Pathogens and Disease, 76, 2018, fty063

doi: 10.1093/femspd/fty063
Advance Access Publication Date: 23 July 2018
Research Article

RESEARCH ARTICLE

Identification of the Apa protein secreted by *Mycobacterium avium* subsp. *paratuberculosis* as a novel fecal biomarker for Johne's disease in cattle

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One sentence summary: The Apa/ModD protein secreted by mycobacterium causing paratuberculosis could be isolated from the feces of infected cattle and may serve as a novel fecal biomarker for diagnosis of Johne's disease
Editor: Patrick Brennan
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ABSTRACT

Paratuberculosis (PTB) or Johne's disease is a chronic intestinal infection of ruminants, caused by *Mycobacterium avium* subsp. *paratuberculosis*. The shedding of mycobacteria in the feces starts at the initial stages and increases with disease progression, suggesting that antigens secreted by mycobacteria could be excreted in the feces. Previously, we demonstrated that the alanine and proline-rich antigen (Apa), a secretory antigen of Map, could be detected in the intestine of cows with PTB using a monoclonal antibody. In this study, we verified whether this protein can be found in consistently detectable levels in the feces of cattle with PTB. Feces were obtained from cows with Johne's disease confirmed by laboratory tests, cows with suspected PTB based on seropositivity and from PTB-free control cows. Samples were immunoprecipitated using anti-Apa monoclonal antibody and analyzed by immunoblot. The Apa was detected as a 60/70 kDa doublet band in all samples obtained from animals with laboratory-confirmed disease and in a substantial proportion of seropositive asymptomatic animals, but not in the control samples. Additionally, the antigen was detected in the feces of animals with Johne's disease by ELISA. This study strongly suggests that Apa is a potential fecal biomarker of Johne's disease that could serve for immunodiagnosis.

Keywords: paratuberculosis; Apa protein; fecal biomarker; immunochemical methods

Received: 7 May 2018; **Accepted:** 14 July 2018
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INTRODUCTION

Paratuberculosis (PTB) or Johne's disease is a chronic granulomatose intestinal infection of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map), leading to the diarrhea and evacuation of bacteria in the feces. The identification and removal of animals shedding the infectious agent before they become significant sources of contamination on a farm is the main measure of disease control at the herd level (Clarke 1997). Isolation of Map cultures from the feces of suspected animals provides a definitive diagnosis of the infection. However, the bacteriologic method is time consuming, as Map is a slow-growing mycobacterial species that needs at least 3 months for growth in specific mycobactin-supplemented medium.

Additionally, the sensitivity of the culture method is low in most asymptomatic animals, varying in the range of only 23%–29% (Nielsen and Toft 2008), because of the intermittent character of bacterial shedding at the subclinical stage of chronic infection. The implementation of molecular methods, based on the detection of mycobacterial DNA by PCR, has allowed for an increase in the sensitivity of the PTB coprodiagnosis (Taddei *et al.* 2004; Schonenbrucher *et al.* 2008), but because of technical difficulties and the high level of resources required for these methods, they are not appropriate for resource-limited settings. In contrast, the good cost-effectiveness and relative simplicity of manipulation associated with immunochemical methods have led to the wide use of serodiagnosis based on ELISA. However, the results of the commercial ELISA are not conclusive for PTB, particularly at early stages of infection characterized by the absence of anti-Map antibodies in the serum (Nielsen and Toft 2006; Facciuolo, Kelton and Mutharia 2013). In addition, the close genetic relationship with other mycobacteria, including those that are ubiquitous in nature, results in serological assays with low specificity Alternatively, the immunodiagnostic approach based on the detection of microbial antigens in fecal samples (ELISA or rapid immunochromatographic assay) has been successfully employed for the non-invasive diagnosis of intestinal infections in humans, such as infections caused by *Helicobacter pylori* (Andrews *et al* 2003; Yang and Seo 2008), *Giardia lamblia*, *Cryptosporidium parvum* (Garcia *et al.* 2003) or *Campylobacter* (Bessède *et al.* 2018).

Antigens that could serve as fecal biomarkers for PTB diagnosis are currently unknown. In our previous study, we demonstrated that the alanine and proline-rich antigen (Apa), a major secretory antigen and cell-surface adhesin of Map, could be detected in the intestinal tissue of cows with Johne's disease by immunohistochemistry employing a specific monoclonal anti-Apa antibody (Souza *et al.* 2011). In this study, we aimed to verify the presence of Apa antigen in the feces of cows with established Johne's disease and chronically infected seropositive cows by employing immunochemical methods, such as immunoprecipitation, western blot and ELISA.

MATERIALS AND METHODS

Sample collection

For this study, we selected fecal samples from three groups of cattle: cows with laboratory-confirmed PTB (clinical disease and/or culture-confirmed infection), cows with suspected PTB (seropositive and seronegative animals from herds with a history of PTB) and healthy cows (PTB-free herd).

The fecal samples of cows with PTB (group 1) were collected in Rio de Janeiro province, Brazil, in 2011 (two cows), and in Buenos Aires province, Argentina, in 2010–2012 (five cows), and were stored frozen at −80°C. The cows from Brazil exhibited signs of advanced Johne's disease, such as irreversible wasting and chronic diarrhea, characteristic signs of intestinal PTB histopathology in post-mortem tests, positive results in serological tests, and the presence of Map in feces and tissue samples (Rodrigues *et al.* 2012). The cows from Argentina were asymptomatic at the time of sample collection, but presented positive results in the serological ELISA test and positive fecal culture, confirmed by PCR analysis for Map (Costanzo *et al.* 2012).

Fresh fecal and blood samples from cows suspected of being infected with PTB (group 2) were collected from individual cows on three dairy farms in the Northern Fluminense region, Rio de Janeiro, Brazil. These farms were previously studied for the seroprevalence of Map in asymptomatic cattle herds (Souza Cabral *et al.* 2015), demonstrating that 8%–13% of the tested subjects were seropositive. This group included eight seropositive cows (total number of seropositive animals at the time of sampling) and seven seronegative cows (randomly selected herd mates over 3 years of age).

Additionally, we collected fecal and blood samples from healthy cows raised in the experimental herd at Universidade Estadual do Norte Fluminense, UENF, that was judged to be free from PTB according to clinical criteria and negative results of regular serological testing for anti-Map (negative control, group 3).

The fecal samples were taken per rectum. Concurrent individual blood samples (10 mL) were obtained from the jugular vein using Vacutainers. All collected samples were placed on ice and transported within a maximum of 6 h to the Laboratory of Biology of Recognition in UENF. Upon receipt at the laboratory, the tubes with clotted blood were centrifuged at 1000 × *g* at 4°C for 10 min, and sera were collected and then stored at −20°C until analysis by ELISA. The feces were aliquoted and then stored at −80°C until immunochemical or bacteriologic testing.

Serodiagnosis

Serum samples were analyzed by performing ELISA ID screen® Paratuberculosis Indirect screening test (ID Vet, Montpellier, France), following the manufacturer's instructions. The test included a pre-absorption step of bovine serum with a suspension of *M. phlei,* which reduces false reactions and increases the test's specificity. Optical density values were transformed to a sample-to-positive (S/P) ratio upon subtraction of the background value (negative control absorbance) from samples and positive controls. According to the interpretation of the kit manufacturer, animals with serum results S/P $\geq$ 70% were classified as positive, those with values of S/P $>$ 60% and $<$70% were considered dubious; $<$60% indicates a negative result.

Isolation of mycobacteria from feces

Feces were thawed at room temperature, and 3 g of each fecal sample was decontaminated with 0.75% (w/v) Hexadecylpyridinium chloride solution for 24 h. After vortexing, the tube was left in a vertical position for 5 min to allow for the sedimentation of big particles. Upper portion of the supernatant was transferred to another tube and incubated for 24 h at room temperature. After centrifugation at 1700 × *g* for 20 min, the supernatant was discarded, and pellets (200 μl) were inoculated onto two Herrold's Egg Yolk Agar medium slants, supplemented with mycobactin J, amphotericin, nalidixic acid and vancomycin (Becton Dickinson, Heidelberg, Germany). Slopes were incubated at 37°C for up to 20 weeks and checked for colony growth at 2-week intervals. Smears were prepared from bacterial colonies, heat-fixed, stained using a Ziehl-Neelsen technique and examined

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microscopically for typical acid fastness and morphology. Each culture with colony growth was subcultured for mycobactin dependency before reporting the culture as positive for Map.

Production of monoclonal and polyclonal antibodies against Apa-Map

In our previous study, we generated a hybridoma producing monoclonal antibodies against the Apa protein of Map (mAb 6F/9) through the immunization of BALB/c mice with recombinant Apa-Map and the standard hybridoma technique (Souza *et al.* 2011). The rApa-Map was produced in *Escherichia coli* as described previously (Gioffré *et al.* 2009). In this study, we used the mAb purified from the hybridoma culture supernatant and the ascites fluid of BALB/c mice.

To produce large volumes of polyclonal anti-Apa antibodies, we employed a non-invasive method of chicken IgY production in the egg yolks of immunized chickens (Almeida *et al.* 2008). For this, six Hysex Brown female chickens, 20-week-old, were used and maintained individually. The experimental protocol was reviewed and approved by the Ethics Committee for the Use of Animals, CEUA, of Universidade Estadual do Norte Fluminense (protocol number 077). For immunization, four chickens were inoculated by injecting 40 μg of rApa-Map protein diluted in PBS into the pectoral musculature with a final volume of 0.5 mL, distributed in two points. The first immunization included complete Freund's adjuvant (Sigma, St. Louis, MO). The second inoculation was made using Freund's incomplete adjuvant at 3-week intervals, followed by three boosters to inoculate the antigen without adjuvant in 2-week intervals. The control group ($n = 2$) was injected with PBS plus the corresponding adjuvant. Eggs were collected 1 week prior to immunization, throughout the immunization period and after an interval of 20 days from the last inoculation, then daily for 4 months. The eggs were washed and stored at 4°C up to extraction of yolk IgY antibodies. The IgY was purified by water-dilution and subsequent ammonium sulfate precipitation of the egg yolk suspension and identified by ELISA and western blotting (WB), as previously described (Almeida *et al.* 2008). Protein content of purified IgY was checked by Bradford protein assay.

Preparation of fecal eluates

Stool eluates were prepared according to a previously described protocol (Espino and Finlay 1994) with modifications. After thawing frozen fecal samples, mixtures were prepared containing 1 part feces to 4 parts PBS, 0.05% Tween 20, pH 7.2 (PBST). The mixtures remained at room temperature for 2 h and mixed every 30 min, using a vortex mixer. Large insoluble material was sedimented by centrifugation at 250 × g for 15 min at 4°C and discarded. Supernatants were further clarified by centrifugation at 5000 × g for 20 min at 4°C. The supernatants were aspirated and stored at −20°C until used for immunochemical testing.

Isolation of Apa-Map protein from fecal eluates by immunoprecipitation and immunoblot analysis

The fecal sample (400 μl eluate of each fecal sample in PBST) was incubated with antibody (6 μl ascites fluid containing 6F/9 mAb) in a microcentrifuge tube, then the Protein A/G-Sepharose beads (Santa Cruz Biotechnology, CA), 10 μl, were added and the volume completed up to 800 μl with PBS, pH 7.2, and placed on rotator for gentle end-over-end mixing, at 4°C for 2 h. Fecal samples obtained from the PTB-free cows were used as a negative control; the filtered supernatant of culture of *Mycobacterium avium* subsp. *hominissuis* strain 104 (Amaral *et al.* 2011), containing secreted Apa, was used as a positive control. The beads were washed in PBS and submitted for SDS-PAGE using the Laemmli buffer system under reducing conditions with a 12% resolving gel.

The resolved proteins were 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 were blocked by incubation with 1% gelatin in 20 mM Tris–HCl (pH 7.5), 0.5 M NaCl buffer (TBS) at room temperature for 1 h. The resulted membrane was assayed by using chicken anti Apa-Map IgY (1:5000) as a primary antibody. After washing, the membrane was treated with a peroxidase-conjugated anti- chicken IgY (1:2000, Invitrogen, Camarillo, CA). The reaction was revealed in the presence of diaminobenzidine and hydrogen peroxide. The molecular size of protein bands was determined by comparing it with a standard prestained molecular weight marker (Amersham, Piscataway, NJ).

Verification of protein glycosylation by PAS staining

The periodic acid-Schiff (PAS) method was used to specifically stain glycosylated proteins. After performing SDS-PAGE, the gel was washed continuously with 40% ethanol and 7.5% acetic acid for 30 min at room temperature. The gel was submerged in 7.5% acetic acid and kept there for 2 h. The gel was transferred to a solution containing 1% periodic acid and 3% acetic acid, and kept immersed for 1 h in dark at 4°C. After washing with 7.5% acetic acid, the gel was incubated in Schiff's reagent at 4°C in dark for 1 h, washed in 0.5% sodium metabisulphate and preserved in 7.5% acetic acid.

Detection of Apa protein by sandwich ELISA

The antibodies produced in this study were used in a sandwich ELISA performed to detect stool antigen. Briefly, a polystyrene 96-well ELISA plate (Nunc, Denmark) was coated with 2 μg mL^{-1} purified anti-Apa 6F/9 mAb in carbonate-bicarbonate buffer (Na_2CO_3 0.015M, $NaHCO_3$ 0.035M, pH 9.6) and kept overnight at 4°C. Excess antibody was removed by washing the plate twice with PBST. Non-specific binding sites on the plate were blocked with 1% gelatin solution in PBS for 2 h at room temperature. The wells were then washed with PBST, and 100 μL of the fecal eluate or the supernatant of *M. avium* subsp. *hominissuis* culture was added in each well and incubated at 37°C for 1 h. The wells were washed thoroughly four times with PBST and then incubated with the detection antibody, 2 μg mL^{-1} chicken anti-Apa-Map IgY, for 1 h at 37°C. After washing, goat anti-chicken IgY labeled with peroxidase (1:2000, Invitrogen) was added and incubated for 1 h at 37°C. The plate was washed again, and the substrate solution, containing o-Phenylenediamine dihydrochloride and hydrogen peroxide, was added. The reaction was stopped by 3M sulfuric acid and measured spectrophotometrically using a microplate ELISA reader at 492 nm. All fecal samples were tested in duplicate. Pooled fecal eluates from three PTB-free cows (group 3) were used as negative controls in each test. The individual samples obtained from six healthy PTB-free cows (negative control) were used to calculate the mean absorbance OD and standard deviation. The ELISA cut-off value was calculated by doubling the mean OD value of the negative control samples.

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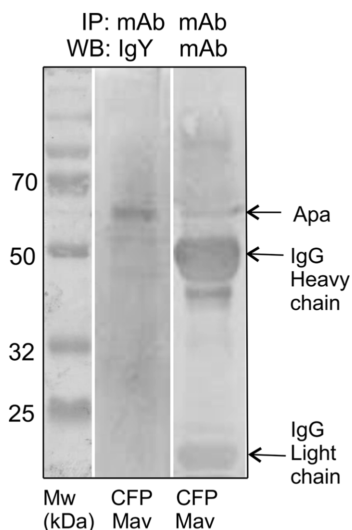

Figure 1. Monoclonal and polyclonal antibodies produced against recombinant Apa-Map protein recognize native Apa protein secreted by *M. avium*. The cell culture supernatant of *M. avium* subsp. *hominissuis* (CFP Mav) was immunoprecipitated by monoclonal 6F/9 antibody (IP: mAb). The precipitated immune complex was submitted to western blot analysis using polyclonal anti-Apa IgY or the 6F/9 antibody (WB: IgY or mAb, respectively). Mw, molecular weight markers.

For the interpretation of results obtained from the group of animals with suspected PTB (group 2), the mean OD value of fecal eluates from the group of animals with PTB (group 1) was used as a positive control. The ELISA OD values were transformed into sample/positive (S/P) ratios by the following equation: S/P = 100 × (OD sample – OD negative control)/Mean OD positive control – Mean OD negative control.

Samples with a ratio ≥ 55% were considered positive, while those with S/P values > 25% and < 55% were considered dubious; <25% was considered negative.

RESULTS

Antibodies produced against recombinant Apa protein of Map recognize native Apa protein secreted by *Mycobacterium avium*

Ability of monoclonal and polyclonal antibodies, induced against rApa-Map, to recognize native Apa protein secreted by *M. avium* in bacterial culture was verified by WB. The monoclonal anti-Apa antibody was produced in our previous study employing conventional hybridoma technology (Souza *et al.* 2011). In this study, we additionally produced polyclonal anti-Apa IgY in egg yolks of chickens immunized with rApa-Map. The ascites fluid containing 6F/9 mAb was used for the isolation of Apa protein from the supernatant of *M. avium* subsp. *hominissuis* culture by immunoprecipitation. The precipitated proteins were submitted to WB analysis, employing the anti-Apa IgY or the mAb. The results presented in Fig. 1 demonstrate that the mAb and polyclonal IgY were able to recognize native Apa protein secreted by mycobacterium in a similar manner, as a band of ~60 kDa (Fig. 1).

Isolation of Apa-Map protein from aqueous eluates of bovine feces by immunoprecipitation

Samples of bovine feces were thawed and resuspended in PBS to obtain aqueous eluates as described in the Materials and Methods section. To verify the presence of Apa protein in the feces of cows with PTB, we first submitted to immunoprecipitation by anti-Apa mAb the fecal samples obtained from cows with laboratory confirmed-PTB (group 1). The precipitated pellets were analyzed by WB. The resulting nitrocellulose membranes were treated with anti-Apa chicken IgY (Fig. 2).

As demonstrated in the Fig. 2A, the Apa protein was detected as a double band of 60 and 70 kDa in all fecal samples obtained from individual cows with laboratory-confirmed PTB, but not in the feces of healthy PTB-free cows (negative control, C1-pooled sample of three PTB-free healthy animals). Analysis of the positive samples without previous IP (the data are presented for sample A1, indicated in Fig. 2A as IP-) demonstrated negative results, suggesting that the Apa protein was presented in fecal eluates in small amounts undetectable by conventional WB methods.

Next, we tested the samples obtained from cows in three herds that were suspected of having PTB (group 2). The results are presented in Fig. 2B. Of the eight seropositive animals included in this group, four (50%) displayed Apa protein in their feces (samples Bv3, I54, I62 and Bv9), the seronegative cows in this group and healthy controls (C2, pooled sample of three PTB-free animals) did not. Recognition of the Apa protein in the sample from animal Bv9 was weaker than in other positive samples; therefore, we examined feces of this animal again (after 6 months). The feces of cow Bv9 were again collected and processed for WB. The results of the second test are indicated by an asterisk (Bv9*) in Fig. 2B. The follow-up study demonstrated the presence of a clearly visible double band at 60/70 kDa,

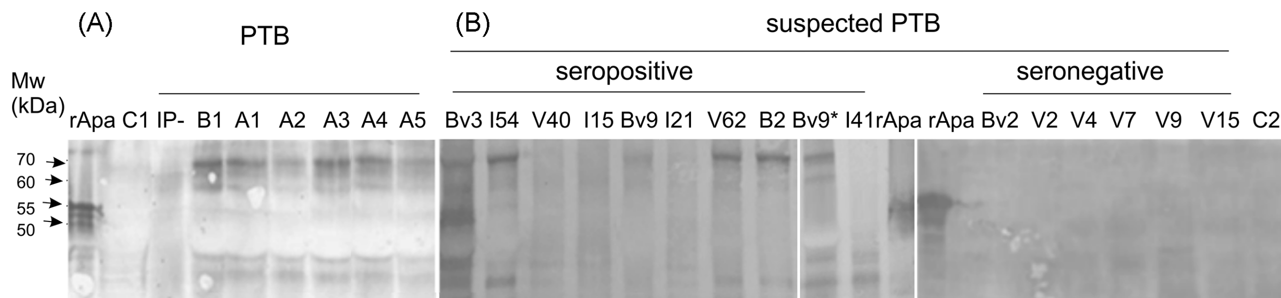

Figure 2. Immunochemical isolation of Apa-Map protein from fecal samples of cows with PTB. Fecal eluates, obtained from the animals with different PTB status, were immunoprecipitated by anti-Apa mAb and submitted to WB analysis. (**A**) Samples from animals with laboratory-confirmed PTB. (**B**) Samples from the cows with suspected PTB, including cows seropositive and seronegative for anti-Map antibodies. The sample from animal B2 with clinical PTB was included as a positive control, and samples from healthy PTB-free animals (C1, C2) were used as the negative control. rApa, recombinant Apa protein.

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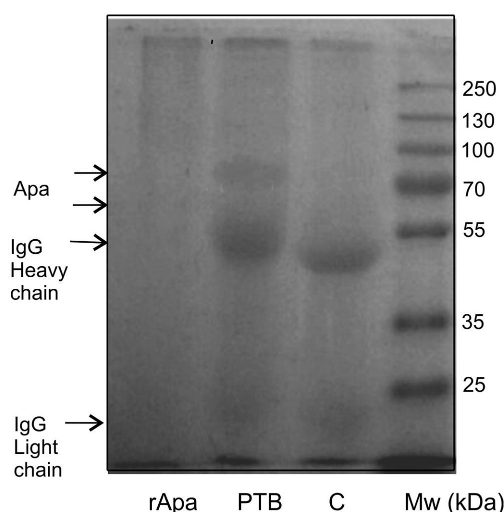

Figure 3. Verification of Apa protein glycosylation by PAS staining. The Apa protein was isolated by immunoprecipitation from the feces of a cow with PTB (PTB), but not from the PTB-free cow (C). Glycosylation was verified by periodic acid-Schiff (PAS) staining for glycosylated proteins. rApa- recombinant Apa protein (negative control). Mw, molecular weight markers.

confirming the presence of Apa-Map protein in the feces of animal Bv9.

Verification of Apa protein glycosylation by PAS staining

We verified whether native Apa protein secreted by Map in bovine intestine and excreted with feces was in glycosylated form. For this, the proteins immunoprecipitated using anti-Apa mAb, obtained from fecal samples of a cow with PTB (the data are presented for cow A1) and a healthy PTB-free cow (C), as well as the rApa-Map, were resolved by SDS-PAGE, and the resulting gel was stained by the PAS method to indicate glycoproteins.

The data presented in Fig. 3 demonstrate that the Schiff reagent reacted with the bands at 70 and 60 kDa, indicating Apa glycoprotein in the sample from the animal with PTB, as well as with two bands at 50 and 23 kDa, corresponding to the heavy and light chains of IgG, respectively, used for immunoprecipitation. The Apa glycoprotein was not detected in the sample from the PTB-free cow (negative control, C). As expected, the rApa-Map protein, produced by *E. coli* in non-glycosylated form, was not stained by PAS.

Detection of Apa protein in fecal samples by sandwich ELISA

The sandwich ELISA technique was adopted using the anti-Apa antibodies produced in our laboratory. Mouse IgG mAb was used as a capture antibody and chicken IgY as the detecting antibody. The fecal eluates, prepared as described above, were tested for binding with anti-Apa antibodies.

As shown in Fig. 4A, all fecal samples obtained from the cows with PTB (group 1) were positive by the sandwich ELISA, demonstrating the presence of Apa protein in the feces of these animals. In the group of seopositive animals with suspected PTB, two of four individual fecal samples that were positive by the IP test (I54 and Bv9) were considered positive in the fecal ELISA as well (S/P > 55%); one sample (I62) displayed a dubious result (S/P > 25% and < 55%) and sample Bv3 was negative. The fecal samples of other animals in the group of cows with suspected PTB (seropositive and seronegative) exibited negative results in the ELISA and IP testing. These results suggest that sensitivity of the fecal anti-Apa ELISA test may be lower than that of the IP test.

Isolation of Map from feces

The fecal samples of cows from the group of animals with suspected PTB (group 2) were cultured on Herrold's egg yolk medium with mycobactin. Of the eight samples obtained from the seropositive animals, only two (samples I54 and Bv9) were positive for Map, demonstrating very slow growth of mycobacterial colonies that were visible only after 14 weeks of incubation. The colonies were tested by Ziehl-Neelsen staining, confirming the presence of acid-fast bacteria. Fecal samples of the seronegative animals from group 2 and from the control group of the PTB-free cows were culture-negative.

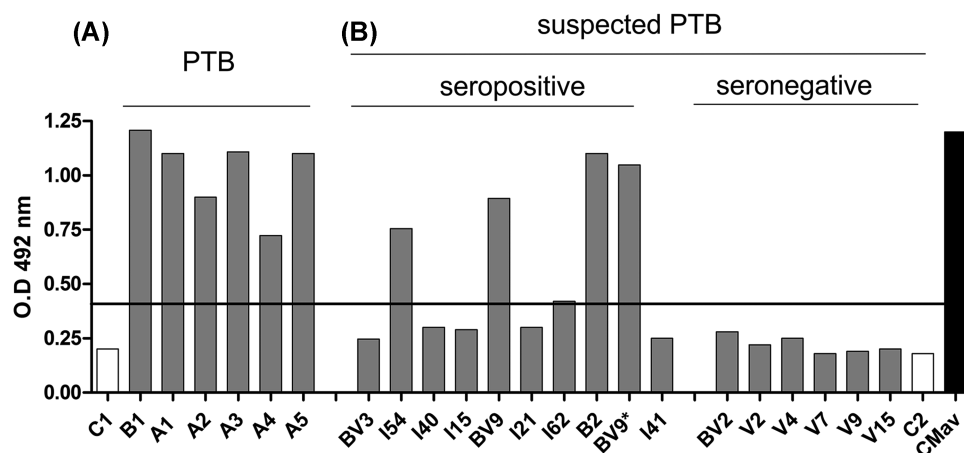

Figure 4. Anti-Apa ELISA reactivity of bovine fecal samples. Fecal eluates were obtained from three groups of cows: animals with laboratory-confirmed PTB (PTB), cows with suspected PTB, including cows seropositive and seronegative for anti-Map antibodies, and healthy PTB-free animals (C1, C2). Samples were tested for binding to anti-Apa antibodies in sandwich ELISA. The cut-off value was calculated as 0.4 (solid horizontal line). OD_{492}—optical density at 492 nm. C1 and C2—two pooled samples from three PTB-free healthy animals (negative control). CMav—supernatant of *M. avium* subsp. *hominissuis* culture (positive test control).

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Table 1. Results of different diagnostic tests for paratuberculosis diagnosis in cattle: conventional tests versus novel immunochemical tests, based on the detection of Apa protein in feces.

			Conventional tests		Novel feces-based tests	
Disease status	Animal n°	Clinical signs	Seroreactivity[a]	Culture[b]	IP[c]	ELISA[d]
PTB (group 1)	A1	–	+	+	+	+
	A2	–	+	+	+	+
	A3	–	+	+	+	+
	A4	–	+	+	+	+
	A5	–	+	+	+	+
	B1*	+	+	+	+	+
	B2*	+	+	+	+	+
Suspected PTB (group 2)	I54	–	+	+	+	+
	I62	–	+	–	+	D
	Bv3	–	+	–	+	–
	Bv9	–	+	+	+	+
	I15	–	+	–	–	–
	I21	–	+	–	–	–
	V40	–	+	–	–	–
	I41	–	+	–	–	–
	Bv2	–	–	–	–	–
	V2	–	–	–	–	–
	V4	–	–	–	–	–
	V7	–	–	–	–	–
	V9	–	–	–	–	–
	V15	–	–	–	–	–
PTB-free (group 3)	C1	–	–	–	–	–
	C2	–	–	–	–	–
	C3	–	–	–	–	–
	C4	–	–	–	–	–
	C5	–	–	–	–	–
	C6	–	–	–	–	–

[a]Seroreactivity- commercial indirect ELISA kit.
[b] Culture- Map isolation from feces or tissues (in animals marked by *).
[c]IP- immunoprecipitation for Apa isolation.
[d]ELISA –anti-Apa sandwich ELISA.
+: positive; -: negative; D: dubious.
*animal subjected to necropsy.

The results of the different diagnostic tests employed in this study for PTB diagnosis in dairy cattle are presented in Table 1. In the group of animals with PTB (group 1, $n = 7$), the results of conventional and novel immunochemical feces-based methods were similar (positive in all tests). In the group of asymptomatic animals with suspected PTB (group 2, $n = 14$), of eight seropositive cows included in this group, only two animals were positive by the fecal culture test. These animals displayed positive results in the novel immunochemical tests (fecal IP test and sandwich ELISA) as well. In addition, two other seropositive cows were positive by the IP test; however, in the ELISA test, one of these animals presented a dubious result and other was negative. The seronegative animals included in group 2 and the PTB-free animals (group 3, $n = 6$) were negative in all tests used in this study.

DISCUSSION

In this study, we verified our hypothesis that mycobacterial Apa/ModD, an alanine and proline-rich glycoprotein, can be excreted in the feces of infected cattle and may serve as a fecal biomarker of Johne's disease.

Monoclonal antibody and polyclonal antibodies against r-Apa-Map were produced in our laboratory and tested for binding to native Apa protein. The antibodies displayed a similar binding pattern, recognizing secreted Apa in *M. avium* culture filtrate as a 60 kDa band in immunoblot testing. However, the specificity of Apa binding by the anti-Apa mAb was greater than that of polyclonal antibodies. In our previous study, we demonstrated that polyclonal anti-Apa antibodies produced against rApa-Map are unable to distinguish different mycobacterial species because of a cross-reaction. In contrast, the mAb 6F/9 was able to bind specifically to the Apa of *M. avium* (including subspecies *paratuberculosis* and *hominissuis*), but not that of *M. tuberculosis* or *M. bovis* (Souza *et al.* 2011). In this study, the mAb 6F/9 was employed for the isolation of Apa protein from feces of cattle with PTB. First, we investigated the feces of animals with Johne's disease confirmed by laboratory tests (fecal culture, serodiagnosis by ELISA and/or post-mortem histopathology test). The feces of these animals were stored frozen for several years. Aqueous eluates of the thawed feces were submitted to immunoprecipitation by the mAb (IP test). WB analysis of the precipitated immune complex by anti-Apa IgY demonstrated the presence of

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Apa protein in all samples. The Apa-Map protein in feces was presented as a double band at 60/70 kDa, whereas the rApa-Map protein displayed double band of 50/56 kDa and the native Apa in supernatant of *M. avium* culture was recognized as a 60 kDa protein. The apparent discrepancy in the molecular weight of the Apa proteins obtained from different sources may be caused by variable levels of post-translational protein glycosylation, which is sensitive to genetic differences among species and environmental niche changes (Lauc and Zoldos, 2010). We verified the glycosylation status of the isolated Apa protein by PAS staining, demonstrating that the immunoprecipitated Apa protein was glycosylated and had been stained by PAS. In contrast, the rApa protein was not stained by PAS, since this modification is lost in recombinant proteins expressed in *E. coli*. In a previous study, the Apa-Map protein detected in supernatant of *M. avium* subsp. *paratuberculosis* culture was presented as a double band around 50/60 kDa (Gioffré *et al*. 2009). The larger molecular weight of Apa-Map isolated from bovine samples may be associated with a higher level of the bacterial protein glycosylation induced by microbe–host interactions.

Next, we verified the presence of Apa protein in fresh bovine feces collected from selected cows on dairy farms that were suspected for PTB infection on the basis of anti-Map seroreactivity in the cattle (Souza Cabral *et al*. 2015). Cows that were positive by the serodiagnostic test (ID Vet ELISA kit, France) at the moment of fecal sample collection and their seronegative herdmates were included in this group.

The results obtained by the IP test demonstrated that four of eight seropositive cows, and none of the seronegative animals, eliminated Apa protein in the feces. Of these four animals positive for the fecal antigen, only two were positive for Map growth in fecal culture as well. These data suggest that the immunochemical test for the isolation of Map antigen may be more sensitive than fecal culture for the isolation of mycobacteria. In contrast to the infectious agent, which, in the chronic phase of PTB, may be shed into the feces at low levels or intermittently, secreted antigens may accumulate in the intestine and be more reflective of many generations of bacteria, not just those bacteria that are present in the feces at the time a sample is taken. The sensitivity of the novel antigen-based immunochemical test in relation to the culture test is yet to be determined.

To make the novel feces-based immunodiagnosis more feasible, we developed a protocol for a plate-based sandwich ELISA test and employed it to verify the presence of Apa in feces. The results obtained in the ELISA were largely similar to those obtained in the IP test. All animals in the PTB group were positive and all animals in the control or the seronegative groups were negative in both tests. In the group of seropositive animals with suspected PTB, Apa was detected in three (two positive and one dubious result) of four animals that were positive for fecal Apa in the IP test. These results suggest that the IP test may be more sensitive than the sandwich ELISA. However, small number of animals evaluated in this study does not allow for a comparison of the sensitivity and specificity of different diagnostic tests, which should be determined in subsequent studies.

In summary, the Apa glycoprotein secreted by the infectious agent of PTB is a mycobacterial antigen excreted in the feces of the infected cattle that may serve as a novel fecal biomarker of Johne's disease. Apa-Map may be detected in the feces using specific antibodies and employing immunochemical methods, such as sandwich ELISA or immunoprecipitation. This study report the presence of Apa protein in the feces of cows with Johne's disease, confirmed by fecal culture, and, additionally, this protein could be detected in a large proportion of seropositive asymptomatic animals, especially those shedding bacteria. The novel antigen-based immunochemical assay described in this work is yet to be clinically validated as a diagnostic test for Johne's disease.

ACKNOWLEDGEMENT

We gratefully acknowledge I. S. Coutinho at the Laboratory of Animal Morphology and Pathology, UENF, for excellent technical assistance and sample collection.

FUNDING

This work was supported by Fundação de Amparo a Pesquisa do Rio de Janeiro- FAPERJ (grant number E-26/190.182/2013 for E.B.L) and Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq (306107/2014-7 for EBL), Brazil.

Conflict of interest. EBL and GSS (on behalf of Universidade Estadual do Norte Fluminense) have submitted a patent related to the ELISA kit for the coprodiagnosis of PTB. The authors have no other relevant affiliations or financial conflicts with the subject matter or materials discussed in the manuscript apart from those disclosed.

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