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Evaluation of antigen specific interleukin-1β as a biomarker to detect cattle infected with *Mycobacterium bovis*

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ABSTRACT

Bovine tuberculosis (bTB) is a major world-wide health problem that has been difficult to control, due to the lack of an effective vaccine and limited ability of the tuberculin skin test (TST) and the ancillary whole blood interferon-gamma (IFN-γ) release assay (IGRA) to detect all infected animals. A 6 h cytokine flow cytometric IFN-γ (CFC) assay was developed in effort to overcome these limitations and expand methods for studying the mechanisms of bTB immunopathogenesis. The present study was conducted to evaluate IL-1β as a biomarker to use in conjunction with the IFN-γ CFC assay to improve the diagnostic accuracy for bTB. Three animal groups with predefined *Mbv* infection status were used for analysis of IL-1β in plasma from whole blood cultures stimulated with ESAT-6/CFP-10 for 20–24 h. Parallel stimulations were performed for enumeration of IFN-γ producing T cells. Data analysis showed that *Mbv* infected animals have a higher frequency of IFN-γ producing $CD4^+$ T cells and plasma IL-1β than animals exposed to non-tuberculous mycobacteria (NTM) or uninfected control animals, with a significant correlation between the two readouts, thus allowing differentiation between the three animal groups. IL-1β has the potential to serve as an additional biomarker for detecting cattle infected with *Mbv*.

1. Introduction

Bovine tuberculosis (bTB) is caused by *Mycobacterium bovis* (*Mbv*); a zoonotic pathogen with a wide host range including humans. *Mbv* is a member of the *Mycobacterium tuberculosis* complex (MTC), a closely related group of mycobacteria that also includes *Mycobacterium tuberculosis* (*Mtb*), the main agent causing human TB [5]. Bovine TB is a major trade barrier for livestock-related products, impairs productivity, and persists as a significant socioeconomic hardship for livestock farmers with estimates of 50 million cattle infected worldwide, costing $3 billion annually [40]. Zoonosis is also a major concern with approximately 3% of all human tuberculosis cases being caused by *Mbv*, with most cases recorded in developing countries [26].

The control and eradication of bTB relies largely on test and slaughter policy and/or abattoir surveillance. In cattle, the principle antemortem tests for bTB diagnosis includes the tuberculin skin test (TST) and interferon-gamma (IFN-γ) release assay (IGRA). Both tests are designed to detect cell mediated immune responses. TST has been in use as the primary screening test prescribed by the Office International des Epizooties (OIE) for the bTB surveillance programs. Despite its widespread use, the TST has limitations regarding sensitivity and specificity [reviewed in Refs. [10,32]]. Because of its better sensitivity as a blood based test [6], the IGRA has been approved by the OIE as an ancillary test to supplement the TST. It has been incorporated into the bTB control programs in many countries such as the US, UK and Australia [10,32,42]. However, it is now apparent that IGRA also has limitations regarding its specificity as a result of using bovine purified

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protein derivatives (PPD-B) and avian PPD (PPD-A) as they share many antigenic components [3,4], some of which are also present in environmental mycobacteria. In addition, the test sensitivity of the IGRA may be reduced by a greater response to PPD-A than to PPD-B, which may occur at an early stage of infection [33] or co-infection with environmental mycobacterial species [2]. Furthermore, responses to the PPDs used in the IGRA fail to differentiate animals naturally infected with *Mbv* from those vaccinated with attenuated *Mbv* bacillus Calmette-Guérin (BCG), since the pathogenic and vaccine strains share common antigens [46]. To overcome these limitations, many studies have focused on identifying new candidate antigens specific to pathogenic mycobacteria to replace PPDs in the IGRA [24]. Early secretory antigenic target protein 6 kDa (ESAT-6) and culture filtrate protein 10 kDa (CFP-10) are antigens encoded by the region of difference 1 (RD1), which is present in the MTC strains and absent in all *Mbv* BCG strains and most nontuberculous mycobacteria strains (NTM) [21]. ESAT-6 and CFP-10 are now well-known front-line diagnostic antigens for bTB detection with the greatest potential for improving the specificity of the IGRA [1,15,24,28]. Using ESAT-6 and CFP-10 instead of bovine and avian PPDs in the IGRA has improved the ability to identify infected animals. However, infected animals may still be missed [1].

Unlike the IGRA, the intracellular cytokine flow cytometric (CFC) assay is designed to detect secretion of IFN-γ at the single cell level [37]. Recently, as a trial to improve detection of infected animals at all stages of infection, we developed and optimized a rapid IFN-γ CFC assay that enables enumeration of IFN-γ producing memory T cells after stimulation of whole blood with PPD-B, PPD-A and ESAT-6/CFP-10 antigens [12]. The assay also allows phenotyping of T cell subsets secreting IFN-γ and assessing their activation and memory status [12].

The IGRA and CFC are single cytokine (IFN-γ) based readout assays. When used as ancillary assays, they increase the sensitivity of the TST. However, the challenge still remains to improve the assays so that 100% of infected animals are detected. Cumulative studies have shown multiple cytokines are secreted along with IFN-γ to orchestrate the immune response to mycobacterial infections. These cytokines could be potential candidates for use in blood-based bTB tests. These cytokines include IL-1β, IL-6, IL-12, IL-10, TNF-α and IL17A [8,27,36,44,45]. IL-1β is considered one of the key proinflammatory cytokines that initiate and mediate the host immune response to mycobacterial infections [14,22,23,30,31]. Although it has been under intense investigation, the potential as a biomarker for detecting cattle infected with *Mbv* has only been explored in one study [16].

As reported here, we extended our studies with naturally infected cattle to determine whether IL-1β can be detected in short term blood cultures and used as an additional biomarker for bTB along with the IFN-γ CFC assay.

2. Materials and methods

2.1. Animals

Three groups of cattle were used in the present study; *Mbv* naturally infected, non-tuberculous mycobacteria (NTM) exposed and uninfected controls. Their bTB infection status was determined primarily by antemortem (TST and IGRA) and retrospectively verified by detection of postmortem gross lesions (GL), bacterial culturing and PCR. The *Mbv* naturally infected cattle group were single cervical tuberculin test (SCT) reactors, IGRA positive (with and without GL), developed colonies of *Mbv* from tissues of all animals and all were IS*6110*-PCR positive. Cattle sensitized with NTM were SCT-reactors, IGRA negative, GL negative and developed colonies of NTM from tissues of all animals and all were IS*1311*-PCR positive. Uninfected cattle were obtained from bTB-free herds, and all were negative for both the TST and IGRA.

All cattle were maintained according to the general guidelines of the Egyptian Organization of Veterinary Services (GOVS) after being tested by TST. All protocols and procedures were approved and guided by Alexandria University under regulations of Egyptian law. All whole blood cultures were set more than 30 days after last skin test and all blood collections were carried out using vacutainer tubes containing sodium heparin (BD Vacutainer), and blood was transported to the lab at ambient temperature for processing within 4 h.

2.2. Antemortem diagnostic tests

The SCT was performed as previously described [1], implemented and recorded by GOVS officers as a regular application of the bTB surveillance program. Briefly, 0.1 ml of tuberculin purified protein (bacterial diagnostic products department, Veterinary serum and vaccine Research Institute, Egypt) was inoculated at the mid-neck of each animal, and after 72 h, the skin-fold thickness was measured. According to the regulations of GOVS, an animal was considered reactive if a swelling of >4 mm or more at the site of PPD-B injection site, <3 mm was considered as negative, and between 3 and 4 mm was interpreted as suspicious. All reactors were scheduled for slaughter at specific designed slaughterhouses. Suspicious-animals were scheduled for a second SCT 60 days later. Animals with an inconclusive skin test were not enrolled in the study.

The IGRA was performed according to the manufacturer's instructions (Prionics AG, Switzerland) using ESAT-6/CFP-10; 5 μg each/ml (Lionex GmbH, Germany). The mean OD_{450} of duplicate plasma samples of Nil and ESAT-6/CFP-10 stimulated blood was determined. Animals were considered bTB positive when samples were reactive and the response to ESAT-6/CFP-10 compared to Nil were ≥0.1 OD as previously described [1].

2.3. Postmortem diagnostic tests

All SCT-reactors were slaughtered, then inspected for typical GL. Specimens were collected from lymph nodes (including retropharyngeal, mesenteric, hepatic, mediastinal, prescapular and prefemoral lymph nodes) and internal organs (including liver, lung and spleen) with and without detectable GL. Tissues were homogenized and decontaminated as previously described [1]. Tissue pellets from each animal were used for bacterial culturing on two Lowenstein–Jensen (Becton Dickinson) slants (one with 0.6% sodium pyruvate and the other with 0.75% glycerol). The presumptive positive colonies were characterized further using a combination of standard biochemical methods including tellurite reduction and niacin accumulation tests, heat-stable (68 °C) catalase and growth on TCH (thiophene-2-carboxylic acid hydrazide) as previously described [1]. Also, tissue samples were subjected to DNA extraction using DNeasy blood and tissue kit (Qiagen) and conventional PCR to determine their positivity for two genes, specifically designed to distinguish *Mycobacterium tuberculosis complex* (MTC) from the *Mycobacterium avium complex* (MAC): IS*6110* (forward: 5′ AGTTTGGTCATCAGCCGTTC-3′ and reverse: 5′-CGAACTCAAGGAGCACATCA-3′), and IS*1311*(forward: 5′ GCGTGAGGCTCTGTGGTGAA 3′ and reverse: 5′ ATGACGACCGCTTGGGAGAC 3′) [12,35,41]. In house defined *Mtb and Mycobacterium avium subspecies paratuberculosis* (*Map*) were included as control strains during the bacteriological and molecular diagnostic tests. Unfortunately, we were unable to include a control strain of *Mbv*.