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► **To cite this version:**

Martin Vordermeier, Stephen V. Gordon, R. Glyn Hewinson. MYcobacterium bovis antigens for the differential diagnosis of vaccinated and infected CATTLE. *Veterinary Microbiology*, 2011, 151 (1-2), pp.8. 10.1016/j.vetmic.2011.02.020 . hal-00701903

**HAL Id: hal-00701903**

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Submitted on 28 May 2012

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## Accepted Manuscript

Title: *Mycobacterium bovis* antigens for the differential diagnosis of vaccinated and infected Cattle

Authors: Martin Vordermeier, Stephen V. Gordon, R. Glyn Hewinson

PII: S0378-1135(11)00101-5  
DOI: doi:10.1016/j.vetmic.2011.02.020  
Reference: VETMIC 5187

To appear in: *VETMIC*



Please cite this article as: Vordermeier, M., Gordon, S.V., Hewinson, R.G., *Mycobacterium bovis* antigens for the differential diagnosis of vaccinated and infected Cattle, *Veterinary Microbiology* (2010), doi:10.1016/j.vetmic.2011.02.020

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**MYCOBACTERIUM BOVIS ANTIGENS FOR THE DIFFERENTIAL DIAGNOSIS OF  
VACCINATED AND INFECTED CATTLE**

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Running title: DIVA reagents for bovine tuberculosis vaccines

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**ABSTRACT**

The urgency for new and improved cattle vaccines and diagnostic reagents for Bovine tuberculosis (TB) has made their development a research priority in Great Britain (GB). Significant progress has been made to develop specific antigens that allow the differentiation of BCG vaccinated and *M. bovis* infected cattle (DIVA test). This has been greatly facilitated by the completion of the genome sequences of *M. tuberculosis*, *M. bovis* and BCG Pasteur and the subsequent application of comparative genome and transcriptome analysis to define DIVA antigens that complemented the prototype DIVA antigens ESAT-6 and CFP-10 by increasing their test sensitivity. Finally, we present an update of our current approaches in this area.

*Keywords:* DIVA, BCG, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, differential diagnosis, antigen reagents

## 1. Development of cattle TB vaccines

Over the last two decades the (tuberculin) test and slaughter strategy failed to prevent a dramatic rise in the incidence of TB in cattle in England and Wales (<http://www.defra.gov.uk/animalh/tb/stats/county.htm>). The urgency for new and improved cattle vaccines and diagnostic reagents has been acknowledged by the United Kingdom government. (<http://www.defra.gov.uk/animalh/tb/vaccination/index.htm>). However, the short-term development of improved cattle TB vaccines faces two major challenges: The identification of vaccine strategies that enhance the efficacy of *M. bovis* bacille Calmette Guérin (BCG), and the development of a diagnostic test that can be used alongside vaccination to differentiate vaccinated and infected cattle (**DIVA** test).

The significant progress in developing TB vaccines for cattle has been reviewed in a number of recent articles (Buddle et al., 2005; Buddle et al., 2006; Hogarth et al., 2006; Hope and Vordermeier, 2005; Vordermeier et al., 2006a; Vordermeier and Hewinson, 2006). Briefly, results in cattle have shown that the most effective vaccination strategy against bovine TB is based on priming the immune system with BCG followed by boosting with subunit vaccines containing protective antigens that are present in BCG (*heterologous prime-boost strategy*), DNA (Skinner et al., 2003; Skinner et al., 2005), protein (Wedlock et al., 2005), or viral subunit vaccines (Vordermeier et al., 2006b; Vordermeier et al., 2004; Vordermeier et al., 2009). Nevertheless, all these strategies resulted in at least a proportion of animals becoming tuberculin-test positive (both in tuberculin skin and IFN- $\gamma$  tests). Thus, the development of

complementary diagnostic reagents allowing the discrimination of infected from vaccinated animals remains an essential requirement so that a test and slaughter approach to TB control can be continued in the face of vaccination.

## **2. Differential diagnosis of infected from vaccinated individuals (DIVA).**

Bovine tuberculosis in cattle is viewed as a spectral disease with cell-mediated immune responses developing early post-infection and serum responses becoming patent at later and advanced stages of disease (Neill et al., 2001; Pollock et al., 2001; Ritacco et al., 1991). Therefore, diagnostic assays like tuberculin skin testing or *in vitro* cytokine detection assays as the Bovigam IFN- $\gamma$  assay (see following paragraph), are employing methods that probe cell-mediated immune responses. Serum tests have in the past, particularly when conducted prior to skin testing (Lyashchenko et al., 2004), generally suffered from poor sensitivity. However, it should be noted that recent advances in this technology using both a wider antigen range and more sensitive detection methods have resulted in several promising new serum assays with increased sensitivity, whose performance needs to be validated in future (e.g. (Green et al., 2009; Whelan et al., 2010; Whelan et al., 2008).

The Bovigam assay is a diagnostic test for bovine tuberculosis based on the measurement of interferon-gamma (IFN- $\gamma$ ) *in vitro* after stimulation of blood with avian and bovine tuberculin PPD (Rothel et al., 1990, 1992). This test is now permitted under European Union (EU) law as an ancillary parallel test to the tuberculin skin test in cattle to improve diagnostic sensitivity and is used

routinely in GB in defined situations. Yet, BCG vaccination compromises tuberculin-based diagnosis strategies in BCG vaccinated animals (Buddle et al., 1999; Vordermeier et al., 1999). The Bovigam test, however, can readily be modified to accommodate specific antigens more suitable as DIVA antigens, and is therefore the method of choice at present for the DIVA approach rather than modified tuberculin-skin testing, which in the past required large and unpractical amounts of recombinant proteins (Pollock et al., 2003), or the inclusion of immunopotentiators to induce measurable skin test responses (Whelan et al., 2003).

Over the past decade significant advances have been made in identifying suitable, more specific, DIVA diagnostic candidates. Target antigens were antigens whose genes are expressed in *M. bovis* yet absent from environmental mycobacteria or BCG (Behr et al., 1999; Gordon et al., 1999; Mahairas et al., 1996). Two of the major antigenic targets identified in both cattle and humans are ESAT-6 and CFP-10 (Pollock and Andersen, 1997a, b). Several other antigens with molecular masses of around 10 kDa and belonging to the ESAT-6 family of proteins have been identified subsequently (for example, Rv0288, and Rv3019c) and have been shown to be equally-well recognized by bovine T cells (Cockle et al., 2006).

CFP-10 and ESAT-6 are encoded by genes located on the RD1 region of the *M. bovis* genome, a locus that is deleted from the genome of all BCG strains (Behr et al., 1999; Garnier et al., 2003; Gordon et al., 1999; Mahairas et al., 1996). When used as a diagnostic antigen, ESAT-6 has been shown to be

able to discriminate between infected and BCG-vaccinated cattle (Buddle et al., 1999; Vordermeier et al., 1999; Vordermeier et al., 2001).

Our approach used synthetic peptides derived from the sequences of CFP-10 and ESAT-6 and showed that these are equally effective as diagnostic reagents. These peptides were formulated into cocktails that gave equivalent responses to the recombinant proteins in naturally infected cattle (Vordermeier et al., 2001). BCG vaccinated cattle did not respond to this peptide cocktail, whilst bovine PPD biased responses were detected in more than 70 % of BCG vaccinated animals (Vordermeier et al., 2001). This peptide cocktail has been recently validated in Defra-funded trials (i.e. specificity trial), and is being used occasionally as part of the GB-accredited routine BOVIGAM IFN- $\gamma$  test to increase test specificity in herds with suspected non-specific skin test reactors (see: <http://www.defra.gov.uk/animalh/tb/control/gamma.htm>).

However, the sensitivity imparted by ESAT-6 and CFP-10 was still below that of tuberculin (see table 1), and in the following sections we will describe approaches used in our laboratory to identify antigens that increased test sensitivity.



### 3. Comparative genome and transcriptome analysis to identify diagnostic antigens.

#### 3.1 Comparative genomics.

The genomes of *M. bovis* (Garnier et al., 2003), *M. tuberculosis* (Cole et al., 1998), *M. bovis* BCG Pasteur (Brosch et al., 2007), *M. avium* subsp. *paratuberculosis* (Li et al., 2005), *M. avium* subsp. *avium*, *M. marinum* (Stinear et al., 2008), *M. microti*, *M. ulcerans*, and *M. smegmatis* have been completed. This allows the search for further differential diagnostic candidates based on comparative genome and transcriptome analyses.

*In silico* analysis can be used to list potentially specific proteins, which can then be assessed experimentally for immunogenicity and specificity. We applied a high-throughput immunological screening system based on pools of chemically synthesized peptides representing the complete amino acid sequences of target proteins. In the first set of experiments we assessed the immunogenicity of the 28 potential antigens encoded in the RD1, RD2, and RD14 gene regions that are deleted from the genome of BCG Pasteur (Brosch et al., 2007; Garnier et al., 2003) in cattle models of *M. bovis* infection and BCG vaccination. Seven antigens (Rv1769, Rv1979c, Rv1986, Rv3873, Rv3873, Rv3878, Rv3879c) were recognized at high frequencies (41-86%) (Cockle et al., 2002). Only Rv1986, Rv3872, and Rv3878 were not recognized by T cells from BCG vaccinated cattle, and are therefore candidates for differential diagnosis (Cockle et al., 2002). Two of the other 4 proteins were recognized by BCG vaccinated animals but not by environmentally sensitized cattle (Rv3873 and Rv3879c).

Other immunogenic antigens identified that could be useful for either diagnosis or as vaccine antigens include proteins belonging to the important ESAT-6 protein family (Pallen, 2002), such as Rv3019c or Rv0288 (Aagaard et al., 2003; Cockle et al., 2006; Vordermeier et al., 2003). In a further refinement, we therefore determined the most frequently recognized peptides from these antigens (Rv3873, Rv3879c, Rv0288, and Rv3019c) and together with peptides from ESAT-6 and CFP-10, formulated them into a peptide cocktail, PhD-1 (Cockle et al., 2006). In a test of naturally infected cattle, this cocktail was significantly better than tuberculin for identifying skin test-negative animals with confirmed bovine tuberculosis (lesioned and/or *M. bovis* culture positive). The specificity of this cocktail was not compromised by *M. bovis* BCG vaccination. However, further studies suggested that this sensitivity benefit in relation to tuberculin and ESAT-6/CFP-10 could largely be realized only in freshly sampled blood, whilst it was less potent in blood that was stored overnight before the blood cultures were established (Vordermeier, unpublished data). This is was mainly due to the fact that overnight blood storage significantly improved the tuberculin specificity whilst it impacted less on sensitivity (Schiller et al., 2009). As now the routine application of the Bovigam assay in GB is based on blood stored overnight, further studies were needed to improve ESAT-6 and CFP-10 sensitivity in this testing scenario (see next paragraph).

### 3.2 Comparative transcriptome analysis.

In an extension of the *in silico* mining effort based on comparative genome analysis, we next integrated genome data and DNA microarray technology to assess the transcriptome of BCG and *M. bovis* after infection of bovine macrophages (*comparative transcriptomics*), the transcriptome being the complete gene expression profile of an organism under defined conditions. In addition, genes that are strongly expressed across different *in vitro* growth conditions (the invarisome, (Sidders et al., 2007)) were also assessed. Only blood that was stored overnight was used to assess these potential antigens, to mimic the assay conditions used at this time in GB in the routine applications of the Bovigam test.

In this analysis, the antigen Rv3615c was recognised by blood cells isolated from more than half of *M. bovis* infected animals tested (Figure 1A and table 1, (Sidders et al., 2008), but not by blood cells from uninfected or BCG vaccinated cattle (Figure 1A, (Sidders et al., 2008). Importantly, Rv3615c was recognized predominantly in the subset of naturally infected animals (i.e. lesioned and/or *M. bovis* culture positive cattle) that escaped detection using ESAT-6/CFP-10 (Figure 1A, (Sidders et al., 2008) and Vordermeier et al., unpublished data). Rv3615c is recognized early after *M. bovis* infection with a response kinetic not significantly different from those of ESAT-6/CFP-10 or tuberculin (Figure 1B). Further field studies have confirmed that Rv3615c complemented ESAT-6 and CFP10 resulting in an increase in test sensitivity without loss of specificity (Table 1).

Consequently, Rv3615c, together with ESAT-6 and CFP-10, is now being assessed in larger numbers of field animals to determine its sensitivity and specificity values more precisely.

#### **4. On-going research in our laboratory**

Our on-going research is aimed to provide a multi-track approach of incremental improvement of current diagnostic tests using targeted and library approaches and this combined with basic research aim to disclose/define categories of infected animals based on infection-stage specific antigens. For example, stage-specific 'latency antigens' of the DosR regulon (Schnappinger et al., 2003) genes were more strongly recognised in latently infected humans compared to patients with active tuberculosis (Demissie et al., 2006; Geluk et al., 2007; Leyten et al., 2006; Lin et al., 2007). Therefore such antigens as well as other hypoxia-induced antigens (Rustad et al., 2008) could define latency in humans. In addition, such antigens could also improve diagnostic sensitivity in cattle or humans. Our preliminary data have demonstrated that a number of such potential latency antigens is being recognised by infected cattle, and further studies will reveal their DIVA potential (Vordermeier, unpublished data). However, our data do not support the notion that such antigens define latency in cattle.

Further, we have recently determined the immunogenicity of the secretome antigens of *M. bovis* (i.e. all antigens known or predicted to be secreted). 382 pools of overlapping peptides spanning 119 *M. bovis* secreted and potentially secreted proteins were prepared and screened *in vitro* using whole blood from

tuberculin-positive reactor cattle using IFN- $\gamma$  production as read-out. All but one of the 15 ESAT-6 proteins tested were recognized by more than 30% of the TB reactor cattle, and 12 of the 25 most commonly recognized secretome antigens overall belonged to this protein family. There was no significant difference in immunogenicity between the ESAT-6 proteins that were components of potentially intact ESX secretory systems and those corresponding to additional partial *esx* loci. Importantly for vaccine design, antigenic epitopes in some highly conserved regions shared by numerous ESAT-6 proteins were identified which do some degree could explain the immunodominance of these highly cross-reactive antigens. However, despite this considerable homology, we also revealed immunodominant peptides located in regions of amino acid variability which are now being assessed for species-specificity and their diagnostic potential (Jones et al., 2010).

We are also evaluating whether the DIVA reagents described in this paper for use in the *in vitro* INF- $\gamma$  test can also serve as skin test reagents. The skin test activity of ESAT-6 to detecting infected cattle had been studied before by us and others (Pollock et al., 2003; Whelan et al., 2003). However, these studies suffered from limitations which would make the described protocols unpractical either due to extremely high protein doses that were required to induce skin responses (Pollock et al., 2003), or the use of an *in vivo* adjuvant (Whelan et al., 2003). In addition, neither study evaluated the DIVA potential of these methods. We therefore re-visited this recently by using cocktails of ESAT-6, CFP-10, MPB83 and Rv3615c proteins or corresponding peptides. Our results showed that both protein and peptide cocktails containing these

antigens were able induce strong skin test responses in infected cattle, but not in BCG vaccinated cattle. The optimal cocktail in this study contained ESAT-6, CFP-10, and Rv3615c, with peptides or proteins giving equivalent responses (Whelan and Vordermeier, submitted for publication).

Whilst IFN- $\gamma$  is a diagnostically useful parameter to measure, additional biomarkers, alone or in combination, could further enhance diagnostic performance of *in vitro* blood based tests. An example of such an alternative biomarker that complemented IFN- $\gamma$  in the diagnosis of human tuberculosis is IFN- $\gamma$  inducible protein 10 (IP-10) (Ruhwald et al., 2008). In contrast, a negative impact of Interleukin-10 on the production of IFN- $\gamma$  in response to tuberculin or ESAT-6 and CFP-10 has been described in cattle which could be overcome by neutralising Interleukin-10 during the whole blood culture (Denis et al., 2007). Therefore, biomarkers could have both negative and positive effects on test performance and it is important to establish a wider immune response quality profile to determine whether additional markers can be defined that could enhance test sensitivity, or alternatively, whether some biomarker responses have to be suppressed or neutralised to alleviate potential negative effects.

We have recently developed a bovine cytokine and chemokine multiplex assay that allows us to address this experimentally. For example, we have applied this novel methodology to assessing the longitudinal immunological and diagnostic consequences of repeated short-interval skin-tests in cattle naturally infected with *Mycobacterium bovis*. This study demonstrated that *in*

*vitro* IL-1 $\beta$  and IFN- $\gamma$  production, for example, are differently influenced by tuberculin skin testing (Figure 2). IFN- $\gamma$  responses are not affected, whilst IL-1 $\beta$  responses decreased along with the progressive skin test desensitisation observed during short interval repeat skin testing (Coad et al., 2010). The reduced IL-1 $\beta$  responses associated with reduced skin test reactivity (i.e. reduced delayed type hypersensitivity) are in agreement with murine studies that have shown that local and systemic inflammatory responses including delayed-type hypersensitivity were reduced in IL1- $\beta$  deficient mice (Dinarello, 2009).

**Acknowledgements.** These studies were funded by the Department for Environment, Food and Rural Affairs (Defra), UK. The authors would like to express our sincere appreciation to the staff of the TB Research Group and the Animal Services Unit at VLA. In addition, we would also like to thank the large number of collaborators that were involved in these studies that are too numerous to name individually.

This manuscript was written based on a plenary session presented by the author at the 5<sup>th</sup> International *M. bovis* Conference. Although comprehensively up-dated with new references, new data and figures, part of the data presented have also been published in *Transboundary and Emerging Diseases* (2009, 56:240-47).

#### **Conflict of Interest Statement**

The authors have not declared any conflict of interest.

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## Figure legends

### **Figure 1. Rv3615c is specific DIVA reagent and can complement ESAT-6/CFP-10 to increase sensitivity.**

**A.** Responses to a peptide cocktail representing Rv3615c in naturally infected animals (*M. bovis*), BCG vaccinated animals (BCG), naïve control cattle (naïve), and a subgroup of naturally infected cattle that did not recognise ESAT-6/CFP-10 (*M. bovis*/E6/C10-negative). The data are expressed as background subtracted optical density ( $\Delta OD_{450}$ ), and the dashed line represents the cut-off for positivity.

**B.** Kinetics of *in vitro* IFN- $\gamma$  responses determined in the blood from experimentally *M. bovis* infected cattle (n=10). Results represent mean  $\Delta OD_{450}$  responses after Rv3615c peptide cocktail stimulation (black) compared to stimulation with ESAT-6/CFP-10 (blue), and PPD-B responses (red).

**Figure 2. Skin test and cytokine responses after repeated short-term skin tests.** Data are presented as *in vitro* PPD-B-induced cytokine responses after performing short interval skin tests, or skin test responses (Skin test = Single intradermal comparative cervical tuberculin test = SICCT; results shown as PPD-B minus PPD-A responses) compared to the responses at the first short-interval skin test (ST1): Responses at ST1 = 1. ST1, ST2, ST3 = first, second and third short interval skin test performed at 60 day-intervals, 23 cattle were tested each time. \*, P < 0.05 (ANOVA test undertaken on raw data, followed by Bonferoni Multiple Comparison post-test).

**Table 1. DIVA reagents: Rv3615c complements ESAT-6 and CFP-10.**

Antigen	SENSITIVITY <sup>a</sup>		SPECIFICITY <sup>b</sup>	
	% (95% CI)	Numbers positive	% (95% CI)	Numbers positive
PPD-B minus A <sup>b</sup>	97 (90.0, 99.3)	82/85	94 (86.4, 98.5)	4/72
ESAT-6/CFP-10 <sup>b</sup>	86 (76.6, 92.5)	73/85	99 (92.5, 99.9)	1/72
Rv3615c <sup>b</sup>	54 (42.9, 65.0)	46/85	100 (95.0, 100)	0/72
Rv3615c <sup>bc</sup> OR ESAT-6/CFP-10 <sup>bc</sup>	94 (86.8, 98.1)	80/85	99 (92.5, 99.9)	1/72

Results are expressed as percentages of animals responding to these antigens in the Bovigam whole blood IFN- $\gamma$  assay. Results represent values from a number of published (Cockle et al., 2006; Sidders et al., 2008; Vordermeier et al., 2001), as well as unpublished studies.

<sup>a</sup> Based on blood samples from reactor animals from farms culture-positive *M. bovis*, with their infection status confirmed by PM and/or culture. <sup>b</sup> Animals from TB-free herds from non-endemic areas. <sup>c</sup> Pools of peptides. <sup>o</sup> Positive to either one of the two cocktails.

Figure 1.

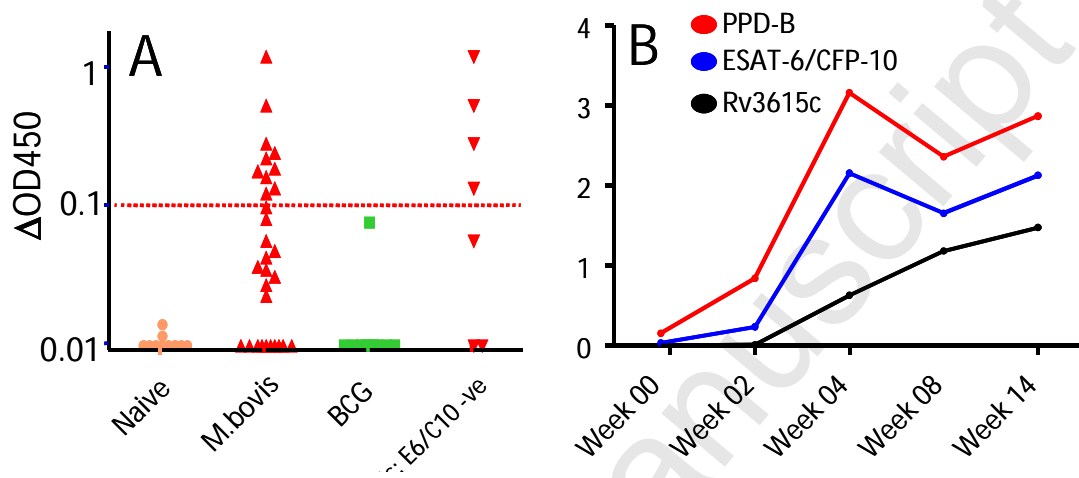


Figure 2.

