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Development and evaluation of two multi-antigen serological assays for the diagnosis of bovine tuberculosis in cattle

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ABSTRACT

There is currently an increased interest in the use of serological approaches in combination with traditional cell-mediated immunity-based techniques to improve the detection of tuberculosis (TB)-infected animals. In the present study, we developed and validated two different serological TB-detection assays using four antigens, MPB70, MPB83, ESAT6 and CFP10, and the tuberculin PPDb. A conventional multi-antigen TB-ELISA method and a novel TB multiplex test, based on Luminex technology, were developed to detect antibodies to multiple antigen targets. The performance levels of the two tests were evaluated and compared using selected panels of samples having known TB states. The TB-ELISA test (containing five antigens, including PPDb) had a sensitivity (Se) of 74.2% and a specificity (Sp) of 94.9%, while the TB-Luminex test had higher Se (79.0%) and Sp (99.1%) rates even when only one reactive antigen was used to classify the test as positive. If a more restrictive criterion, requiring two positive antigens to classify the test as positive, was used, then the TB-ELISA's Sp rate increased to 99.8% but the Se decreased to 61.3%, while the TB-Luminex test's Sp rate increased to 100% but the Se decreased to 51.2%. TB-ELISA and TB-Luminex were applied to a panel of 257 sera collected from bTB-positive herds, as determined by a post-mortem inspection. They showed good performance levels, identifying 49 (80.3%) and 48 (78.7%), respectively, of 61 samples that had tested positive by the intradermal tuberculin (IDT) test and/or interferon-gamma assay. In addition, TB-ELISA and TB-Luminex were able to identify 60 and 42 samples as positive, respectively, out of the 196 samples that tested negative to IDT and interferon-gamma at the time of serum collection. Subsequent IDT tests performed after 1–2 months, confirmed the positivity of 18 samples, indicating the strategic value of having two serological assays to detect TB-infected herds that were not reactive to initial IDT testing, thereby allowing for the rapid control of outbreaks and eradication of the disease.

1. Introduction

Bovine tuberculosis (bTB) is a chronic inflammatory disease mainly caused by *Mycobacterium bovis* and, to a lesser extent, by *Mycobacterium caprae*. The infection can affect several domestic animal and wildlife species and has a zoonotic relevance owing to its potential transmission to humans. Consequently, bTB is subject to official eradication campaigns, which mainly utilize a test and slaughter policy.

Because bTB infection predominantly triggers cell-mediated immunity (CMI) during its early and intermediate phases, the main diagnostic techniques, the intradermal tuberculin (IDT) test and interferon-gamma (IFN-γ) assay, used worldwide in eradication programs are based on the detection of the CMI response to a bovine purified protein derivative (bPPD).

The IDT is recognized by the World Organization of Animal Health and the European Commission as the primary screening test, either in the form of the single intradermal tuberculin or the comparative intradermal tuberculin test, for the detection of tuberculosis in cattle (de la Rua-Domenech et al., 2006; Bezos et al., 2014).

The IFN-γ assay has been included in the World Organization of Animal Health's Terrestrial Manual since 1996 and accepted for use as an ancillary test to the IDT test in the European Union since 2002

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[Council Directive 64/432/EEC, amended by (EC) 1226, 2002] to maximize the detection of TB-infected animals in a herd or in a region (EFSA, 2012).

As the disease progresses, there is both a decrease in CMI and a development of serological responses (Pollock et al., 2001; McNair et al., 2007; Schiller et al., 2011). In cattle experimentally infected with *M. bovis,* a low, or absent, serological immune response occurs in the initial stages and then increases substantially as the infection progresses (Ritacco et al., 1990; Fifis et al., 1994; Amadori et al., 2002; Waters et al., 2006). Therefore, antibody (Ab) detection has not played an important role in eradication programs nor has it been included as an official diagnostic tool (Bezos et al., 2014). However, it has been hypothesized that in the advanced and generalized stages of TB infections, a certain proportion of anergic animals may be present, which are potentially highly infectious and do not produce a reaction to CMI-based tests. These animals could relapse and cause future recurrent TB outbreaks. Thus, the first serological assays were developed during the 1990's but were generally lacking in sensitivity (18%–73%). Additionally, their specificity could be affected by the presence of other mycobacterial infections, such as paratuberculosis (Johne's disease; Pollock et al., 2005).

Recent studies have renewed the interest in serological assays as diagnostic tests to detect infected animals that were not detected by IDT and the IFN-γ assays (Green et al., 2009; Whelan et al., 2010; Waters et al., 2011). In particular, the identification of more specific diagnostic antigens (such asMPB70, MPB83, ESAT6, CFP10 and others) and the introduction of multiplex assay strategies allow for the development of new tests.

In particular, MPB70 and MPB83 are among the most relevant antigens in the *Mycobacterium tuberculosis* complex. MPB70 is a soluble secreted protein expressed by *M. bovis* (Harboe et al., 1990; Lin et al., 1996; Lightbody et al., 2000), and MPB83, which has been detected as being serodominant (van Pinxteren et al., 2000; Waters et al., 2006; Lyashchenko et al., 2008), is a glycosylated lipoprotein on the surface of the mycobacterial membrane. ESAT6 and CFP10 (Pollock and Andersen, 1997; van Pinxteren et al., 2000) are antigens expressed in *M. bovis* but absent from environmental mycobacteria and *M. bovis* bacillus Calmette–Guerin that have been proposed as relevant differentiating infected and vaccinated animal test candidates (Vordermeier et al., 2011) and used as alternative antigens to the PPDs for blood stimulation in the IFN-γ assay (Bezos et al., 2014).

During experimental infections, the early detection of antibodies has been observed with MPB83 3–4 weeks after infection (Waters et al., 2006; Waters et al., 2010), while responses against ESAT6 and MPB70 were detected after 12 and 20 months, respectively, after infection (Fifis et al., 1992; Lyashchenko et al., 1998). In experimentally infected animals, the serological response increases after performing intradermal tests (anamnestic effect), leading to the increased sensitivity of these techniques (Harboe et al., 1990; Lightbody et al., 1998; Waters et al., 2011).

A variety of enzyme-linked immunosorbent assays (ELISAs) have been developed that use single (Cho et al., 2007) or multiple antigen combinations (Koo et al., 2005; Waters et al., 2011; Souza et al., 2012) in indirect or competitive designs. In general, these assays are simple, rapid and inexpensive, but they are generally designed to measure a single or few biomarkers in a sample.

Antibody responses to *M. bovis* are not uniform, and there is no evidence of a dominant persistent response to a single antigen across infection stages. A multiplex assay may overcome this issue and detect animals at different stages of infection (Whelan et al., 2008). To increase sensitivity, several multiplex assays were developed based on different approaches, including the multi-antigen print immunoassay, which consists of a cocktail of antigens applied to nitrocellulose membranes in narrow bands that are incubated with serum samples and subjected to immunodetection (Lyashchenko et al., 2000), a multiplex chemiluminescent immunoassay, which simultaneously detects antibody directed to > 20 antigens in a single well of a 96-well plate (commercialized as Enferplex TB) (Whelan et al., 2008; Whelan et al., 2010), and the lateral-flow rapid test, which is based on immunochromatographic techniques (Bermudez et al., 2012).

An alternative technology designed for multiplex testing is the Luminex platform (Elshal and McCoy, 2006) that is based on the use of polystyrene beads filled with two fluorescent dyes at different ratios, resulting in 100 sets of microspheres, each with its own unique spectral address. The surface chemistry of the beads facilitates the covalent conjugation of various capture molecules, such as protein antigens, and, hence, the setup of the assays is similar to that of the ELISAs.

The application of Luminex technology to the serological detection of antibodies to multiple antigen targets in a single assay is well documented for a variety of pathogens (Dias et al., 2005; Clavijo et al., 2006; Khan et al., 2006; Anderson et al., 2011) These examples predominately use recombinant generated proteins as capture antigens.

As a National Reference Center for bTB, we were interested in establishing serological assays that would be used in parallel with IDT and IFN-γ tests to maximize the overall detection rate of infected animals. The aim of this work was to develop and evaluate the applicability and performance of an ELISA method and Luminex technology to detect antibodies targeted to *M. bovis/M. caprae* using a panel of antigens, including MPB70, MPB83, ESAT6 and CFP10 proteins.

Furthermore, the diagnostic values of the two serological tests were evaluated under specific epidemiological circumstances in a region of South Italy (Calabria) where TB is still endemic, and, in most cases, the detection of TB outbreaks occurred at slaughterhouses through the post-mortem inspection of animals negative for IDT tests. The aim was to verify the ability of serology-based methods, combined with cell-mediated official diagnostic tests, to recognize anergic animals and to maximize the detection rate of infected cattle, which will allow for the more rapid control of outbreaks and eradication of the disease.

2. Material and methods

2.1. M. tuberculosis antigens

The genes encoding the MPB70, MPB83, ESAT6 and CFP10 proteins of *M. bovis* were independently cloned into the vector pQE30 (Qiagen, Hilden, Germany) and expressed in *Escherichia coli* as NH_2-terminally polyhistidine-tagged fusion proteins. Protein purification was performed in denaturing condition (8 M urea) using a Ni-NTA affinity column (Qiagen), according to manufacturer's instructions. ESAT6 and CFP10 were further submitted to two purification steps, anion exchange chromatography and gel filtration (Colangeli et al., 1998). Recombinant proteins were quantified using a Bicinchoninic acid (BCA) Protein assay kit (BIO-RAD, Hercules, CA, USA) and by the Coomassie Blue staining of polyacrylamide gels (Laemmli, 1970).

The bPPD was produced by the Istituto Zooprofilattico of Umbria and Marche (IZSUM, Perugia) according to standard procedures [Commission Regulation (EC) No 1226/2002, Annex B].

His-tag recombinant Rv3615c and Rv3020c were supplied by Lionex Diagnostics and Therapeutics (Braunschweig, Germany), and expressed and purified in *E. coli*.

2.2. Serum samples

A total of 1440 sera were collected to study the tests' performance levels. They were classified according to bTB and, when available, Johne's disease (JD) status (Table 1s) into the following four different groups:

- Group 1: 162 sera from bTB positive herds/unknown JD status. All sera were collected 15–20 d after a single IDT test from bTB-positive herds located in North (Trentino Alto Adige, Veneto and Lombardia) and South (Calabria) Italy from *M. bovis/M. caprae* culture-positive