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Expression and purification of diagnostically sensitive mycobacterial (*Mycobacterium bovis*) antigens and profiling of their humoral immune response in a rabbit model

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ABSTRACT

The incidence of bovine tuberculosis (bTB) is increasingly giving rise to large economic losses in the agricultural industry. The current methods used for detection and control of bTB (skin test and interferon-gamma) lack desired sensitivity and specificity. Therefore, the development of a rapid and reliable bTB serological based assay is urgently required. An antibody assay using combinations of strain-specific mycobacterial antigens could resolve both specificity and sensitivity issues. We analyzed the ability of a series of selected mycobacterial antigens to outline a humoral immune response in a rabbit model experimentally challenged with different mycobacterium. Antibodies specific for three antigens, MTB40, ESAT6 and CFP10, were present in serum 2 weeks post-challenge (early indicator), while two other antigens, Rv3870 and Rv1580c, could be detected from 8 to 11 weeks post-challenge. These selected mycobacterial antigens did not exhibit any cross-reactivity with avian PPD and only a very low positivity with bovine PPD. This data suggests that this panel of strain-specific mycobacterial antigens could be used for identification of *Mycobacterium bovis* infection in serum samples. The combinatorial application of these antigens could form part of a serum field test which may assist the future diagnosis of TB.

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1. Introduction

Mycobacterium bovis (*M. bovis*) is the main etiological agent of bovine tuberculosis (bTB), a serious infectious disease that gives rise to large economic losses. The presence of a zoonotic reservoir of bTB and widespread occurrence in cattle from the developing world signify a potential risk to public health (Ameni et al., 2006). The bacteria attack the respiratory system by entering the lungs, multiplying and spreading to the nearby lymph nodes. It is one of the most difficult animal health problems that the farming industry faces worldwide. In the UK, some 5.88 million bTB tests are performed annually costing the taxpayer approximately £114m (Defra, 2005, 2007). Bovine TB varies regionally within the UK, with worst incident rates observed in areas such as South Wales, Cornwall and Gloucestershire (Defra, 2005, 2007). Moreover, of immediate concern, is an estimate that the incidence of bTB is increasing at a rate of 2.5% per year in previously uninfected herds (Defra, 2005, 2007; Gilbert et al., 2005).

The disease control programme for bTB operated by a number of countries (such as UK and Ireland) are based on a test and removal strategy utilising the intradermal skin test, which relies on a purified protein derivative (PPD) prepared from *M. bovis* strain AN5, to elicit an immune response in infected cattle (Caffrey, 1994; Monaghan et al., 1994; Cousins and Roberts, 2001). Disease control based on the intradermal skin test can be complimented by the gamma-interferon test, which measures the specific T cell response when exposed to PPD material (Rothel et al., 1990; Wood et al., 1990; Defra, 2005, 2007; Vordermeier et al., 2004; Miles and Booty, 2005). The gamma-interferon test can be utilised as a parallel diagnostic assay for herd screening (based on regional policy) but has limited high-throughput potential. Moreover, due to their modes of action, the sensitivity and specificity of both the intradermal skin test and the gamma-interferon test will continue to raise issues. Various studies have shown that the intradermal skin test identifies around 5–30% false negatives i.e., wrongly identified as disease-free, and 1–5% false positives i.e., uninfected animals wrongly identified as diseased (Monaghan et al., 1994; Vordermeier et al., 2004; Whelan et al., 2004; de la Rua-Domenech et al., 2006). There is also likelihood that more false positive reactors are identified when the gamma-interferon test is applied, as opposed to the skin test, due to the higher sensitivity of this test

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when compared with skin test (Monaghan et al., 1994; Vordermeier et al., 2004; Miles and Booty, 2005; Whelan et al., 2004; WHO, 1994). Several antigens such as ESAT6 and CFP10 have been incorporated into various versions of gamma-interferon test in an effort to improve its diagnostic capability (Alito et al., 2003; Mustafa, 2005). Nonetheless, the gamma-interferon test is significantly more expensive than the skin test therefore it is considered as unsuitable as a screening test (Defra, 2003, 2005; More and Good, 2005).

Developing an antibody assay using strain-specific antigens, covering all stages of *M. bovis* infection, has the potential to resolve both specificity and sensitivity issues. To date however, limited progress has been made in developing an antibody assay that identifies infected animals at all stages of infection (Cousins and Florisson, 2005; Aranaz et al., 2006; de la Rua-Domenech et al., 2006). This is attributable in part to the finding that the antibody response to *M. bovis* is complex with no evidence of a uniform response to a single antigen at any stage of infection (Lyashchenko et al., 1998). Furthermore, cross reactions induced by different mycobacteria strains and environmental mycobacteria such as *Mycobacterium microti* and *Mycobacterium africanum*, and the conflicting requirements between specificity and sensitivity of the test antigens, all accrue to the difficulties in establishing a serological protocol for bTB (Defra, 2006). Therefore cocktails of different strain-specific antigens, rather than reliance upon a single antigen, are required for the development of an antibody assay capable of accurate diagnosis for large numbers of cattle from diverse genetic backgrounds (Wood et al., 1992; Frothingham and Meeker-O'Connell, 1998).

The application of a TB rabbit model in bTB infection studies has been utilised previously. Infected animals develop pulmonary cavities consistent with the disease pathology in cattle demonstrating its usefulness as a research disease model (Gupta and Katoch, 2005; Manabe et al., 2008). In this study, we outline the humoral immune response profiles of mycobacterium challenged rabbits to a panel of recombinant mycobacterial antigens. These antigens have the potential of distinguishing wild-type TB infected animals from animals exposed to environmental mycobacteria or PPD (through the intradermal skin test) by identifying antibodies raised in response to *M. bovis*, PPD tuberculin and other environmental mycobacteria. We examined the strain specificity of these antigens by testing sera from rabbits experimentally challenged with mycobacterial antigens, avian PPD, bovine PPD and heat-inactivated *M. bovis* wild-type; profiling the humoral immune responses and thus evaluating their diagnostic potential.

2. Materials and methods

2.1. Cloning of mycobacterial antigens

DNA encoding target mycobacterial antigens (Table 1) were identified and analyzed using bioinformatics software (Vector NTI® Suite, Invitrogen, CA). Primers were designed to amplify selected antigenic fragments/full-length proteins of each target antigen by PCR. Each purified PCR product was digested with appropriate restriction enzymes and cloned into the pQE-30 bacterial expression vector (Qiagen), incorporating an N-terminal hexahistidine tag [$(His)_6$-tag] to enable downstream purification. Positive clones of each target antigen were identified by colony PCR and characterised by sequencing using Big Dye Terminator chemistry and automated DNA sequencer (ABI Prism 3100).

2.2. Protein expression and purification of mycobacterial antigens

The recombinant mycobacterial antigens were purified via the incorporated N-terminal hexahistidine tag using immobilized metal ion chromatography (IMAC). A verified clone of each target antigen was transformed into competent *Escherichia coli* cells and cultured until reaching mid-log phase (A_{550} = 0.5; 37 °C). Expression of mycobacterial antigen was induced by the addition of IPTG (1 mmol/L) and propagated for a further 4 h before harvesting. Cell pellets were resuspended and lysed in 50 mmol/L NaH_2PO_4 (pH 8.0) containing 8 mol/L urea, 300 mmol/L NaCl, and 10 mmol/L imidazole. The crude denatured lysate was clarified by centrifugation (10,000*g*, 60 min at 4 °C) before application to an immobilized metal affinity chromatography (IMAC) column charged with Ni^{2+} ions (HiTrip 1 mL column; GE Healthcare). Non-specifically bound material was washed from the column using 50 mmol/L NaH_2PO_4 (pH 8.0) containing 8 mol/L urea, 300 mmol/L NaCl, and 20 mmol/L imidazole followed by on-column refolding by reduction of the urea from 8 to 0 mol/L over 200 column volumes. Refolded column-bound material was washed with a further 20 column volumes of 50 mmol/L NaH_2PO_4 (pH 8.0), 300 mmol/L NaCl, and 20 mmol/L imidazole then eluted with 50 mmol/L NaH_2PO_4 (pH 8.0), 300 mmol/L NaCl, and 250 mmol/L imidazole. Protein fractions were collected, desalted into PBS, and analyzed by SDS–PAGE and Western blotting to determine purity and integrity. Concentrations of each mycobacterial antigen were determined using BCA method (Pierce) and stocks of each purified mycobacterial antigen were stored at −20 °C before use.

2.3. Animals

Twelve female New Zealand white rabbits were obtained from SPF facilities at Charles River and used at 8 weeks of age. All animals were housed in appropriate biological containment and work was carried out in accordance with the Animal (Scientific Procedures) Act 1986, following local ethical review.

2.4. Mycobacteria

Heat-inactivated *M. bovis* wild-type isolate AF2122/97, PPD *M. bovis* AN5 (0.5 mg/ml) and PPD *Mycobacterium avium* (0.5 mg/ml) were obtained from Veterinary Laboratories Agency (Weybridge, UK), subsequently frozen at −20 °C, and used for all challenges.

Table 1
Antigenic and strain-specific recombinant mycobacterial antigens used in this study.

Antigen	NCBI accession no.	Strain differentiation			
		M. bovis wild-type AF2122/97	*M. bovis* AN5 (PPD)	*M. avium* (PPD)	*M. microti*
MTB40	CAD96080	+	+	–	–
Rv3870	CAD96086	+	+	–	–
*ESAT6	CAD96091	+	+	–	–
CFP10	CAD96090	+	+	–	–
Rv1580c	CAD96274	+	–	+	–

* ESAT6 is a recombinant tandem repeat.

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