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Experimental *Corynebacterium pseudotuberculosis* primary infection in goats: kinetics of IgG and interferon-γ production, IgG avidity and antigen recognition by Western blotting

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Abstract

Corynebacterium pseudotuberculosis is the cause of caseous lymphadenitis (CLA) in small ruminants, a chronic granulomatous disease that provokes significant zootechnics losses to ovine and goat breeders in northern Brazil. The present work was conducted to analyse aspects of humoral and cellular immune responses after experimental infection. Eight goats were infected intradermally with a single dose of virulent *C. pseudotuberculosis* strain and specific IgG, interferon-γ (IFN-γ) production as well as IgG avidity and antigens pattern recognition dynamics against an excreted–secreted antigen were recorded during 20 weeks. At the end of the follow-up, animals were slaughtered and necropsied. Although no animals showed apparent clinical signs of infection at the end of the trial, IFN-γ response, even more so than the humoral response, differentiated animals into two groups of high or medium/low response. The time-course of IFN-γ production presented a short-lived primary response on day 5 after infection of animals of both groups, and a strong and long lasting secondary response starting on day 16 after infection in the high response group. The indirect ELISA used was able to detect a positive antibody titre between 6 and 11 days after infection in the two groups. IgG avidity index oscillated initially between 15 and 45%, and showed approximately 5% units increment during the 20 follow-up weeks. With only one individual exception, the qualitative antigens pattern recognition showed on day 11 after infection remained constant through the experiment. IgG avidity is highly correlated with IgG production, but could not be related with specific immunodominant bands. Both humoral and cellular responses kinetics presented a similar pattern of activation/deactivation but necropsy results suggested that the IFN-γ test would be a very specific marker of CLA status.
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Keywords: Goats; *Corynebacterium pseudotuberculosis*; Interferon-γ; Serology; Kinetic; Caseous lymphadenitis

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1. Introduction

Caseous lymphadenitis (CLA) is a worldwide chronic infectious disease of small ruminants caused by a Gram positive rod, *Corynebacterium pseudotuberculosis*. In goats, the disease is characterised by the formation of pyogranulomas mainly in superficial lymph nodes and more rarely in visceral nodes and lungs (Ayers, 1977; Batey et al., 1986). The economic losses attributed to CLA are related to the reduction of wool and meat production (Paton et al., 1998), an increased rate of culling (Stoops et al., 1984) and condemnation of carcasses and skins in abattoirs (Stanford et al., 1998). Because *C. pseudotuberculosis* presents a broad spectrum of excreted–secreted and somatic proteins (Ellis et al., 1991c), as well as being a facultative mononuclear phagocytes intracellular pathogen (Tashjian and Campbell, 1983), immunity to *C. pseudotuberculosis* is complex and involves both cell-mediated and humoral immune responses (Ellis et al., 1990). Nevertheless, numerous studies point to a cellular mediated response, of mainly Th1 type, more than the humoral response as being protective in sheep and goats (Lan et al., 1998; Pepin et al., 1997; Alves and Olander, 1999; Simmons et al., 1998; Eggleton et al., 1991). An eradication programme based on the serodiagnostic detection and further elimination of positive individuals have been described with apparent success (Schreuder et al., 1994; Dercksen et al., 1996). Nevertheless, all antibodies detection tests failed to distinguish between truly infected or cured animals, or detect animals with small lesions since antibodies titre and infection intensity have been show not to be correlated (Ellis et al., 1990). Only recently, the use of IFN-γ response as cell-mediated immunity detection for *C. pseudotuberculosis* infection has been described (Regis, 2001; Prescott et al., 2002) and seems to be a very promising diagnostic tool.

Although different studies with experimental models of *C. pseudotuberculosis* primary infection still have described histopathologic events and cytokines associated production during pyogranuloma formation (Pepin et al., 1991, 1997; Ellis et al., 1991c; Lan et al., 1998) as well as specific antibodies profile (Pepin et al., 1993), little is known about the antigenic recognition and IgG avidity kinetic as well as about IgG and IFN-γ production in association with the kinetic of the infection.

The aim of the present study is to follow the kinetic of different aspects of humoral (specific immunoglobulins production, IgG avidity, antigens recognition) and cell-mediated (IFN-γ production) responses after experimental primary infection with *C. pseudotuberculosis* in goats.

2. Materials and methods

2.1. Animals, experimental infection, samples collection

Eleven cross-bred female goats, 12–18 months old, were bought 6 months before the beginning of the experiment from a goat producer located in a historically endemic region for CLA. The animals did not present clinical signs of the disease and were seronegative by ELISA. They were dewormed 3 weeks before the experimental infection. The infection was carried out in eight goats (four males and four females) through a double inoculation of 10^7 cfu *C. pseudotuberculosis* wild strain in the inguinal region (intradermally on the right side and trickle on scarified skin on the left side). Blood samples were collected from the jugular vein for serological tests and IFN-γ assay on the day of inoculation and then on 5, 11, 16, 21, 28, 42, 56, 84, 112 and 140 days post-infection (PI). On days 145–155 PI, all the animals were sacrificed and submitted for necropsy. Three goats were similarly inoculated with PBS and observed during the 21 first days PI, being considered as uninfected controls.

2.2. Preparation of excreted–secreted antigen in synthetic medium

Excreted–secreted antigen was prepared as described by Moura-Costa et al. (2002) with slight modifications. Briefly, a wild strain of *C. pseudotuberculosis* from the culture collection of the Laboratory of Microbiology of ICS/UFBA, and isolated from caseous material in the State of Bahia, was grown in chemically defined medium containing 4.22 g/l $Na_2HPO_4{\cdot}7H_2O$, 0.14 g/l KH_2PO_4, 0.25 g/l NH_4Cl, 5 mg/l Ca_2Cl, 50 mg/l $MgSO_4$, 12 g/l glucose, 2% MEM aminoacid solution, 2% MEM non-essential aminoacid solution, 2% MEM vitamin solution (Life Technologies, USA), incubated at 37 °C during 72 h and

continuously stirred. The culture supernatant was concentrated 50–70 times by liophilization and resuspended in Tris buffer 20 mM, pH 7.2. The protein content was determined by the Bio-Rad protein assay system.

2.3. IFN-γ assay

The IFN-γ assay (Bovigam^{TM}), originally developed to detect bovine IFN-γ (Rothel et al., 1990), is also suitable for ovine and caprine IFN-γ (Rothel et al., 1990; Emery et al., 1990). The test was performed as recommended by the manufacturer (CSL, Australia), using secreted–excreted antigen of *C. pseudotuberculosis* cultured in minimum medium (Moura-Costa et al., 2002), that showed strong and specific IFN-γ stimulation activity in CLA infected animals (Regis, 2001). Briefly, on the day of sample collection, 1.5 ml of heparinised blood were incubated at 37 °C for 24 h in a humidified atmosphere of 5% CO_2 in a 24 wells tissue culture tray with either no antigen (PBS), stimulated with Pockweed (PWM) (2.5 μg/ml) (Regis et al., 2002) or with the secreted–excreted antigen (20 μg/ml). Blood was centrifuged and IFN-γ detected in the separated plasma by sandwich EIA. To facilitate comparison between days of measurement, OD results were converted to IFN-γ indices using the following formula:

$$\text{IFN-}\gamma\,\text{indice} = \frac{\text{OD SEA sample} - \text{OD EIA negative control}}{\text{OD EIA positive control} - \text{OD EIA negative control}}$$

2.4. ELISA techniques

ELISA for specific antibodies was performed as previously described (Moura-Costa, 2001). Blood samples were allowed to clot during 3–4 h at room temperature, centrifuged and stored at −20 °C until required. Microtiter plates (Costar, USA) were coated with 50 μl per well of filtrated titled 1:100 BHI supernatant, then left overnight at 4 °C. After one wash with PBS–Tween 0.05%, plates were blocked with 200 μl 5% dry skimmed powder milk in PBS–T during 2 h at 37 °C. Plates were subsequently incubated with 50 μl of 1:100 diluted sera during 1 h at 37 °C, washed five times with PBS–T and incubated with 50 μl of secondary conjugated antibody (rabbit anti-goat Ig, DAKO, Denmark) diluted 1:10000 in 1% dry skimmed powder milk PBS–T during 1 h at 37 °C. Plates were then washed five times in PBS–T and developed with *o*-phenylenediamine dichloride (OPD). Optical density was measured at 492 nm with an ELISA plate reader (Bio-Tek Instruments, USA). Cut off value was determined by ROC curve analyse using sera from 31 positive cultured animals and 52 non-clinically infected animals coming from the same endemic region. The cut off value was defined in 0.25 OD 492 nm.

Avidity ELISA, based on end-point titration (Hedman et al., 1993), was carried out with a previous standardisation of sera working dilutions, molarity and incubation times of urea. We used a simplified calculation method to estimate IgG titration curves with or without urea from only two sera dilutions data as suggested by Korhonen et al. (1999). However, unlike theses last authors who used a logistic model, we applied a power function that better modelled the shapes of our IgG titration curves. Briefly, microtiter plates (Costar, USA) were coated and blocked as described for the ELISA for specific antibodies. Tested sera were diluted 1:25, 1:100, 1:400 and 1:800 and incubated in duplicate with 50 μl per well for 1 h at 37 °C. After incubation with the serum, the plates were washed five times with PBS–T. Then, wells incubated previously with 1:25 and 1:400 diluted sera were incubated for three time for 5 min with 50 μl of urea 6 M and washed three more times. The other wells, incubated previously with 1:100 and 1:800 diluted sera, were washed three times for 5 min with PBS buffer. Afterwards, the wells were incubated 1 h at 37 °C with peroxidase-conjugated rabbit anti-goat IgG (Sigma, USA) diluted 1:25000 and washed five times with PBS–T. Plates were developed with OPD and read at 492 nm. The avidity index (AI) was calculated as the ratio of IgG titers obtained from a fixed absorbance (50% of maximum absorbance without urea), using the formula: AI = (titer with urea/titer without urea) × 100.

2.5. Immunoblotting

The antigenic fractions for immunoblotting were obtained from BHI 48 h culture filtrate concentrated by precipitation in 30% saturated ammonium sulphate and desalinised with Tris buffer 20 mM by exclusion chromatography (BioGel column, Bio-Rad, USA). The protein content was determined by the Bio-Rad

protein assay system. Antigenic proteins were separated by one-dimensional polyacrylamide gel electrophoresis under denaturing conditions. We used a discontinuous SDS-PAGE system with a 4% stacking gel and a 12% running gel. Proteins were transferred to nitrocellulose membranes (Hybond, Amersham, USA) and were blocked with 5% dry skimmed milk in 0.05% PBS–Tween overnight at 4 °C. Afterwards, the membranes were incubated in 1:50 diluted sera in PBS–Tween buffer containing 1% dry skimmed milk, during 1 h at 37 °C, washed five times in 0.05% PBS–Tween buffer and then incubated during 1 h with horse-radish peroxidase-conjugated rabbit with anti-goat immunoglobulins (DAKO A/S, Glostrup, Denmark) diluted 1:100 in PBS–Tween. The membrane strips were washed five times in 0.05% PBS–Tween and developed with substrate developer solution (4-chloro-1-naphthol 0.3% diluted 1:5 in PBS, hydrogen peroxide). The reaction was stopped by a final rinse in distilled water and the strips were dried and photographed.

2.6. Post-mortem examination

On day 150 post-experimental infection, the animals were anaesthetised with pentobarbital and exsanguinated. At necropsy, all organs were macroscopically evaluated for abscesses and all detectable lymph nodes were removed, opened to assess the presence of caseous material and fixed in formalin for histopathologic examination.

2.7. Statistical analysis

Correlations between antibody ELISA OD, Bovine IFN-γ OD results and AI were estimated by non-parametric Spearman statistic (level of significance $P < 0.05$) and measure of association between the variables "qualitative antigenic recognition" and "AI" were carried out by Cramer's V statistic.

3. Results

3.1. Clinical evolution and post-mortem examination

All inoculated animals presented an acute inflammatory response at both sites of inoculation with more intense signals of tumefaction on the left side (inoculation trickled on scarified skin) compared to the right side (inoculation intradermally). After 10–14 days PI, inflammation signals were no longer visible. Although clear indications of humoral immune responsiveness of all animals were registered by ELISA or immunoblotting, clinical disease was not severe, and no animal, during the 140 days PI follow-up, presented swelling superficial lymph nodes. At slaughter, five of the eight infected animals showed at least one lymph node with caseous material (Table 1), from which *C. pseudotuberculosis* was isolated thereafter, and no animal presented obvious

Table 1
Summary of post-mortem examination results and IgG and IFN-γ responses at slaughter of experimental animals

Animals	Post-mortem examination	ELISA (cut off 0.25 OD 490 nm)	IFN-γ (bovine IFN-γ corrected OD 450 nm)
Infected animals			
185	No abscess	+	0.033 (−)
186	Aortic lombar LN, left parotid LN	+	0.526 (+)
187	No abscess	−	0.093 (−)
189	Right retropharyngeal LN	−	0.969 (+)
190	Inoculation site	+	1.168 (+)
191	No abscess	+	0.489 (+/−)
192	Left inguinal LN	+	2.246 (+)
199	Right mandibular LN	+/−	0.473 (+/−)
Uninfected animals			
17	No abscess	−	0.04 (−)
18	No abscess	−	0.047 (−)
19	No abscess	−	0.124 (−)

internal abscesses. The three uninfected animals neither exhibited clinical or serologic modifications during the 28 days follow-up.

3.2. Antibody and IFN-γ responses

Specific humoral immune response against excreted–secreted *C. pseudotuberculosis* antigens are shown in Figs. 1 and 2. A similar pattern of IgG production, with clear individual intensity variations, can be seen in all animals, with a maximum titre reached between days 11 and 21 PI and a further regular decline until the end of the experiment. All animals are considered serologically positive from days 6 to 11 PI and six out of the eight remained positive through out the 20 weeks follow-up. All three control animals remained ELISA negative until the end of the observational period (data not shown).

Results of bovine IFN-γ corrected OD 450 for infected goats are shown in Fig. 1. The IFN-γ pattern response allow us to differentiate animals into two groups of high (animal nos. 189–192) or medium/low (animal nos. 185–187 and 199) response. The time-course of IFN-γ production presented a short-lived primary response on day 5 PI for animals of both groups, and a strong and long lasting secondary response starting on day 16 and declining from days 42 to 56 after infection in the high response group. "IFN-γ low producer" group exhibits only a short-lived peak on day 5 after infection and thereafter display no more significant antigen specific cell-mediated stimulation. Uninfected animals did not exhibit any IFN-γ response even on day 5 after PBS inoculation (data not shown). It is noteworthy that the "lowest IFN-γ producers" of each group (animal nos. 185 and 187 from "low group" and no. 191 from "high group") were also without any caseous abscesses detected at slaughter (Table 1).

3.3. Avidity ELISA and immunoblotting analysis

IgG AI oscillated initially between 15 and 45%, and showed an approximately 5–10% units increment during the 20 follow-up weeks (Fig. 2). As can be observed, the AI variability was much more dependent upon individual characteristics than on maturation affinity acquired over a period of time.

With the exception of animal 190 which recognised only two immunodominant bands (62 and 66 kDa) on day 11 PI and more five bands thereafter, the qualitative antigens pattern recognition showed at day 11 PI remained constant through the experiment (Fig. 3). Immunoblot analysis demonstrated that six proteins of 26, 30, 37, 41, 62 and 66 kDa are predominantly recognised (Table 2) and that sera detection intensity peaked on day 11 PI and decreased thereafter.

3.4. Correlation and association measurements

Considering all infected animal results, IgG ELISA OD and bovine IFN-γ OD results are weakly but significantly correlated ($\text{rho} = 0.35, P = 0.001$). This result is exclusively explained by the IFN-γ high producer group results ($\text{rho} = 0.49, P = 0.001$) comparatively to the IFN-γ low producer group results which presented a very poor correlation ($\text{rho} = 0.15, P = 0.33$).

IgG ELISA OD values showed to be highly correlated with AI ($\text{rho} = -0.60, P < 0.001$), while there was no relationship between this last criteria and bovine IFN-γ OD values ($\text{rho} = -0.17, P = 0.31$).

Likewise, no association between the mainly immunoreactive bands (26, 30, 34, 37, 41, 62 and 66 kDa) and avidity results was detected (Cramer's V value $= 0.14, P = 0.99$).

Table 2
Summary of antigenic recognition by experimentally infected animals

Antigen (kDa)	Animals								%[a]
	185	186	187	189	190	191	192	199	
67	X	X	X		X	X	X	X	88
63	X	X	X	X	X	X	X	X	100
55		X	X			X			38
49	X	X	X			X			50
44	X		X				X		38
41	X	X	X			X	X	X	75
37	X	X	X	X	X	X	X		88
34	X	X	X	X	X	X	X		88
33			X	X	X				38
30		X	X	X	X	X	X	X	88
26		X	X	X	X	X	X	X	88

[a] Proportion of experimental animals which recognise the characterised antigen.

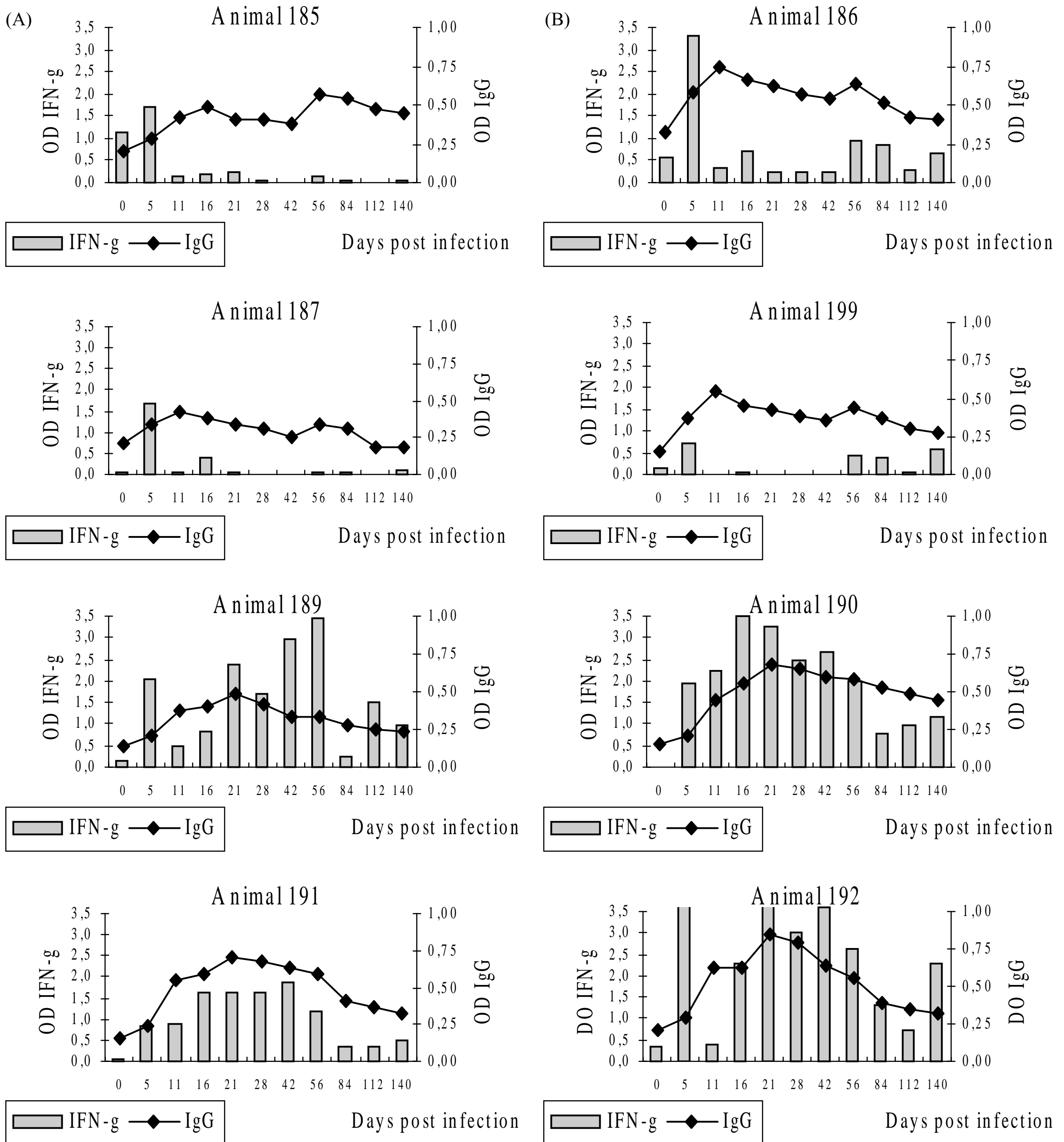

Fig. 1. Kinetics of specific bovine IFN-γ ELISA and antibodies ELISA responses to *C. pseudotuberculosis* excreted–secreted antigens of experimentally infected goats during the period of experimentation.

4. Discussion

The present study was conducted to analyse aspects of humoral and cellular immune responses of goats experimentally infected with *C. pseudotuberculosis*. Although clinical disease provoked by our experimental infection model appeared to be less severe than in other published reports (Ribeiro et al., 1991;

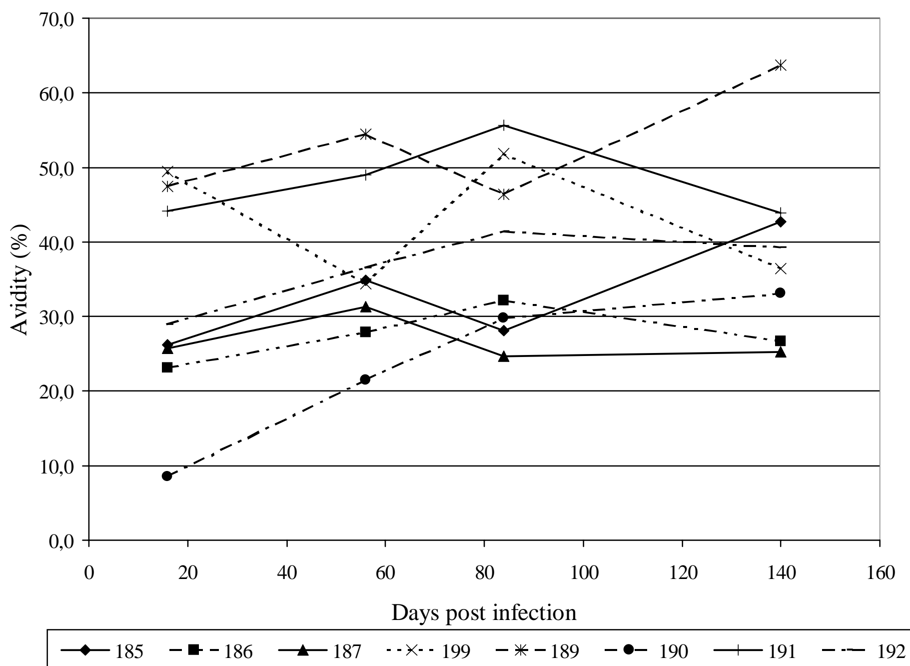

Fig. 2. IgG avidity change from eight experimentally infected goats with *C. pseudotuberculosis*.

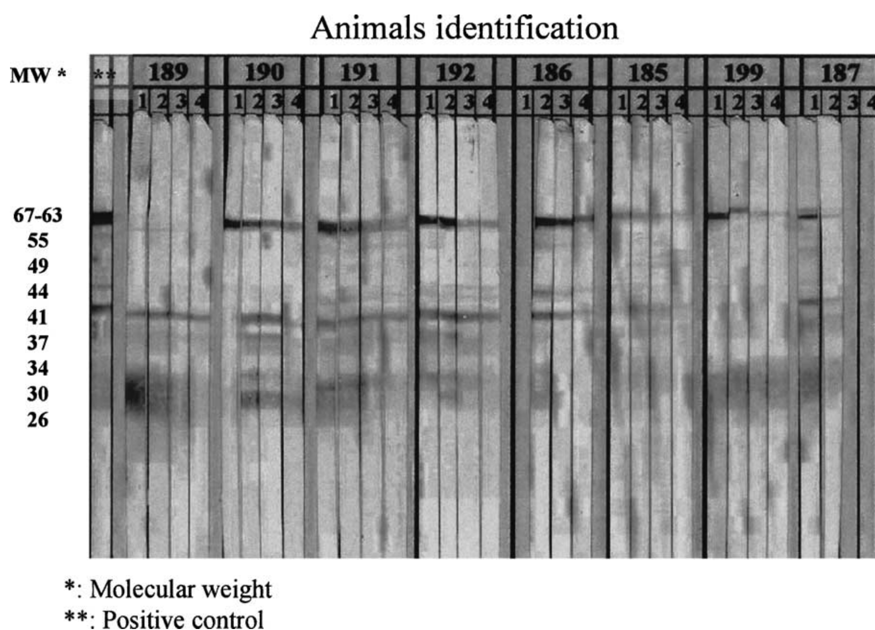

Fig. 3. Immunoblot of ammonium sulphate-precipitated filtrate from 48 h *C. pseudotuberculosis* culture with sera of eight experimentally infected goats, at days 11, 28, 56 and 140 after infection.

Pepin et al., 1991; Walker et al., 1991), our study confirm the involvement of both IgG and IFN-γ responses and some conclusions could be drawn.

Using an excreted–secreted fraction of *C. pseudotuberculosis* with a commercially available bovine IFN-γ kit detection, we confirm results recently published by Prescott et al. (2002) with sheep, that showed good reliability of this approach in detecting CLA infection in goats. Comparatively with the Canadian results (Prescott et al., 2002) which used whole cell high pressure disrupted antigen, the excreted–secreted antigen seems to be much more reactive since commonly were obtained OD values two to three times higher than IFN-γ kit positive control. This could be related with earlier studies carried out in our laboratory, that did not register significant IFN-γ stimulation using a whole cell sonicated antigen whereas the excreted–secreted one was very reactive and specific (Regis, 2001). Moreover, similar results with the same antigens have been found in lymphoproliferation assays (Regis, unpublished data).

Interestingly, this adapted goat specific IFN-γ detection test was able to describe the well-documented IFN-γ dependent response after intracellular micro-organisms infection. That is to say, a short-lived response observed immediately after infection, which may reflect innate IFN-γ response involving mainly NK cells (Anegón et al., 1988) and a secondary durable IFN-γ response observed from day 16 after infection which corresponds to the acquired T cell-mediated immune response (Pepin et al., 1997). Our results need to be confirmed, but would divide equally infected goats (by post-mortem examination) into at least two IFN-γ reactivity patterns groups and contrarily to Prescott et al. (2002) results, does not show apparent correlation between severity of infection and IFN-γ response.

An explanation for the apparent correlation between a very low level of detectable IFN-γ and protection registered at necropsy (no abscesses) may have resulted from sequestration of most *C. pseudotuberculosis* sensitised T cells into secondary lymphoid organs or at the sites of aggression, as has been suggested by Buddle et al. (1995). These latter authors had shown that IFN-γ production from whole blood culture of BCG vaccinated calves after specific antigen stimulation had a distinct pattern according to the protection status of the animals after challenge. Indeed, animals with no *Mycobacterium bovis* cultured at necropsy, but with positive intradermal test, had no IFN-γ response after challenge while partially protected animals showed a significant increase in IFN-γ production. If such results could be confirmed, it would indicate that IFN-γ assay is a useful diagnostic tool for active infection.

All infected animals presented specific antibody response detected by indirect ELISA assay. These results corroborated previous studies which had shown high sensitivity of this anti-excreted–secreted antigens ELISA (Carminati et al., 2002). On the other hand, this test showed high capability in detecting specific antibodies in the very early steps of infection, as all animals have detectable specific antibodies between 6 and 11 days after infection, earlier therefore than other published results with well known less sensitive tests (synergistic haemolysis inhibition test and bacterial agglutination test) (Kuria et al., 2001). However, as our experimental infection procedure provoked only a sub-clinical infection state, as could be expected and already described (Shigidi, 1979; Ellis et al., 1990), this indirect diagnostic tool showed a lack of specificity, as two out of the three animals without granuloma at slaughter were still positive by ELISA.

From immunoblot results analysis, some features could be pointed out. All infected animals sera recognised proteins of approximately 30 and 41 kDa on Western blot. These molecular masses may correspond to those of the phospholipase D exotoxin of *C. pseudotuberculosis* (Carne, 1940) and of the CP40 antigen previously described by Walker et al. (1994). Most infected animals sera also recognised proteins of 26, 37, 63 and 67 kDa which were previously described (Ellis et al., 1991b; Braithwaite et al., 1993). It is noteworthy to stress on the high and persistent immunoreactivity of both 63 and 67 kDa antigens. As suggested by Ellis et al. (1991b), these molecular weights may correspond to several heat shock proteins described in Corynebacteria related bacteria like Mycobacteria, or alternatively, might correspond to the two major secreted proteins of *Corynebacterium glutamicum*, PS1 and PS2 (Joliff et al., 1992; Peyret et al., 1993). Interestingly, the PS1 protein appears to have a significant N terminal region similarity with the secreted antigen 85 protein complex of *Mycobacterium* (Joliff et al., 1992), and

the PS2 protein was shown to be the only one S-layer protein of *C. glutamicum*, making it potentially involved in the antigenicity/pathogenicity of this bacteria. We observed that all these bands, with a little individual variability, are immediately recognised and none of them seems to be indicative of disease progression or outcome. This is in agreement with previous studies conducted as well as with *C. pseudotuberculosis* in naturally infected sheep (Ellis et al., 1991a), which did not detect apparent relationship between CLA lesion development and humoral response against specific antigens, as also with other goats experimentally infected models (March et al., 2002; Conde et al., 2001). Third, the strong intensity of the bands immediately after infection (11 days) are in accordance with our ELISA results, which also peak on day 11 PI, and confirm the high sensitiveness of the immunoblotting technique.

Avidity ELISA results are indicative of primary infection with no strong evidence of expected avidity gain with the chronicity of the disease. In a surprising way and with the necessary caution due to our limited sample size, avidity value variability through the infection seems to be much more representative of individual genetic background than a disease time-course indicator. Moreover, the strong negative correlation encountered between avidity level and antibody level reinforce this hypothesis and may explain why Ellis et al. (1990) find no relationship between circulating antibodies (ELISA OD values) and CLA lesion development. Also, our study was not able to associate avidity results, neither with a specific antigen recognised by immunoblot, nor with disease status.

Our study confirm the potentiality of the excreted–secreted fraction of *C. pseudotuberculosis* as a component of vaccine as it is strongly recognised by both humoral and cells mediated aspects of immune response in infected goats. Nevertheless these results do not match with protection data induced by toxoid vaccine which are somewhat disappointing especially in goats (Brown et al., 1987). We could hypothesise that formaldehyde treatment used in toxoid vaccine may alter some antigenic determinants involved in cells mediated defence mechanisms, and Prescott et al. (2002) results, that show that whole blood of bacterin-toxoid vaccinated goats does not produce expressive IFN-γ quantities after antigenic stimulation would support this assumption. Further studies employing not inactivated excreted–secreted fraction as a vaccine in goats would be interesting.

Although we had no persuasive explanation about the detection of low and high IFN-γ producing goats, more studies have to be carried out with our IFN-γ response stimulating antigen in order to confirm such results and establish a cut off value (Prescott et al., 2002), since this test would be a very specific marker of CLA active disease.

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