Short communication

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

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\textbf{A R T I C L E  I N F O}

\textbf{Keywords:}
- Paratuberculosis
- Antibody
- IgG2
- Calves
- Experimental infection

\textbf{A B S T R A C T}

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 sub-clinical paratuberculosis. Calves were challenged with Map strain \textit{S900-RFLPA} (n = 3) or Map strain \textit{S900-RFLPC} (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.

\section{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).

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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 (Sonicors Vibra-cell model 600W, Sonics & Materials Inc., Newton, MA, USA).

Recombinant p34 (p34r) 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 (Hsp65r) 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 AAA GGC TAA AAT TCT GCA 3’) oligonucleotide primers contained BamHI and EcoRI restriction sites, respectively, allowing for directional cloning of the ampiclon into the polylinker site of the expression vector pRSET-A (Invitrogen, Carlsbad, CA, USA). The recombinant plasmid was transformed into Escherichia coli BL21 pLyS5 (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 IS900-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-RFLPA (MA; n = 3), calves infected with Map strain IS900-RFLPC (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, Euthanyl, 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 IS900- 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 OD600 cMap, 40 μg/mL of PPA, p34r or Hsp65r 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, p34r and Hsp65r 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 OD600) and then incubated at 4°C overnight (p34r and Hsp65r). 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 antimouse 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 OD490 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-Protein 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% Coomasie 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.
Strain IS- and F57-PCR. The mean CFU Data are expressed as with (+) or without (-) macroscopic growth. In all positive cases, the identity of Map was confirmed by IS-Confirmation of Map infection.

Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Calf No.</th>
<th>Feces</th>
<th>Days post-infection</th>
<th>Lymph nodes</th>
<th>Days post-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>MA</td>
<td>926</td>
<td>–</td>
<td>+ (80 ± 0)</td>
<td>+ (40 ± 57)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>928</td>
<td>–</td>
<td>+ (80 ± 0)</td>
<td>+ (80 ± 0)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>931</td>
<td>–</td>
<td>+ (80 ± 0)</td>
<td>–</td>
<td>+ (80 ± 0)</td>
</tr>
<tr>
<td>MC</td>
<td>930</td>
<td>–</td>
<td>+ (160 ± 113)</td>
<td>–</td>
<td>+ (760 ± 509)</td>
</tr>
<tr>
<td></td>
<td>932</td>
<td>–</td>
<td>+ (200 ± 170)</td>
<td>–</td>
<td>+ (40 ± 57)</td>
</tr>
<tr>
<td>MI</td>
<td>920</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>933</td>
<td>–</td>
<td>–</td>
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<td></td>
<td>934</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tbody>
</table>

Data are expressed as with (+) or without (-) macroscopic growth. In all positive cases, the identity of Map was confirmed by IS900- 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-RLFPA (MA; n = 3), calves infected with Map strain IS900-RLFPLC (MC; n = 2) and mock infected (MI; n = 3).
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 ~80kDa (IgG) and ~50kDa (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 50kDa (two wide bands) was identified in MC sera. Other authors have also described Map-specific response (IgG) to antigens of ~50, 42 and 25kDa as

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**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_RFLPA (MA; n = 3), calves infected with Map strain IS900_RFLPC (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: ▲MC > MA > MI; *MC and MA compared with MI; ¥MC compared with MI; #MC compared with MA and MI.

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**Table 2** Identification of Map-infected calves by ELISA.

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Days post-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20  30  50  60  80  110  150  180</td>
</tr>
<tr>
<td>PPA IgG</td>
<td>0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5</td>
</tr>
<tr>
<td>PPA IgG1</td>
<td>0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5</td>
</tr>
<tr>
<td>PPA IgG2</td>
<td>1/5 Ne 1/5 3/5 3/5 3/5 3/5 3/5 3/5 3/5</td>
</tr>
<tr>
<td>cMap IgG</td>
<td>0/5 0/5 1/5 3/5 3/5 2/5 2/5 2/5 2/5</td>
</tr>
<tr>
<td>cMap IgG1</td>
<td>0/5 0/5 2/5 2/5 2/5 2/5 2/5 2/5 2/5 1/5</td>
</tr>
<tr>
<td>cMap IgG2</td>
<td>0/5 0/5 2/5 2/5 2/5 2/5 2/5 2/5 2/5 1/5</td>
</tr>
<tr>
<td>p34r IgG</td>
<td>1/5 1/5 1/5 1/5 1/5 1/5 1/5 2/5 2/5 1/5</td>
</tr>
<tr>
<td>p34r IgG1</td>
<td>0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5 0/5</td>
</tr>
<tr>
<td>p34r IgG2</td>
<td>1/5 1/5 1/5 1/5 1/5 1/5 1/5 2/5 2/5 2/5</td>
</tr>
<tr>
<td>Hsp65r IgG</td>
<td>Ne 1/5 0/5 Ne 0/5 Ne 0/5 Ne 0/5 Ne 0/5 Ne</td>
</tr>
<tr>
<td>Hsp65r IgG1</td>
<td>Ne 1/5 0/5 Ne 0/5 Ne 0/5 Ne 0/5 Ne 0/5</td>
</tr>
<tr>
<td>Hsp65r IgG2</td>
<td>Ne 0/5 0/5 Ne 0/5 Ne 1/5 Ne 1/5 Ne 1/5</td>
</tr>
<tr>
<td>LAM IgG</td>
<td>Ne Ne 0/5 Ne 1/5 Ne 2/5 Ne 2/5 Ne 2/5</td>
</tr>
<tr>
<td>LAM IgG1</td>
<td>Ne Ne 0/5 Ne 0/5 Ne 0/5 Ne 0/5 Ne 0/5</td>
</tr>
<tr>
<td>LAM IgG2</td>
<td>Ne Ne 0/5 Ne 0/5 Ne 1/5 Ne 1/5 Ne 1/5</td>
</tr>
</tbody>
</table>

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), p34r (recombinant p34 protein), Hsp65r (recombinant 65-kDa heat-shock protein) and LAM (lipoarabinomannan)/ELISA. Ne: not evaluated.
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.

**References**


