopp et al., 2012; OIE, 2018). As a zoonotic disease bTB poses a public health threat, especially in the low- and middle-income countries (LMIC) like in Ethiopia (Ashford et al., 2001).

Many developed countries have controlled bTB in their livestock populations using detection and slaughter of reactor animals (Schiller et al., 2010; Buddle et al., 2013). However, in many LMIC, the implementation of such control is economically and societally unacceptable and different control strategies need to be considered. Vaccination is an alternative control strategy and BCG is the only currently feasible vaccine. Research trials evaluating of the efficacy of BCG vaccination against *M. bovis* infection in cattle have demonstrated promising results (Vordermeier et al., 2009; Ameni et al., 2010; Vordermeier et al., 2016a; Ameni et al., 2018; reviewed by Buddle et al., 2018). However, BCG vaccination sensitizes vaccinated animals to react to tuberculin-based tests, such as SICCT or SIT test (Vordermeier et al., 2001; Whelan et al., 2010) as BCG was originally derived from *M. bovis*, compromising their specificity. Therefore, there is a need for a diagnostic test that differentiates *M. bovis* infected from BCG-vaccinated animals (DIVA role).

Significant progress has been made applying defined antigens as DIVA tests for cattle (Vordermeier et al., 1999; Vordermeier et al., 2002; Vordermeier et al., 2011; Jones et al., 2012; Vordermeier et al., 2016b; Srinivasan et al., 2019), using mycobacterial antigens present in field strains of *M. bovis* but absent in the BCG vaccine. These antigens include early secretory antigen target-6kDa (ESAT-6) and culture filtrate protein-10kDa (CFP-10) (Pollock and Anderson, 1997; Vordermeier et al., 1999; Vordermeier et al., 2001). Their encoding genes are located within the region of difference 1 (RD1) of the *M. bovis* genome, a region was deleted from all BCG strains (Gordon et al., 1999; Garnier et al., 2003). As a result, T-cells of the BCG-vaccinated and/or non-infected cattle do not recognize ESAT-6 and CFP-10. However, the use of the cocktail of these two antigens showed a lower capacity in detecting infected animals compared with tuberculin (Sidders et al., 2008; Vordermeier et al., 2011). In attempts to overcome this limitation, the antigen Rv3615c was discovered to be a useful additional DIVA antigen to complement ESAT-6 and CFP-10 (Sidders et al., 2008).

This protein cocktail has previously been evaluated as a blood and skin test reagent, mainly in *Bos taurus* breeds such as Holstein-Friesians but not in zebu cattle or cross-breeds between zebus and taurine cattle. In the present study, protein cocktail-based DIVA skin test (DSTc) was evaluated in Zebu-Holstein Friesian crossbreed cattle under field condition.

2. MATERIAL AND METHODS

2.1. Study animals and husbandry

The study was conducted on Holstein Friesian x Zebu crossbred calves and cows. The calves were all male and recruited from bTB free dairy farms within two weeks of age. Up on arrival at our animal facility the calves were screened by the whole blood interferon-gamma release assay (IGRA) to demonstrate freedom from infection (data have not been shown). The cows were recruited from a known bTB herd and were again tested by both IGRA and SICCT and confirmed to be positive for bTB. The

naïve calves and the cows were kept in separate barns at the National Animal Health Diagnostic and Investigation Center at the Sebeta, Ethiopia. The calves were fed on pasteurized partially skimmed milk, hay and concentrate. The cows were fed on hay and concentrate. Both the calves and cows watered *ad libitum*.

2.2. Study design, plan and setting

It is a cross-sectional study in which the performance of the DSTc was evaluated in comparison with SIT and SICCT tests. First, the diagnostic specificity of DSTc was tested on 74 calves. The calves were randomly assigned into BCG vaccinated and control groups using a lottery method. Accordingly, 35 calves were vaccinated subcutaneously by 1 x 10^6 CFU of BCG Sofia (InterVax Ltd, Toronto, Canada) at the two weeks while the remaining 39 were kept unvaccinated. After 6 weeks vaccination, both the vaccinated and unvaccinated calves were tested by DSTc and SICCT test. The 68 reactor cows were tested by both DSTc and SICCT test. In addition to DSTc, an independent study was undertaken to assess the diagnostic performance of the recombinant fusion protein of ESAT, CFP-10 and Rv3615c as DIVA skin test (DSTf) in 30 known bTB positive cows for comparison with the DSTc and SICCT test. The 30 cows were tested by DSTf and DSTc test at the same time on one side of the neck while SICCT was applied on the other side of the neck of the study animals.

2.3. Antigens

The cocktail protein-based DST (DSTc) consisted of the ESAT-6, CFP-10, and Rv-3615c antigens of *M. tuberculosis* and *M. bovis*. The individual recombinant protein was produced by Lionex GmbH, Braunschweig, Germany. When preparing the DSTc, equal amounts of each freeze-dried protein were combined in a PBS solution containing 100µg/ml of each protein (300µg total protein/ml). The DSTc

solution was stored at -80°C until needed. The DSTf reagent was supplied as solution by Lionex (300µg total protein/ml) and stored at 4°C until being use. Bovine and avian purified protein derivatives (PPD-B and PPD-A) were obtained from Thermo-Fisher (Lelystad, the Netherlands).

2.4. Protein-based DIVA skin test (DST)

All study animals (BCG vaccinated and control calves; bTB positive cows) were injected intradermally with 0.1ml DSTc (30µg total protein per dose) into the middle of the right side of the neck. For the comparison between DSTc and DSTf, 0.1ml DSTc (30µg protein per dose) was injected 10cm below the crest and 0.1ml DSTf was injected 12cm below DSTc on a vertical line. Skin thicknesses were measured before inoculation and at 72 hours post inoculation. The measurements were done by the same operator using the same digital caliper in every testing. Results are expressed as the difference in skin fold thickness (in millimeter) before administration of the antigens and 72 h post the administration of the antigen. Skin reaction was considered positive if the increase in skin thickness at the DSTc or the DSTf site was greater than or equal to 2mm (Casal et al., 2012; Vordermeier et al., 2016b).

2.5. Single intradermal cervical comparative tuberculin test

The SICCT test was performed on the left side of the study animals in the middle of the neck. After preparation of the injection site, 0.1ml PPD-A (3,000IU/ml; Prionics, Lelystad, The Netherlands) was inoculated 10cm below the crest and the same volume of PPD-B (2,500IU/ml; Prionics, Lelystad, The Netherlands) was injected at a site 12cm apart from PPD-A injection site in vertical line in reactor cows. The skin thicknesses were measured just before injection and at 72 hours post injection by the same operator using the same digital caliper and the results were presented as change in skin thickness (mm) between the two readings. In case of the SIT test, skin reaction was defined as positive when the

increase of skin thickness at PPD-B site was greater than or equal to 4mm, otherwise considered as negative. For the SICCT test, the differences in the increase of skin thickness at the bovine and avian PPD injection sites were considered. An animal was considered to be positive when the increase in skin thickness at the bovine PPD site was greater than the increase in skin thickness at the site of the avian injection by at least 4mm. If the differential increases between the two sites were equal to or less than 1mm, or between 1mm and 4mm, the animal was considered negative or doubtful, respectively (OIE, 2018).

2.6. Data analysis

Data analysis was performed using Prism 8 (GraphPad Software). The skin fold thickness increase was summarized using median and 95% confidence interval of median (95% CI) after assessment of normality of the data. Wilcoxon matched-pair signed rank test was performed for comparison of skin reactions induced by two defined antigens while using the Friedman test (repeated measures non-parametric analysis of variance) with Dunn's multiple comparison test for more than two defined antigens. In addition, Spearman rank test was used for evaluation of the relations of the degree of skin thickness induced by different antigens. A comparison of the DSTc relative sensitivity was scrutinized using the Fisher exact test. Kappa test was made to evaluate the diagnostic agreement between DSTc and that of the SICCT or SIT test. In all cases, a 95% CI and a significant level of 5% were used to express statistical significance.

3. RESULTS

3.1. Performance of DSTc as a DIVA test in vaccinated and unvaccinated calves

Six weeks post-vaccination, the BCG-vaccinated (n= 35) and unvaccinated control (n = 39) calves were skin tested with DSTc and avian and bovine PPD (PPD-A, PPD-B). All BCG vaccinated calves were DSTc-negative (Figure 1A, median of increase in skin thickness: 0.40mm, 95% CI = 0.05–0.89). In contrast, all of the vaccinated calves responded to PPD-B (SIT-positive. Figure 1A, median reaction sizes PPD-B: 10.91mm, 95% CI = 8.50–13.67). Furthermore, 82.9% (95% CI = 65.7–92.4) of the vaccinated calves were SICCT-positive test (Figure 1A, median PPD-B minus PPD-A: 6.60mm, 95% CI = 4.87–7.85). Comparative analysis indicated that the skin thickness induced by DSTc in calves was significantly lower than the skin thicknesses induced either by PPD-B (p<0.001) or by PPD-A (p<0.001). These data demonstrated the superior specificity of DSTc compared to SIT or SICCT. Therefore, DSTc demonstrated its DIVA utility in crossbred cattle in Ethiopia. As observed with the BCG vaccinated calves, none of the unvaccinated calves showed a skin reaction difference of 2 mm or higher and they were therefore classified as DSTc negative (Figure 1B; median reaction size: 0.72mm, 95% CI = 0.33–0.99). Similarly, none of the unvaccinated calves were positive either in the SIT (median reaction size 0.64mm, 95% CI = 0.30–0.90) or the SICCT (median reaction size: 0.57mm, 95%Cl = 0.32- 0.85; Figure 1B).

3.2. Diagnostic performance of DSTc in bTB infected cows

The diagnostic sensitivity of the DSTc was tested in 68 naturally infected SIT-positive from confirmed bTB positive herds. The recorded median of skin thickness increase was 6.25mm (95% CI = 5.30–6.76) at the DSTc injection site while the median skin thicknesses were 10.82mm (95% CI = 9.55–13.63) at PPD-B and 4.48mm (95% CI = 3.59 – 5.03) at PPD-A sites. The result indicated that the median

increase of skin thickness to DSTc injection was significantly lower (p<0.001) than the median in skin thickness at the injection site of PPD-B (Figure 1C). On the other hand, by considering SICCT results, the median of the differences in skin thickness at the injection site of PPD-B and the injection site at PPD-A was 7.10mm (95% CI = 5.54–8.40) which did not statistically (p>0.05) differ from the median skin thickness at the injection site of DSTc. Thus, there was no difference in thicknesses induced by DSTc and SICCT test.

As summarized in Table 1, out of 68 animals, 65 were positive to the DSTc, resulting in a relative sensitivity of 95.6% (95% CI = 86.9 – 98.6%). Similarly, the relative sensitivity of SIT was 98.2% (67/68 animals positive, 95% CI = 89.83 – 99.80). On the other hand, only 51 of the 68 reactors were identified as positive by the SICCT test and hence its relative sensitivity was 75.0% (95%CI = 63.1-84.1%). As the result, the relative sensitivity of SICCT test was significantly (p<0.001) lower than that of DSTc.

The diagnostic agreement of DSTc and SIT test as well as that of DSTc and SICCT test were evaluated on 107 cattle (68 reactor cows and 39 calves). Out of those cattle, 65 were DSTc-positive while the remaining 42 were negative for the DSTc test (Table 1). The test agreement of DSTc with the SIT and the SICCT tests were 98.13% and 85.98%, respectively. Thus, a strong agreement (k = 0.961, p<0.001) was recorded between the DSTc and the SIT test, while moderate agreement (k = 0.743, p < 0.001) was recorded between the DSTc and the SICCT test.

3.3. Diagnostic performance of the DST fusion protein (DSTf) in reactor cattle

The diagnostic performance of the DSTf was evaluated in 30 bTB infected cattle. The reactivity of the skin to injection with DSTf was compared with the skin reactivity after injection with DSTc, PPD-B, and PPD-A. The results of this experiment are presented in Figure 2. The median of skin thickness increases at the DSTf injection site was 5.89mm (95% CI = 5.43–7.08) compared to 5.38mm (IQR = 4.53–8.85) at the DSTc site. The medians of skin thicknesses were 6.32mm (95% CI = 5.52–7.25) at the PPD-A site and 9.97mm (IQR = 8.35–13.03) at the PPD-B site. Multiple comparison analysis revealed that the skin thickness caused by DSTf inoculation was statistically lower (p<0.001) than that caused by PPD-B inoculation (Fig 2). On the other hand, the thickness caused by inoculation of DSTf was similar to the difference in skin thicknesses of the PPD-B site and PPD-A site (median differences between PPD-B and PPD-A readings was 3.55mm, IQR = 3.34–5.39, Fig 2). Likewise, there was no significant difference (p>0.05) in skin thicknesses caused by inoculation of DSTf and DSTc (Fig 2). A statistically, a strong correlation (r = 0.753; 95%CI =0.532–0.879) was recorded between the DSTf and DSTc as demonstrated in Fig. 3.

r=0.753, p<0.001

4. DISCUSSION

The present study was conducted to evaluate the performance of DIVA skin test (DST) based on the *M. tuberculosis* complex proteins ESAT-6, CFP-10 and Rv3615c using either their cocktail (DSTc) or their fusion (DSTf) in differentiating *M. bovis* infected and BCG vaccinated cattle. The study was conducted on 74 non-infected calves, and 68 known bTB infected cows from herds with confirmed bTB prevalence, and both were Zebu-Holstein Friesian crossbreed. The calves consisted two groups i.e. 35 BCG-vaccinates and 39 non-vaccinates. The result of the DSTc were analyzed and evaluated for its performance as DIVA skin test. Furthermore, the performance of DSTc was compared with that of the

SIT and SICCT tests in 68 known bTB positive cattle. Such evaluation of DIVA reagents was repeatedly performed in taurine breed cattle in developed countries settings (Whelan et al., 2010; Casal et al., 2012; Jones et al., 2012; Vordermeier et al., 2016a, b) earlier. However, this is the first study to evaluate DSTc and DSTf proteins in zebu-taurine crossbred cattle in the context of developing countries; hitherto only a peptide cocktail of these antigens tested in bTB reactor cattle in Ethiopia (Srinivasan et al., 2019).

The relative specificity of DSTc was evaluated in 35 non-infected and BCG-vaccinated calves, which were recruited from known bTB free dairy herds and also re-affirmed to be free of bTB by IGRA. Six weeks after subcutaneous inoculation with $1x10^6$ CFU dose of BCG, they did not react to intradermal injection of the DSTc, making relative specificity of the DSTc was 100% (95% CI = 90.0-100). Similar to the present result, previous studies reported that antigenic protein- or peptide-based intradermal DSTc did not induce detectable skin reactions in BCG-vaccinated taurine cattle (Whalen et al*.,* 2010; Jones et al*.,* 2012; Vordermeier et al*.,* 2016b). Similarly, all the 39 non-vaccinated calves did not react to all the three (DSTc, SIT and SICCT) tests and the specificity of DSTc in non-vaccinated calves was 100%. Furthermore, DSTc detected 65 of the 68 known bTB positive cows while SICCT test detected 51 of them. Thus, the sensitivity of DSTc was 95.6% while the sensitivity of the SICCT test was 75%, suggesting DSTc has higher sensitivity than SICCT. Recently, Srinivasan et al. (2019) recoded similar level of sensitivity of peptide cocktail-based DIVA skin test in known bTB positive cattle in Ethiopia although our sample size was substantially larger. The sensitivity of DSTc in the present study was higher than the sensitivities of DSTc reported by other studies (Casal et al*.,* 2012; Jones et al., 2012; Vordermeier et al., 2016a), here in crossbreed cattle, which encourages the future application of DSTc in conjunction with BCG usage.

In addition to estimation of the sensitivity and specificity of DSTc, Kappa statistics was used to evaluate its agreement with SIT and SICCT tests. The agreement between DSTc and SIT test was strong while on the other hand a moderate agreement was recorded between the DSTc and SICCT test. In another field evaluation, a moderate agreement was recorded between DSTc and the SIT test in 23 reactors (Casal et al*.,* 2012). However, since the true disease status of the test animals was not known, it is difficult to make a conclusive remark on the sensitivities and specificities of DSTc unless gold standard test (TB lesion and or *M. bovis* isolation) is used. Therefore, there is a need for further evaluation of the sensitivities and specificities of DSTc on a large number of cattle using the appropriate gold standard test.

With regard to the intensity of the skin thickness induced by the injection of DSTc, the magnitude of skin thicknesses induced by DSTc and PPD-B were compared in known bTB positive cows and it was observed that the median of skin thickness induced by DSTc injection (6.3mm) was lower than those induced by PPD-B (10.8mm). This observation agreed with the observations made earlier by other studies elsewhere in taurine cattle (Whelan et al*.,* 2010; Jones et al*.,* 2012; Srinivasan et al*.,* 2019). The stronger skin reaction to PPD-B could be because of that PPD-B consists of a more diverse range of immunogenic proteins (Borsuk et al., 2009); whereas the DSTc contains only the mycobacterial proteins ESAT-6, CFP-10 and Rv3615c. Moreover, a dose of tuberculin solution contains greater protein content than a dose of the DSTc (Yang et al., 2013). The strong skin reaction following PPD-B could also be due to less purification compared to highly purified DSTc. Except Casal et al*.* (2012) who recorded comparable medians skin thicknesses by injection of DSTc and PPD-B, other researchers recommended the possibility of strengthening the skin reaction to DSTc by adding Rv3020 (Jones et al*.,* 2012; Vordermeier et al*.,* 2016b).

In most of the earlier studies, the experiments evaluating DIVA tests were conducted on whole blood-based IFN-γ assays for easiness of the protocol to accommodate modifications Vordermeier et al*.,* 2016b). However, the use of these DIVA reagents in the IFN-γ assay will be difficult to implement in areas with economic and technical constraints (Ameni et al*.,* 2000). In contrast, the DIVA skin testing is a simpler technically and can easily be applied in the field in same way as the tuberculin skin test. Thus, a recombinant fusion protein containing ESAT-6, CFP-10, and Rv3615-c (DSTf) was produced in a similar presentation as PPD-B containing 1.2 ml (12 doses) per vial. In the present field trial in reactor cattle, the DSTc and DSTf demonstrated comparable medians of skin thickness in 30 known TB positive cattle. Like DSTc, the DSTf induced skin reaction equivalent to the final skin thickness induced by SICCT test. Therefore, these observations encourage the use of DSTc or DSTf for the diagnosis of bTB in cattle.

5. CONCLUSION

This is the first study to investigate the performance of the DIVA skin test based on a cocktail/fusion protein of three mycobacterial antigens (ESAT-6, CFP-10 and Rv3615c) in zebu-taurine crossbred cattle. The data showed high sensitivity of DSTc in known bTB positive cows and its high specificity in BCG vaccinated bTB free calves after six weeks of BCG vaccination. The data generated by the two DST preparations (cocktail and fusion) were comparable. Thus, the findings of this study demonstrated the potential utility of DSTc or DSTf to support BCG vaccine-based bTB control policies although additional extended field evaluation of these tests are important for re-affirmation of the observations of this study.

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DECLARATION OF COMPETING INTERESTS

The authors declare that there are no competing interests.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by Ethical Review Board (IBR) of the Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Addis Ababa, Ethiopia (Reference No. IRB/07/2014).

DATA AVAILABILITY

The raw data of used for this study has been submitted with the manuscripts for availability for the researchers on the basis of request and acknowledgements.

ABBREVIATIONS

BTB: Bovine tuberculosis; BCG: Bacillus Calmette Guerin; culture filtrate protein 10 kDa; DIVA: differentiate infected from vaccinated; DST: DVIA skin test; DSTc: cocktail proteins based DST; DSTf: fusion proteins based DST; ESAT-6: early secretory antigen target 6 kDa; CFP-10: LMIC: low- and middle-income countries; PPD-A: avian purified protein derivative; PPD-B: bovine purified protein

derivative; RD1: region of difference 1; SIT: single intradermal tuberculin test; SICCTT: single intradermal cervical comparative tuberculin test

AUTHORS' CONTRIBUTIONS

BB performed field data collection and laboratory analysis, did the data analysis and drafted the manuscript. AZ contributed in the laboratory work of the experiment. AW contributed in the field data collection and laboratory work. SB contributed in editing and correcting the manuscript. RGH contributed in designing of the experiment and in editing the manuscript. JW contributed in the designing the experiment and in reviewing the manuscript. HMV contributed in the designing of the experiment and editing the manuscript. GA contributed in designing of the research, supporting in analysis and its interpretation of the data and in reviewing the manuscript. All authors read and approved the final manuscript.

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