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Research paper

Apa antigen of *Mycobacterium avium* subsp. *paratuberculosis* as a target for species-specific immunodetection of the bacteria in infected tissues of cattle with paratuberculosis

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ABSTRACT

Comparative genomics of *Mycobacterium* spp. have revealed conservative genes and respective proteins differently expressed in mycobacteria that could be used as targets for the species-specific immunodiagnostics. The alanine and proline-rich antigen Apa is a mycobacterial protein that present significant variability in primary sequence length and composition between members of *M. avium* and *M. tuberculosis* complexes. In this study, the recombinant Apa protein encoded by the MAP1569/*ModD* gene of *M. avium* subsp. *paratuberculosis* (Map) was used to generate a panel of monoclonal antibodies which were shown to recognize the most important veterinary pathogens of the *M. avium* complex, specifically Map and *M. avium* subsp. *hominissuis*, and which did not cross-react with *M. bovis* or *M. tuberculosis*. The produced antibodies were demonstrated to be a useful tool for the species-specific immunofluorescence or immunohistochemical detection of Map in experimentally infected cell cultures or intestinal tissues from cattle with bovine paratuberculosis and, additionally, they may be employed for the discrimination of pathogenic *M. avium* subspecies via Western blotting.

1. Introduction

Conserved mycobacterial antigens differently expressed in *Mycobacterium* spp. are a potential targets for immunodiagnosis of important veterinary infections, such as bovine tuberculosis and paratuberculosis, caused by *Mycobacterium bovis* and *Mycobacterium avium* subsp. *paratuberculosis* (Map), respectively. The alanine and proline-rich secreted Ag, Apa, is a conserved mycobacterial protein that presents significant variability in primary sequence length and composition (GenBank's protein database), as well as in post-translational modifications of the protein, in the different mycobacterial species. In a recent study, we demonstrated that the Apa protein secreted by Map is represented by a 50/60 kDa polypeptide dimer, whereas that of *M. bovis* is 45/47 kDa (Gioffré et al., 2009). These structural differences could affect immunogenic and virulence properties of the Apa Ag, which is a fibronectin-attachment protein (FAP) that contributes to the infection of target cells by bacteria (Wieles et al., 1994; Schorey et al., 1996; Secott et al., 2004). Indeed, a strong difference between the response of *M. bovis*-infected animals and that of Map-infected animals against the recombinant Apa of Map (r-Apa-Map)

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showed an important inter-species difference in the immunological properties of this antigen (Gioffré et al., 2009).

The described structural differences of the Apa protein in different mycobacterial species could be used as a target for bacterial detection and identification using specific antibodies. Polyclonal serum produced against the Apa-Map protein was found to be a useful tool for immunodetection of the Apa Ag secreted by different mycobacteria (Gioffré et al., 2009); however, it was unable to differentiate mycobacterial species because of cross-reactivity. The production of monoclonal antibodies (mAbs) against distinct epitopes of the antigen could significantly increase the specificity of Map recognition and improve the currently used immunohistochemical (IHC) method of mycobacterial detection in infected intestinal tissues of paratuberculous cattle. The IHC test using commercially available polyclonal serum against Map was demonstrated to be as sensitive as the in situ hybridization test using the IS*900* probe to detect Map in intestinal tissues, and, additionally, more sensitive and much more specific than Ziehl–Neelsen staining (Delgado et al., 2009). Nevertheless, false positive results could not be completely excluded from the former tests because of the possible cross-reactivity with other mycobacterial species (Cousins et al., 1999; Martinson et al., 2008), such as *M. bovis*, the causative agent of bovine tuberculosis, including its intestinal form. Recent study of intestinal tissue samples randomly collected from slaughtered cattle in an abattoir in Pakistan and analyzed by polymerase chain reaction (PCR) using primers targeting IS*900* and IS*1311* for Map detection and primers targeting genomic DNA fragment specific for *M. bovis* demonstrated that 70% of the intestinal samples positive for acid-fast staining were infected by Map, whereas *M. bovis* was detected in almost 30% of the positive samples (Khan et al., 2010). These observations demonstrate the importance of differential diagnostics of paratuberculosis and intestinal form of tuberculosis in the cattle and necessity of methods to distinguish between the mycobacterial pathogens. The IHC is the method of choice for the specific detection of mycobacteria in situ. The specificity of bacterial recognition could be increased through the production of antibodies that are able to discriminate different species and subspecies of mycobacteria.

In the present study, we aimed to generate mAbs against the recombinant Apa protein of Map (r-Apa-Map), select those specifically able to recognize Map, but not *M. bovis,* and employ these antibodies for the immunodetection of Map in tissue samples from cattle with paratuberculosis.

2. Materials and methods

2.1. Mycobacterial antigens

Recombinant Apa protein of Map (r-Apa-Map) and whole cell lysates (WCL) obtained from bacterial cultures of Map, *M. avium* subsp. *hominissuis* (Mav), *M. tuberculosis* (Mtb) and BCG were used as a mycobacterial Ags. The r-Apa-Map protein was produced in *E. coli* as described previously (Gioffré et al., 2009). WCL Ags were obtained from the following mycobacterial strains: a field strain of Map characterized by IS*900*-PCR and its dependence on mycobactin for growth (Gioffré et al., 2009); a field strain of Mav, strain P104, isolated from infected pig (Oliveira et al., 2003) and *M. bovis* BCG vaccine strain Moreau (from Butantan Institute, Sao Paulo, Brazil). Bacterial cells from an exponential-phase growing mycobacterial cultures were subjected to Fast-Prep (Qbiogene, Solon, OH, USA) bead beater disruption in Lysing Matrix B, and resulted bacterial proteins were precipitated by acetone (Gioffré et al., 2009). Secreted proteins were obtained by filtering the culture supernatant with a 0.22 μm filtration unit Nalgene (Nalge Nunc), precipitated by acetone, resuspended in PBS and kept at −80 °C until use (Gioffré et al., 2009). Biochemically purified Mtb32/Apa *M. tuberculosis* protein (Apa-Mtb), as well as WCL Mtb H37Rv strain reagents and rabbit anti-Apa Mtb serum, were kindly provided by Dr. J. Belisle, Colorado State University (USA), according to the NIH contract HHSN266200400091C.

2.2. Hybridoma generation and Ab production

MAbs and polyclonal sera were produced using standard protocol. Briefly, 6-week-old female BALB/c mice were immunized three times intraperitoneally (i.p.) with the recombinant Apa-Map protein (40 μg per injection) at 14-day intervals. The Ag was emulsified in incomplete Freund adjuvant (Sigma, St. Louis, MO). Three weeks after the last injection, the immune mice were boosted by i.p. injection of 30 μg of r-Apa- Map and spleens were obtained four days later. Alternatively, immune sera were collected seven days later. Spleen cells were isolated from the spleens of immunized animals and fused with NS0 myeloma cells to obtain hybridomas. Ag-specific-Ab-secreting cells were detected by ELISA, cloned at least twice by limiting dilution and grown in DMEM–F12 medium supplemented with 10% FCS (Gibco, BRL, EUA) and as ascite-producing tumors in the peritoneal cavities of pristane-primed BALB/c mice. MAbs containing in hybridoma culture supernatant and ascite fluid were collected and characterized for specificity of Apa Ag recognition by Western blotting technique. The produced mAbs were immunotyped in ELISA using isotype kit I (Pierce, Rockford, IL) to define the heavy chains. All experimental protocols involving animals were approved by the Animal Care and Usage Committee of Universidade Estadual do Norte Fluminense.

2.3. Western blot and immunoprecipitation assay

The protein concentration in the mycobacterial cell extracts (WCL), culture filtrates (CFP) and purified Apa proteins were determined using the Bradford method. The antigens were resolved by 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA) using a Bio-Rad Trans-Blot Cell tank transfer unit at 150 mA for 2 h in buffer, containing 25 mM Tris–HCl (pH 8.0), 0.19 M glycine and 20% (v/v) methanol. Non-specific sites in the blot were blocked by incubation for 1 h with 5% dried non-fat powdered milk in 20 mM Tris–HCl (pH 7.5), 0.5 M NaCl buffer (TBS) at room temperature. The resulted membrane was assayed by using polyclonal murine anti-Apa-Map sera

(1:2000), hybridoma culture supernatants (1:1) or ascite fluid containing anti-Apa-Map Mabs (1:3000) as a primary antibody. After washing, the membrane was treated with a peroxidase-conjugated anti-murine Ig serum (1:2000, Invitrogen, Camarillo, CA, USA). The reaction was revealed in the presence of diaminobenzidine, DAB (Sigma). The molecular size of proteins was determined by comparing it with a standard prestained molecular weight marker (Amersham RPN 800, Piscataway, NJ, USA).

In immunoprecipitation experiments, the CFP or WCL samples obtained from cultures of Map or *M. bovis* BCG, 500 μg, were first incubated with specific anti-Apa Ab, using murine ascite Mab 6F/9 (3 μl) or rabbit anti-Apa Mtb serum (15 μl) followed by protein A/G Plus agarose (Santa Cruz Biotechnology, CA, USA) at 4 °C for 1 h each. After centrifugation, the immunoprecipitates were washed in lysis buffer before addition of 2× gel sample buffer, heated to 95 °C for 4 min, and subsequently analyzed by Western Blot employing 12% SDS-PAGE. Membranes were probed with the murine anti-Apa-Map serum and then with the peroxidase-conjugated anti-murine Ig serum

2.4. Cell culture infection with M. avium and immunofluorescence analysis

Murine Raw 264.7 macrophages were seeded in sterile glass chamber slides (Corning, NY, USA), 5×10^5 cells, and cultured overnight in DMEM–F12 medium supplemented with 10% FCS. The cells were infected with *M. avium* subsp. *hominissuis* strain P104 at a MOI 1:50 and BCG at a MOI 1:10 for 3 h, washed, incubated for additional 72 h and fixed with 4% paraformaldehide for 1 h at room temperature. Fixed cells were permeabilized with 0.5% Triton 20 for 30 min, washed in PBS and stored at 4 °C in PBS until ready to use. The cells were blocked in 2% (wt./vol.) BSA (PBS–BSA) and treated with either anti-r-Apa-Map ascite mAb 6F/9, dilution factor 1:3000, anti-r-Apa-Map murine serum, 1:2000 or with rabbit anti-Apa-Mtb serum, 1:20 for 1 h at 37 °C. The slides were washed, and primary Ab was detected with fluorochrome-conjugated goat anti-mouse IgG or goat anti-rabbit IgG sera (Molecular Probes, OR, USA). The cells were visualized by differential interference contrast (DIC) and fluorescence microscopy using a Axioplan-Zeiss microscope conjugated with AxioCam. The images were analyzed employing AxionVision program (Zeiss, Germany).

2.5. Tissue samples from paratuberculous cattle and immunohistochemical (IHC) analysis

After the necropsy of four cows suspected of paratuberculosis that originated from one herd in the Rio de Janeiro province, Brazil, ileum samples were obtained and submitted to histopathological analysis. All of the suspected animals presented clinical signs of advanced disease: irreversible wasting and diarrhoea. Furthermore, the diagnosis of paratuberculosis in these cases was confirmed by faecal culture and PCR analysis (Rodrigues, 2005; Ristow et al., 2007). Organ specimens were fixed in 10% neutral phosphate-buffered formalin (pH 7.4) and then embedded in paraffin wax by conventional methods. Tissue sections cut at 4 μm were laid on untreated glass slides for Ziehl-Neelsen (ZN) staining or on silan-coated slides (Sigma-Aldrich, MO, USA) for IHC. The ZN staining was performed according to standard methods.

IHC was performed on serial sections of the ileum for the localization of mycobacterial antigens by using an indirect streptavidin–biotin method with a commercially available peroxidase kit (Dako LSAB2 System, peroxidase, Dako, Carpentaria, CA). Briefly, the tissue sections were deparaffinized with xylene and ethanol rinses and endogenous peroxidase activity was blocked via immersion in aqueous solution of 3% hydrogen peroxide (Vetec, RJ, Brazil) for 30 min. Antigenic recovery was performed by incubation of the samples in citrate buffer (2.1 g of citric acid (Vetec, RJ, Brazil) in 1 liter of distilled water, pH 6.0), at 98 °C for 20 min. The sections were saturated with PBS supplemented with 0.02% goat serum and 1% BSA (Sigma–Aldrich, MO, USA) in Tris-buffered saline (TBS) (50 mM Tris–HCl, 150 mM NaCl) for 30 min at 37 °C.

The sections were then incubated with anti-Apa-Map murine serum (1:500) or ascite mAb (1:1000), or with rabbit anti-Apa-Mtb serum (1:20), diluted in TBS with 0.1% BSA, incubated at 4 °C overnight, and then washed by TBS. Ag–Ab complexes were detected with goat anti-mouse IgG biotinylated Abs by incubation for 30 min at room temperature, followed by treatment with streptavidin-peroxidase conjugate kit LSAB-HRP+ (Dako) for 30 min. The peroxidase activity was detected with buffered substrate solution and DAB (Dako). The resulted specimens were counterstained with Harris hematoxylin and mounted with Permount® (Sigma–Aldrich, MO, USA) for histological examination.

3. Results

3.1. Characterization of mAbs produced against the r-Apa-Map protein by a Western blot assay

The fusion of immune splenocytes with NS0 myeloma cells, according to the standard protocols of hybridoma technology, resulted in six stable hybridomas that were positive in ELISA screening against the r-Apa-Map. Isotype analysis revealed that all of the produced hybridomas secreted IgG2a H chain Abs.

In order to verify whether or not the mAbs produced against the recombinant Apa-Map protein were able to recognize the native protein in the Map bacteria, and whether or not this recognition was species-specific, we evaluated the Ag-binding capacity of the produced antibodies, mAbs and polyclonal serum, by Western blot analysis of the native mycobacterial Ags: whole-cell lysates (WCL) of *M. avium* subsp. *paratuberculosis* (Map), *M. avium* subsp *hominissuis* (Mav), *M. tuberculosis* (Mtb), BCG, and purified Apa from Mtb.

As shown in Fig. 1, all of the tested Abs, mAbs (Fig. 1B) and polyclonal serum (Fig. 1A), recognized the recombinant Apa-Map protein as a major dimer of two bands of approximately 50 and 60 kDa. A similar double band was detected in the WCL-Map sample demonstrating that the Abs produced against the recombinant protein were able to bind the native form of the protein in the lysed bacteria. The produced Abs recognized the Apa protein in the WCL-Mav as well, although the pattern of protein recognition

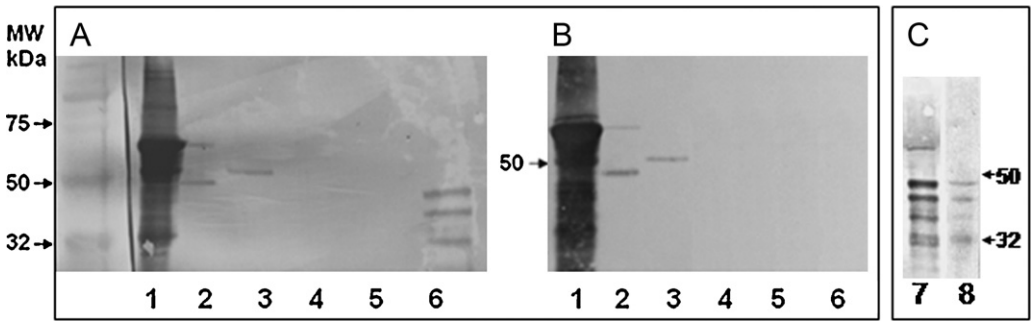


Fig. 1. Recognition of native Apa protein of *M. avium* subsp. *paratuberculosis* (Map), *M. avium* subsp. *hominissuis* (Mav), and *M. tuberculosis* (Mtb) or *M. bovis* BCG by Western blot (WB). Purified Apa proteins or mycobacterial whole cell lysates (WCL) were submitted to WB, and resulted membranes were treated with murine serum (A) or ascite mAb 6F/9 (B), produced against r-Apa-Map protein. (C) The proteins of BCG culture filtrate or BCG-WCL were immunoprecipitated by rabbit anti-Apa-Mtb serum, the precipitates were submitted to WB, and membranes were treated with murine anti-r-Apa-Map serum. 1, r-Apa-Map (1 μg); 2, WCL-Map (20 μg); 3, WCL-Mav (20 μg); 4, WCL-BCG (20 μg); 5, WCL-Mtb (20 μg); 6, purified Apa-Mtb (2 μg); 7, CFP-BCG (500 μg); 8, WCL-BCG (500 μg). MW, molecular weight marker.

was different: only one polypeptide band of 50–55 kDa. These data demonstrate that both polyclonal serum and mAb were able to differentiate *M. avium* bacteria at the subspecies level by the apparent differences in the molecular weight of the Apa polypeptides expressed in these bacteria. In contrast to the *M. avium* results, neither of the tested anti-Apa-Map Abs detected the Apa protein in the WCL obtained from the Mb BCG or H37Rv Mtb strains. Nevertheless, a sample of the purified Apa-Mtb protein was recognized by the anti-Apa-Map serum, but not by the mAbs, demonstrating a cross-reactivity of the polyclonal Abs (Fig. 1, lane 6). Negative results of Apa detection in the WCL-Mtb and WCL-BCG samples (Fig. 1A, lanes 4 and 5) suggest that the Apa epitopes targeted by the serum Abs were less abundant in the WCL of these bacteria in comparison to those in WCL-Map. In the additional experiments, utilization of the specific polyclonal anti-Apa-Mtb serum for immunoprecipitation of the protein from the WCL-BCG confirmed the presence of Apa protein in the BCG lysate which was recognized by the polyclonal anti-Apa-Map Ab (Fig. 1C, lane 8), but not by the mAb (data not shown). Amount of the Apa protein immunoprecipitated from the sample of culture filtrate proteins of BCG culture, CFP-BCG (Fig. 1C, lane 7), was more pronounced than that in the WCL-BCG immunoprecipitate (Fig. 1C, lane 8), suggesting high level of the Apa secretion by these bacteria.

The obtained data demonstrated that Apa proteins expressed in different species of mycobacteria display some common epitopes recognized by the polyclonal serum, whereas the mAbs obtained in this work were produced against distinct epitopes. It should be noted that the Apa protein is differentially expressed in the Mtb and Map bacteria. In the Mtb sample, it was represented by two major bands of 45 and 47 kDa polypeptides, and one minor band of 32 kDa, which was probably a proteolytic fragment, in contrast to the 50 and 60 kDa dimer in Map (Fig. 1A, lanes 6 and 2). All the tested mAb samples obtained from the six different hybridomas generated in this work were specific to Apa-Map/Apa-Mav, but not to Apa-Mtb/Apa-Mb, and demonstrated similar recognition patterns of the protein in the respective *M. avium* sub-species. Therefore, we presented the data obtained with one of these antibodies, 6F/9 mAb, in this work.

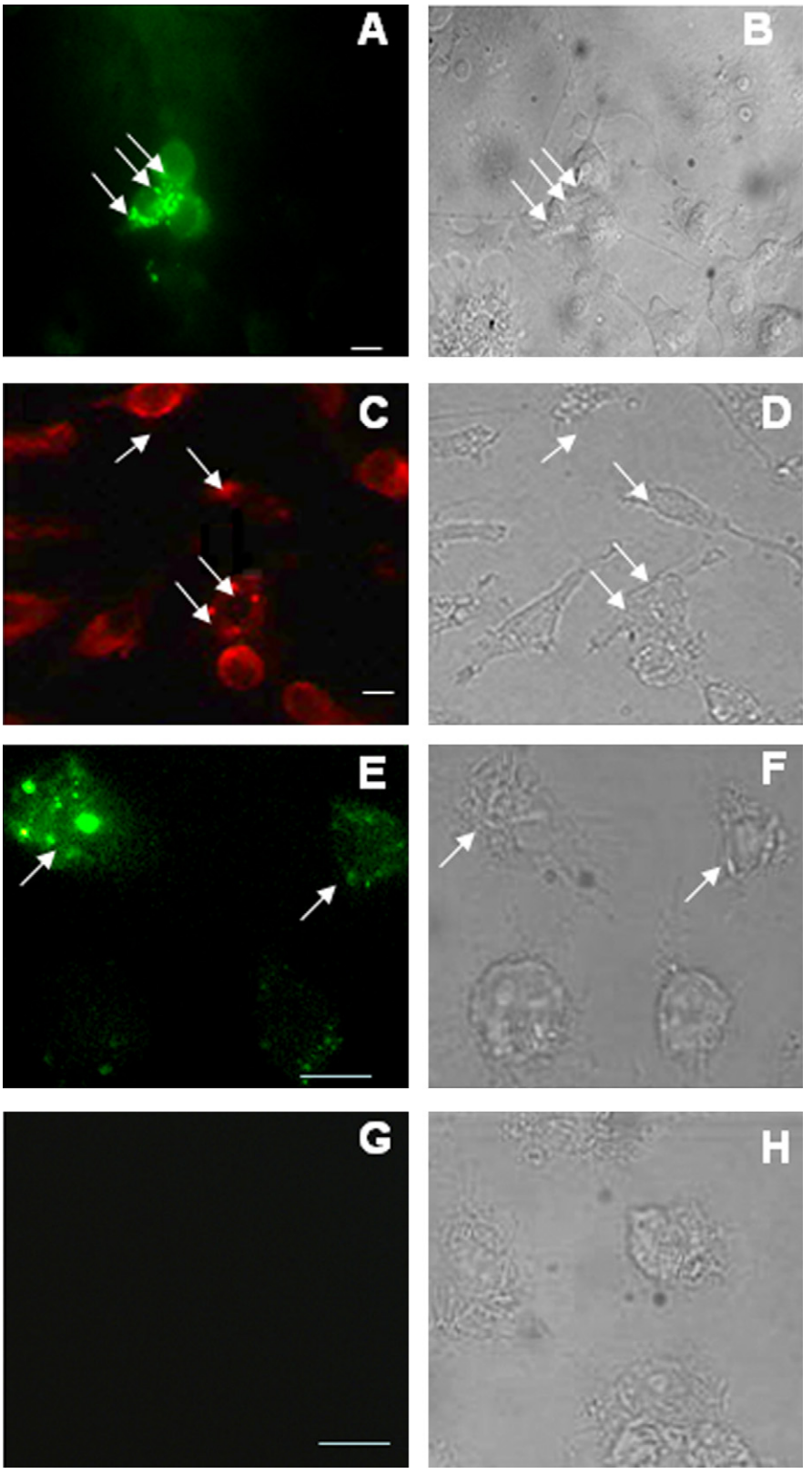
3.2. Immunofluorescence detection of the mycobacteria in infected macrophages

In order to determine whether or not the produced antibodies might be useful for the immunodetection of the whole bacteria, Raw 264.7 murine macrophages were infected with *M. avium* or *M. bovis* BCG and an indirect immunofluorescence assay accompanied by DIC microscopy was performed. Both, the polyclonal serum Abs and mAbs generated against r-Apa-Map stained equally well in intracellular Mav bacteria (Fig. 2). No staining was observed in neighbouring uninfected cells when the cultures were treated with mAbs, although cells treated with the polyclonal anti-Apa serum showed an increase in background fluorescence (Fig. 2C). Intracellular BCG were detected by the anti-Apa-Mtb serum (Fig. 2E), but not by the anti-Apa-Map mAbs which were able to recognize Mav (Fig. 2G), demonstrating species-specificity of the mAbs generated in this study.

3.3. Immunohistological detection of Map in intestinal tissues from infected animals

In order to verify whether or not the produced Abs were suitable for the immunodetection of Map in tissues

Fig. 2. Immunofluorescence detection of intracellular *M. avium* subsp. *hominissuis* (Mav) or *M. bovis* BCG in an experimentally infected culture of RAW 264.7 murine macrophages. The cells were infected with Mav strain P104 (A, B, C and D) or BCG (E, F, G and H) for 72 h, fixed and treated with either anti-r-Apa-Map ascite mAb and secondary anti-mouse IgG-FITC Ab (A, B, G and H); with murine anti-r-Apa-Map serum and secondary anti-mouse IgG- PE Ab (C and D); or with rabbit anti-Apa-Mtb serum and anti-rabbit IgG Ab conjugated with Alexa fluor 488 (E and F). The cells were visualized by using DIC (B, D, F and H) or immunofluorescence microscopy (A, C, E and G). Arrows indicate the locations of the mycobacteria. The bar indicates 5 μm for all of the panels.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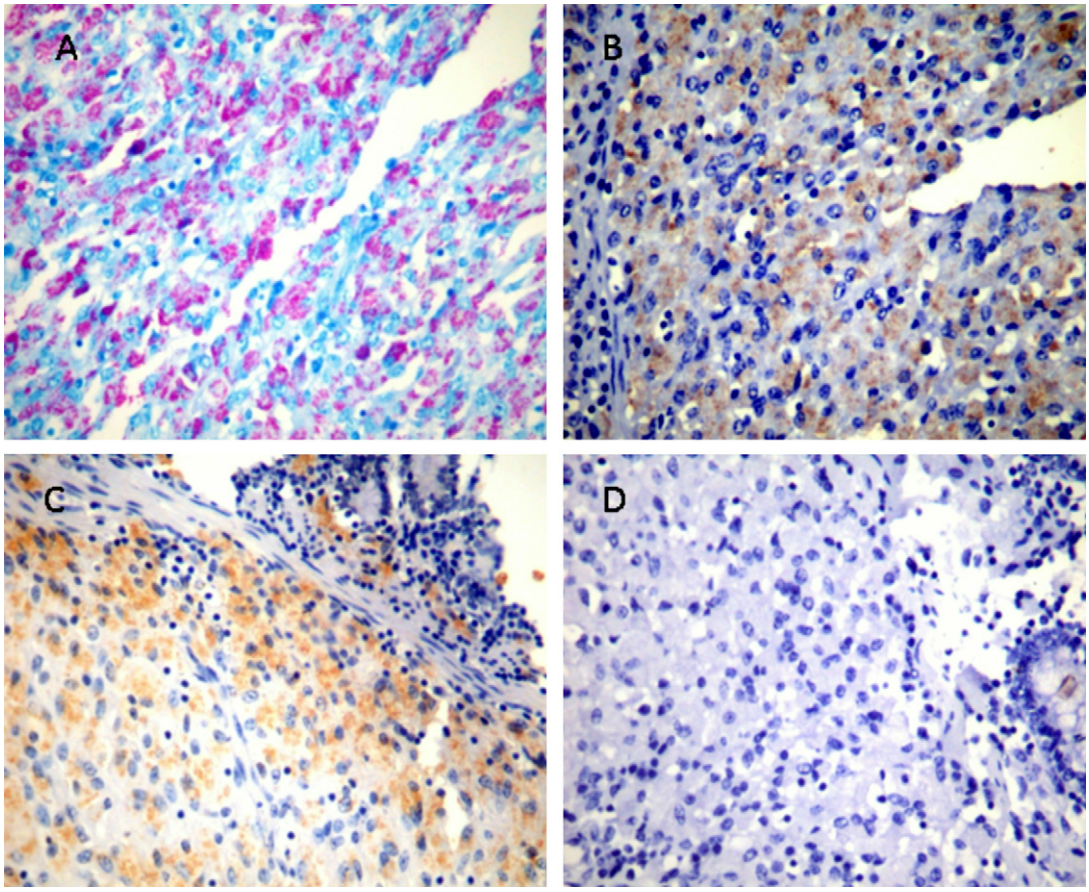

Fig. 3. Detection of Map in tissue sections immunostained with anti-rApa-Map mAb or serum. Sections of the ileocaecal mucosa from a cow with the pluribacillary form of paratuberculosis were subjected to the ZN staining (A) and an immunohistochemical procedure with anti-r-Apa-Mav ascite mAb 6F/9 (B) or polyclonal serum (C) as described in Section 2. The negative control sample was treated with secondary anti-mouse Ig reagents (D). Original magnification: ×40.

of animals with paratuberculosis, we tested intestinal tissue specimens obtained from cattle with advanced disease confirmed by positive faecal culture. Sections of paraffin-embedded ileum specimens were used for this retrospective analysis. As expected, histological analysis and ZN staining (Fig. 3A) demonstrated that the examined animals presented the pluribacillary form of the disease, according to the types of lesions and mycobacterial loads observed in the intestinal biopsies. In the pluribacillary form, the lamina propria of the intestinal mucosa contained numerous macrophages infiltrated with mycobacterial clumps (Fig. 3A). Serial sections were comparatively stained by the ZN method and treated with mAbs (Fig. 3B) or polyclonal anti-Apa serum (Fig. 3C). Immunolabelling by mAbs was strong and demonstrated the same pattern of bacterial localization in the tissue as that revealed by the ZN staining, whereas no labelling was observed in the negative control sample (Fig. 3D). There was no obvious difference in the immunolabelling provided by mAbs and those with the polyclonal antiserum. The tested Abs yielded negative responses in the intestinal tissues obtained from healthy animals.

4. Discussion

In this study, we examined whether or not a conserved mycobacterial protein, alanine and proline-rich antigen – Apa, known to present significant variability in primary sequence length and composition between different mycobacterial species, could be used as a target for the immunodetection of bacteria belonging to two important families of mycobacterial pathogens, *M. tuberculosis* and *M. avium*. Our specific interest was directed towards the differential recognition of *M. avium* subsp. *paratuberculosis* (Map) and *M. bovis* (Mb) that is important for the differential diagnoses of paratuberculosis and the intestinal form of tuberculosis in ruminants.

For this, it was necessary to produce Abs that specifically recognized the Apa protein and verify whether or not this protein, which is known as a secreted mycobacterial Ag mainly present in cultured supernatant filtrates of different mycobacterial species (Romain et al., 1993; Gioffré et al., 2009), could be used as a marker of the whole bacterium. The mAbs, as well as polyclonal serum, were produced against the previously described recombinant Apa protein of Map (Gioffré et al., 2009), and tested for recognition of

the whole bacterium or the bacterial lysate in immunochemical assays.

From the immunoblot experiments with whole cell lysates (WCL) of the mycobacterial species of interest, it can be concluded that all of the tested anti-Apa-Map antibodies, mAbs and polyclonal serum, recognized the native Apa of Map and Mav, providing evidence of determinants shared between the subspecies of *M. avium*. Nevertheless, the recognition pattern of Apa-Mav (one band of 51–55 kDa) differed from that of Apa-Map (50 and 60 kDa dimer), which could be related to the different sizes of the protein in these two subspecies (385 aa in Mav and 368 aa in Map) and/or with the glycosylation status of the polypeptides. These data demonstrate that in the Western blot experiments, the Abs produced were able to differentiate *M. avium* bacteria at the subspecies level, revealing the apparent differences in the molecular weight of the Apa polypeptides in these bacteria. Thus, this immunochemical method could be useful for identification of the bacterial cultures of Mav and Map via analysis of the Apa protein in the samples of lysed bacteria.

It is noteworthy that the specificity of the mAbs was higher than that of polyclonal serum since the latter antibodies recognized Apa protein of the bacteria of Mtb complex, that was not detected by the Mabs. The recognition pattern of the Apa-Mtb protein (45 and 47 kDa polypeptide dimer) differed from that of Map (50 and 60 kDa dimer). Additionally, the intracellular amount of Apa in the *M. avium* species was greater than in the BCG bacteria which were able to high-level secretion of the protein. The observed differences in the protein expression could be attributed to relatively low level of homology in the coding sequences of the *apa/ModD* gene between species of *M. tuberculosis* and *M. avium* complexes which is not more than 67% (GenBank).

In order to verify the utility of the produced Abs for labelling the whole bacterium in infected cells and tissues, we performed an immunofluorescence test of cultured macrophages experimentally infected with Mav and BCG and an immunohistopathological analysis of intestinal tissues obtained from cattle with advanced paratuberculosis. Data obtained in the immunofluorescence test demonstrated that the produced mAbs labelled Mav, but not BCG, whereas the polyclonal sera, in accordance with the WB data, labeled both bacteria. The most important observation was that the produced Abs were able to detect Map in the infected intestinal tissues of the paratuberculous cows, providing convincing evidence that these reagents could be useful for the research and diagnosis of Johne's disease. Additionally, the Abs able to differentiate Map and Mb could be used for the differential diagnoses of bovine paratuberculosis and the intestinal form of tuberculosis through the immunohistopathological analysis of the suspected intestinal lesions, that should be verified better in a separate study.

To the best of our knowledge, this is the first report demonstrating the successful generation of mAbs able to specifically recognize pathogens of the Mav complex, but not those of the Mtb complex. In the previous study, Horn et al. (1996) produced mAbs against Apa protein of BCG able to specifically recognize the Mtb complex bacteria, i.e. Mtb and Mb, but not those of the Mav complex or other environmental mycobacteria. Both studies confirm the presence of differential epitopes in Apa that are orthologous in these mycobacterial species and could be targeted by mAbs. Additionally, Wu et al. (2005) demonstrated that the existing differences in nucleotide sequences of the C-terminus of the *apa* gene (*fap* gene element) of Map allowed reliable differentiation between cultured strains of Map and other subspecies of the *M. avium* complex by PCR targeting of this region (Wu et al., 2005). These data suggest that elusive anti-Apa mAbs able to differentiate the subspecies of *M. avium* can be generated.

In conclusion, the data obtained in this work demonstrate that the Apa protein is a good target for the specific immunodetection of distinct mycobacterial species/subspecies.

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No conflicts of interest exist.

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