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Development of immune-diagnostic reagents to diagnose bovine tuberculosis in cattle

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ABSTRACT

Bovine tuberculosis remains a major economic and animal welfare concern worldwide. As part of control strategies, cattle vaccination is being considered. This approach, used alongside conventional control policies, also requires the development of vaccine compatible diagnostic assays to distinguish infected from vaccinated animals (DIVA). In this review we discuss recent advances in DIVA development based on the detection of host cellular immune responses by blood testing or skin testing approaches.

1. Introduction

1.1. Bovine tuberculosis

Bovine tuberculosis (TB) is a chronic disease of cattle caused by mycobacteria from the *Mycobacterium tuberculosis* complex, with *Mycobacterium bovis* the predominant causative agent. The economic impact of this disease relate to a reduction in productivity in severely affected animals, movement controls, testing, culling of affected animals and restriction on trade. The global economic impact of bovine TB is estimated at US$3 billion annually (Waters et al., 2012). *M. bovis* is also infectious to humans and prior to mandatory pasteurisation in many countries, *M. bovis* accounted for about one fourth of TB cases in children (Roswurm and Ranney, 1973). Important wildlife reservoirs of *M. bovis* infection include the badger in the UK and Ireland, brushtail possum in New Zealand, wild boar and deer in Spain and white-tailed deer in the USA (de Lisle et al., 2002).

1.2. Surveillance tests

The tuberculin skin test is the primary surveillance test for the diagnosis of bovine TB. In Europe, bovine PPD is applied in the mid-cervical region (cervical single intradermal test; SIT), while in the Southern Hemisphere and North America, bovine PPD is applied in the caudal fold of the tail (caudal fold test). In the UK and Ireland, the SIT has a poor specificity and a single intradermal comparative cervical test (SICCT) is used, where avian PPD, prepared from a culture of *Mycobacterium avium*, and bovine PPD are injected into adjacent sites in the mid-cervical region and increases in skin swelling between the two sites are compared at 72 h post-injection (de la Rua-Domenech et al., 2006; Monaghan et al., 1994). More recently, the whole blood interferon-γ (IFN-γ) release assay has been developed and utilised as an ancillary test for re-testing skin test reactor cattle and improved detection of *M. bovis*-infected cattle. The test is based on sensitised lymphocytes from *M. bovis*-infected animals releasing a cytokine, IFN-γ, when re-exposed *in vitro* to *M. bovis* antigens. The specificity of the response is achieved by comparing levels of IFN-γ released from whole blood cultures stimulated with bovine PPD and avian PPD or using specific *M. bovis* proteins such as ESAT-6 and CFP-10 (reviewed in Buddle et al., 2009; de la Rua-Domenech et al., 2006).

Control programmes cannot rely on test and slaughter of reactor animals alone and it is critical to supplement these measures with new control strategies, including vaccination of cattle against bovine TB together with the use of a DIVA test to differentiate vaccinated from infected animals (DEFRA, 2014).

2. Vaccine development

2.1. Bacille Calmette and Guérin (BCG)

BCG is a live attenuated strain of *M. bovis*. Calmette and Guérin first reported in 1911 that BCG induced protection in cattle against

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experimental challenge with *M. bovis* and trials were undertaken in a number of different countries in the first half of the 20th century to determine the efficacy of BCG vaccine for cattle. While studies investigating protection against experimental challenge provided encouraging results, more variable efficacies were reported in field trials (reviewed in: Skinner et al., 2001; Waters et al., 2012). However, over the past 20 years, a large amount of knowledge has been gathered regarding the use of BCG in cattle through the harmonisation of challenge models, BCG strains, and doses. BCG vaccination has been shown to be effective when administered at relatively low doses (10^4–10^6 CFU) subcutaneously (Buddle et al., 1995) or at higher doses (10^8 CFU) via the oral route (Wedlock et al., 2011) and by different daughter BCG strains (Pasteur and Danish) (Hope et al., 2011; Wedlock et al., 2007).

2.2. Development of vaccines that improve on BCG protective efficacy

Despite these recent encouraging results, the fact still remains that BCG vaccination confers variable protection in cattle and humans both at the population and individual animal levels. Therefore, major efforts to improve the protection imparted by BCG vaccination are under way. Currently there are several strategies for the development of vaccines that confer greater protection than BCG against TB in both humans and cattle. One strategy is to increase the level of protection afforded by BCG through the use of supplemental or boosting vaccines; the other strategy is to develop vaccines that replace BCG altogether. One of the most effective vaccination protocols 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). Subunits have been based on DNA (Maue et al., 2007; Skinner et al., 2003, 2005) or virally vectored booster vaccines (Dean et al., 2014; Vordermeier et al., 2006, 2004, 2009). A variation of this theme is the simultaneous vaccination with BCG and subunit vaccines (Wedlock et al., 2008). Other approaches consist of replacing BCG in favour of attenuated *M. bovis* strains, or genetically-modified BCG strains with improved immunogenicity (Blanco et al., 2013, 2012; Buddle et al., 2002; Waters et al., 2009), to over-express antigens such as Ag85B (Blanco et al., 2012; Rizzi et al., 2012), or to improve the efficacy of BCG itself through genetic modifications that increase its immunogenicity (e.g. Sander et al., 2015). We would like to highlight that the majority of the vaccines described in this section (e.g. Sander et al., 2015; Wang et al., 2004) were developed as vaccines to protect humans against *M. tuberculosis*. Thus, the bovine TB vaccine development field has vastly benefited by aligning the bovine vaccine development efforts with the international human TB vaccine programme.

As most of these vaccines or vaccine strategies depend on BCG or are based on genetically-modifed *M. bovis* or BCG, whose application will negate the specificity of tuberculin-based tests, the development of so-called DIVA (Differentiating Infected from Vaccinated Animals) tests will be required for countries which intend to use vaccination of cattle alongside conventional test and slaughter control strategies. The following sections will describe the approaches taken to develop vaccine-compatible DIVA reagents.

3. DIVA test development

Unfortunately, BCG vaccination sensitises cattle to bovine tuberculin, which compromises the use of the tuberculin skin tests and the Bovigam IFN-γ IGRA (Berggren, 1981; Buddle et al., 1995, 1999; Vordermeier et al., 1999). However, both test formats can be modified by replacing bovine tuberculin with specific antigens suitable as a DIVA. Due to the easy availability of large volumes of blood from cattle coupled with the high-throughput cost-effective use of pools of chemically synthesized peptides representing the complete amino acid sequences of target proteins, the whole blood IGRA is ideal for antigen mining studies required to identify potential DIVA candidates. The elucidation of the genomes for relevant mycobacterial species (including *M. bovis* (Garnier et al., 2003), *M. tuberculosis* (Cole et al., 1998), *M. bovis* BCG (Brosch et al., 2007), *M. avium* subsp. *avium*, and *M. avium* subsp. *paratuberculosis* (Li et al., 2005)) and the advent of microarray technology has revolutionised antigen mining strategies.

3.1. Comparative genomic analysis

Comparative genomic analysis has been used to identify *M. bovis* genes that are deleted in BCG (either as individual gene deletions or present in deleted gene regions), or that contain mutations resulting in either truncations or modified amino acid sequences after frame-shifting. Two of the major antigenic targets identified in cattle and humans are ESAT-6 and CFP-10 (Pollock and Andersen, 1997a,b; Ravn et al., 1999; van Pinxteren et al., 2000; Vordermeier et al., 2001). Their genes are located on the RD1 region of *M. bovis* and *M. tuberculosis*, which is deleted from the genome of all BCG strains (Mahairas et al., 1996). When used as diagnostic antigens, both ESAT-6 and CFP-10 have the capacity to differentiate *M. tuberculosis* infected from BCG vaccinated humans (e.g. Arend et al., 2000; van Pinxteren et al., 2000). Similarly, ESAT-6 has been shown to discriminate between *M. bovis* infected and BCG vaccinated cattle (Buddle et al., 1999; Vordermeier et al., 1999). When peptides derived from CFP-10 were formulated into a cocktail with those from ESAT-6, a greater number of infected cattle could be identified compared to using the peptide cocktail for either ESAT-6 or CFP-10 alone, demonstrating the potential to increase test sensitivity by using a combination of the two antigens (Vordermeier et al., 2001). Importantly, test specificity was maintained when this ESAT-6/CFP-10 peptide cocktail was used in BCG vaccinated cattle (Vordermeier et al., 2001).

Although demonstrating high specificity in BCG vaccinates, the sensitivity of the ESAT-6/CFP-10 peptide cocktail in identifying infected cattle is below that of tuberculin, thus necessitating the need to identify additional antigens to increase overall test sensitivity. The DIVA potential of other gene products encoded in the RD1 region and two other regions (RD2 and RD14) deleted in BCG (Brosch et al., 2007; Garnier et al., 2003) has also been assessed (Cockle et al., 2006, 2002). Although several additional peptides were identified that were recognised by infected animals but not by BCG vaccinates, a cocktail of these peptides demonstrated no added sensitivity benefit in relation to the ESAT-6/CFP-10 peptide cocktail when used under the routine conditions of the Bovigam assay in the UK (i.e. using blood that was stored overnight before the blood cultures were established). Thus, alternative approaches to comparative genomic analysis were needed to identify potential DIVA antigens to complement ESAT-6/CFP-10 and increase overall test sensitivity.

3.2. Comparative transcriptome analysis

This approach has been used to explore the link between gene expression level and antigenicity, with the aim of identifying novel reagents with DIVA potential. DNA microarray analysis quantifying the level of gene expression on a genome-wide scale was used to identify *M. tuberculosis* and *M. bovis* gene products consistently expressed at high levels under a variety of culture conditions (known as the abundant invariome) (Sidders et al., 2007). Genes showing high levels of homology (>98%) between *M. tuberculosis* and *M. bovis* orthologues, but low identity with *M. avium*,

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