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Short communication

Early IgG2 in calves experimentally infected with *Mycobacterium avium* subsp. *paratuberculosis*

Bárbara Fernández[a,b], Silvia Beatriz Colavecchia[a], Giselle Gabriela Ingratta[a], Ana Jolly[a], Ana Stempler[a], María Laura Fortuny[a], Fernando Alberto Paolicchi[c,d], Silvia Leonor Mundo[a,b,*]

[a] Universidad de Buenos Aires, Facultad de Ciencias Veterinarias, Cátedra de Inmunología, Av. Chorroarín 280, C1427CWO, Buenos Aires, Argentina
[b] Universidad de Buenos Aires, Facultad de Ciencias Veterinarias, Instituto de Investigación y Tecnología en Reproducción Animal (INITRA), Av. Chorroarín 280, C1427CWO, Buenos Aires, Argentina
[c] Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria Balcarce, Laboratorio de Bacteriología, Ruta 226, Km 73.5, Provincia de Buenos Aires, Balcarce B7620BEN, Argentina
[d] Universidad Nacional de Mar del Plata, Facultad de Ciencias Agrarias, Departamento de Producción Animal, Mar del Plata, Ruta 226, Km 73.5, Provincia de Buenos Aires, Balcarce B7620BEN, Argentina

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ABSTRACT

The diagnosis of the early stages of paratuberculosis, caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map), is a cumbersome task. In this study, an experimental Map-infection model of calves was used to improve the knowledge of early antibody response and to evaluate different in-house ELISAs in the detection of subclinical paratuberculosis. Calves were challenged with Map strain $_{IS900\text{-}RFLP}$A (n = 3) or Map strain $_{IS900\text{-}RFLP}$C (n = 2) (Argentinean isolated strains) or mock infected (n = 3), and their specific humoral response was evaluated. The diagnostic ELISA (IgG against Map protoplasmic antigen; PPA) could not detect the infection throughout the experimental period (180 days post-infection; dpi), whereas the IgG2/PPA-ELISA was able to identify infected calves at least once during the experiment. In addition, the use of crude Map extract detected most of the infections from 60 dpi onwards. Antibodies were also characterized by immunoblot: IgG2-reactivity to antigens of molecular weight lower than 50 kDa was detected in all infected calves. The experimental Map-infection model of calves used allows the study of the early humoral immune response in paratuberculosis. The evaluation of IgG2 specific to antigens lighter than 50 kDa emerges as an interesting alternative in calves naturally infected with paratuberculosis.

1. Introduction

Paratuberculosis, which is caused by *Mycobacterium avium* subsp. *paratuberculosis* (Map), is a chronic granulomatous enteritis disease affecting ruminants. The primary mechanism for transmission of Map is the fecal-oral route and the most susceptible animals are calves. The subclinical stage is predominated by a cell-mediated immune profile, which contributes to bacteria containment and delays disease progression. After a long incubation period, the infection of same cattle develops into the clinical stage, which is characterized by a humoral profile. However, the existence of simultaneous cellular and humoral responses has also been described (Ganusov et al., 2015; Koets et al., 2015). In addition, several studies have demonstrated an active role of antibodies against Map (Hostetter et al., 2005; Mundo et al., 2008; Jolly et al., 2011; Koets et al., 2015; Jolly et al., 2016).

During the subclinical stage, cattle usually shed lower levels of Map, but, since they constitute the largest part of Map-infected herds, detection of these animals is of great importance for paratuberculosis control. However, the current available diagnosis techniques have low sensitivity to detect subclinically Map-infected cattle. The sensitivity of the fecal culture is between 23 and 74%, whereas that of the IFN γ assay is between 13 and 85% (Nielsen and Toft, 2008). The sensitivity of the ELISA is high to detect clinically Map-infected cattle but low to detect subclinically infected ones (OIE, 2014). Hence, new approaches and tools are needed to detect immune responses to Map soon after infection (when the animals do not yet exhibit clinical signs).

* Corresponding author at: Universidad de Buenos Aires, Facultad de Ciencias Veterinarias, Cátedra de Inmunología, Av. Chorroarín 280, C1427CWO, Buenos Aires, Argentina.
E-mail addresses: bfernandez@fvet.uba.ar (B. Fernández), scolave@fvet.uba.ar (S.B. Colavecchia), gingratta@fvet.uba.ar (G.G. Ingratta), ajolly@fvet.uba.ar (A. Jolly), anastempler@hotmail.com (A. Stempler), mlfortuny@yahoo.com.ar (M.L. Fortuny), paolicchi.fernando@inta.gob.ar (F.A. Paolicchi), smundo@fvet.uba.ar (S.L. Mundo).

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Map has a complex cell wall (Mikkelsen et al., 2011; Leite et al., 2015). Among its molecular components, the p34 protein, which is exposed on the cell surface, has been identified as a Map-specific antigen for bovine B cells (De Kesel et al., 1993; Mundo et al., 2008). On the other hand, lipoarabinomannan (LAM), which is the main glycolipid antigen on the mycobacterial envelope, has been used to develop a diagnostic test (Jark et al., 1997). In addition, specific antibodies against 65-kDa heat-shock proteins (Hsp65), which are cytosolic antigens, have been detected in both subclinically and clinically infected animals (el-Zaatari et al., 1995; Koets et al., 2001).

The aim of the study was to improve the knowledge of early antibody response in an experimental Map-infection model of calves and to evaluate different in-house ELISAs for the detection of subclinical paratuberculosis.

2. Materials and methods

2.1. Antigens

The Map strain ATCC 19698 was grown as described previously (Mundo et al., 2008) and heat-inactivated (85 °C for 30 min). This crude Map extract (cMap) was either used for ELISA or sonicated (MapS extract) for use in immunoblot. Sonication was performed at a constant power of 400 W for 35 min on ice (Sonics Vibra-cell model 600 W, Sonics & Materials Inc., Newton, MA, USA).

Recombinant p34 (p34 r) were obtained as described previously in Mundo et al. (2008). The fragment encoded nucleotides 1239–1638 of the sequence described by Gilot et al. (1993; Gene Bank Accession X68102). Recombinant Hsp65 of Map K10 (Hsp65 r) was produced according to the method described in Mundo et al. (2008). The chosen fragment encoded nucleotides 197–868 of the sequence described by Colston et al. (1994; Gene Bank Accession X74518.1). The sense (5' CTC GGA TCC GCG GTA AAG GTC ACG TTG G 3') and antisense (5' GTC GAA TTC GAT GAT CAG CAG CGG CTT G 3') oligonucleotide primers contained BamHI and EcoRI restriction sites, respectively, allowing for directional cloning of the amplicon into the polylinker site of the expression vector p*RSET-A* (Invitrogen, Carlsbad, CA, USA). The recombinant plasmid was transformed into *Escherichia coli* BL21 *pLysS* (Stratagene, La Jolla, CA, USA). LAM extract was obtained as described previously by Jolly et al. (2011).

2.2. Animals and study design

The experimental design has been previously described in detail in Colavecchia et al. (2016). Briefly, eight male Holstein calves (42 to 49 days old) born to mothers with neither clinical signs nor serological evidence of paratuberculosis, from a dairy herd of the Pampa region of Argentina in which no clinical cases of paratuberculosis have been reported over the past 5 years, were challenged with two strains of Map isolated from feces of adult cattle with paratuberculosis from Argentina. The Map strains were characterized by MIRU-VNTR as INMV 1 and by IS*900*-RFLP as A and C (Moreira et al., 1999). Calves were experimentally infected *per os* with 250 mg of total wet weight live Map (total average of 6.8×10^9 CFU), on two consecutive days. The groups were: calves infected with Map strain $_{IS900\text{-}RFLP}$A (MA; n = 3), calves infected with Map strain $_{IS900\text{-}RFLP}$C (MC; n = 2) and mock infected calves (MI; n = 3). Delayed-type hypersensitivity (DTH) responses with PPD were determined at 160 days post-infection (dpi), and positive results were obtained in both challenged groups (Colavecchia et al., 2016). Calves were euthanized at 180 dpi, using an intravenous injection of barbiturate (pentobarbital sodium, Euthanyle, Brower, Buenos Aires, Argentina). Health status was monitored by clinical inspection, hemogram and serum biochemistry as well as liveweight estimation (calculation based on chest circumference). The experimental protocol was approved by the institutional committee for the use of experimental animals belonging to the School of Veterinary Medicine, Buenos Aires University, Argentina (approval number 2010/27).

2.3. Diagnosis of paratuberculosis

To confirm the infection of the challenged calves, ELISA as well as fecal and jejunal mesenteric lymph node culture were carried out. Blood samples were collected from the jugular vein. IgG specific to Map protoplasmic antigen (PPA; Allied Monitor Inc., Fayette, MO, USA) was evaluated in sera by using our in-house ELISA (Fernández et al., 2012). Samples of feces from the rectum (throughout the experimental period) and of jejunal mesenteric lymph nodes (at 180 dpi) of each animal were collected and stored at −20 °C. Samples were cultured in Herrold's egg yolk medium enriched with mycobactin J (Allied Monitor) and pyruvate (Sigma-Aldrich Corp., St. Louis, MO, USA) (OIE, 2014). The number of CFU/g of feces or lymph nodes was counted. The identity of Map isolates was confirmed by IS*900*- and F57-PCR (Collins et al., 1993; Vansnick et al., 2004; Gilardoni et al., 2016).

2.4. Characterization of the antibody response

IgG, IgG1 and IgG2 against various antigens of Map were evaluated by ELISA. Flat-bottomed 96-well Microlon polystyrene plates (GBO, Greiner Bio-One, Monroe, NC, USA) were coated with 50 µL of either: 0.5 OD_{600} cMap, 40 µg/mL of PPA, p34 r or Hsp65 r diluted in 0.05 M sodium carbonate buffer pH 9.6 (4 °C, 16 h), or 500 µg carbohydrate/mL of LAM in PBS (4 °C, 48 h). The plates were washed three times with rinsing buffer (0.05% Tween 20 in PBS) and blocked with 10% skimmed milk in PBS. All subsequent incubations were performed at 37 °C for 1 h and, after each incubation, plates were washed three times with rinsing buffer. Prior to the PPA, p34 r and Hsp65 r assays, pre-absorbing steps were included to reduce the presence of cross-reactive antibodies. Sera were pre-adsorbed with *Mycobacterium phlei* as described previously (Fernandez et al., 2012) (PPA), or diluted 1:50 with PBS containing *E. coli* (2 OD_{600}) and then incubated at 4 °C overnight (p34 r and Hsp65 r). Samples in 5% skimmed milk PBS were added to the plates. The antibodies used were: HRP-conjugated goat anti-bovine IgG (KPL, Kirkegaard & Perry Laboratories Inc., Gaithsburg, MD, USA; 1:4,000), HRP-conjugated sheep anti-bovine IgG1 (Bethyl Laboratories Inc., Montgomery, TX, USA; 1:500), and mouse monoclonal anti-bovine IgG2 (Sigma-Aldrich; 1:10,000) followed by HRP-conjugated goat anti-mouse IgG (KPL; 1:1,000) diluted in 5% skimmed milk PBS. Plates were developed using ortho-phenylenediamine dihydrochloride (Sigma-Aldrich) in citrate buffer (Sigma-Aldrich) and read in an OpsysMR spectrophotometer (Dynex Technologies, Chantilly, VA, USA). Results were expressed as mean OD_{490} values corrected by the conjugated negative control. Mean p34r and Hsp65r OD values were corrected by the OD obtained for *E. coli* lysates. The cut-off points used were the mean OD obtained for MI group plus three SD. Sera from a naturally infected and a non-infected cow were included in each plate as positive and negative control respectively.

The calves' specific isotype responses against MapS extract were analyzed by immunoblot, at 0 and 150 dpi. SDS-PAGE was performed under reducing conditions in a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories Inc., Hercules, CA, USA) on 12% polyacrylamide gels, following the manufacturer's instructions. A total of 5 µg of MapS extract protein was used per lane and stained either with 0.1% Coomassie blue for protein analysis or with modified Silver Stain (Bio-Rad) for carbohydrate detection, as suggested by Fomsgaard et al. (1990). Electrophoretic transfer onto nitrocellulose membranes (Trans-blot transfer medium, Bio-Rad) was carried out in a Trans-Blot SD cell (Bio-Rad) following the manufacturer's instructions. Membranes were blocked with 10% skimmed milk in PBS and then incubated with the sera diluted 1:20 in 1% skimmed milk in PBS. Subsequently, the following antibodies were added: HRP-conjugated goat anti-bovine IgG (KPL, 1:2,000), HRP-conjugated sheep anti-bovine IgG1 (Bethyl Laboratories Inc., 1:500), and mouse monoclonal anti-bovine IgG2

(Sigma-Aldrich, 1:1,000) followed by HRP-conjugated goat anti-mouse IgG (KPL, 1:500). All incubations were performed at 37 °C for 1 h, and each step was followed by three washes in 1% skimmed milk and 0.05% Tween 20 in PBS. The reaction was developed using 0.6 mg/mL DAB (HRP Color Development Reagent 3,3-diaminobenzidine, Bio-Rad) and 1 μL/mL H_2O_2 100 vol. in TBS buffer (pH 7.5). As a positive control, serum from a clinically ill, naturally infected cow was included in each immunoblot.

2.5. Data analysis

STATISTIX 10 (Analytical software, Tallahassee, USA) was used to analyze results of the humoral immune response evaluated by ELISA. Natural logarithms of the mean OD values obtained were compared between groups on each day. Data were studied with ANOVA followed by Tukey's test. Kruskal-Wallis test followed by pairwise comparisons was run when the variances were not homogeneous. The level of significance was set at a p-value < 0.05.

3. Results and discussion

During the study, neither clinical abnormalities (in the hemogram or serum biochemistry) nor liveweight abnormalities were detected (Supplementary Figure and Supplementary Table 1).

3.1. Confirmation of Map infection

Early diagnosis of paratuberculosis is difficult. In spite of the low and intermittent fecal shedding described in subclinical paratuberculosis, the reduction of Map-viability due to storage at −20 °C and the decontamination procedure (Khare et al., 2008; Nielsen and Toft, 2008; OIE, 2014; Corbett et al., 2018), cultures of feces were positive (Table 1). The isolation of Map from feces of all infected animals at 3 dpi implies the "pass through" phenomenon (Corbett et al., 2018) as well as the inoculum viability. In addition, most of the infected calves (four out of five) shed Map between 30 and 110 dpi. On the other hand, in agreement with previous reports (Koo et al., 2004; Sweeney et al., 2006; Eisenberg et al., 2011), in one infected calf (No. 931, MA) fecal Map-shedding was not detected after 3 dpi.

The infection of all Map-challenged calves was confirmed *post-mortem* by lymph node culture (Table 1).

3.2. IgG2 dominates the early humoral response against Map in calves

The in-house diagnostic ELISA (IgG/PPA) could not detect Map in any of the infected calves throughout the experimental period (Fig. 1a). In agreement with this, several previous studies have reported that experimentally Map-infected calves are generally ELISA-negative (Supplementary Table 2) (Waters et al., 2003; Koo et al., 2004; Eda et al., 2006; Koets et al., 2006; Speer et al., 2006; Eisenberg et al., 2011; Stabel et al., 2011; Subharat et al., 2012; Mortier et al., 2014). Our IgG/cMap-ELISA showed that on average the MA and MC sera were above threshold at 60 dpi. In addition, this ELISA allowed the identification of significant increases in antibody levels in sera of Map-infected calves from 80 dpi onwards ($p < 0.05$). The detection of MA positive animals was transient (two out of three at 60 and 80 dpi; none at 110 dpi), while MC calves were identified until 160 dpi ($p < 0.005$). Consequently, the IgG/cMap-ELISA appears to work much better with Map strain $_{IS900\text{-}RFLP}$C than Map strain $_{IS900\text{-}RFLP}$A.

IgG isotypes against Map were evaluated (Fig. 1b and Table 2). It seems that IgG1 would not be relevant in the early response to Map. On the other hand, specific IgG2 was detected in sera from all infected calves, regardless of the strain used for the challenge, and, interestingly, the analysis of PPA-specific IgG2 (but not IgG or IgG1) allowed the detection of Map in all experimentally infected calves at least at one point throughout the experiment. This result is in accordance with our previous report, in which we highlighted the utility of the IgG2/ELISA-PPA to identify subclinically ill, naturally Map-infected cattle (Fernández et al., 2012). The use of IgG2 therefore, seems to improve the detection of subclinically ill, naturally and experimentally infected cattle. In addition, the use of crude Map extract increased the recognition of infected animals, giving positive results in most of the cases from 60 dpi onwards. In contrast, Schillinger et al. (2013) studied Map-specific isotypes in challenged calves and detected IgG1 at 300 dpi (two out of 12 calves) by flow cytometry. Specific IgG2 however, was not detected until 392 dpi (final time point). Taking into account that we studied the early immune response in calves at the subclinical stage, our results are in line with those of Koets et al. (2001), who described increases in PPD-specific IgG2 in sera from subclinically ill, naturally infected cattle.

The low sensitivity of the current ELISA diagnostic test for paratuberculosis is well recognized, and thus researches have begun to search novel antigens to improve diagnosis (Gioffré et al., 2009; Bannantine et al., 2017; Li et al., 2017). Our results demonstrate that antigens present in the bacterium other than PPA should be identified to develop an IgG2/ELISA to enhance the detection of subclinically Map-infected cattle.

Regarding the specific response to the three Map immunodominant antigens selected (p34 r, Hsp65 r and LAM), none of them resulted in a better detection of infected calves than the complex antigens PPA or cMap (Table 2). In a previous study, we evaluated the specific humoral immune response to p34 r in clinically ill, naturally infected cattle and found that this antigen was immunodominant (Mundo et al., 2008). In this study, we only detected specific antibodies (mainly IgG2) in two out of five infected calves and both of them had been infected with Map strain $_{IS900\text{-}RFLP}$A. This may indicate that p34 r is not a good antigen to

Table 1
Confirmation of Map infection.

Groups	Calf No.	Feces Days post-infection							Lymph nodes Days post-infection
		0	3	30	60	110	150	180	180
MA	926	–	+ (80 ± 0)	+ (40 ± 57)	–	–	–	–	+ (7 ± 2)
	928	–	+ (80 ± 0)	+ (80 ± 0)	+ (40 ± 57)	–	–	–	+ (137 ± 40)
	931	–	+ (80 ± 0)	–	–	–	–	–	+ (337 ± 159)
MC	930	–	+ (160 ± 113)	–	+ (760 ± 509)	+ (40 ± 57)	–	–	+ (287 ± 19)
	932	–	+ (200 ± 170)	–	+ (40 ± 57)	–	–	–	+ (3 ± 0)
MI	929	–	–	–	–	–	–	–	–
	933	–	–	–	–	–	–	–	–
	934	–	–	–	–	–	–	–	–

Data are expressed as with (+) or without (-) macroscopic growth. In all positive cases, the identity of Map was confirmed by IS*900*- and F57-PCR. The mean CFU counted per gram of feces or jejunal mesenteric lymph node samples ± standard deviations are detailed between parentheses. Groups: calves infected with Map strain $_{IS900\text{-}RFLP}$A (MA; n = 3), calves infected with Map strain $_{IS900\text{-}RFLP}$C (MC; n = 2) and mock infected (MI; n = 3).

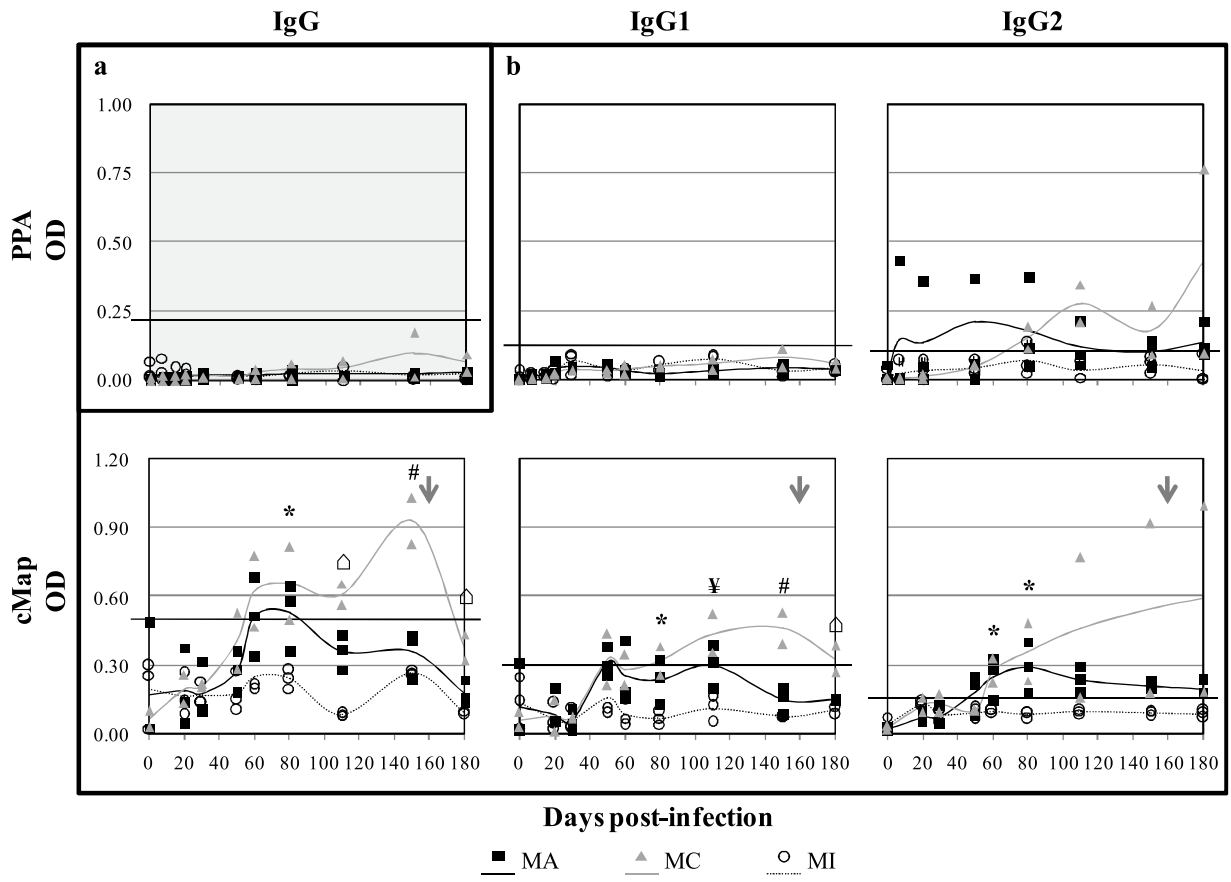

Fig. 1. Characterization of the antibody response by ELISA.
a) Paratuberculosis diagnosis by ELISA (IgG against Map protoplasmic antigen; PPA).
b) IgG, IgG1 and IgG2 specific to PPA and cMap (crude Map extract). Data are expressed as mean optical density (OD) of each calf (squares, triangles and circles) and group (lines). Groups: calves infected with Map strain $_{IS900\text{-}RFLP}$A (MA; n = 3), calves infected with Map strain $_{IS900\text{-}RFLP}$C (MC; n = 2) and mock infected (MI; n = 3). The cut-off points used were 0.205 OD (**a**, Fernandez et al., 2012) or the mean OD obtained for negative animals plus three standard deviations (**b**). Arrows: delayed-type hypersensitivity response with PPD. Significant difference ($p < 0.05$) between groups: $^{\frown}$MC > MA > MI; *MC and MA compared with MI; $^{¥}$MC compared with MI; $^{\#}$MC compared with MA and MI.

Table 2
Identification of Map-infected calves by ELISA.

ELISA		Days post-infection							
		20	30	50	60	80	110	150	180
PPA	IgG	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**
PPA	IgG1	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	IgG2	1/5	Ne	1/5	Ne	4/5	3/5	3/5	3/5
cMap	IgG	0/5	0/5	1/5	3/5	3/5	2/5	2/5	0/5
	IgG1	0/5	0/5	2/5	2/5	2/5	4/5	2/5	1/5
	IgG2	0/5	0/5	2/5	4/5	5/5	4/5	5/5	5/5
p34r	IgG	1/5	1/5	1/5	1/5	1/5	2/5	2/5	1/5
	IgG1	0/5	1/5	0/5	0/5	0/5	0/5	0/5	1/5
	IgG2	1/5	1/5	1/5	1/5	1/5	2/5	2/5	2/5
Hsp65r	IgG	Ne	1/5	0/5	Ne	0/5	Ne	0/5	Ne
	IgG1	Ne	1/5	0/5	Ne	0/5	Ne	0/5	Ne
	IgG2	Ne	0/5	0/5	Ne	0/5	Ne	1/5	Ne
LAM	IgG	Ne	Ne	0/5	Ne	1/5	Ne	2/5	Ne
	IgG1	Ne	Ne	0/5	Ne	0/5	Ne	0/5	Ne
	IgG2	Ne	Ne	0/5	Ne	0/5	Ne	1/5	Ne

Number of positive calves out of the total experimentally infected (n = 5) evaluated by IgG, IgG1 and IgG2 specific to PPA (Map protoplasmic antigen), cMap (crude Map extract), p34 r (recombinant p34 protein), Hsp65 r (recombinant 65-kDa heat-shock protein) and LAM (lipoarabinomannan)/ELISA. Ne: not evaluated.

detect Map at this early stage, since only one out of two strains studied induced the production of these antibodies. The antibody response against Hsp65 and LAM in subclinically infected cattle has been previously described (el-Zaatari et al., 1995; Koets et al., 2001; Waters et al., 2003). In this study, we were able to detect specific antibodies only in two out of five infected calves in each case.

As expected, SDS-PAGE demonstrated that the MapS extract obtained was composed of a mixture of proteins and carbohydrates as revealed by both stains (Fig. 2a). Irrespective of the isotype detected, the use of crude Map extract was better at identifying infected calves than the other Map antigens. Therefore, we further characterized the humoral immune response as evidenced by immunoblot applying a denatured and sonicated Map, in an attempt to identify immunodominant bands (Fig. 2b). The positive control included (i.e. serum of a clinically ill, naturally infected cow) allowed the validation of the IgG and IgG1 tests but the IgG2 assay showed no band. These data are in agreement with our previous findings using ELISA (Fernández et al., 2012). Our results suggest that cross-reactive antibodies are involved in the recognition of antigens of ˜80 kDa (IgG) and ˜50 kDa (IgG and IgG2) as this response was seen in sera from the three groups at both times. Nevertheless, specific response to Map antigens was detected in the experimentally infected calves. Specific IgG reactivity to antigens of molecular weight lower than 50 kDa (two wide bands) was identified in MC sera. Other authors have also described Map-specific response (IgG) to antigens of ˜50, 42 and 25 kDa as

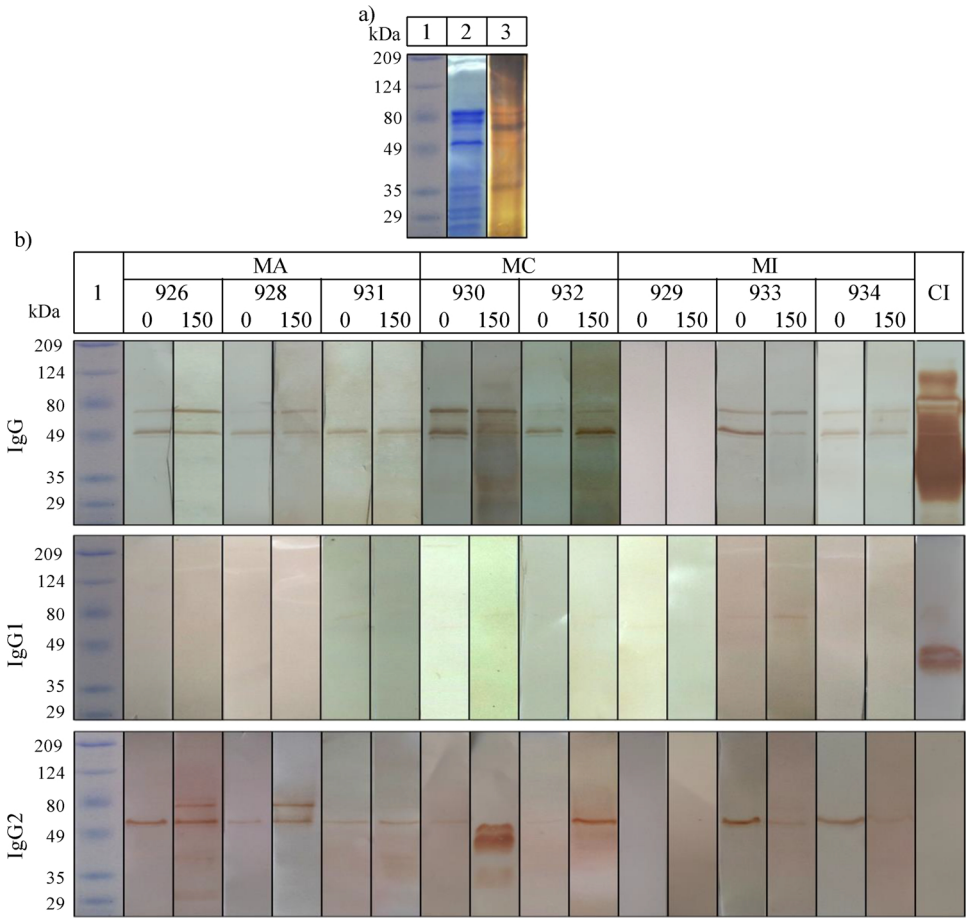

Fig. 2. Characterization of Map sonicated (MapS) extract-specific antibodies.
a) SDS-PAGE of MapS extract. Lane 1: Molecular weight marker (Bio-Rad). Lane 2: Coomassie blue-stained (protein analysis); Lane 3: silver-stained (carbohydrate detection).
b) Immunoblot. IgG, IgG1 and IgG2 against MapS extract in sera were evaluated at 0 (before infection) and 150 dpi. Groups: calves infected with Map strain $_{IS900\text{-}RFLP}$A (MA; n = 3), calves infected with Map strain $_{IS900\text{-}RFLP}$C (MC; n = 2) and mock infected (MI; n = 3). CI: clinically ill, naturally infected cow.

evaluated by immunoblot (Waters et al., 2003; Stabel et al., 2011). The study of IgG2 showed a band of ˜80 kDa in MA sera and various different patterns (< 50 kDa) in sera from all infected calves. It must be noted that, at this subclinical stage, IgG2 allowed the identification of all infected calves, but failed to detect Map in the positive control serum. The antigens involved in our results could be: LAM (˜40 kDa), p34 (˜34 kDa) or other unexplored ones such as Malate dehydrogenase (˜35 kDa), MAP0210c (˜34 kDa), Fructose 1,6-bisphosphate Aldolase (˜34 kDa), MAP_3007 (˜30 kDa) or MAP1386c (˜28 kDa) (Willemsen et al., 2006; Piras et al., 2015). Piras et al. (2015) have identified most of these antigens as immunoreactive and proposed studying them in depth.

In a previous study, we evaluated DTH responses with PPD at 160 dpi (Colavecchia et al., 2016). Twenty days later (180 dpi), we found decreasing levels of IgG and IgG1 and constant or increased levels of IgG2. In contrast, Kennedy et al. (2017) described higher levels of IgG post-DTH. The influence of DTH on the humoral immune response should be further evaluated.

In the above-mentioned previous report (Colavecchia et al., 2016), we demonstrated that MA showed higher cellular immune response by DTH than MC. The results of the present study identified lower levels of humoral response in MA when compared to MC. These results are similar to those described by Gulliver et al. (2015) in naturally infected sheep, in which the severity of the cutaneous DTH reaction was linked to lower levels of systemic specific antibodies.

The experimental Map-infection model of calves used allows studying the early humoral immune response in paratuberculosis. The serological test (IgG/PPA-ELISA) was not able to detect the infection, whereas the study of IgG2 was. Thus, the evaluation of IgG2 specific to Map antigens lighter than 50 kDa emerges as an interesting alternative in calves naturally infected with paratuberculosis.

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Declaration of Competing Interest

The authors declare having no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.109886.

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