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# Peptides specific for *Mycobacterium avium* subspecies *paratuberculosis* infection: diagnostic potential

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***Mycobacterium avium* subspecies *paratuberculosis* (*Map*) is the causative agent of Johne's disease (JD). Current serological diagnostic tests for JD are limited by their sensitivity when used in sub-clinical stages of the disease. Our objective was to identify peptides that mimic diagnostically important *Map* epitopes that might be incorporated into a new-generation JD diagnostic. Four peptides were isolated from a phage-displayed random peptide library by screening on antibodies derived from *Map*-infected goats. The peptides were recognised by antibodies from *Map*-infected goats but not by antibodies from uninfected goats. The peptides elicited immune responses in rabbits, which reacted strongly with *bona fide Map* antigens proving the peptides were true epitope mimics. To assess the diagnostic value a panel of goat sera was screened for reactivity's with peptides. The peptides were recognised by antibodies from a proportion of goats infected with *Map* compared with control animals with a diagnostic specificity of 100% and the sensitivity ranged from 50 to 75%. Combinations of any two peptides improved sensitivity 62.5–87.5% and 100% sensitivity was achieved with three of the four peptides in combination. These data suggest peptides representing diagnostically important *Map* epitopes could be incorporated into a sensitive diagnostic test.**

*Keywords*: diagnosis/Johne's disease/peptide mimics/phage-display

## Introduction

Paratuberculosis or Johne's disease (JD) is an infectious incurable chronic wasting disease, which affects cattle, sheep and goats worldwide resulting in large economic losses (Benedictus *et al.*, 1987). Johne's disease is caused by infection with *Mycobacterium avium* subspecies *paratuberculosis* (*Map*) which after initial infection can remain sub-clinical or lead to clinical symptoms typical of enteritis. *Map* is usually transmitted by contact with contaminated faeces and *Map* infections can be transmitted from mothers to off-spring through contaminated colostrum or milk or across the placenta into the foetus before the calf is born (Chiodini *et al.*, 1984).

Early detection of the disease is therefore important in the management and vital for prevention of the spread of the disease. Diagnosis based on faecal culture requires long incubation times and has low sensitivity. Diagnostic tests are available which generally have high specificity; however, their sensitivity in the early or sub-clinical stages of the disease is relatively low (Stewart *et al.*, 2004, 2006, 2007; Collins *et al.*, 2005). During the early stages of JD, often in the sub-clinical period, a cell-mediated immune response develops. There are diagnostic tests to measure the cytokine interferon-γ (IFN-γ) but the test is unreliable in sub-clinical animals aged below 1 year (Huda *et al.*, 2003).

A more widely used diagnostic test is based on detection of antibodies in serum or milk that are produced during the pre-clinical and clinical stages of the disease to *Map* antigen. Many commercial diagnostic enzyme-linked immunosorbent assays (ELISA's) require an extract of the native *Map* antigen which consists of a mixture of proteins coated onto ELISA wells. However, this crude mixture may also incorporate unwanted specificities and cross-reactions with other *Mycobacteria*; and as a consequence the majority of diagnostic ELISA kits require a serum absorption step to remove antibodies that cross-react with *Mycobacterium phlei* (Yokomizo *et al.*, 1985). A recent study reported that this absorption step dramatically improved the specificity of diagnosis of JD in four commercial kits (>99%) but the diagnostic sensitivity ranged from 30.2 to 41.5% (Fry *et al.*, 2008). This serum absorption step resulted in a high specificity; however, there is a requirement for development of more sensitive diagnostic tests for JD. An alternative could involve the replacement of intact antigen mixture with one or more peptides representing diagnostically important epitopes. These peptides, referred to as peptide mimotopes or epitope mimics, would bind to antibody paratopes and mimic the linear or conformational/discontinuous epitopes present on the native *Map* antigens. The use of peptides for diagnosis may also increase the sensitivity and specificity of the ELISA by avoiding unwanted specificities present in whole antigen complexes (Kouzmitcheva *et al.*, 2001). Peptides can be synthesised reproducibly, relatively cheaply and applied to ELISAs with ease.

We have shown previously it is possible to generate peptides representing epitopes to Epstein–Barr virus (EBV). Peptides that mimic EBV epitopes were selected from a

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phage-displayed peptide library by selection against monoclonal antibodies or purified immunoglobulin G (IgG) from an EBV-immunised rabbit. Using a combination of peptides it was possible to replace the native antigen for diagnosis of EBV, with high sensitivity (54–94%) and specificity (100%; Casey *et al*., 2006, 2009).

In our previous study we induced an immune response to raise polyclonal antibodies to the EBV pathogen (Casey *et al*., 2009) as an alternative strategy in the present study serum from goats that have been naturally infected with *Map* (and showing signs of JD) was used. A series of pre-clearance and selection steps has been developed in order to isolate peptides recognised by naturally occurring *Map* antibodies from animals with JD. They should represent the immunodominant epitopes involved in the production of a typical humoral immune response in animals infected with *Map* and suffering from JD.

To establish the function of each peptide isolated in this study we immunised animals and studied the resulting antibody response. The anti-peptide antibodies were strongly reactive with native *Map* antigens, indicating the peptides closely resemble the 3-D shape of the epitopes and are therefore true epitopic mimics. The peptides were also shown to have diagnostic potential by their ability to be recognised by antibodies produced in animals infected with JD. Each peptide potentially represents a different epitope and should be recognised by different subsets of antibodies. A combination of peptides has the potential to replace complex *Map* antigen preparations in current diagnostics. The use of peptides may also alleviate the need for a serum absorbent step that is needed to remove antibodies to other *Mycobacterium species* and consequently result in a diagnostic test with greater specificity and sensitivity for JD.

## Materials and methods

### *Goats infected with* Map

Goat serum samples were obtained from two animals referred to as goat-1 (G1) and goat-2 (G2) prior to (pre-challenge) and after infection (post-challenge) with *Map*. All animal experimentation was conducted at CSIRO Animal Health Laboratory, Geelong, Australia (Stewart *et al*., 2006). Briefly four doses of *Map*-cultured bacteria ($1 \times 10^{10}$–$2 \times 10^{10}$) were administered orally at weekly intervals for 4 weeks, blood samples were collected for a period of 55 months post-infection. Serum samples were also obtained from goats infected with macerated gut mucosal tissue from naturally occurring JD for diagnostic screening (Stewart *et al*., 2006).

### *Purification of goat serum*

Serum was diluted 1:4 in 20 mM sodium phosphate buffer pH 7, filtered through a 0.22 µm filter and the IgG fraction purified using a 1 ml HiTrap protein G HP column (GE Biosciences). The IgG was eluted with 0.1 M glycine buffer pH 2.7, fractions were collected, neutralised with 1 M Tris pH 9 and dialysed into phosphate-buffered saline (PBS). The absorbance at 280 nm was measured and the IgG concentration calculated using the extinction coefficient for antibodies 1.45.

### *Phage library and panning*

To select JD specific peptides we screened our random 20 amino acid peptide library displayed on M13 bacteriophage (provided by AdAlta Pty Ltd). The phage peptide library contains $>5 \times 10^8$ individual peptides and has been screened successfully on many occasions to isolate biologically functional peptides (Casey *et al*., 2004, 2006, 2008a,b, 2009; Harris *et al*., 2005, 2009; Read *et al*., 2009). Our random peptide library was screened for peptides specific for IgG preparations purified from two individual goats challenged with *Map* (goat-1: G1 and goat-2: G2), using a selection strategy similar to our previous studies with the addition of negative selection steps to remove unwanted antibody specificities (Casey *et al*., 2004, 2006, 2009). Briefly post-challenged IgG was coated onto microtitre wells (10 µg/ml) and panning was performed as detailed previously with several modifications (Casey *et al*., 2006). For goat G2 only a pre-clearance step was introduced using the pre-challenge sera in order to select for phage binding specifically to post-challenge IgG. This negative selection step involved amplifying the phage that did not bind to the pre-immune serum coated onto the ELISA wells. Wells were washed four times for pre-clearance in round five the washes were retained and amplified for selections on post-challenge IgG. No pre-clearance was introduced for selections involving goat G1.

### *Peptide synthesis and conjugation to bovine serum albumin*

Peptides were synthesised to >85% purity by GLBiochem (Shanghai, China). B7 peptide contained a disulphide bond at positions eight and 18. E1 and B7 peptides were soluble in PBS, B6 in dimethylformamide and J10 in 0.1 M acetic acid. Peptides were also synthesised with three additional glycines to act as a spacer and a cysteine residue at the *C*-terminus for conjugation to bovine serum albumin (BSA), using a similar method we have used previously described in detail by Casey *et al*., 2006.

### *Immunization of rabbits with BSA-peptide conjugates*

Four New Zealand white rabbits were immunised intramuscularly with 200 µg of BSA-conjugated peptides emulsified with Montanide ISA7 adjuvant (Aucouturier *et al*., 2002). One rabbit was used per conjugated peptide (E1, J10, B6 and B7). Two further boosts with the same amount of conjugated peptide were administered in monthly intervals.

### *Enzyme-linked immunosorbent assays*

*Map* antigen ELISA's were performed using the commercial PARACHEK® test (Prionics AG, Switzerland) using the manufacturer's protocol, with the following modifications. ELISA tests were performed in duplicate and various dilutions of sera were used. For each test the kit control positive and negative samples were employed.

Phage ELISA's were performed by coating 5–10 µg/ml of IgG in PBS to a microtitre plate (Nunc, Maxisorp) overnight at 4°C and subsequent blocking with 5% milk powder/PBS (blocking buffer) for 2 h. Blocked wells were washed twice with 200 µl/well PBS. Phage dilutions (100 µl) were prepared in PBS and transferred in duplicate to the blocked washed wells and incubated for 1 h with shaking. The wells were washed five times with PBS containing 0.05% Tween 20 (PBSTween) and 100 µl of anti-M13 antibody conjugated



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to horse-radish peroxidase (HRP, GE) at a 1/5000 dilution in PBST was added to each washed well. After 1 h incubation and washes as above the bound phages were detected using o-phenylenediamine substrate (OPD, Sigma) according to the manufacturer's protocol.

Similar ELISA's were designed for measuring anti-peptide antibodies in rabbit sera or antibodies in goats challenged with *Map*. Bovine serum albumin-conjugated peptides were immobilised onto microtitre plates at 5 μg/ml wells were also coated with BSA alone at the same concentration as control wells. Preliminary experiments revealed that no additional blocking step was necessary prior to adding the first antibody layer. Dilutions of rabbit or goat sera were prepared in PBS and added at 100 μl per well in duplicate or triplicate for 1 h shaking. The same washing scheme as above (five times PBST) was employed followed by the addition of 100 μl per well of anti-rabbit or anti-goat HRP (Chemicon; 1/2000 dilutions) prepared in PBST. The remainder of the assay was carried out as above, using 3,3',5,5'-tetramethylbenzidine substrate prepared using the manufacturer's recommendations (TMB, Sigma). All ELISA's were repeated to ensure consistent results

## Results

### *Reactivity of antibodies from* Map*-infected goats*

Two goats, the subjects of a *Map* antigen challenge study produced high titres of anti-*Map* antibodies measured using the commercial PARACHEK® test (Stewart *et al*., 2006) (Fig. 1a). Goat-1 (G1) serum had a higher titre of anti-*Map* antibodies than goat-2 (G2) serum. The corresponding pre-challenged sera showed comparatively low binding to *Map* proteins. Pre- and post-challenge sera were purified by affinity chromatography to isolate the IgG fractions. Post-challenge IgG showed high reactivity to *Map* antigen when compared with the relatively low signal for the pre-challenge IgG (Fig. 1b). The titre of *Map*-reactive antibodies in G1 was higher when compared with G2 (Fig. 1b). These IgG preparations of anti-*Map* antibodies from G1 and G2 were used to identify peptide mimics of *Map* proteins.

### *Peptides specific for post-challenge* Map *IgG*

To isolate peptides mimicking *Map* epitopes two separate panning experiments were performed using post-challenge IgG from goat-1 (G1) and goat-2 (G2). The IgG preparations were coated onto a solid phase and up to five rounds of panning using a phage displayed 20-mer random peptide library performed. There was enrichment in both (G1 and G2) post-challenge *Map* IgG preparations of phage peptides after four rounds of panning. Pools of phage in each round of panning specific for G1 IgG (Fig. 2a) showed low reactivity against the pre-infection challenge IgG. Whereas for G2 IgG a large proportion of the phage reacted with the pre-challenge IgG in round four indicating a large proportion of phage peptides were reactive with non-*Map*-specific serum IgG's. This phage pool was pre-cleared with pre-challenge G2 IgG resulting in a significant decrease in the ELISA signal to the pre-challenge IgG when compared with the high reactivity of with post-challenge G2 IgG (Fig. 2b).

Individual phage colonies (>10) from rounds four and five were grown and analysed for their reactivity with G1 or

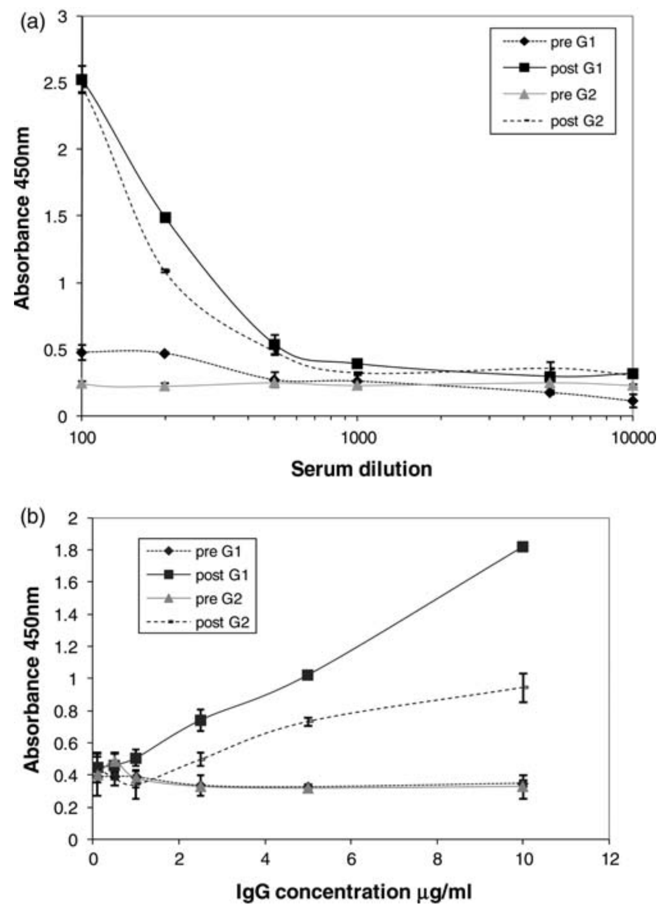


**Fig. 1** Reactivity of antibodies from two goats (G1 and G2) challenged with *Map*. (**a**) The pre-challenge and post-challenge sera for goats G1 and G2 were analysed for binding to the original antigen using the commercial PARACHEK® test kit. (**b**) The IgG fractions of pre- and post-challenge sera were purified using Protein G chromatography and the reactivity of the IgG fractions was analysed using the PARACHEK® test. Error bars indicate ranges of individual values.

G2 pre- and post-challenge IgG. Phage clones with high reactivity to post-challenge IgG and low reactivity with pre-challenge sera were sequenced. Individual phage clones sequenced in round four that reacted strongly to the post-challenge G1 IgG all had an identical sequence (peptide E1, Fig. 3a). Three peptides with different amino acid sequences in round five that reacted strongly with post-challenge G2 IgG were identified (peptides J10, B6 and B7; Fig. 3a).

There was no significant similarity between the peptide sequences and a basic local alignment search tool (NCbi) search of the peptides was performed to assess the degree of similarity with *Map* or related antigens. For peptides J10, B6 and B7 there was no significant identity in the database. E1 peptide revealed homology to a region in the mycocerosic acid synthase (*Mas*) of *Mycobacterium tuberculosis (Mtb)*. Amino acid residues 4–12 KDYQRNRGPSKFTGLQPSVL of E1 showed eight out of nine identical residue matches (underlined region) with *Mas* QRSRGPSKF at residues 2007–2015. Only one difference was found (shaded region) which was an interchange of Asparagine (N) with Serine (S) of *Mas*. The *Mas* sequence (total 2111 amino acids) was analysed for predicted transmembrane helices (Fig. 3b). Two separate models predicted the region of similarity (residues 2007–2015) to be non-cytoplasmic and therefore likely to be



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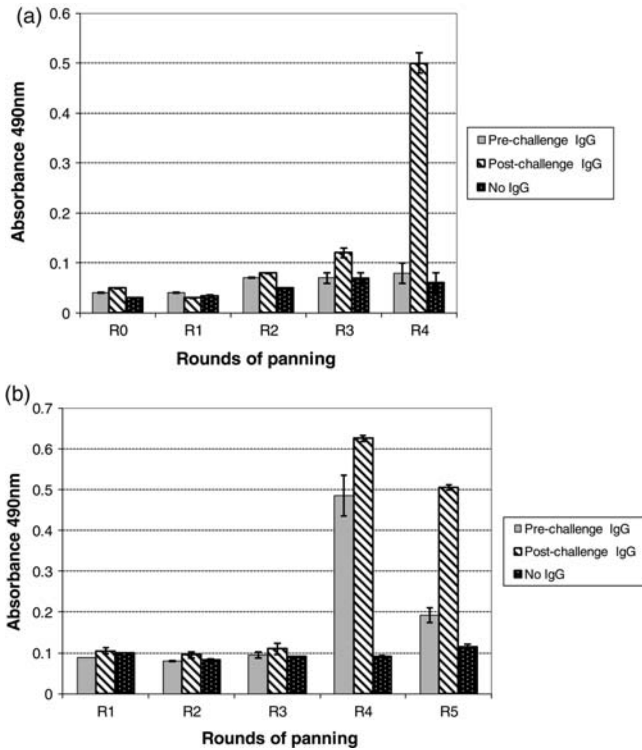


**Fig. 2** Panning the phage peptide library for clones that are recognised by affinity purified antibodies from two individual goats (G1 and G2) infected with *Map*. The reactivity of selected phages from each round of panning to pre- and post-challenge IgG is shown by ELISA. (**a**) Four rounds of panning were performed for goat G1. (**b**) Five rounds of panning were performed for goat G2 including pre-clearance in round five to remove phage reactive with pre-challenge IgG. Error bars indicate ranges of individual values.

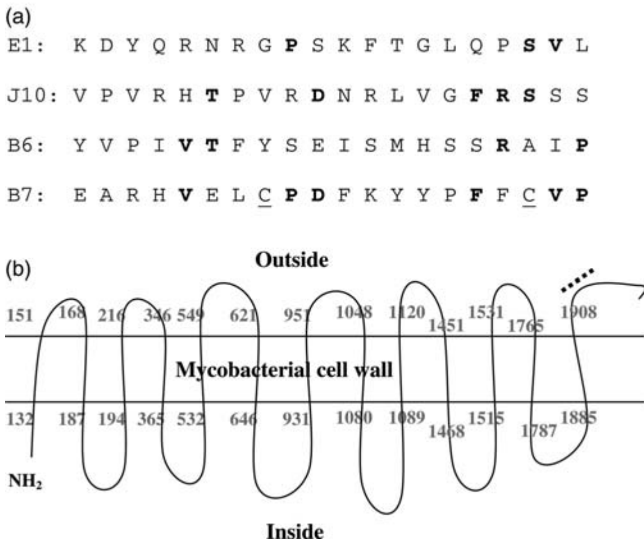


**Fig. 3** (**a**) Amino acid sequences of peptides, identical positional residues are shown in bold, cysteine residues are underlined. (**b**) A transmembrane helices prediction model predicts the region of *Mas* residues 2007–2015 (QRSRGPSKF) with homology to residues 4–12 of E1 peptide (QRNRGPSKF) is predicted to be exposed on the outer membrane of *Mas* shown by the dotted line.

exposed on the surface of the bacterium. It is conceivable therefore that this region could be a shared membrane epitope common to *Map* and *Mas*.

### *Peptide mimotopes induce* Map*-specific antibodies*

In order to establish whether E1, J10, B6 and B7 peptides represent authentic epitopes on a *Map* antigen rabbits were immunised with the synthetic peptide-BSA conjugates. High titres (1:5 000–1:10 000) of peptide-specific antibodies were generated (Fig. 4). The immune peptide sera also reacted strongly with the native *Map* antigen in the PARACHEK® test, whereas the pre-bleed was not reactive with *Map* (Fig. 5).

These data show that the peptide mimics selected in this study are antigenic mimics of *Map* antigen(s) and they are also immunogenic mimics evidenced by the induction of an immune response that is equivalent to a proportion of the immune response in animals infected with *Map*.

### Map *peptide mimics as diagnostic reagents for JD*

To assess the diagnostic potential of the peptide mimics, the ability of *Map* antibodies to recognise the peptide mimotopes was assessed. A clinical study was carried out comprising four goats infected with *Map* in a challenge experiment (G1–4), four goats infected with gut tissue from animals with natural JD (G5–8) and five non-infected control goats (C1–5; Stewart *et al*., 2006). The absorbance readings from infected and control goats were subtracted from the pre-challenge readings and plotted (Fig. 6). The horizontal line represents the cut-off level which is defined as the mean of the negative population (control animals) plus three standard deviations (SDs). Values above this line were considered positive and below the line negative. Background levels were observed for all of the samples when analysed on BSA (the peptide conjugated carrier) alone (Fig. 6e) indicating the signal to noise level of the carrier molecule was low.

All four peptides showed low reactivity with antibodies in the control goats, indicating no false-positive readings and 100% diagnostic specificity of each peptide in this study (Fig. 6, Table I). E1 peptide was pre-dominantly recognised by antibodies in the sera from one *Map*-infected goat (G1), this sera was obtained from goat-1 (G1) which was originally used for selection of E1 peptide. E1 peptide was also recognized by antibodies in two other tissue-infected goats (G7 and G8) and one other bacterially infected goat (G3; Fig. 6a). The resulting sensitivity of E1 peptide alone was 50% (Table I). Peptide J10 produced the highest diagnostic sensitivity (75%; Table I) of all the peptides as it was recognised by serum antibodies in six of eight of the goats infected with *Map* (Fig. 6b). J10 was isolated from goat-2 (G2) antibodies and sera from the same goat in Fig. 6 was analysed. In contrast to E1 peptide being recognised most strongly by the original selection sera, higher levels of detection were obtained by antibodies in three different goats (G3, G6 and G8) compared with the original selection sera (G2). Peptide B6 was recognised by sera from four out of eight goats in this study including G2 the original selection sera (Fig. 6c, Table I). However, the absorbances were lower than for the other peptides. The weak recognition of peptide B6 may reflect the low solubility of this peptide; B6 was insoluble in PBS and required solubilisation in the solvent dimethyl formamide which might have reduced its reactivity in the assay. The sensitivity of B6 for diagnosis of JD in this study was 50%. Peptide B7 was recognised by antibodies in G2 sera and slightly lower levels of detection were observed



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**Fig. 4** Immunisation of rabbits with peptides conjugated to BSA produced high titres of anti-peptide antibodies to (**a**) E1, (**b**) J10, (**c**) B6 and (**d**) B7 peptides. Rabbit pre-bleed (Pre-b) and immune sera (Imm) were analysed for binding to the respective peptides conjugated to BSA. Rabbit pre-bleed and immune sera were also analysed for binding to BSA alone as a control; Pre-b (BSA) and Imm (BSA). Error bars indicate ranges of individual values.

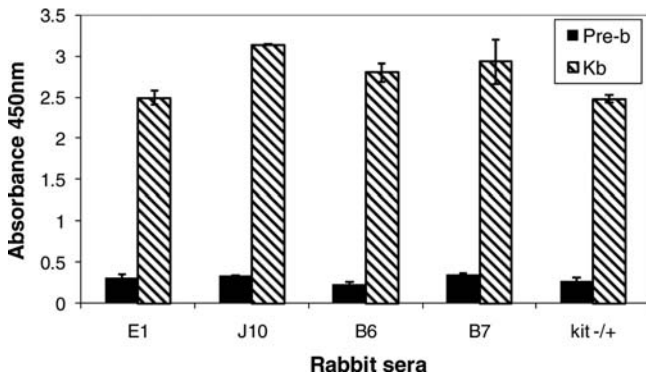


**Fig. 5** Immune responses of rabbits immunized with peptides conjugated to BSA as a carrier to the PARACHEK® antigen. Kit+ and kit− are the positive and negative control test sera from the PARACHEK® kit. Pre-b refers to pre-bleed serum samples and Kb refers to the immune rabbit serum samples. Serum was diluted 1:50 and the assay was repeated twice to ensure reproducibility.

by antibodies in a further four goats (Fig. 6d). The sensitivity of B7 was 62.5% (Table I).

A combination of most of the peptides resulted in an increase in the diagnostic sensitivity. B6 combined with B7 peptide did not improve the sensitivity for *Map* infection. The best combination of two peptides was E1 and J10, E1 and B7 or J10 and B7 indicating these peptides represent different diagnostically important epitopes. A combination of all three of these peptides (E1, J10 and B7) resulted in 100% detection of serum antibodies in all of the goats infected with *Map* in this study.

## Discussion

Diagnostic tests for JD involve detection of *M. avium* subspecies *paratuberculosis* or *Map*-specific antibodies from the serum of infected animals. Current commercial diagnostics have poor sensitivity and require a serum absorption step to remove cross-reactive antibodies. As possible alternatives for complex antigens we have selected JD antigen mimics and propose these peptides could be used to produce a more sensitive uniform diagnostic test.

To identify JD specific peptides we selected peptides representing antigen epitopes by screening a phage-peptide library against IgG preparations derived from goats infected with *Map*. The peptides (E1, J10, B6 and B7) were recognised specifically by sera from *Map* infected goats but not by pre-challenge sera. Phage-displayed selection technology does not require knowledge of the original antigen (Folgari *et al*., 1994; Casey *et al*., 2006) and in this study we have



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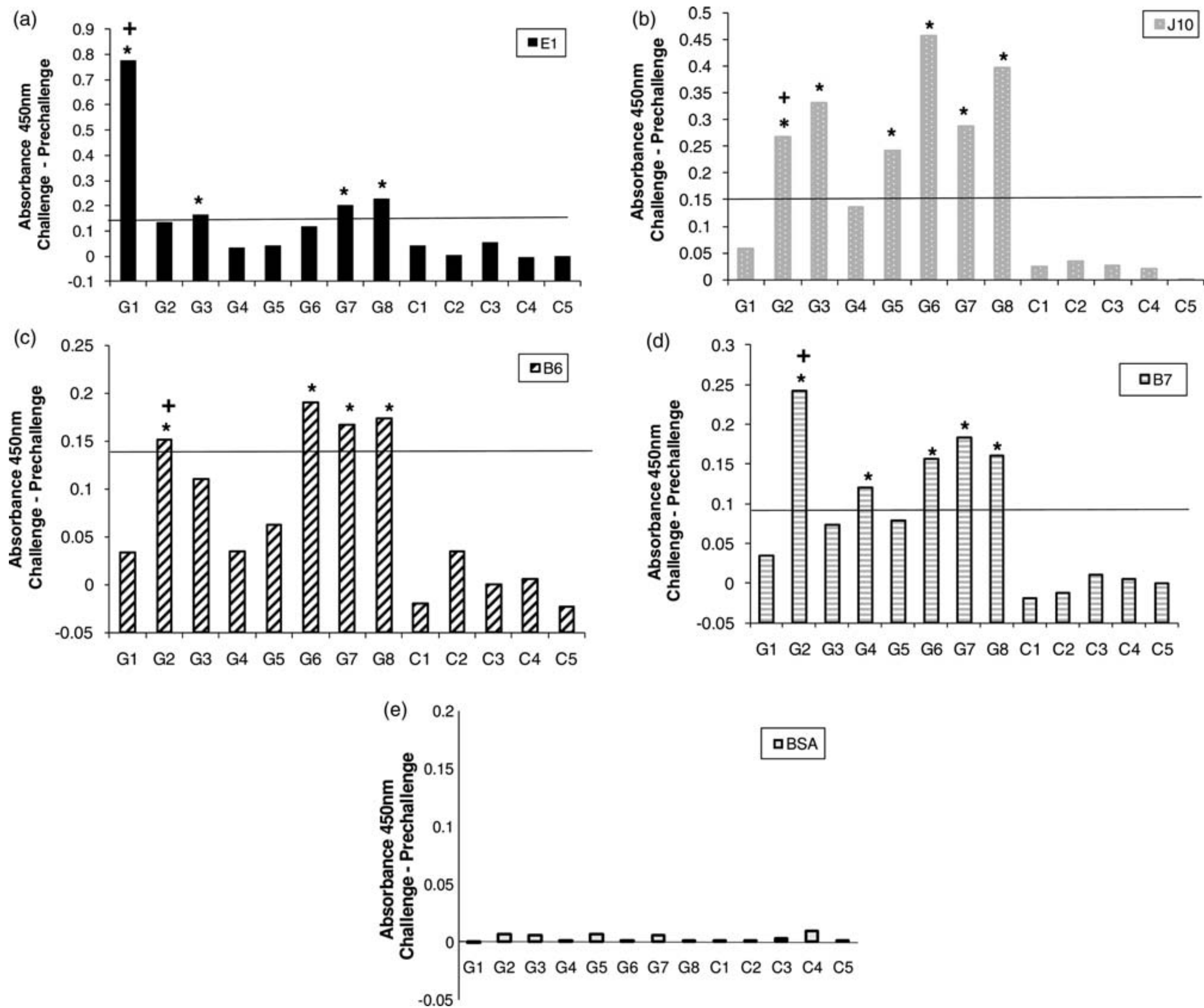


**Fig. 6** Evaluation of peptides conjugated to BSA as JD diagnostics (**a**) E1, (**b**) J10, (**c**) B6, (**d**) B7 peptides and (**e**) BSA alone was used as a control. Serum from goats involved in *Map*—challenge experiments was allowed to react with the peptides and bound antibodies were detected with anti-human HRP. Goats (G1–4) were challenged with bacterial *Map* and goats G5–8 were challenged with *Map* infected gut tissue, C1–4 were untreated control goats. The absorbance reading from infected and control goats were subtracted from the pre-challenge readings and plotted (mean values of three experiments in triplicate). The horizontal line represents the cut-off level equivalent to three standard deviations above the mean of the negative population. Bars with * indicate positive signals above the cut-off level. Bars with+ indicate this was the original selection sera used for isolation of the peptide.

selected peptides against polyclonal antibodies produced in response to infection with *Map*, without prior knowledge of the original antigen(s) to which the polyclonal antibody response to *Map* infection was raised. *Map* infection stimulates the humoral immune response to generate antibodies to multiple surface-exposed epitopes including carbohydrates, which form a large component of the mycobacterial cell wall. *Map*-infected sera from JD infected goats are therefore likely to represent mixed populations of antibodies from surface-exposed epitopes on *Map*, some of which maybe immunodominant epitopes. We have harnessed this selection system to identify peptides representing these important epitopes and further analysed their diagnostic potential.

This technology is well established and has largely been explored for identification of protective epitopes. Peptides that represent protective epitopes and can induce a neutralising immune response could form the basis of a peptide vaccine. Since the pioneering study by Prezzi *et al.* to select peptides specific for polyclonal sera from patients with Hepatitis C (Prezzi *et al.*, 1996), this technology has been extended to identify peptides specific for other diseases such as Lyme disease (Mathiensen *et al.*, 1998), Hepatitis A (Larralde *et al.*, 2007), cystic hydatid disease (Read *et al.*, 2009) and Cœliac disease (Zanoni *et al.*, 2006). Although these studies suggest a diagnostic approach this application remains largely unexplored. Here and in our previous study (Casey *et al.*, 2009) we promote the identification of peptides recognised by IgG from diseased individuals. This is an attractive application as this technology is potentially applicable to any disease which produces disease-specific antibodies in particular where diagnostic tests are lacking in sensitivity or specificity, and therefore this approach could lead to the generation of superior peptide-based diagnostic tests.

Ideally a diagnostic test would cover as many immunodominant epitopes as possible for complete coverage of the



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**Table I.** Evaluation of peptides alone and in combination for diagnosis of JD

| Peptide combinations | % sensitivity[a] | % specificity[b] |
|---|---|---|
| One peptide | | |
| E1 | 50 | 100 |
| J10 | 75 | 100 |
| B6 | 50 | 100 |
| B7 | 62.5 | 100 |
| Two peptides | | |
| E1 + J10 | 87.5 | 100 |
| E1 + B6 | 75 | 100 |
| E1 + B7 | 87.5 | 100 |
| J10 + B6 | 75 | 100 |
| J10 + B7 | 87.5 | 100 |
| B6 + B7 | 62.5 | 100 |
| Three peptides | | |
| E1 + J10 + B6 | 87.5 | 100 |
| E1 + J10 + B7 | 100 | 100 |
| E1 + B6 + B7 | 87.5 | 100 |
| J10 + B6 + B7 | 87.5 | 100 |
| Four peptides | | |
| E1 + J10 + B6 + B7 | 100 | 100 |

[a]Based on positive identification of serum antibodies above the cut-off level (refer to Fig. 6).
[b]There were no false-positive results.

immune response in every *Map*-infected individual with subclinical or clinical JD. Our results elude to coverage of a range of different epitopes mimicked in particular by peptides J10, E1 and B7. These peptides are recognised by different sub-populations of antibodies that commonly occur after infection with *Map* (Fig. 6), and when combined 100% sensitivity was achieved. All the peptides in this study were specifically recognised by *Map* antibodies and the epitopes of *Map* the peptides represent are JD disease specific. This is highly beneficial since the commercial PARACHEK® test and most of the current JD diagnostics require a serum absorption step prior to the assay to remove antibodies that cross-react with *M. phlei* (Yokomizo *et al*., 1985). The use of peptide mimotopes to JD alleviated the need for this pre-clearance step as 100% specificity was observed with no need for an *M. phlei* antigen serum absorption step.

Optimization of the display of peptide ligands to allow maximum capture of antibodies on a solid phase could improve the sensitivity of diagnosis. Here we chose to couple the peptides to a carrier molecule BSA, which we have previously shown to be superior to use of peptides alone (Casey *et al*., 2006, 2009). A short linker sequence was added at the *C*-terminus to space the peptide away from the *C* -terminal thiol group used in conjugation to BSA. This was an attempt to resemble the orientation of the peptide on bacteriophage leaving the N-terminus of the peptide available for reaction with antibodies. One or more peptides could also be genetically fused to lattices as solid supports (Tschiggerl *et al*., 2008), attached to dendrimers (Lee *et al*., 2005), latex or nitrocellulose (Cardosa *et al*., 1995) which may be more amenable to an on-site dip-stick type rapid diagnostic. In a follow-up study a combination of peptides E1, J10 and B7 attached individually or in combinations to various solid supports could be performed to optimise the presentation of the peptides with a larger cohort of samples to further analyse the diagnostic potential of these peptides.

Sequence analysis revealed that one of the peptides E1 had similarity in 8 out of 20 residues with an *Mtb* protein, *Mas* Mycocerosic acids are cell wall lipid precursors that are found only in pathogenic mycobacteria and are required for virulence (Sirakova *et al*., 2003). It is therefore feasible we have selected a peptide (E1) mimicking an extracellular region of *Mas* and the same E1 peptide also mimics an epitope of a *Map*. Detailed sequence analysis of *Map* identified >3000 genes with homologues to the human pathogen *Mtb* the causative agent of human tuberculosis (TB; Li *et al*., 2005). Further studies to characterise the significance of this region of similarity between *Map* and *Mas* could prove that if this epitope is shown to be disease specific it may be possible to develop a diagnostic test for both JD and TB.

We have shown here it is possible to simultaneously select peptides representing different epitopes and these could be useful for diagnosis. However, the identity of the epitope is unknown; recently peptides were identified to a known immunodominant epitope present at the C-terminus of p34, a component of *Map* (Ostrowski *et al*., 2003). Future epitope mapping studies could reveal that one or more of the peptides described in this study may represent immunodominant epitopes of p34 or other epitopes exposed on the bacterial surface.

All the peptides were able to generate an immune response which produced anti-peptide antibodies that recognised the original *Map* antigen. This test was carried out to confirm the peptides were true epitope mimics since antibodies can be raised against a single epitope of *Map* and these antibodies can bind to the same epitope in the context of the original antigen. Studies could be performed to further characterise the epitopes and observe whether a protective immune response could be generated after immunisation. This may also have implications for a future peptide-based vaccine for JD.

The results presented in this report highlight the potential of synthetic peptides to substitute for the cognate antigen in diagnostic tests. The peptides may also provide useful tools for the discovery of surface-exposed antigens which constitute important components of the antibody-mediated immune response to JD or could be explored as a potential vaccine component for JD.

In this study we have demonstrated proof-in-principle, and the potential for a future diagnostic in this pilot study. A follow-up study using a greater number of infected animals and controls, animals at different stages of disease and optimisation of the support matrix for high levels of sensitivity would reveal the benefits of each peptide alone and in a combination and their diagnostic efficacy.

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