Antigenic profiles of recombinant proteins from *Mycobacterium avium* subsp. *paratuberculosis* in sheep with Johne’s disease

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Abstract

Methods to improve the ELISA test to detect *Mycobacterium avium* subsp. *paratuberculosis* have been explored over several years. Previously, selected recombinant proteins of *M. avium* subspecies *paratuberculosis* were found to be immunogenic in cattle with Johne’s disease. In the present study, antibody responses of infected and healthy sheep were evaluated using 18 purified recombinant proteins in an ELISA-based format for the serodiagnosis of ovine paratuberculosis. These selected recombinant proteins represent heat shock proteins, hypothetical proteins and cell surface proteins of *M. avium* subsp. *paratuberculosis*. Whereas, Map0862 (a gene uniquely present in *M. avium* subsp. *paratuberculosis*) and Map3786 encoded protein solicited the strongest antibody response in infected sheep. The protein encoded by Map2116c showed the weakest antibody response among the animals tested. Although none of the recombinant proteins detected all 11 infected sheep singly, antibodies to Map0862 were detected in 9 of 11 (81%) infected sheep. Furthermore, ovine responses to these selected antigens were assessed temporally over the course of 1 year during which we found a spiking effect rather than an incremental increase of antibody reactivity. This study evaluated multiple *M. avium* subsp. *paratuberculosis* recombinant proteins in an ELISA-based format for sheep.

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Keywords: *Mycobacterium avium* subsp. *paratuberculosis*; Johne’s disease; Sheep; Antigens

1. Introduction

Johne’s disease results from a chronic infection with *Mycobacterium avium* subsp. *paratuberculosis* that affects farmed ruminants such as cattle, sheep and goats. In the United States alone, Johne’s disease accounts for US$ 220 million in annual loss to the agricultural economy (Stabel, 1998; Sweeney, 1996). However, because most *M. avium* subsp. *paratuberculosis* infections are pre-clinical and therefore may go undiagnosed for several years, the actual cost to producers is probably much higher. Despite its heavy economic burden on the agricultural industry, there are still no practical chemotherapeutic agents or efficacious vaccination programs against Johne’s disease. In order
to effectively combat Johne’s disease, there is an urgent need for the development of improved (i.e. relatively inexpensive, easy to implement, rapid, sensitive and specific) diagnostic tests. The use of an ELISA test for identifying infected herds and as a screening test to identify infected animals is one of the control strategies recommended by the National Johne’s Disease Voluntary Control Program of the United States.

In a recent study, diagnostic sensitivity of the fecal culture test (using Herrold’s-egg-yolk agar-centrifugation technique) was estimated to be 75% (Wells et al., 2006). Although the sensitivity of fecal culture outperform any other available diagnostic tests (e.g. ELISA and PCR tests), it is relatively expensive (US$ 30/test) and requires 5–16 weeks or more for cultivation (Lambrecht et al., 1988; Stabel, 1998). Among various serological tests for Johne’s disease, ELISA-based tests are widely used and can be conducted rapidly and require limited expertise. However, they suffer from a low to moderate sensitivity-based performance. Sensitivities of ELISA tests for detecting M. avium subsp. paratuberculosis infections range from 13.9 to 27.8% as compared to fecal-culture-positive animals and 6.9 to 16.9% when compared to tissue-culture-positive animals (McKenna et al., 2005).

One currently developed ELISA test for M. avium subsp. paratuberculosis is based on a protoplasmic antigen detection method by Yokomizo et al. (1983). However, these tests require pre-absorption of non-specific antibodies with M. phlei (Yokomizo et al., 1983) and still exhibit low sensitivity (Whitlock et al., 2000). Lipooarabinomannan (LAM), a component of the cell wall of mycobacteria, has also been tested in an ELISA format for the diagnosis of Johne’s disease (Jark et al., 1997; McNab et al., 1991; Reichel et al., 1999). The LAM fraction contains antigens that specifically react with sera from Map-infected cattle and is specific to Map and not M. phlei (Reichel et al., 1999). However, when the serum was absorbed with extracts of M. avium subsp. avium, the specific antibody reaction got abolished showing cross-reactivity between LAM antigens of Map and M. avium subsp. avium (Reichel et al., 1999).

Its has been more than 20 years since the hunt for an ideal serological test to monitor Johne’s disease began. The antigens used in conventional ELISAs generally consist of complex, ill-defined mixtures of proteins, lipids and carbohydrates including purified protein derivative (Yokomizo et al., 1983) or lipooarabinomannan (Jark et al., 1997) that is obtained by extensive physical and chemical disruption of M. avium subsp. paratuberculosis cells. The use of harsh conditions to extract mycobacterial antigens may be partially responsible for the low levels of diagnostic sensitivity and specificity of conventional ELISAs. In the early 1990s, Sugden et al. (1991) developed an ELISA based on two proteins purified by anion and HPLC chromatograph. This ELISA appeared promising, showing 100% sensitivity for one of the proteins, but it could not be isolated in enough quantities or with the consistent reproducibility necessary for large-scale use. Nonetheless, these data show that promising single antigens are present in M. avium subsp. paratuberculosis and they could be used in developing an improved ELISA test. More recently, a formaldehyde extract (Speer et al., 2006) and an EtOH-vortex antigen extract (Eda et al., 2006) containing mostly surface proteins of M. avium subsp. paratuberculosis have shown a much improved sensitivity level of over 95%.

In Sardinia, an island in the Mediterranean, the human population is 1.6 million and yet more than 3.0 million sheep are farmed. This demographic setting therefore opens the possibility of extensive M. avium subsp. paratuberculosis contamination and increased potential exposure to Sardinian residents. In fact, data show that M. avium subsp. paratuberculosis is present in the intestinal tissue of more than 70% of Sardinian patients suffering from Crohn’s disease (Sechi et al., 2005). Furthermore, various supporting facts such as the Sardinians being genetically segregated population (Passarino et al., 2001) and the presence of mutations in the NOD2 (Sechi et al., 2005) and nrampl (Sechi et al., 2006b) loci strenthen the hypothesis of this association. There is no mandatory reporting of Johne’s disease in Sardinia, unlike bovine TB. Therefore, no official data are present describing ovine Johne’s disease in Sardinia. However, an unofficial survey showed that nearly half of the 50 farms tested carried at least one animal positive for paratuberculosis.

Realizing paucity of commercial ELISA development out of specific recombinant antigens, we tested 18 purified recombinant antigens representing M. avium subsp. paratuberculosis proteins in an ELISA format using sera from infected and healthy sheep. To our knowledge, this is the most comprehensive M. avium subsp. paratuberculosis antigen screen to date. Results from this study show that Map0862 is the best candidate antigen to be used in an ELISA-based test for ovine Johne’s disease.

2. Material studied, area descriptions, methods, techniques

2.1. Animals

A total of 11 adult sheep (5 males and 6 females of sarda breed), originating from a known paratuberculosis
infected Sardinian farm, were used in this study. All sheep were monitored for paratuberculosis on the basis of monthly clinical signs, fecal shedding and serological response (Table 1). Healthy controls (n = 10, 5 males and 5 females of sarda breed) that were negative to M. avium subsp. paratuberculosis (Map) by AGID, fecal culture and ELISA were recruited from a farm free of Map infection on the basis of flock health history and location (Nurra) (5 years documented history of AGID, ELISA and fecal culture negativity by the Istituto di Patologia Generale of the Sassari University, and no positivity for Map infection in, either bovine or ovine herds, in the area). Approval from the Sassari University animal experiment Ethical Committee was obtained.

2.2. Serum samples

Blood was collected from M. avium subsp. paratuberculosis-infected sheep at 1–2 week intervals over a period of 1 year. Additionally, sera were obtained from 10 sheep tested negative for M. avium subsp. paratuberculosis by AGID, ELISA and fecal culture. A total of 221 sera samples were aliquoted and stored frozen at −20 °C for short-term storage (<6 months) and −80 °C for long-term storage (>6 months).

2.3. Recombinant antigens

Clones producing recombinant M. avium subsp. paratuberculosis proteins, were generated using methods previously described by Nagata et al. (2005) and Reichel et al. (1999). Briefly, all recombinant fusion proteins except Map3968, Map3184 and Map1518 contained maltose-binding protein (MBP) as the tag used in affinity purification. Map3968 (HBHA) was cloned into the pET15 his-tag expression plasmid as described previously by Sechi et al. (2006a) and Map3184 (Map39) and Map1518 (Map41) were constructed as described by Nagata et al. (2005). The MBP fusions were produced by cloning the M. avium subsp. paratuberculosis gene of interest into the pMAL-c2 expression vector (New England Biolabs, Beverly, MA, USA). The upstream and downstream oligonucleotides for each amplification used in the pMAL-c2 cloning are listed in Table 2. After overnight ligation at 16 °C, the products were transformed into competent E. coli DH5α (Invitrogen, Carlsbad, CA, USA). Constructs from selected transformants in each experiment were authenticated by DNA sequencing. Each fusion protein was overexpressed using 0.3 mM IPTG and purified by either maltose affinity chromatography using an amylase resin supplied by New England Biolabs or by nickel chromatography using standard protocols (1 mM IPTG for HBHA antigen). Detailed methods used for the induction and affinity purification of MBP/Map fusion proteins are previously described by Bannantine and Paustian (2006).

2.4. ELISA assay

An indirect ELISA was optimized by using 5 μg/ml of M. avium subsp. paratuberculosis recombinant antigen, a checkerboard dilution pattern of randomly selected sera were obtained from all 11 infected sheep and pooled sera from 10 healthy sheep versus various concentrations of Map antigens and 1:2000 labeled secondary antibody. These conditions were chosen after checkerboard titration to achieve the best signal:noise ratio. Flat-bottom 96-well plates (Maxisorp; Nunc-Immunoplate, Roskilde, Denmark) were coated with 50 μl of each antigen diluted in 0.05 M Carbonate–Bicarbonate Buffer, pH 9.5 (Sigma–Aldrich) and
incubated overnight at 4 °C. Next day, plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T washing buffer) and blocked with 200 μl of 1% bovine serum albumin (Sigma–Aldrich) in PBS-T for 1 h at room temperature (RT). Sera from sheep with paratuberculosis and controls were diluted in PBS-T and added to each well. After 2 h of incubation at RT, plates were washed four times with PBS-T and 100 μl of optimally diluted (1:2000) conjugated anti-sheep immunoglobulin G (IgG)-alkaline phosphatase antibody (Sigma–Aldrich) was added to wells followed by 1 h of

### Table 2
*Mycobacterium avium* subsp. *paratuberculosis* recombinant proteins

<table>
<thead>
<tr>
<th>Genea</th>
<th>Gene sizeb</th>
<th>Amino acids</th>
<th>Functional category</th>
<th>Primers used to clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Map0852</td>
<td>546</td>
<td>All 181</td>
<td>No hits</td>
<td>For: ATCCTTAGAtcgatatcggggtggcgaagttg&lt;br&gt;Rev: GCAGCAAGCTTcaacctctccccacccctcag</td>
</tr>
<tr>
<td>Map0853</td>
<td>660</td>
<td>All 219</td>
<td>Hypothetical protein</td>
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</tr>
<tr>
<td>Map0855</td>
<td>926</td>
<td>308</td>
<td>No hits</td>
<td>For: ATCCTTAGAtcgatatcggggtggcgaagtt&lt;br&gt;Rev: GCAGCAAGCTTcaacctctccccacccctcag</td>
</tr>
<tr>
<td>Map0857c</td>
<td>318</td>
<td>All 105</td>
<td>Hypothetical protein</td>
<td>For: ATCCTTAGAtcgatatcggggtggcgaagtt&lt;br&gt;Rev: GCAGCAAGCTTcaacctctccccacccctcag</td>
</tr>
<tr>
<td>Map0862</td>
<td>989</td>
<td>329</td>
<td>No hits</td>
<td>For: ATCCTTAGAtcgatatcggggtggcgaagtt&lt;br&gt;Rev: GCAGCAAGCTTcaacctctccccacccctcag</td>
</tr>
<tr>
<td>Map0865</td>
<td>1272</td>
<td>All 423</td>
<td>Hypothetical 30.9 kDa protein</td>
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<td>Map0961c</td>
<td>719</td>
<td>239</td>
<td>Hypothetical protein</td>
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<tr>
<td>Map1087</td>
<td>441</td>
<td>All 146</td>
<td>Probable peptide transport permease</td>
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<td>Map1518</td>
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<td>Map1643</td>
<td>2277</td>
<td>759</td>
<td>Isocitrate lyase (beta) module</td>
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<td>Map2116c</td>
<td>1269</td>
<td>All 422</td>
<td>Cell invasion protein (mce3)</td>
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<td>Map2157</td>
<td>1221</td>
<td>All 406</td>
<td>IS900</td>
<td>For: ATCCTTAGAtcgatatcggggtggcgaagtt&lt;br&gt;Rev: GCAGCAAGCTTcaacctctccccacccctcag</td>
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<td>Map2737</td>
<td>723</td>
<td>All 240</td>
<td>Dihydroxyphthalate decarboxylase (flhI)</td>
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<tr>
<td>Map3184</td>
<td>1175</td>
<td>391</td>
<td>PPE-family protein</td>
<td>For(^c)&lt;br&gt;Rev(^c)</td>
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<tr>
<td>Map3786</td>
<td>1086</td>
<td>362</td>
<td>Unknown hydrophobic protein</td>
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</tr>
<tr>
<td>Map3833c</td>
<td>626</td>
<td>208</td>
<td>Hypothetical protein</td>
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</tr>
<tr>
<td>Map3840</td>
<td>1872</td>
<td>All 623</td>
<td>70 kDa heat shock protein</td>
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</tr>
<tr>
<td>Map3968</td>
<td>588</td>
<td>195</td>
<td>Heparin binding hemagglutinin protein</td>
<td>For: ATGAAAGGAA&lt;br&gt;Rev: CTACTTCTGGgtgaccttcttggc</td>
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<tr>
<td>MBP-LacZ</td>
<td>1389</td>
<td>462</td>
<td>Control protein</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>

\(^a\) The *M. avium* subsp. *paratuberculosis* genome sequence project designation (Li et al., 2005).

\(^b\) Gene size is reported in base pairs.

\(^c\) Nagata et al. (2005).
incubation at RT. Plates were washed four times in PBS-T and 200 μl of p-nitrophenylphosphate (SIGMA FAST™ pNPP tablets Sigma–Aldrich) was used as substrate for alkaline phosphatase (AP) enzyme. Plates were incubated at 37 °C in the dark until color developed and were read three times at 405 nm wavelength at 2-min intervals by using VERSATunable Max microplate reader (Molecular Devices, USA). Negative sera and antigen controls were included in each plate. Where the antigen of interest was fused with the affinity tag maltose binding protein (MBP), sera were tested against the LacZ-MBP control to evaluate seroreactivity. Results were expressed as optical density (OD) values.

2.5. Agar gel immunodiffusion (AGID)

The test was performed according to Whittington et al. (2003).

2.6. Histology

The histology methods used in this study were described previously by Sechi et al. (2006a). Staining of tissues was done using Ziehl–Nielsen stain.

2.7. Statistical analysis

Statistical analysis was performed by the Chi-square test with Bonferroni’ correction.

2.8. Fecal culture

Fecal samples were collected from the rectum with single-use, clean obstetric sleeves and immediately transferred to sterile whirl pack bags. Two grams of feces were mixed with 35 ml of sterile water, shaken vigorously and placed in a mechanical rocker for at least 30 min. The suspension was then allowed to stand at RT (room temperature) for another 30 min, after which 5 ml of supernatant were mixed with 0.75% hexadecylpyridinium chloride in brain heart infusion broth and incubated overnight at 35–37 °C for decontamination. The samples were then centrifuged at 900 × g for 30 min. After being processed and concentrated, the pellets resuspended were analysed for the presence of acid-fast bacilli after Ziehl–Nielsen staining of smears. The pellet resuspended in 1 ml of antibiotic mix containing amphotericin B (100 mg/ml), nalidixic acid (100 mg/ml) and vancomycin (50 mg/ml), and incubated overnight at 35–37 °C for final fungal decontamination. The samples were then divided in two parts used for DNA extraction and for inoculation into the MGIT 960 non-radioactive culture system (Becton Dickinson) where Mycobactin J and Egg yolk emulsion (20 μl) had previously been added. The medium was then incubated into the MGIT instrument until growth was detected. All samples were inoculated also in four tubes of Herrold egg yolk medium (HEYM) (three with Mycobactin J and one without Mycobactin J) and MGIT 960.

2.9. PCR amplification

A PCR targeting IS900 was designed. Primers p89 and p92, described in a previous work (Sechi et al., 2005) were used to amplify a Map specific 284 bp fragment. The reaction mixture (final volume of 50 μl) comprised primers at a concentration of 0.5 μM. Expand High fidelity reaction buffer 1×, 200 μM (each) dNTPs and 3 U of Expand High Fidelity Taq polymerase (Expand High Fidelity PCR system; Roche, Lewes, United Kingdom). Cycling conditions were 1 cycle of 94 °C for 3 min and 36 cycles of 94 °C for 40 s, 62 °C for 40 s and 72 °C for 40 s. Followed by a final step of 72 °C for 5 min. Amplified fragments were visualized with ethidium bromide on 2.5% agarose-1000 gel (Life Technologies, Grand island, NY, USA) and purified with a Qiapquick and Qiagen gel extraction kit (Quiagen, Crawley, United Kingdom).

3. Results

3.1. *M. avium* subsp. *paratuberculosis*-infected sheep

Progression of Johne’s disease was monitored in sheep for up to 1 year. Of the 11 infected sheep enrolled in this study, all fecal PCR positive for Map, six succumbed to Johne’s disease before the end of the study (Table 1). Four of these six animals (1109, D1, 255 and 245) showed progressive weight loss and diarrhea. Detailed necropsy examinations were conducted on tissues taken from these animals and intestinal lesion type was categorized as either paucibacillary (tuberculoid) or multibacillary (lepromatous), depending on the presence and abundance of acid-fast bacilli in histological sections of the small intestine and terminal ileum (Fig. 1).

3.2. Recombinant proteins

A total of 18 *M. avium* subsp. *paratuberculosis* recombinant proteins were used in this study (Table 2).
Nine of these represent hypothetical proteins that have no assigned function. Five of these nine hypothetical proteins (Map0852, Map0853, Map0855, Map0857c and Map0862) contain no similarity with other mycobacteria or any sequence in public databases, making them potentially unique diagnostic proteins (Table 2). The remaining 10 genes encode a heat shock protein (Map3840), putative virulence functions (Map1518, Map1643, Map2116c, Map3184 and Map3968), IS900 element (Map2157) and metabolic functions (Map0961c, Map1087 and Map2737). As a control, the MBP-LacZ peptide was used in all assays.

3.3. Evaluation of recombinant proteins by ELISA

All 18 recombinant proteins were tested for antibody reactivity in sera from 11 infected sheep collected over a 1-year time period. In addition, each protein was analyzed with pooled sera from 10 healthy sheep to assess non-specific reactivity (Table 3, organized by statistical significance). Finally, the MBP-LacZ control protein was used to determine if any reactivity was associated with the maltose binding protein affinity tag. When ELISA data were arranged for individual animals, sheep 762 showed the strongest responses to most of the antigens with 16 of 18 proteins. This animal was among the group that succumbed to JD before the 1-year study period ended (Table 1); however, not all animals that died showed strong antibody responses to these proteins. The weakest serology among the dead sheep was of animal number 1109, which responded to only 3 of the 18 proteins. The pool of sera from healthy animals gave a consistent low response when compared to the response of infected sheep (Table 3).

The M. avium subsp. paratuberculosis-specific protein encoded by Map0862 was the strongest antigen in 3 of the infected sheep (H65, 1127 and 1197) and among the antigens which were recognized differentially by the sheep (Table 3). Furthermore, OD_{405 nm} readings above 0.4 for Map0862 were obtained for 6 of the 10 infected sheep. The Map1518 and Map2116c recombinant gene products appeared to be the poorest antigens with observed OD_{405 nm} readings in the range of that observed for the MBP-LacZ control protein (Table 3). No single recombinant protein was consistently the strongest antigen among all the animals. An arbitrary cut-off of 0.3 was selected for the antigens that reacted with the control sera with a median OD_{405 nm} between 0.1 and 0.22 whereas an arbitrary cut-off of 0.4 was selected for the antigens that reacted with the control sera with a median OD_{405 nm} between 0.23 and 0.34. Map2737 and Map3786 were the strongest antigens differentially recognized. Map2737...
encodes for a dihydroxyphthalate decarboxylase which is present in different mycobacteria and other species so it will be difficult to use it as a specific antigen. Map3786 also responded to control cows (Bannantine, personal communication) and thus was not considered further.

3.4. Temporal analysis of humoral immune response in sheep

In general, antibody responses varied considerably over the course of the study period. Antibody levels for recombinant proteins generally did not show an increase over the course of time, but rather showed a spiking effect where there was a marked increase followed by a decrease (Fig. 2). Animal H58 in particular revealed this spiking effect at three different stages over the course of the year with several different recombinant proteins (Fig. 3). These data emphasize the importance of repeated sample collection for immunological assays used to detect JD.

4. Discussion

Antibody responses in sheep with Johne’s disease have previously not been examined using defined recombinant proteins. This study represents initial efforts to characterize the ovine JD antibody response to selected "M. avium" subsp. "paratuberculosis" proteins and identify novel antigens in this host. The "M. avium" subsp. "paratuberculosis" genome has revealed a total of 35 predicted coding sequences that are not present in available sequence databases, suggesting that these genes are unique and specific to "M. avium" subsp. "paratuberculosis" (Bannantine et al., 2002). Of these 35 genes, 6 were evaluated in this study and a major finding is that 2 of these show promise as diagnostic antigens in sheep (Map0862 and Map0865). Interestingly, Map0862, along with Map0865, are present uniquely in "M. avium" subsp. "paratuberculosis" (Paustian et al., 2005) and are part of a large 15.3 kb insertion known as LSP 4 (Semret et al., 2005). Antibodies against Map0862 have been previously observed in infected cattle (Paustian et al., 2004) while Map0865 has not been evaluated for immunogenicity in this host. The results from the present study in sheep also suggest Map0862 to be a good antigen as it was detected in 91% of the infected animals. Both Map0862 and Map0865 have no known functions predicted from sequence similarity searches but the results from this study suggest that they are clearly expressed by "M. avium" subsp. "paratuberculosis" during infection.

Therefore, in the light of the findings from this ovine study and those previously carried out using

Table 3
Comparison of pooled samples from infected and uninfected animals arranged by statistical significance

<table>
<thead>
<tr>
<th>Antigen</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
<th>C&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cut-off</th>
<th>P-value&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Corrected P-value&lt;sup&gt;d&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Map2737</td>
<td>0.44071</td>
<td>0.185</td>
<td>0.3</td>
<td>0.0001</td>
<td>0.0018</td>
</tr>
<tr>
<td>Map3786</td>
<td>0.60847</td>
<td>0.344</td>
<td>0.4</td>
<td>0.0001</td>
<td>0.0018</td>
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<td>Map0852</td>
<td>0.47699</td>
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<td>0.3</td>
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<td>0.28804</td>
<td>0.4</td>
<td>0.002</td>
<td>0.036</td>
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<td>Map0862</td>
<td>0.55213</td>
<td>0.25649</td>
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<td>0.002</td>
<td>0.036</td>
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<td>Map0865</td>
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<td>0.23558</td>
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<td>Map3833c</td>
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<td>0.25887</td>
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<tr>
<td>Map1518</td>
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<td>0.24167</td>
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<sup>a</sup> P = OD<sub>405</sub> mean values of ovines with paratuberculosis.
<sup>b</sup> C = OD<sub>405</sub> mean values of controls.
<sup>c</sup> P-value obtained from Chi-square test.
<sup>d</sup> P-value obtained from Chi-square test with Bonferroni’s correction.
cattle (Paustian et al., 2004), Map0862 appears to be a strong prospect as an ideal diagnostic antigen in an ELISA-based test. However, we advise that additional proteins must be evaluated in a similar manner alongside Map0862 before it may be considered the best antigen. Moreover, antigens encoded by Map0862 and Map0865 were highly recognized among the animals that died regardless of their degree of intestinal lesions and presence of diarrhea (Table 1). While at the same time, not all living animals had strong antibody response to these antigens. For example, animal H58 did not recognize the Map0862 recombinant, but animals K39 and 1127, which showed no diarrhea, no animal leanness, no cachexia or fecal shedding had a strong response to this protein. While further studies using, additional animals are clearly needed, these findings nonetheless lay down a solid foundation towards the development of feasible ELISA-based assays.

Fig. 2. Temporal analysis of antibody reactivity with selected recombinant proteins. ELISA data shown are OD_{405 nm} readings obtained from serum samples collected from three individual sheep during the 1-year study period.
5. Conclusions

Unfortunately, no single antigen was found to be immunodominant among all the infected animals, and perhaps more importantly, no single antigen showed a consistently strong antibody response over the 1-year study period in any single animal. These data reinforce the need to incorporate multiple antigens in future antigen-based diagnostic tests, not only to ensure all infected animals are detected, but to increase the chance of detecting antibodies to \textit{M. avium subsp. paratuberculosis} at any time during the infection. Data from this study suggest that an antigen mixture prepared for ELISA that contained proteins encoded by Map2737, Map0862, Map0865 and Map0852 should detect all infected animals in this study. But even this cocktail may not be sufficient to detect all infected sheep in a larger cohort study or in large sheep herds, especially if collecting only a single blood sample. Immunogenity of Map proteins during natural infection resulted, in some animals, in fluctuation of positive to negative results which could represent an actual fluctuation in antibody production by the sheep. Considering that fluctuation of antibody detection are a potential source of false-negative results, multiple tests over time increase the chance of detection of an infected animal.

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References


