FULL ARTICLE

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Interferon gamma kinetics associated with immunodiagnostic techniques in experimental goats infected with biofilm producer and non-biofilm producer strains of *Corynebacterium pseudotuberculosis*

Cinética de interferon gamma associado a testes de imunodiagnóstico em caprinos experimentalmente infectados com cepas Corynebacterium pseudotuberculosis produtora e não produtora de biofilme

Maria da Conceição Aquino de Sá[1]; José Tadeu Raynal[1]; Maria Emília Alcantara[1]; Gilvan Anésio Ribeiro Lima[1]; Ramon Mendes dos Santos[1]; Marcos Borges Ribeiro[1]; Soraya Castro Trindade[2]; Mateus Matiuzii Costa[3]; Roberto Meyer[1]

[1]Universidade Federal da Bahia, Instituto de Ciências da Saúde, Laboratório de Imunologia e Biologia Molecular, Salvador – BA, Brazil
[2]Universidade Estadual de Feira de Santana, Feira de Santana – BA, Brazil
[3]Universidade Federal do Vale de São Francisco, Petrolina – PE, Brazil

ABSTRACT

Caseous lymphadenitis can be diagnosed using serological tests, mainly the enzyme-linked immunosorbent assay (ELISA). In this work, we evaluated interferon-gamma production in goats infected and not infected with *Corynebacterium pseudotuberculosis*, correlating it with the ELISA diagnostic test and analyzing the cellular and humoral responses, respectively. Eighteen Canindé goats experimentally infected with biofilm-producing and non-biofilm-producing strains were used. The sensitivity of the antigens was above 80%, with seroconversion observed from 14 days post-infection. A significant band between 64 and 70 kDa was shown in Western blotting. The production of IFN-y shows a tendency towards greater production in goats infected with the biofilm-producing strain stimulated by the TPP antigen of the same strain. The results help better understand the cellular and humoral response and antigenicity of the strains under study, correlated with the production of IFN-y, which plays a vital role in infection.

Keywords: Antigenicity. Goat. Humoral immunity. Cellular immunity. Caseous lymphadenitis.

RESUMO

O diagnóstico da linfadenite caseosa pode ser realizado através de testes sorológicos, principalmente o ensaio imunoenzimático (ELISA). Neste trabalho avaliamos a produção de Interferon gama em caprinos infectados e não infectados com *Corynebacterium pseudotuberculosis* correlacionando com o teste diagnóstico ELISA, analisando respectivamente as respostas celular e humoral. Foram utilizados 18 caprinos da raça Canindé infectados experimentalmente com as cepas produtoras de biofilme e não produtoras de biofilme. A sensibilidade dos antígenos foi acima de 80%, com soroconversão observada a partir de 14 dias pós infecção. No Western blotting, apresentou uma banda expressiva com tamanho entre 64 e 70 kDa. A produção deIFN-y mostra uma tendência de maior produção nos caprinos infectados com a cepa produtora de biofilme, estimulada pelo antígeno TPP da mesma cepa. Os resultados auxiliam para uma melhor compreensão sobre a resposta celular e humoral e antigenicidade das cepas em estudo, correlacionadas com a produção do IFN-y que tem um importante papel na infecção.

Palavras-chave: Antigenicidade. Caprinos. Imunidade humoral. Imunidade celular. Linfadenite caseosa.

Correspondence to:
Maria da Conceição Aquino de Sá
Universidade Federal da Bahia, Instituto de Ciências da Saúde, Laboratório de Imunologia e Biologia Molecular
Av. Reitor Miguel Calmon, s/n, Canela
CEP: 40110-902, Salvador – BA, Brazil
e-mail: aquino.maria@estacio.br

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Introduction

Caseous Lymphadenitis (CL) is a disease responsible for substantial economic losses in herds of goats and sheep. Therefore, various studies have been developed to prevent this disease (Carminati et al., 2003; Meyer et al., 2005; Moura-Costa et al., 2008; Baird & Malone, 2010; Farias et al., 2018). One of the preventive measures would be an early diagnosis, which may be obtained through serological tests with adequate predictive values, sensitivity, and specificity. For this purpose, it is necessary to use standardized methods for determining immunodominant bacterial antigens (Hoelzle et al., 2013). The choice of these standardized antigens goes through a strict selection process, and various combinations of antigenic peptides and other macromolecules that potentiate immunogenicity are observed. In this sense, because of its complex biochemical composition, biofilm could be a suitable candidate for preparing this type of antigen, composed of bacterial biofilms as a promising source for obtaining proteins with diagnostic potential (Vogt et al., 2016).

These antigens may be obtained using conventional microbiologic methods, resulting in variations in the profiles of proteins secreted in each new lot produced. Alternatively, high-purity protein could be obtained using recombinant DNA technology, minimizing this variability but with increased cost (Rezende et al., 2016).

The dosage of interferon-gamma (IFN-γ) has also been shown to be promising for the identification of CL. However, there are no commercially available tests specifically for dosing this cytokine in small ruminants, so using reagents produced for other species is necessary. Given this obstacle, studying cell membrane proteins is promising because the antigens produced appear to be better conserved, making it possible to cross the response between species (Rebouças et al., 2011).

In the search to identify immunodominant antigens whose host response could be related to the production of IFN,, the present study analyzed the humoral immune response against antigens of the membrane obtained from strains of biofilm producers and non-biofilm producers and compared them with their inductions in the production of IFNγ.

Materials and Methods

Animals

The Ethics Committee authorized this study on the Use of Animals of the Institute of Health Sciences (CEUA - ICS) Protocol Number 123/2017.

In this experiment, 18 goats of the Canindé breed, with a mean age of six months, were used. They came from areas considered non-endemic for CL and negative serology, maintained at the Experimental Station – UFBA of Salinas de Margarida-BA, localized at latitude 12°52'16" and longitude 38°45'52".

Before they arrived at the experimental unit, the animals were submitted to anamnesis and clinical exam (Cunningham, 2014) and to a serological test for CL (Carminati et al., 2003) to ensure seronegativity at the beginning of the experiment. After this, they were treated with anti-helminthic medication. Three groups were formed: a Control (Group 1), with six females inoculated only with 0.9% saline solution; a group with six males inoculated with the strain Cap3W (Group 2); and a group with six males inoculated with the strain CapJ4 (Group 3). The animals were inoculated subcutaneously in the suitable pre-scapular regions with 1 mL of saline solution containing $2x10^6$ CFU of *C. pseudotuberculosis*.

Antigens of *C. pseudotuberculosis*

Two isolates of *C. pseudotuberculosis* obtained from goats in the Region of Petrolina-PE (BRAZIL) were used: strain CapJ4 (GenBank No. CP026499), biofilm producer, and strain Cap3W (GenBank No. CP026500), negative for biofilm production (Sá et al., 2021). The strains were cultivated to produce antigens according to the method of Carminati et al. (2003). Second cultivation was performed according to Sampaio et al. (2019) and Raynal et al. (2018) to extract hydrophobic somatic components with organic solvent (surface antigens - F3). The strains CapJ4 and Cap3W were cultivated in BHI medium, and after centrifugation at 4 °C, at 6,000 rpm for 20 min, and washing the *pellet* twice with phosphate-buffered saline (PBS), the bacterial masses were adjusted to 2 g, re-suspended in 10 mL PBS and sonicated

at 60 Hz (five cycles of 60 s). The product was submitted to a protocol for extraction of low hydrophobicity membrane proteins, using 30 volumes of 9% of 1-butanol for 3 h with agitation (150 rpm) at ambient temperature, followed by 6000 rpm at 4 °C for 20 min. The procedure was applied another two times, and each extraction's supernatants were dried by rotary evaporation (50 °C). The resultant protein extracts of each replica were re-suspended in PBS, and their protein concentrations were determined by the modified Lowry method (Kit of reagents DC, Bio-Rad Laboratories) (Raynal et al., 2018). For the production of proteins secreted by the three-phase partition (TPP) method for the two strains, the protocol for extraction and concentration of proteins established (Paule et al., 2004) was used. The production of antigens secreted in chemically defined synthetic medium (CDM) for the two strains described used the protocol described by Moura-Costa (2002).

Blood collection

Blood samples were collected from each goat before the inoculations (time zero) and at time intervals of 7, 14, 28, 45, 60, 90, 120, and 180 days after inoculation by venal puncture of the external jugular vein in tubes without coagulant and with heparin for cell culture. After day 180, the animals were sacrificed for *post-mortem inspection.*

Standardization of surface antigens in indirect ELISA system

For the detection of IgG-specific anti-*C. pseudotuberculosis* antibodies, the immunoenzymatic indirect (ELISA) test described by Guimarães et al. (2011). The plates were sensitized with the surface antigen solutions (F3) of the two strains evaluated, and the two antigens cultivated in CDM were adjusted to concentrations of 0.5 µL/mL. The plates were read in a spectrophotometer (*Thermo Scientific Multiscan* 60) using a 492 nm light filter. The tests were conducted in duplicate.

Characterization of the electrophoretic profile of antigen recognition by IgG

The fractions obtained were submitted to electrophoresis procedures in a 12% polyacrylamide gel with sodium dodecyl-sulfate (SDS-PAGE), according to Rebouças et al. (2013). The pool of goat serum from the three groups used in the experiment was tested in all the collection time intervals by using F3 of the strain Cap3W and F3 of strain CapJ4 as antigens for sensitizing the membrane.

Quantification of production *in vitro* of IFN-γ by blood cells

Quantification of the *in vitro* production of Interferon-gamma (IFN-γ) was developed according to Rebouças et al. (2011).

Statistical analysis

The ELISA test's cut-off point values, sensitivity, and specificity were defined using the *receiver-operating characteristic* (ROC) curve.

The Kruskal-Wallis with Dunn`s post-tests were used for the non-paired groups and the Friedman with Dunn`s post-tests for the paired groups.

The statistical tests were performed in the SPSS v. 23 program, considering the level of significance of 95%.

Results

Elisa indirect

The Kinetics of Immunoglobulin G (IgG) anti-*C. Pseudotuberculosis* production was observed throughout the time interval of 180 days of the experiment with the use of indirect ELISA, performed with five different antigens: the total bacterial extract (BHI) used as standard, two surface antigens (F3Cap3W and F3CapJ4) and two antigens secreted and produced by processing with CDM (CDMCap3W and CDMCapJ4). The protein concentration of the antigens in reference may be observed in Table 1. The sensitivity and specificity values are demonstrated in Table 2. For the antigen considered standard (BHI), the choice of optical density (OD) of 0.230 was considered the cut-off point.

The absorbance values varied among the different antigens (Figures 1 to 5): For all the antigens tested in the serums of the animals of the noninfected group (Group 1), a response was observed close to that of the results demonstrated in the serums tested with the BHI antigen. This antigen was prepared with bacteria T1, producing biofilm throughout the 180 days of observation. Furthermore, in this group, the levels of IgG against the antigen CDMCapJ4 were lower

Table 1 – Protein quantification of the fraction of antigen from surface (F3) extracted with 9 butanol, chemically defined synthetic medium (CDM), and three-phase partition (TPP), concentration measured in mg/mL

Samples	F3 p/ ELISA	F3 P/ WB	TPP	CDM
Cap3W		0.556	0.666	5,789
CapJ4	0.173	1.282	1.730	4.405

Table 2 – Values obtained using ROC curve for indirect ELISA test, with the different antigens used (BHI, F3Cap3W, MQDCap3W, F3CapJ4, and CDMCapJ4)

Antigens	Cut-off	Sensitivity (%)	Specificity (%)
BHI	0.230	96.3	95.8
F3Cap3W	0.162	92.5	81.2
CDMCap3W	0.145	83.8	75.0
F3CapJ4	0.287	97.5	93.7
CDMCapJ4	0.111	96.3	93.7

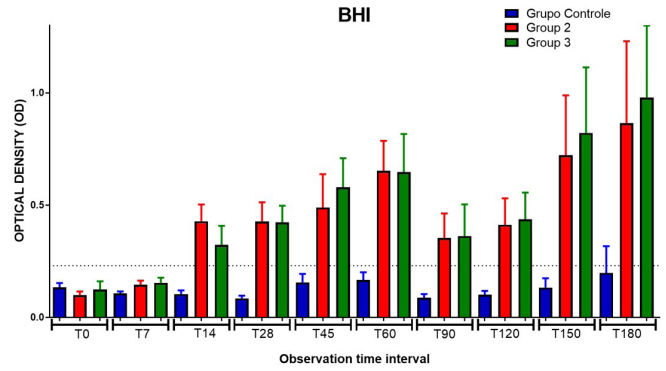

Figure 1 – Kinetics of IgG-specific anti-C *pseudotuberculosis* production in goats during a time interval of 180 days of observation. Control Group (n=6) - without experimental infection; Group 2(n=6) - infected with *C. pseudotuberculosis* non-biofilm producer; and Group 3 (n=4) - infected with *C. pseudotuberculosis* biofilm producer. The traced line represents an optical density (OD) cut-off point of 0.230. Using 96-well polystyrene plates sensitized with BHI antigen.

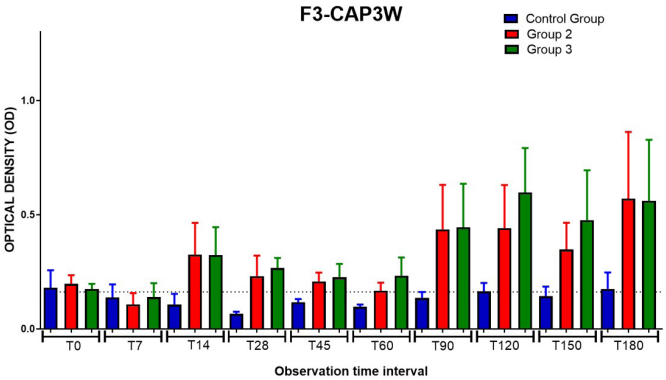

Figure 2 – Kinetics of IgG-specific anti-C *pseudotuberculosis* production in goats during a time interval of 180 days of observation. Control Group (n=6) - without experimental infection; Group 2(n=6) - infected with *C. pseudotuberculosis* non-biofilm producer; and Group 3 (n=4) - infected with *C. pseudotuberculosis* biofilm producer. The traced line represents an optical density (OD) cut-off point of 0.162. ELISA sensitized with antigen F3Cap3W.

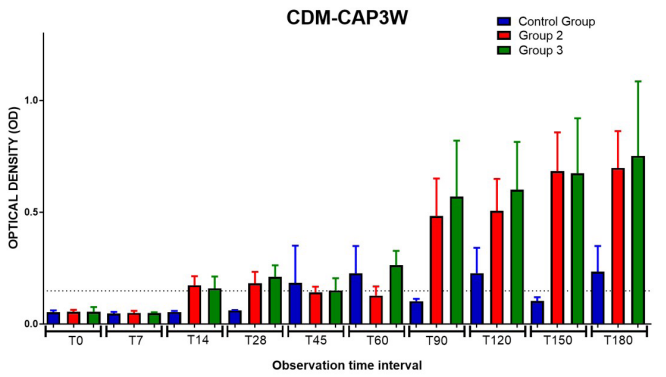

Figure 3 – Kinetics of IgG-specific anti-C *pseudotuberculosis* production in goats during a time interval of 180 days of observation. Control Group (n=6) - without experimental infection; Group 2(n=6) - infected with *C. pseudotuberculosis* non-biofilm producer; and Group 3 (n=4) - infected with *C. pseudotuberculosis* biofilm producer. The traced line represents an optical density (OD) cut-off point of 0.145. ELISA sensitized with antigen CDMCap3W.

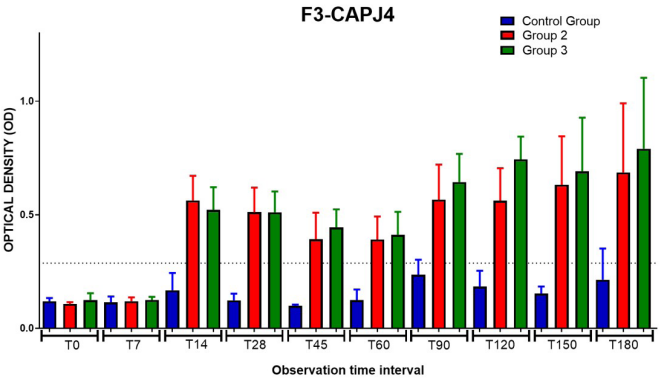

Figure 4 – Kinetics of IgG-specific anti-C *pseudotuberculosis* production in goats during a time interval of 180 days of observation. Control Group (n=6) - without experimental infection; Group 2(n=6) - infected with C. pseudotuberculosis non-biofilm producer and Group 3 (n=4) - infected with C. pseudotuberculosis biofilm producer. The traced line represents an optical density (OD) cut-off point of 0.287. ELISA sensitized with antigen F3CapJ4.

in comparison with the antigen F3CapJ4 in all the time intervals evaluated. Whereas for the antigen Cap3W, the IgG levels were higher for the CDM antigen than for the antigen F3. This result could be observed in all the time intervals (points) of observation throughout the 180 days. At some collection times, an increase was observed, caused

by some non-specific reaction, which is also reflected in the infected groups.

The highest levels of seroconversion for Group 2 were found for the BHI antigen. This seroconversion began after 14 days of observation, while in Group 3, it increased continually as the infection became chronic.

In all the groups, seroconversion was observed when the antigens F3Cap3W and CDMCap3W were tested, compared with the antigen F3CapJ4. This, in turn, induced higher values of IgG than those of CDMCapJ4.

Moreover, Group 2 showed a more significant response than Group 3 in the first days of infection.

Western blotting

In the investigation of the recognition of the antigens F3Cap3W and F3CapJ4 in a pool of serums from the three comparison groups (Figures 6 and 7), IgG identified molecules with molecular weights from 6 to 115 kDa.

The time zero of infection showed no antigenic recognition of the two antigens described. The profile of bands was coincident for the two antigens in most time intervals of the experiment. The most significant band recognized in all the pools of serums appeared between 64 and 70 kDa, and a band between 30 and 37 kDa was also recognized in all Groups 2 and 3 pools.

Quantification of production *in vitro* of IFN-γ by blood cells

In the observation of *in vitro* production of IFN-γ, the peripheral blood cells stimulated by the antigen TPPCapJ4 showed the highest concentrations of this cytokine compared to those stimulated with the other antigens.

In Group 3, these concentrations were higher than in the other comparison groups (Figure 8).

Discussion

Standardization of the immunoenzymatic assay (ELISA) performed favored the obtainment of satisfactory sensitivity and specificity values (Table 2), in agreement with the results of (Rezende et al., 2016), who obtained a sensitivity of 92.5% and specificity of 95% using recombinant antigens. This agreement could have resulted from the fact that the original strains of the antigens of this study were the

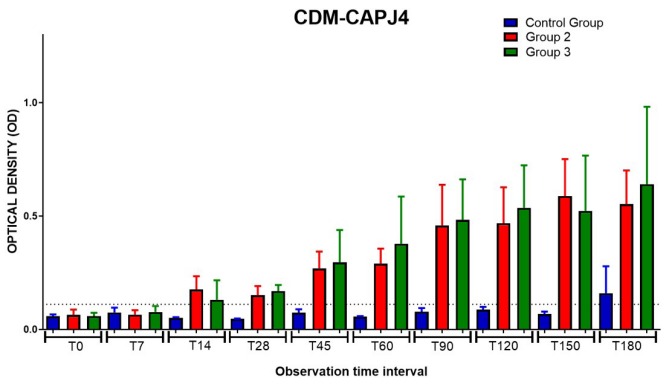

Figure 5 – Kinetics of IgG-specific anti-C *pseudotuberculosis* production in goats during a time interval of 180 days of observation. Control Group (n=6) - without experimental infection; Group 2(n=6) - infected with *C. pseudotuberculosis* non-biofilm producer and Group 3 (n=4) - infected with C. pseudotuberculosis biofilm producer. The traced line represents an optical density (OD) cut-off point of 0.111. ELISA sensitized with antigen MQDCapJ4.

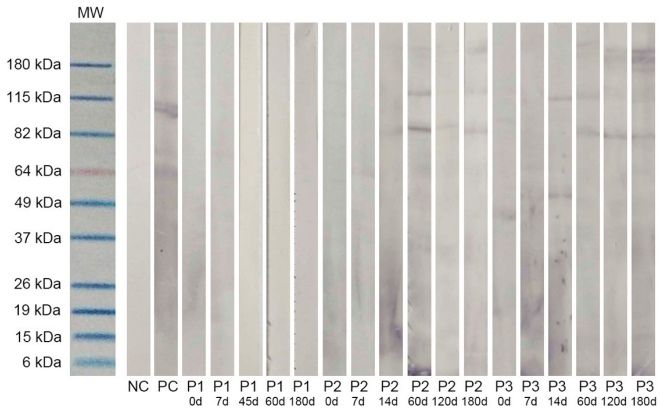

Figure 6 – Western blotting membrane with a profile of reactivity for surface Antigen F3CCap3W against a pool of serum from goats of Control Group (P1); Group 2 (P2), infected with *C. pseudotuberculosis* non-biofilm producer and Group 3 (P3) - infected with *C. pseudotuberculosis* biofilm producer. NC: (negative control); PC: (positive control); MW (molecular Weight).

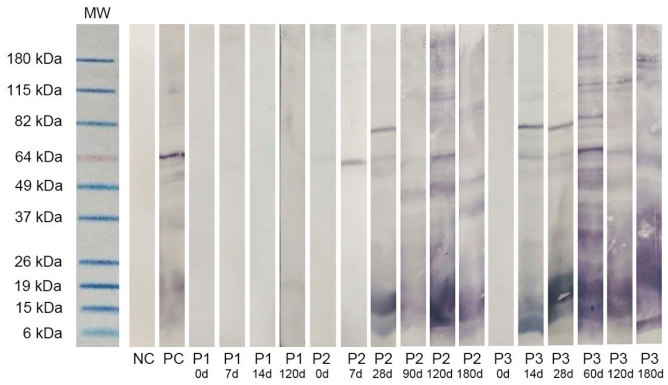

Figure 7 – Western blotting with a profile of reactivity for surface Antigen F3 CapJ4 against a pool of serum from goats of Control Group (P1); Group 2 (P2), infected with *C. pseudotuberculosis* non-biofilm producer and Group 3 (P3) - infected with *C. pseudotuberculosis* biofilm producer. NC: (negative control); PC: (positive control); MW (molecular Weight).

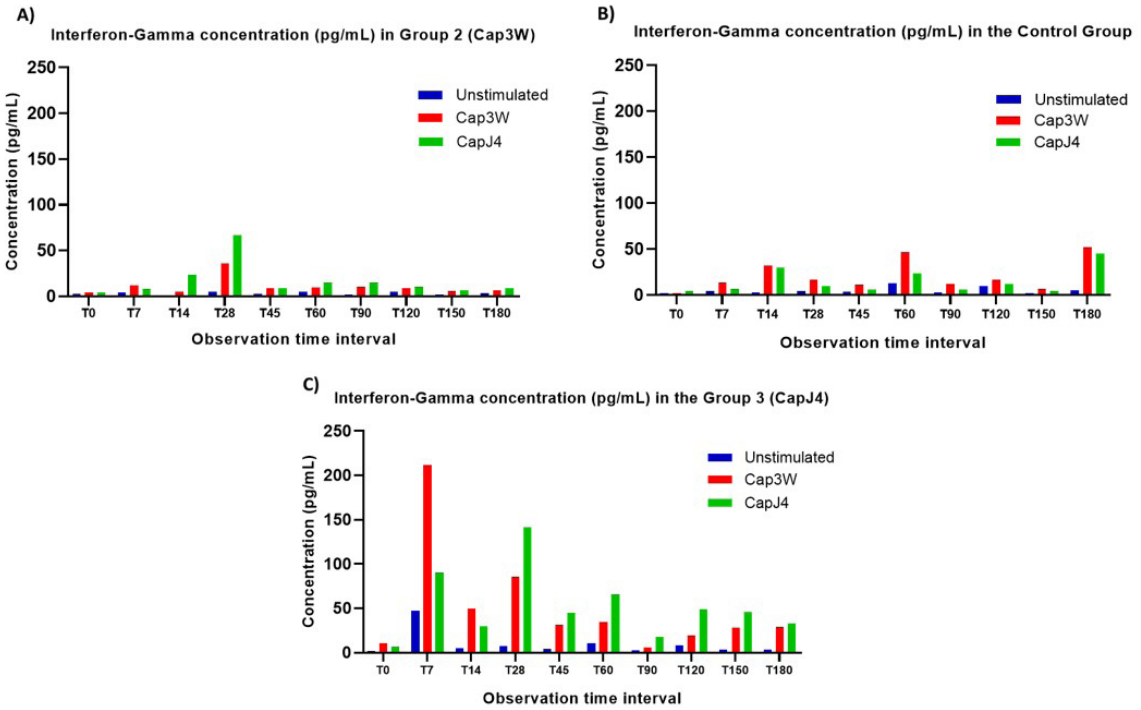

Figure 8 – Production *in vitro* of IFN- γ in the culture of whole blood from the goat of Control Group (n=6) (A); Group 2 (n=6) (B); Group 3 (n=4), under the stimulus of antigens TPPCap3W- *C. pseudotuberculosis* non-biofilm producer and TPPCapJ4 - *C. pseudotuberculosis* biofilm producer.

same. However, the use of recombinant antigens for an immunodiagnostic test, because of their cost, could make this unfeasible for use in the field, particularly in small-sized properties (Barral et al., 2019).

The search for an antigenic extract for use in the indirect ELISA test must be subject to various types of care taken regarding quality control (sensitivity, specificity, and predictive values) to avoid tests with false results, making it unfeasible to use them. According to (Rebouças et al., 2013), the indirect ELISA test can be used in serological tests for detecting Caseous Lymphadenitis, resulting in an efficient control and eradication program because the reference test could accurately detect the infected animals.

Therefore, discovering antigenic properties is essential for more precise tests (Rezende et al., 2016). Of the antigens tested, F3CapJ4 attained high sensitivity (96.3%) and specificity (95,8%) values, so that it most resembled the values described for sensitivity (93.5%) and specificity (100%) of ELISA prepared with the BHI antigen in a previous study (Carminati et al., 2003). It must be emphasized that the bacteria preparing the BHI antigen (T1) is also a biofilm producer. Preliminary studies of *C. amicolatum* have indicated that the biofilm may be very important in stimulating the immune system by releasing pro-inflammatory cytokines in T cells (Olender et al., 2019).

Other studies involving the standardization of antigens for the ELISA test in the diagnosis of CL have observed variations in the sensitivity indexes (from 80% to 97%) and specificity (from 95% to 100%) (Carminati et al., 2003; Paule et al., 2004; Rebouças et al., 2013; Galvão et al., 2017; Farias et al., 2018; Calderón et al., 2022).

This response inconsistency may be explained by the variations in the antigens tested, mainly with the different choices of strains and protocols for obtaining the supernatants of the culture of *C. pseudotuberculosis*. In this sense, searching for a specific antigen is fundamental, given that serological tests are the best alternative for diagnosing CL. This is because they are easy to perform, scale, and low-cost for application in herds or those without a history of disease (Farias et al., 2020).

However, antigens are still not readily available for reference laboratories for animal evaluation, particularly to meet the requirements of small municipalities, where most small producers are concentrated (Farias et al., 2018). These factors analyzed in conjunction justify the need for studies involving other strains and different protocols for the extraction of antigens, as in the case of the analysis of membrane fractions (Raynal et al., 2018; Rebouças et al., 2020).

Studies with the production of new antigens are necessary to improve diagnostic tests and increase the reliability of the results (Marques et al., 2019). A study using the BHI antigen detected a prevalence of 34.4% in different herds in the Northeast of Brazil (Farias et al., 2018), demonstrating

the importance of serological tests in controlling CL since many animals present no symptoms.

Concerning the kinetics of production of IgG antibodies, it was observed that for all the antigens tested, an earlier response was observed in the serums coming from the group infected with the bacteria CapJ4 (biofilm producer), in comparison with the group infected with the bacteria Cap3W (without biofilm production), mainly in the chronic period of the infection. There is a gradual increase in the kinetics of IgG from 14 days of infection, decreasing after 60 days of infection, and increasing its levels again after 150 days post-inoculation of the bacteria. This variation in immune response, showing different peaks in the production of antibodies, may be related to the capacity of the bacteria to survive prolonged periods even in the presence of the immune response of animals, which is mainly of the cellular type, notably Th1, since this microorganism has a facultative intracellular nature, and induces interferon-gamma production, in addition to other cytokines (Paule et al., 2003; Barral et al., 2022).

Western blotting

To confirm the results obtained in the ELISA test, *Western blotting* revealed various bands in the F3Cap3W and F3CapJ4 fractions, especially one with 64 kDa significantly observed in all the pools. Previous studies have also shown evidence of the presence of this band in this and other strains of *C. pseudotuberculosis* (Paule et al., 2003, 2004; Rebouças et al., 2013; Santana-Jorge et al., 2016; Raynal et al., 2018, 2022) and have suggested that this could concern protein NanH (Neuramidase H), an extracellular neuraminidase described as having a molecular weight of 74 kDa, which could be involved in recognition of sialic acid on the surface of the host (Trost et al., 2010). However, Farias et al. (2020), when testing rNanH, did not obtain good sensitivity and specificity in the tests using goat serums, which shows the importance of the interaction of other molecules, such as co-stimulators for immunogenicity of an antigen of fractions.

Quantification of production *in vitro* of IFN-γ by blood cells

The antigens tested in the present study are of great use for developing immunodiagnostic tests, with the potential for producing vaccines and as a necessary strategy for controlling CL. For *C. pseudotuberculosis*, an intracellular microorganism, stimulation of the immune response of the profile of Th1 responses, with the production of its signature cytokine IFN-γ, is the desired mechanism because, among its other functions, this activates the macrophages in the process of phagocytosis (Meyer et al., 2005; Abbas et al., 2015). The results found here suggest that the biofilm-producer bacteria have mechanisms that favor the increase in production of INF-, and therefore, this is not only a critical bacterial virulence factor but also an essential trigger for activation of the cellular response (Galvão et al., 2017). In this sense, the formation of biofilm must be considered an important virulence factor (Forestier et al., 2017), and the understanding of the complexity of interactions that biofilm promotes for escaping from the immune response of the host could help with the search for new, more efficient vaccination strategies.

Conclusion

In conclusion, all the antigens tested in immunodiagnostics were recognized, and the production of antibodies 7 days after inoculation increased, showing a more ascendant curve after day 14. However, it was clear that the antigen of the strain CapJ4 biofilm producer had a greater capacity for stimulating the humoral immune system, as confirmed by Western blotting. In addition, a capacity for stimulating macrophages was observed, considering the elevation in dosage of interferon-gamma in the acute phase of infection, demonstrating an essential role of the cytokine in the immunological response to infection by *C. pseudotuberculosis.*

Conflict of interest

The authors declare that they have no conflict of interest.

Ethics Statement

This article was approved by the Ethics Committee for Animal Use (CEUA) of the Federal University of Bahia (UFBA) under protocol number 123/2017.

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