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Evaluation of the single cervical skin test and interferon gamma responses to detect *Mycobacterium bovis* infected cattle in a herd co-infected with *Mycobacterium avium* subsp. *paratuberculosis*

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ABSTRACT

This study reports the performance of the single intradermal tuberculin (SIT) test and the interferon-gamma (IFN-γ) assay for *Mycobacterium bovis* in a cattle herd with high prevalence of paratuberculosis (PTB). A total of 58/350 animals were selected for necropsy based on one or more of the following criteria: positive to SIT, IFN-γ, a breeding cow that seroconverted to PTB and showed signs compatible with a wasting disease. Infection status was determined by *post mortem* diagnostic tests that included histopathology examination, mycobacterial cultures and PCR identification for *M. bovis* and *Mycobacterium avium* subsp. *paratuberculosis* (MAP). In 7/58 animals primary tuberculosis (TB) lesions, affecting only the retropharyngeal and/or mediastinal lymph nodes, were found; 3/7 animals were found SIT positive. PTB was confirmed in 35/58 animals, of which 30 had seroconverted and 14 had typical clinical signs. 45/58 animals were IFN-γ^+ using the most stringent criterion (cut-off point ≥ 0.05); however, IFN-γ test was only positive in 33 animals when using a higher threshold (cut-off point ≥ 0.1). Three animals co-infected also showed extensive TB and diffuse PTB lesions. These results show that the combined use of SIT and IFN-γ, as interpreted using official guidelines, detected all confirmed cases of TB. Individually, the sensitivity of the SIT was inadequate to diagnose TB-positive animals with an advanced stage of PTB. The large number of IFN-γ^+ animals with no visible TB lesion could be due, in part, to some protection conferred by prior infection with MAP.

1. Introduction

The quality of screening and diagnostic tests for detecting animals infected with *Mycobacterium bovis* has always been a major challenge in the diagnosis and control of bovine tuberculosis (bTB). Unfortunately, none of the tests currently available for bTB diagnosis allows complete

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discrimination of animals with and without disease. The single intradermal tuberculin (SIT) tests applied to the base of the tail or the cervical region, are still the most widely used standard assays worldwide for detecting infected cattle, and although they have high specificity with a reported median of 96.8% from seven field trials, their sensitivity has typically been much lower with a median value of 83.9% (review by De la Rua-Domenech et al., 2006). In Europe, the preferred site of injection has been the skin of the mid-cervical region, and when both the bovine and avian tuberculin are used side-by-side, the test is referred to as the comparative cervical tuberculin (CCT). This test gains specificity over the SIT by overcoming some of the problems associated with non-specific reactions by the presence of other non-tuberculous mycobacteria, and gains sensitivity when compared with the caudal SIT because the cervical region appears to be 2–3 times more sensitive than the tail area (Monaghan et al., 2004; De la Rua-Domenech et al., 2006).

On practical considerations, if the goal is to eradicate the disease, as it has been for decades in European countries (Schiller et al., 2011), the ability of a diagnostic test to detect all infected animals (that is, not missing truly infected individuals by giving false negative results) would be more important than erroneously yielding some false positives (De la Rua-Domenech et al., 2006). In an attempt to gain net sensitivity, ancillary assays such as the *in vitro* antigen-specific IFN-γ assay (Wood and Jones, 2001), and more recent and promising multiplex enzyme-linked immunosorbent assay (Whelan et al., 2011), could be used simultaneously with the *in vivo* CCT to promptly detect bTB and speed the removal of infected animals from the herd. Using two of the above assays simultaneously, an animal would be considered to be positive for TB if it yields a positive result on any one or both of the tests, but in order to be considered negative it would have to test negative on both assays. In Spain, the IFN-γ assay and the SIT have been the two *ante mortem* diagnostic tests of the National Eradication Campaign, in accordance with the European policy (Council Directive 64/432/EEC).

A major limitation to the interpretation of the *in vivo* and *in vitro* TB assays has been its cross-reactivity with responses induced by exposure to non-tuberculous mycobacteria, including *Mycobacterium avium* subsp. *paratuberculosis* (MAP), which is endemic in many cattle herds in areas of countries like Spain (Aranaz et al., 2006). The standard IFN-γ assay has used purified protein derivatives (PPD) produced from *M. bovis* (PPD-B) and from *M. avium* (PPD-A) as the stimulating antigens. PPD-B contains immunogenic proteins that are also present in non-tuberculous mycobacteria (Waters et al., 2004) which would compromise the specificity of the assay. Particularly, this specificity has been discussed when the most strict criterion (OD cut-off point PPD-B $\geq$ negative control + 0.05) has been used to classify animals as positive (Aranaz et al., 2006).

Few studies have been carried out in field conditions to characterize the immune response and lesion profile in herds dually infected with *M. bovis* and MAP. Experimentally, it has been shown that prior exposure to *M. avium* can induce a level of protection against *M. bovis* (Hope et al., 2005). So more studies where pathological changes are correlated with the results of diagnostics tests in co-infected animals in field conditions are needed.

The purpose of this study was to determine the performance of the two currently approved European Union tests for TB diagnosis, the SIT and IFN-γ, in a cattle herd with high prevalence of paratuberculosis (PTB).

2. Materials and methods

2.1. Cattle

This study was performed in a fighting cattle herd, an autochthonous breed of cattle bred for their behavior rather than for milk or meat production. The herd is located in southeastern Spain and has been under official eradication campaigns for TB for over 15 years using only the SIT test with negative results every year. During the 4 year period of the study (2006–2010), the population consisted of 350 heads with an annual replacement of 10%. The official program requires that at least one SIT test is performed every year, and that all positive reactors must be culled (European Council Directive 64/432/CEE and National Royal Decree 2611/96). However, more frequent repeat testing is recommended in endemic herds, allowing for at least 2 month intervals to avoid potential interference from the last tuberculin injection. The herd had also been suspected endemic for PTB with occasional animals showing diarrhea and/or caquexia unresponsive to symptomatic treatment; the PTB status was unknown because it was not subjected to an official eradication program and no diagnostic tests prior to the study period were performed.

2.2. Inclusion criteria

Throughout the study period a herd of 350 bovines of between 2 and 12 year of age, with most animals being below 4 years of age, were subjected to the official diagnostic tests for *M. bovis* (SIT and IFN-γ assays) and simultaneously tested for antibody ELISA against MAP. Testing was repeated four times in the study period with more than 90 days between tests to allow for desensitization of the last intradermal tuberculin injection as recommended by the EU (European Council Directive 64/432/CEE and National Royal Decree 2611/1996). Blood samples were taken immediately before the PPD-B intradermal injections to avoid any potential interference of the tuberculin test with the IFN-γ assay. A total of 58 animals were selected for sacrifice and necropsy based on one or more of the three following criteria: animals were positive to the SIT test, animals were positive to the IFN-γ assay and or animals were positive to ELISA PTB.

2.3. SIT test

SIT test was performed by public veterinary inspectors following the European Commission guidelines (EU council directive 64/432/CEE and National Royal Decree 2611/1996) using PPD-B (CZ Veterinaria). The skin thickness were evaluated before and after animals were injected intradermally with 0.1 ml (0.1 mg, 2500 CTU) of PPD-B on the left