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The Use of a Mixture of esat-6 and cfp-10 Protein as Antigens in ELISA for the Diagnosis of Bovine Tuberculosis in South China

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Abstract: Several Enzyme-Linked Immunosorbent Assay (ELISA) methods exist to diagnose Bovine Tuberculosis (BTB). In this study, an ELISA method was established with a mixture of esat-6 and cfp-10 as the diagnostic antigens. ELISA tests on 160 bovine serum samples (80 from cattle with BTB; 80 from healthy cattle) demonstrated that the sensitivity was 76.3% (61/80) and the specificity was 97.5% (78/80). A further screening test using ELISA and PPD was conducted with 335 serum samples collected at random from eight dairy herds (5 problematic herds and 3 BTB-negative herds) in Guangdong, China. In the problematic herds, the positive ELISA rate was 7.9% (16/202) for anti-esat-6 and cfp-10 antibodies and 5.4% (11/202) in the PPD. This study demonstrated that ELISA with the use of the esat-6 and cfp-10 mixture of antigens is simple and sensitive and can be used to analyze large numbers of samples for the serodiagnosis of BTB.

Key words: Bovine tuberculosis, esat-6, cfp-10, samples, sensitive, ELISA

INTRODUCTION

Bovine Tuberculosis (BTB) is a zoonotic disease that is seriously detrimental to the dairy industry. The disease can occur sporadically and is difficult to eradicate. The introduction of milk pasteurization in developed countries has dramatically reduced the rates of transmission of TB from cattle to humans (Hardie and Watson, 1992) but the causative agent of BTB, *M. bovis*, remains responsible for up to 5-10% of human TB cases (De Kantor *et al*., 2008). Strict import and export regulations and extensive movement restrictions, combined with skin test and slaughter policies have significantly reduced the incidence of BTB. However, it is now clear that more efficient diagnostic tools for eradication, control and surveillance programs as well as new and improved vaccination strategies are required to control BTB.

BTB control programs are largely based on a test-and-slaughter policy using assays that detect Cell-mediated Immunity (CMI). Therefore, skin testing with bovine Purified Protein Derivative (PPD) has been used for many years in control programs. The PPD test has several advantages such as inexpensive reagents and procedural simplicity. However, PPD testing is labor-intensive and since, it shares many antigenic components with avian PPD (PPDA), specificity can be compromised (Amadori *et al*., 2002). Hence, serological diagnostic methods with high specificities and sensitivities are required to overcome these limitations.

The Enzyme-Linked Immunosorbent Assay (ELISA) is a widely used in vitro diagnostic test due to its rapidity, simplicity and low cost. ELISA methods for antibody detection in BTB have been extensively assessed with reported sensitivities that vary from 50-75% while specificities range from 90-95% compared to PPD testing (Silva, 2001). ELISAs have been suggested as an ancillary parallel test for skin test-negative cattle in herds with confirmed chronic infections or TB outbreaks (De La Rua-Domenech *et al*., 2006).

Esat-6 is the best-documented TB antigen for ELISA to date with sensitivities that range from 66-78% reported in Europe. We previously found an overall specificity of 91% and a sensitivity of 69% which is at the lower end of the reported interval due to variations between sites. The gene encoding cfp-10 (esxB) is co-transcribed with esat-6 encoding the *esxA* gene (Berthet *et al*., 1998); the two encoded proteins have been shown to form stable heterogeneous secreted signaling complexes in myco-

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bacteria (Zhang *et al*., 2010). This co-localization is likely to explain why cfp-10 results in the same strong responses as esat-6 in skin test-positive animals and with minimal background in TB-free animals. All studies have clearly identified the two antigens esat-6 and cfp-10 are the most immune dominant of those tested in experimentally infected cattle. Furthermore both can be used to distinguish between BCG-vaccinated and *M. bovis* infected cattle.

In order to reduce serological crossreactions of ELISAs and increase their accuracy, recent studies have focused on the development of tests using several purified and species-specific antigens. Although, esat-6 and cfp-10 have been evaluated for the diagnosis of mycobacterial infections in human, very few have been developed in cattle. The purpose of this study was to evaluate the use of esat-6 and cfp-10 as the antigen in ELISA for the diagnosis of bovine tuberculosis.

MATERIALS AND METHODS

Serum samples: Bovine tuberculosis-positive bovine serum samples (80) were measured with the use of ELISA based on the antigen mixture described above to determine the sensitivity of the assay. Healthy bovine serum samples (80) were measured to determine the specificity of the assay.

Serum samples were collected from a total of 335 cows from eight milk farms around the city of Guangzhou, China; five of these had a previous history of BTB and three were TB-negative herds for >5 years. The prevalence of TB in each herd was determined by PPD. Cows that had been calved or tested <6 months prior to this study were excluded.

ELISA: The esat-6 and cfp-10 fusion gene fragments were amplified from the genomic DNA of a Mycobacterium bovis reference strain and inserted into the expression plasmid pET32a (+) to generate the recombinant plasmid pET-esat-6 and pET-cfp-10. The recombinat expression plasmid was transformed into *E. coli* BL21 (DE3). The fused protein esat-6 and cfp with His-tag were expressed after inducing with IPTG and purified with affinity chromatography (Wang *et al*., 2006). To test the antibody reactivity of the purified proteins, Costar ELISA plates were coated with serial dilutions of the esat-6 and cfp-10 proteins (50 μL well^{-1}). The serum were used at 1:100 dilution with PBST (PBS with 0.1% Tween 20; 100 μL well^{-1}). Plates were incubated for 30 min at 37°C and then washed 4 times in 300-350 μL Phosphate Buffered Saline containing 0.05% Tween 20 (PBST). Then 100 μL of HRP-conjugated goat anti-bovine immunoglobulins were used at 1:2500 dilution for 30 min at 37°C and the four wash cycles were repeated. Finally, 100 μL of chromogenic substrate was added and incubation took place at room temperature (20-25°C) for 15 min in the dark. The reaction was stopped adding 2 M sulfuric acid and the plate was read with a spectrophotometer at 492 nm. Results were read on a Dynatech MR 7000 ELISA reader (Bio-Ran model 680).

All reaction mixtures were performed in triplicate and the average values were used for recording and calculations. Cut-off points based on OD readings were calculated using Receiver Operator Characteristic curves (ROC) analyses. Sera were considered reactive when OD results were greater than the cut-off value (OD>0.373). Those samples with an absorbance higher or equal to the cut-off value were considered reactive.

RESULTS AND DISCUSSION

Eighty serum samples from cattle with bovine tuberculosis and 80 serum samples from healthy cattle demonstrated that the sensitivity of the ELISA was 76.3% (61/80) and the specificity was 97.5% (78/80; Table 1).

In the screening test, using 335 serum samples from eight dairy herds, the positive rates in the five problematic herds were 7.9% (16/202) for anti-esat-6 and cfp-10 antibodies ELISA and 5.4% (11/202) in the PPD (Table 2). In the present study two highly specific antigen genes, esat-6 and cfp-10 were recombined.

The sub-sequent specificity of the ELISA was 97.5% which was comparable to the reported specificity of ELISA based on a single protein (94.4%) (Silva, 2001). The sensitivity was 76.3% which was higher than the figure of 30%, reported by Ritacco who used PPD as a ELISA diagnostic antigen. Both figures were also higher than the

Table 1: Specificity and sensitivity analysis of ELISA

Bovine tuberculosis positive	ELISA positive	Bovine tuberculosis negative	ELISA negative
80	61	80	78

Sensitivity = 61/80% = 76.3%; specificity = 78/80% = 97.5%

Table 2: Comparison of ELISA using individual serum samples and tuberculin skin test on the dairy cows from problematic and negative herds

	Positive samples (n)			Positive samples (n)	
Farms	Cattles (n)	ELISA	(%)	PPD	(%)
A	42	4	9.5	4	9.5
B	56	3	5.4	2	3.8
C	37	6	16.2	4	10.8
D	35	1	2.9	0	0.0
E	32	2	6.3	1	3.1
F	43	0	0.0	0	0.0
G	38	0	0.0	0	0.0
H	52	0	0.0	0	0.0
Total	335	16	7.9	11	5.4

18 and 47.5%, respectively reported by Wood. Also, the sensitivity and specificity of ELISA based on the mixture of esat-6 and cfp-10 proteins were significantly higher than the results of ELISA using esat-6 and cfp-10 separately (Buddle *et al*., 1995; Kanaujia *et al*., 2003). The study indicated that the ELISA method using the fusion protein esat-6 and cfp-10 as the antigen had a wide antibody reactivity spectrum which increased the test sensitivity.

Several advantages of the use of ELISA for the diagnosis of BTB were identified; sampling requires only a single visit by the veterinarian to the farm and handling of the animals but blood sampling can be repeated as often as necessary without altering the immune status of the animal. The interpretation is based on numerical values and is more objective than observation of skin swelling.

The detection of specific antigens has always been important for the diagnosis of BTB. In particular, secretory proteins in *M. bovis* culture filtrates have been intensively investigated. Many studies have demonstrated that the small molecular weight secretory protein, esat-6 is only expressed in the *M. tuberculosis* complex (including *M. tuberculosis*, *M. bovis* and *M. africanum*) and several other pathogenic mycobacteria but not in Bacillus Calmette-Guerin (BCG) and most non-pathogenic mycobacteria (Sorensen *et al*., 1995; Harboe *et al*., 1998). In addition, esat-6 has many T-and B-cell epitopes and thus is strongly immunogenic (Brandt *et al*., 1996) which suggests that esat-6 is a good candidate for a diagnostic antigen. An ELISA assay based on esat-6 demonstrated a high correlation between esat-6 antibody levels and BTB infection. However, only 53% (30/57) of infected animals were positive with esat-6-ELISA (Kanaujia *et al*., 2003).

As an intracellular parasitic bacterium, *M. bovis* exhibits large individual differences in antibody reactivity and has a wide antibody reactivity spectrum; hence, single antigens cannot reflect the antibody level and infection status *in vivo*. A mixture of antigens of *M. bovis* may dramatically increase the sensitivity of serological diagnostic methods (Lyashchenko *et al*., 2000; Greenwald *et al*., 2003).

The highly immunogenic nature of esat-6 and cfp-10 has previously been reported across a broad range of species in cluding mice, guinea pigs, humans and cattle (Van Pinxteren *et al*., 2000). Their potential to differentiate between BCG infection and *M. tuberculosis* or *M. bovis* infection has been recognized for several years and a number of reports have demonstrated high degrees of specificity and sensitivity with the use of these two proteins in cattle and humans (Pollock *et al*., 2000; Aagaard *et al*., 2003). In order to enhance the performance of the esat-6 and cfp-10 antigen mixture by adding multiple antigens, the sensitivity would need to be improved without impairing the high specificity.

CONCLUSION

Compared to the PPD skin test as the standard method, this ELISA method showed a high sensitivity and specificity. This may represent a promising supplementary method to the PPD skin test or as an efficient tool for epidemiological surveys of cattle and wild animals. Given the high specificity and sensitivity obtained with the esat-6 and cfp-10 mixture and the ability of CMI-based assays to detect early BTB infections, this method may have great value for the routine testing of cattle. On the basis of this study, we suggest that a specific mixture of esat-6 and cfp-10 should be tested in the near future with large numbers of field animals.

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