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REVIEW

An update on *Mycobacterium avium* subspecies *paratuberculosis* antigens and their role in the diagnosis of Johne's disease

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Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is responsible for Johne's disease (JD) or paratuberculosis. Diagnosis of MAP infection by measuring host cell-mediated and humoral immune responses has been a major focus in MAP research. For this purpose, several MAP antigens such as secreted protein, cell envelope protein, cell-mediated immune and lipoprotein antigens have been identified and tested to measure their diagnostic utility with varying degree of success. Identifying the optimal antigen or antigen combinations for diagnosis of infected animals is hindered by the complex nature of the disease, prolonged subclinical infection, the differential expression of antigens and scarcity of well characterized MAP-specific epitopes making selection of a single MAP antigen very difficult. Thus, multiplexing of antigens with larger scale and longitudinal studies may lead to development of cost-effective next generation serodiagnostics. This mini review focuses on the role of different MAP antigens in the diagnosis of JD.

Keywords Johne's disease · Cell-mediated · Secreted antigens · Cell envelope antigens

Introduction

Johne's disease (JD) or paratuberculosis (Ptb) caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is an incurable, chronic, granulomatous enteritis of ruminants and other animals (Tripathi 2005). The disease is prevalent in world-wide and incurs significant economic losses to cattle and small ruminant industries due to reduced body weight gain, premature culling, reduced carcass value, lowered productivity and cost of stock replacement (Barkema et al. 2018; Garcia and Shalloo 2015). In addition, possible involvement of MAP in human Crohn's disease and other autoimmune diseases is a great public health concern (Slavin et al. 2018). Susceptible young animals, especially neonates and under 6 months of age are infected via ingestion of MAP through the fecal–oral route and it takes several years to progress into clinical disease. During this long subclinical phase, infected animals are apparently healthy, shed MAP intermittently in the feces, thereby spreading the infection to young susceptible animals. Differentiating these infected animals from non-infected animals is very difficult due to lack of sensitive and specific diagnostic assays (Li et al. 2017). Nonetheless, timely diagnosis and subsequent implementation of management practices are vital to the control of JD.

Currently, JD is diagnosed by detection of MAP nucleic acid in the clinical samples by polymerase chain reaction, by isolation of organisms in the feces and by measuring the host immune responses (Sonawane and Tripathi 2013). Among these, diagnosis of MAP infection by measuring host immune responses such as cell-mediated and humoral immune responses is a major focus of MAP research. For technical reasons, immune response based tests are easy to perform, adaptable and become part of routine laboratory diagnosis. Serological tests particularly ELISAs are low cost, highly specific and sensitive, easy to perform and rapid (Chaubey et al. 2016). A common feature of the current JD ELISAs, is the use of crude MAP cellular extracts, lipoarabinomannan and surface proteins as antigens (Pradenas et al.

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2009). In order to improve the sensitivity and specificity of these assays, identification of well defined antigens is necessary. Moreover, these antigens should be MAP-specific and to be able to detect animals in the subclinical stages of infection when MAP shedding and immune responses are not so evident. Several MAP antigens have been discovered but only a few have been characterized and tested for their clinical utility (Mikkelsen et al. 2011b). In this short communication, we review major MAP antigens with their diagnostic potentiality.

Secreted antigens

Secretory proteins of MAP play a vital role in bacterial virulence, intracellular survival and proliferation in macrophages by altering the host immune response and signal transduction pathways (Slavin et al. 2018). Also, secreted proteins are considered to be more immunogenic due to their interactions with host antigen-receptors of the innate and adaptive immune responses and their availability to cells of the immune system. In a study, immunoblot analysis of serum samples from JD test-positive animals by fecal culture showed higher immunoreactivity to secreted proteins than to proteins of intracellular origin (Cho and Collins 2006). A total of 14 antigenic secretory proteins were identified by 2-DE immunoproteomic analysis and these antigens could be useful for the development of improved serodiagnostics for JD (Cho et al. 2006). Serum samples from a JD clinical case screened against a genomic expression library led to identification of three secretory immunogenic proteins, namely; MAP2609, MAP2942c and MAP0210c. Subsequent analysis with a limited number of serum samples from MAP-positive cases revealed that these proteins were able to bind to MAP antibodies (Willemsen et al. 2006). MAP culture filtrate analysis by proteomic approaches identified a total of 121 proteins. From these proteins, 25 were considered to be specific to MAP (Leroy et al. 2007). Five proteins (MAP1693c, MAP2677c, MAP3199, MAP0586c and MAP4308c) were further tested in ELISA with a panel of serum samples from MAP test-positive (n = 19) and test-negative (n = 48) cases. Three (MAP1693c, MAP4308c and MAP2677) out of five proteins showed a sensitivity (Se) and specificity (Sp) of 73.68% and 91.67%, respectively (Leroy et al. 2007).

Antibody responses against Ag85A (MAP0216), Ag85B (MAP1609c), Ag85C (MAP3531c) and SOD (MAP0187c) recombinant protein antigens were tested in serum samples from cattle (n = 80). MAP0216, MAP1609c, MAP3531c and MAP0187c respectively showed diagnostic sensitivity of 66.7, 71.7, 68.3 and 80.8% and diagnostic specificity of 81.8, 86.4, 81.8 and 90.9% (Shin et al. 2004). An ELISA using MAP culture filtrates protein antigens was used to test 856 positive and 359 negative cattle sera. The test yielded a higher sensitivity (56.3%) in comparison to five commercially available paratuberculosis ELISAs and the specificity (99%) was equivalent to commercial ELISA kits. This finding revealed that MAP secretory antigens might be useful in the development of low cost and high-throughput serodiagnostic test to support paratuberculosis control program (Shin et al. 2008). Another study in which MAP culture filtrate was analyzed against serum (n = 14) samples from JD test-positive cattle by fecal culture and ELISA showed that proteins of various molecular weights including 28 kDa [i.e. two co-migratory proteins (MAP1981c and MAP0471)], 47 kDa [i.e. two co-migratory proteins (MAP0196c and MAP1569)] and 52 kDa (MAP0196c) were found to be strongly immunogenic and incorporation of these antigens in an ELISA may aid in JD diagnosis and control (Facciuolo et al. 2013).

Five recombinantly expressed stress-associated secretory proteins including MAP2411, MAP2281c, MAP0435c, MAP0593c and MAP1027c were tested for their immunogenicity by ELISA with sheep serum from JD test-positive and healthy controls, proved inconclusive (Gumber et al. 2009). PtpA (MAP1985) and PknG (MAP3893c) are secretory proteins, essential for MAP survival and are immunogenic (Slavin et al. 2018). Bovine sera from farms with high and low MAP prevalence herds, and farms that were JD test-negative were assessed for antibodies against PknG (MAP3893c) recombinant protein in an ELISA format. Results showed that specific antibody responses were only detected in cattle from infected herds (Bach et al. 2018b).

Another study evaluated serodiagnostic utility of recombinant MAP PtpA (MAP1985) protein with 1001 MAP-infected and 230 uninfected cow sera samples in an indirect ELISA and results were compared with commercial ELISA. The PtpA ELISA had a diagnostic sensitivity of 22.7%, whereas the commercial ELISA had a sensitivity of 4.5% (Bach et al. 2018a). The study suggested that PknG and PtpA could be useful biomarkers for the serological diagnosis of early stage of MAP. Alanine and proline-rich antigen (Apa- MAP1569) is a secretory protein that has been consistently detected in feces of MAP test-positive cattle by ELISA suggesting that Apa could be a potential immunodiagnostic biomarker for MAP infection (de Souza et al. 2018). Cattle sera from heavy, moderate and low fecal MAP shedders were strongly reactive to MAP secretory proteins of 25, 32, 34, 35, 36, 37, 42 and 45 kDa in 1-DE immunoproteomic analysis suggesting that a cocktail of secretory protein antigens are potential serodiagnostic candidates for JD detection (Pradenas et al. 2009). Serum IgG response against 10 MAP secretory recombinant proteins was tested in calves experimentally infected with MAP, of which 6 recombinant proteins namely; MAP2786c, MAP3428c, MAP0471, MAP2785c, MAP0196c and MAP1718c showed significantly ($P < 0.05$) elevated levels of serum IgG levels

at two month post-infection (Facciuolo et al. 2016). A cocktail ELISA (C-ELISA) containing six recombinant secretory protein antigens were tested on sera from 150 sheep, 125 buffaloes, 300 cattle and 723 goats and results were compared with indirect ELISA with semi purified protoplasmic antigens. The cocktail ELISA showed diagnostic sensitivities (Se) of 98.8, 94.6, 95.9 and 98.7% in sheep, buffaloes, cattle and goats, respectively, and diagnostic specificity (Sp) was 100% in four ruminants studied (Chaubey et al. 2018).

Based on various studies, multiplexing of native MAP secretory protein antigens for the development of serological diagnosis is a viable option. However, it involves handling of live MAP cultures for secretome preparation that could be an occupational hazard to laboratory workers. Lack of standard procedures and protocols for optimizing and /or generating culture filtrate proteins could affect the quality and quantity of the secretory protein preparation, which can affect the test results. For instance, a study compared the secretory protein preparation from Middlebrook 7H9-OADC (7H9) versus Watson and Reid (WR) media and reported that protein preparation from WR medium was of better quality and more immunogenic than those prepared from 7H9 medium (Pradenas et al. 2009). Greater resources are required to obtain sufficient quantities of secretory proteins to develop serological diagnostics on a larger scale, which would be another issue that needs to be addressed.

Cell envelope antigens

Cell envelope proteins (cell wall and membrane) play critical roles in the bacterial pathophysiology during attachment, colonization and infection of target tissues/cells. As such, these cell envelope proteins are accessible to the host immune system and are considered to be very immunogenic (Leite et al. 2015). A study that screened a set of monoclonal antibodies generated against MAP identified several cell membrane antigens including MAP1025 (proline rich antigen), MAP1272c (NlpC/P60 domain protein), MAP2698c, MAP2121c (major membrane protein) and MAP4145 that might be useful in the development of point of care diagnostics for Johne's disease (Bannantine et al. 2018). A panel of sera from MAP infected (n = 30) and non-infected (n = 30) cattle was tested with MAP1197 antigen in an ELISA (MapechA ELISA) and it had a 96.7% of diagnostic sensitivity and specificity (Nagata et al. 2013). Moreover, serum antibodies against MAP1197 were detected in calves experimentally infected (8 wks PI) with MAP without any cross reactivity against *M. avium* hyper-immune sera (Nagata et al. 2013). An ELISA using recombinant MAP3968 detected higher levels of antibodies in serum samples collected from MAP PCR test-positive animals than test-negative animals (Sechi et al. 2006). However, MAP3968 elicited cross reactivity with *M. bovis* test-positive animals, which may affect the diagnostic specificity of this antigen. A total of 57 outer membrane proteins (OMPs) of MAP have been identified by computational approach, of which 43 were species-specific to MAP and contained B and T cell epitopes. These novel antigens may be useful in the development of rapid diagnostic tests (Rana et al. 2015).

A study involving cell surface "shaving" of live MAP cells with trypsin and subsequent proteomic analysis to identify proline–proline–glutamic acid protein families (PPE) on the MAP cell wall revealed two PPE proteins (MAP1506 and MAP3420c) that were surface-exposed. MAP3420c was found to induce humoral immune responses in MAP-infected animals (Newton et al. 2009). Ten antigenic MAP envelope proteins (Leite et al. 2015) were identified using blue native PAGE and subsequent 2-DE PAGE analyses. Five of these proteins (MAP2121c, MAP2120c, MAP0630c, MAP2855 and MAP3290c) reacted strongly in immunoblotting and an IFN-γ release assay using serum and whole blood samples from Holstein dairy cows naturally infected with MAP (Leite et al. 2015). Other studies on animals experimentally and naturally infected with MAP showed strong humoral immune responses to two cell wall proteins namely; MAP1204 and MAP1087 (Bannantine et al. 2008). However, these antigens were not MAP species-specific and showed cross-reactivity with serum samples from *M. tuberculosis*-positive cases (Bannantine et al. 2008).

An ELISA developed with a recombinant immunodominant 34 kDa extracellular protein, which is MAP species-specific correctly identified 18 MAP-positive and 50 MAP-negative cattle (Malamo et al. 2006). Analysis of immunogenicity of 3 recombinantly expressed MAP purified protein derivatives (PPD) i.e. MAP1138c, MAP1718c and MAP3515c revealed that MAP1138c had greater serum antibody response in JD test-positive than in test-negative animals, despite being conserved among the mycobacterial species (Santema et al. 2009). Proteomic characterization of purified protein derivatives from MAP (Johnin PPD or PPDj), *Mycobacteriumavium* subspecies *avium* (PPDa) and *Mycobacteriumbovis* (PPDb) showed that PckA (MAP3646), Mdh (MAP2541c), Tig (MAP2282c) and Eno (MAP0990) proteins were specific to PPDj, however, their immunogenicities were not evaluated (Wynne et al. 2012).

Recently 15 MAP-specific cell envelope protein antigens (MAP3698c, MAP0150c, MAP3651c, MAP2541c, MAP0827c, MAP2452c, MAP1164, MAP4129, MAP2121c, MAP0614, MAP1205, MAP1609c, MAP2698c, MAP0035, MAP1233) were identified by comparative proteomic analysis but their diagnostic utility was not tested (Karuppusamy et al. 2018). ELISA assessment of serum samples from 180 cows for the diagnostic utility of four MAP antigens (MAP2942c, MAP2609, MAP1569 and MAP1272c) revealed diagnostic sensitivities between 48.3 and 76.7%,

and specificities between 96.7 and 100% (Li et al. 2017). Furthermore, a cocktail of these antigens showed a diagnostic specificity of 96.7% and a sensitivity of 88.3% (Li et al. 2017). Another study screened MAP-specific coding sequences and found that 87 sequences were specific to MAP. Among these, three sequences were recombinantly expressed and evaluated in an ELISA format with 18 JD-positive and 48 negative serum samples to measure MAP-specific antibodies. Two of these recombinant proteins namely antigen 6 and MAP1637c showed a sensitivity of 72 and 82%, respectively with a specificity of 98% for both (Leroy et al. 2009). Although, cell envelope antigens are promising candidates for the development of rapid, sensitive and specific diagnostic tests, there are certain technical and practical difficulties in the preparation of antigens. These include: (i) sub-cellular fractionization to isolate the envelope is very tedious due to the complex nature of the mycobacterial cell, (ii) lack of availability of specific markers to assess the purity of envelope fractions to rule out significant contamination from other cellular fractions and (iii) MAP is a very slow growing pathogen, therefore, it is difficult to purify sufficient quantities of cell envelope antigens in a large scale to develop a screening test for JD.

Lipoprotein antigens

Lipid conjugated macromolecules are major constituents of the mycobacterial cell wall and are accessible to the host immune system for producing antibodies against them. For example, the lipoarabinomannan and lipid-free arabinomannan are strong immunogenic macromolecules on the MAP cell wall, and are also widely conserved in mycobacteria (Olsen et al. 2002). Lipopentapeptide (L5P) is MAP species-specific and immunogenic. To support this contention, L5P was tested with serum samples from MAP or, *M. bovis* or *Mycobacterium avium* subsp. *avium* (MAV)-infected cattle and MAV and *M. intracellulare* (member of *M. avium* complex)-positive human cases. Results revealed that L5P was more sensitive and specific in a diagnostic test of MAP infection in comparison to a MAP-PPDj-based diagnostic test (Biet et al. 2008). Lipid Para-LP-01 is a major lipopeptide component of MAP cell wall that shows seroreactivity with MAP-positive serum samples (Eckstein et al. 2006).

PstA (MAP1242), another MAP-specific lipoprotein is involved in MAP invasion, biofilm formation and immunogenicity. Immunoblot analysis of serum from a cow subclinically infected with MAP strongly reacted with synthetic PstA peptides in comparison to cows in clinical stages of JD, whereas serum from uninfected cows were non-reactive (Wu et al. 2009). This antigen appears to have potential for its use in the diagnosis of subclinical cases of MAP-infected animals. The diagnostic utility of L5P, Para-LP-01 and PstA needs to be thoroughly evaluated with appropriate sample sizes (Mikkelsen et al. 2011b). Lipoproteins are present in significant amounts in mycobacterial cells, for instances in *M. tuberculosis*, it accounts for 2.5% of total proteome (Becker and Sander 2016). Lipid-conjugated macromolecules are cell wall anchored in the mycomembrane or to the cytoplasmic membranes and can be extracted from the lysed cells using detergents or extensive washes with organic solvents to remove the impurities (Nakayama et al. 2012). Purification is often difficult with low yields and thus would affect the consistency, quality and quantity of lipoproteins for the development of diagnostic assays. Alternatively, these protein antigens may be recombinantly expressed, but retention of antigenicity and post translational modifications in comparison to the native lipoproteins could be an issue. Nevertheless, additional studies are needed to explore the utility of MAP lipoproteins for the development of diagnostics.

Cell-mediated immune antigens

In general, CMI-based assays suffer from low sensitivity and specificity due the antigens used in the assay and most studies have been conducted with purified protein derivatives (PPDs). Stimulation of whole blood samples with a PPD preparation from *M. avium* and a subsequent IFN-γ release assay had a sensitivity of 36.8% and58.3% for focal and multifocal lesions of MAP-infected animals, respectively (Vazquez et al. 2013). The IFN-γ release assay had very low specificity (68.9% to 76.4%) in the identification of MAP infection suggesting that measurement of CMI response is not adequate for detection of MAP-infected animals (Vazquez et al. 2013). Several studies have been conducted with more specific antigens to measure the CMI response. MAP specific recombinant protein antigens MAP3184 and MAP1518 were used to stimulate peripheral blood mononuclear cells (PBMCs) isolated from experimentally MAP infected and non-infected cattle to measure IFN-γ and both proteins induced strong IFN-γ responses in infected animals compared to controls (Nagata et al. 2005). The diagnostic potential of these candidate antigens should be determined in larger scale studies with field samples. Fourteen novel recombinant proteins were tested to measure CMI responses in blood samples and their specificities in the diagnosis of early stages of MAP infection in 26 heifers from a MAP-infected herd (Mikkelsen et al. 2011a). Of the 14 recombinant proteins, three latent proteins including putative carbonate dehydrogenase (LATP-1), putative PhiRv2 prophage integrase (LATP-2), and a hypothetical conserved protein (LATP-3) showed specific and consistent IFN-γ responses in MAP-infected animals (Mikkelsen et al. 2011a).

Studies on immunogenicity of 30 MAP-specific proteins for the induction of CMI responses revealed that three proteins, viz MAP1297, MAP1365 and MAP3651c induced high IFN-γ levels in subclinically infected sheep in comparison to the healthy animals (Hughes et al. 2013). Olsen et al. (2000) used MAP alkyl hydroperoxide reductase C and D (Ahp C-Map1589c and D-MAP1588c) to strongly induce IFN-γ production and MAP-specific CMI suggesting their potential utility in testing for JD (Olsen et al. 2000). Two MAP-specific recombinant protein antigens namely; MAP3547c and MAP0586c were tested to measure their diagnostic potential in an IFN-γ release assay in blood samples collected from MAP-infected, -negative and *M. bovis*-infected herds. MAP3547c and MAP0586c recognized 25.6% and 39.0% of animals, respectively, from the MAP-infected group of animals above 18 months old (Dernivoix et al. 2017). Souriau et al. (2017) tested 21 MAP antigens (five lipids and 16 recombinant proteins) by interferon gamma release assay (IGRA) for diagnosis of MAP infection in goats. Of these, 10 antigens (MAP1653, MAP1589c, MAP3527, MAP3936, MAP0210c, MAP3651c, MAP2020, MAP4000c, MAP1050c and MAP3840) showed a diagnostic sensitivity between 25 and 75% in younger goats (<10-months-old). In 20-month-old goats, seven antigens (MAP1653, MAP1589c, MAP3527, MAP0210c, MAP3651c, MAP2020 and MAP1050c) showed 21–33% diagnostic sensitivity and $\geq 90\%$ specificity (Souriau et al. 2017). Two MAP-specific recombinant proteins i.e. MAP0268c and MAP3651c elicited CMI responses in samples from subclincally infected sheep, and experimentally and naturally infected cattle (Hughes et al. 2017).

Heat shock proteins such as HspX, Hsp65, GroES (MAP4264), Hsp70, MAP3840 and MAP3841 are strongly immunogenic, but the fact that they are conserved in other bacteria make them unsuitable for MAP diagnosis (Olsen et al. 2002). In silico screening of B cell and T cell epitopes of MAP proteins up-regulated under in vitro stress conditions revealed that MAP2698c, MAP2312c, MAP3651c, MAP2872c, MAP3523c, MAP0187c, MAP3567 MAP1168c and a hypothetical protein carry large numbers of B cell and T cell epitopes (Gurung et al. 2012). Some of the epitopes are specific to MAP suggesting their utility in the study of T and B cell-mediated immune responses within infected hosts and serodiagnostic assays (Gurung et al. 2014).

Measuring the CMI could be an additional tool for the diagnosis and control of JD. While, the elevated levels of CMI responses may be an early indicator for MAP infection or exposure, but varied immune response at different time points is a major challenge for the use of this assay as a consistent indicator of MAP infection. This also complicates the interpretation of interferon gamma release assay (IGRA); for instance animal may be test-positive due to recent infection or exposure but infection may or may not progress into clinical cases. Similarly, if animal is test-negative, it doesn't mean that animal is free from infection. Therefore, further longitudinal studies with greater numbers of IGRA test-positive samples in conjunction with fecal culture and ELISAs are needed to confirm the utility of various protein antigens in the diagnosis of MAP infection based on CMI assays. Considering the complex nature of the disease, the prolonged subclinical infection and the differential expression of antigens poses a difficulty in the selection of one type (secreted/cell envelope, lipoproteins/CMI) antigen(s) over others, but a mixture of different types of antigen detecting different stages of MAP infection could be appropriate sensitive for diagnosis of JD. A study involving cocktail of seven different types of antigen showed high diagnostic sensitivity (Mon et al. 2012). Therefore, multiplexing of antigens from different fractions would be more appropriate than a single type antigen. However, isolation of these proteins form MAP in the native form is time consuming and would need lot of resources. Consequently, use of bioinformatics, computational biology and generation of chimeric proteins could be explored to find out additional novel immunogenic antigens.

Conclusion

Several MAP antigens have been identified and evaluated for their diagnostic utility with varying degree of success. Precisely, as of now, more than 800 MAP proteins have been recombinantly expressed to discover diagnostic antigens, of which more than 200 proteins have been tested to measure their antigenicity, which represents only 20% of the MAP proteome (Li et al. 2017). However, to date, no obvious fully characterized and evaluated MAP species-specific antigens are available for either detection of cell-mediated immune response (IFN-γ) or antibody-mediated immune response for diagnosis of MAP infection. This is due to the fact that the majority of MAP antigenic epitopes are shared with other closely related mycobacterial species. As the disease presents immunologic and pathologic spectra, there is no single MAP-specific antigen that could detect all infected animals at different stages of infection, thus posing a considerable challenge in the selection of suitable antigens for incorporation into JD diagnostic tests. Hence, major breakthroughs in diagnostic assays, which could differentiate MAP-infected from non-infected animals in early stages of infection is yet to come. Recent advancement in computational biology, gene editing and synthetic gene biology could appropriately used to identify novel MAP antigens. Upon identification, these antigens could be recombinantly expressed in mycobacteria or other suitable hosts to retain their antigenicity or biological activity. Multiplexing of such antigens in larger scale and longitudinal studies could lead to the development

of cost effective next generation diagnostics for JD for the field use.

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Compliance with ethical standards

Conflict of interest Authors do not have any conflicts of interests.

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