Humoral and innate response in goats proteins immunized with vaccine against Corynebacterium pseudotuberculosis

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IgG,
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Summary
The objective of this study was to evaluate the humoral immune response and the innate response of goats immunized with attenuated vaccine against *Corynebacterium pseudotuberculosis* prepared from the strain 1002. One hundred thus, goats were divided into 5 groups (n=20 animals/group). Each group was vaccinated as follows: G control: saline solution; G1 - 10^7 CFU/mL; G2 -10^7 CFU/mL re-vaccinated within 21 days; G3 – 10^6 CFU/mL; G4 – 10^6 CFU/mL revaccinated within 21 days. Blood samples were collected monthly over 12 months and serology was performed through indirect ELISA. In order to verify the innate response through of the dosages of acute phase proteins (ceruloplasmin and haptoglobin), samples of five animals from each group were evaluated on days0, 7, 14, 21, 28 days for the groups G1 and G3, and on days 0, 21, 28, 56 days for the groups G2 and G4. The results showed humoral response activation with the production of immunoglobulins above the cut-off point in all groups. The results showed that strain 1002 vaccine induced the antibodies production by the goats' humoral immune system and that the increase in serum concentrations of haptoglobin and ceruloplasmin may be related of the innate immune response.

Introduction

The caseous lymphadenitis (CLA), is one of the most important diseases related to small ruminants' health. Caused by *Corynebacterium pseudotuberculosis* (CP), it results in reduction of weight gain, interferes with the reproductive efficiency, causes early disposal of animals, decrease in production of wool and milk, as well as condemnation of carcasses, leather and whool depreciation (Izgur *et al.* 210; Ilhan,2016).

Treating the CLA in sheep and goat herds is practically unfeasible because the bacterium is facultative intracellular surviving and multiplying inside the macrophages.

After the cell death, there is the of a necrotic lesion with thick fibrous capsule which hinders the penetration of the majority of antibiotics. Vaccination is still the best way to control the disease (Belchior *et al.* 2006; Dorella *et al.* 2006).

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The defense mechanisms of the immune system to fight the invasion of CP have been of constant interest for researchers aiming at identifying strategies to control the disease through vaccines and diagnostic methods. Initially, it was believed that the cell-mediated immunity was the sole immune defense mechanism against the agent, but it is known that the immune reaction to this pathogen involves both participation of cell-mediated immunity and humoral response with the production of specific antibodies, acting together to try to eliminate the bacterium (Ruiz *et al.* 2007).

The acute phase proteins are constituents of the non-specific innate response, produced mainly by hepatocytes and are involved in inflammation, homeostasis regulation, bacterial growth restriction, before a more effective response of the immune system. Studies on the variation of this innate response through the dosage of acute-phase proteins in the vaccine stimulation are still infrequent in the literature and in the caprine species (Rudoler *et al.* 2015).

In this context, the objective of this study was to evaluate the response of humoral immune system and the response of acute-phase proteins in goats immunized against CP, using an attenuated experimental vaccine prepared from strain 1002.

Materials and methods

One hundred healthy goats were enrolled in the study, aged from two to three years, serologically negative and without defined breed.

The 100 animals were divided into 5 groups of 20 animals each, which received an experimental vaccine prepared from the 1002 live attenuated strain of CP. The vaccine was injected subcutaneously, at a dose of 0,1 mL for each animal.

The groups were prepared as follows: group 1 was vaccinated on the day zero with 10^7 CFU/mL; group 2 vaccinated on the day zero with 10^7 CFU/mL and revaccinated within 21 days; group 3 vaccinated on the day zero with 10^6 CFU; group 4 vaccinated on the day zero with 10^6 CFU/mL and revaccinated within 21 days and control group G which was injected saline solution.

Blood samples were carried out over 12 months.

The procedures performed with the animals were submitted to and approved by the Ethics Committee for Animal Use under the number 23007.016893/2013-73 Federal University of Recôncavo of Bahia.

For the humoral immune response evaluation in goats' sera, ELISA immunoassay was used. Polystyrene Elisa microplates were coated with 100µl of somatic antigens of CP, diluted 1:100 in carbonate-bicarbonate buffer 0.05M, pH 9.6 and incubated at 4 ºC for 18 hours. Then, plates were washed twice with PBS containing 0.1% Tween-20 (PBS-T20). The plates were blocked with 200µl/well of PBS-T20 containing 5% of skimmed milk, during 2 hours at 37ºC. After this period, the plates were washed twice with PBS T-20. Fifty µl/well of test sera diluted 1:100 in PBS-T-20 were added and plates were incubated for 1 hour at 37 ºC. After plates were incubation for 1 hour, 5 washing cycles were performed with PBS-T-20 and 50µl of goat anti-immunoglobulin of rabbit were added to the plates, conjugated to peroxidase (Dako, Santa Clara, CA, USA), diluted to 1:10,000 in PBS-T-20. The plates were incubated at 37 ºC for 45 minutes, and then washed five times in PBS-T-20 and incubated with 50µl well of revealing solution (10 mL of phosphate buffer pH 5.1 citric orthophenylenediamine + 4mg + 4µl H202). The reaction was stopped with 25 µl of sulfuric acid (H_2SO_4) 4N. The Optical Density (OD) reading was performed in an ELISA reader, using a 490nm wavelength filter. The cutoff point considered in the experiment was 0.310 based on indirect somatic ELISA test for goats standardized by Zerbinati *et al.* (2007). Five serum samples from each group were used for the determination of the electrophoretic profile of proteins (ceruloplasmin, haptoglobin), by the method of polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE). The G1 and G3 vaccinated groups were evaluated on days 0, 7, 14, 21 and 28 while the G2 and G4 revaccinated groups were evaluated on days 0, 21, 28 and 56.

For the fractioning of different protease serum constituents, electrophoretic running was performed on the respective serum samples in polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE), according to the technique described by modified Laemmli (1970). Ten µL of blood serum, diluted in 30 µL of phosphate buffer saline (PBS) and 20 µL gel mix were heated for 10 minutes. An aliquot of 5 µL of each sample of blood serum was deposited in duplicate together with the reference standard samples (a marker of molecular weights) in the gel cavities. After the proteins fractioning, the gel was stained for 10 minutes in a solution of 0.25% Coomassie Blue.

A methanol-based decolorizing solution was used to remove the excess of dye. The molecular weight and the concentrations of the protein fractions were determined by computerized densitometry, from the samples scanning.

For the molecular weight calculation, markers of molecular weights of 200, 116, 97, 66, 55, 45, 36, 29, 24 and 20 kDa were used, in addition to purified proteins. For the densitometric evaluation of protein bands reference curves were manufactured, from the reading

of the standard marker.

In the immunoglobulins evaluation, a descriptive analysis was performed of the data and subjected to the analysis of linear mixed model with repeated measures in time considering the dosage as fixed effect and random effect.

For the protein evaluation, data were analysed by means of a factor model considering the following variables: dosage, time and their interactions. For multiple comparisons of means, the Tukey test was applied at 5% level of significance.

Results

No significantly different (p=0.06) concentrations of immunoglobulins in all the immunized groups were observed when used the indirect ELISA during the 12 months of observation, however, vaccinated and re-vaccinated immunoglobulin concentrations are different (p<0.05) different from the control group.

In group 1 (10^7 CFU/mL), an early production of antibodies above the cut-off point was observed at 28 days, and a peak of production in the values of OD at 140 days (0.508). This group had a mean (M) and standard deviation (SD) of 0.399 ± 0.085 over the period. A decline of production was observed at 196 days, with varying levels of OD, until the end of the period of observation, as shown in figure 1.

In group 2 (10^7 CFU/mL revaccinated at 21 days), an early production was also observed at 28 days, above the cut-off point set at 0.310 OD, with peaks of production of 0.610 in the values of OD at 140 days.

The mean and standard deviation, observed in this group was 0.384 ± 0.101. It was noted a decrease in values of OD below the cutoff point at 280 days, with an elevation of these levels to 0.308, remaining until the end of the observational period, as shown in figure1.

In group 3 (10^6 CFU/mL), it was noted at 28 days a beginning of production, as well as in groups 1 and 2. At 168 days, a peak of production of 0.516 in the OD values was observed. At 140 days, a decrease in serum levels was observed close to the cutoff point in contrast to what was observed in group 1 and 2 in the same period. Values close to the cut-off point were also observed at 196 days with following periods of increasing and decreasing values until the end of the experimental period, as shown in figure 2. The mean and standard deviation, observed in this group was 0.375 ± 0.105 over the observaton period. In group 4 (10^6 CFU/mL revaccinated at 21 days) a beginning of immunoglobulins production was also observed at 28 days, with a peak of production of 0.528 in the OD values at 84 days. Values close to the cut-off point were recorded in different periods of observation: on days 112, 196 and 336, with periods of following increase as shown in figure 2. The mean and standard deviation obtained in this group were 0.361 ± 0.108.

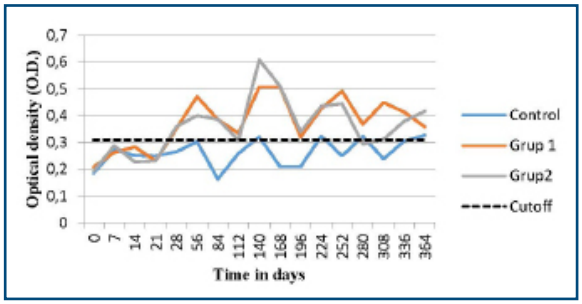

Figure 1. *Means of dosage of total IgG using somatic Corynebacterium pseudotuberculosis antigen in indirect ELISA: control group (saline solution), G1 (dose 10^7 CFU/mL) and G2 (dose 10^7 CFU/mL revaccinated at 21 days).*

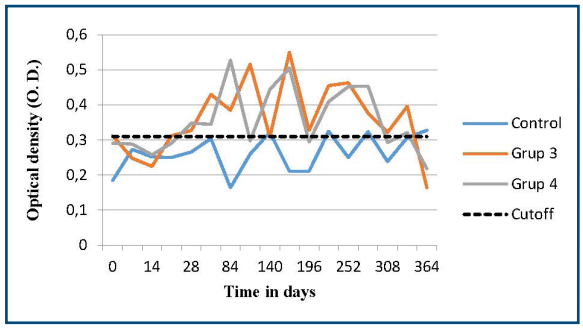

Figure 2. *Means of dosage of IgG total using somatic Corynebacterium pseudotuberculosis antigen in indirect ELISA: control group (saline solution), G3 (dose 10^6 CFU/mL) and G4 (dose 10^6 CFU/mL revaccinated at 21 days).*

Regarding the acute phase proteins, analyzing the serum concentrations of ceruloplasmin, it was possible to observe that at 21 days, the group 1 presented statistically significant higher means than the group 3, but equal to the control group (Table 1). During the measurement period for this group, no differences were observed among the experimentation days. Whereas at 14 days, group 3 showed concentrations 1.5 times higher than group 1, although these means were not statistically different. Considering the time factor, no variations were observed in the means of group 3.

Table I. *Means of serum concentrations of ceruloplasmin g/L groups of Corynebacterium pseudotuberculosis vaccinated animals, G1(10^7 CFU/mL) and G3(10^6 CFU/mL).*

	Days of experiment (time)					
Groups	**0**	**7**	**14**	**21**	**28**	**CV%**
Control	0.190^{Bb}	0.782^{ABa}	0.868^{ABa}	1.240^{Aa}	$0.624A^{Ba}$	63.87
G1	0.847^{Aa}	0.655^{Aa}	0.652^{Aa}	0.881^{Ba}	0.579^{Aa}	38.59
G3	1.540^{Aa}	0.778^{ABa}	1.020^{ABa}	0.713^{Ba}	0.561^{Ba}	56.28

Means followed by upper case letters equal in the same column and lowercase letters on the same line do not differ among themselves by Tukey test at 5% probability CV = coefficient of variation.

Regarding the revaccinated animals, group 2 showed, on day 21, serum concentrations of ceruloplasmin 1.5 times, when compared with the period before vaccination, with reduction of these concentrations at the end of the measurement periods (Table 2). Group 4 showed concentrations very similar to group 2 on day 21, but no change in relation to the moment before vaccination and also with a decrease in the concentrations in the final periods.

Table II. *Means of serum concentrations of ceruloplasmin g/L groups of Corynebacterium pseudotuberculosis revaccinated animals at 21 days, G2(10^7 CFU/mL) and G4(10^6 CFU/mL).*

	Days of experiment (time)				
Groups	**0**	**21**	**28**	**56**	**CV%**
Control	0.190^{Bb}	1.240^{Aa}	0.624^{ABa}	0.880^{ABa}	74.31
G2	0.664^{Aa}	1.014^{Aa}	0.842^{Aa}	0.695^{Aa}	46.9
G4	1.172^{Aa}	1.198^{Aa}	0.515^{Aa}	0.694^{Aa}	51.46

Means followed by upper case letters equal in the same column and lowercase letters on the same line do not differ among themselves by Tukey test at 5% probability. CV = coefficient of variation.

Observing the serum concentrations of haptoglobin, groups 1 and 3 exhibited at 7 days, concentrations higher and different from those of the control group, although not differing among themselves (Table 3).

Group 1 presented means 3.6 times higher at time 7(seven) in relation to 0 (zero). The statistical analysis ($p<0.05$) revealed no differences among the groups and throughout the period evaluated.

Table III. *Means of serum concentrations of haptoglobin g/L groups of Corynebacterium pseudotuberculosis vaccinated animals, G1(10^7 CFU/mL) and G3(10^6 CFU/mL).*

	Days of experiment (time)					
Groups	**0**	**7**	**14**	**21**	**28**	**CV%**
Control	0.232^{Ba}	0.364^{Ba}	0.520^{ABa}	0.344^{ABa}	0.829^{Aa}	74.07
G1	0.216^{Ba}	0.783^{Aa}	0.426^{ABa}	0.614^{ABa}	0.692^{Aa}	47.91
G3	0.661^{Aa}	0.626^{Aa}	0.624^{Aa}	0.314^{Aa}	0.500^{Aa}	47.71

Means followed by upper case letters equal in the same column and lowercase letters on the same line do not differ among themselves by Tukey test at 5% probability. CV = coefficient of variation.

In the groups of revaccinated animals, it was observed that at 21 days their means differed from the control group. Group G2 had 2.4 times higher concentrations of haptoglobin levels than the control group in this period, while G4 presented an increase of 3.2 times in their concentrations, in relation to the control group, as shown in table 4. The statistical analysis ($p<0.05$) revealed no differences throughout the evaluated period.

Table IV. *Means of serum concentrations of haptoglobin g/L groups of Corynebacterium pseudotuberculosis revaccinated animals at 21 days, G2(10^7 CFU/mL) and G4(10^6 CFU/mL).*

	Days of experiment (time)				
Groups	**0**	**21**	**28**	**56**	**CV%**
Control	0.232^{Aa}	0.344^{Ab}	0.829^{Aa}	0.284^{Aa}	86.45
G2	0.332^{Aa}	0.835^{Ba}	0.963^{Aa}	0.376^{Aa}	67.94
G4	0.939^{Ba}	1.113^{Ba}	0.608^{ABa}	0.354^{Ba}	50.11

Means followed by upper case letters equal in the same column and lowercase letters on the same line do not differ among themselves by Tukey test at 5% probability. CV = coefficient of variation.

Discussion

All the animals were allocated in the same physical space, endemic for CP, and physical contact occurred among the individuals of the different groups throughout the experimental period. They were subjected to the same water and sanitary regime, the same risk of contamination from various etiologic agents, being managed in their natural climatological and edaphological habitat. In other words, they were in the same conditions to the majority of animals kept in extensive husbandry systems in semi-arid regions (Almeida *et al.* 2010).

The immunoglobulins production curve showed a dynamic way in all groups, with peaks of production and decrease of immunoglobulins throughout the experimental period, not following the expected standard described in the literature (Castelan *et al.* 2008) that would be a curve of antibody production with low initial production, followed by a growth and stability, with sharp drop after a period of time. These variations in the immunoglobulins curve may be explained by the influence of environmental factors, the nature of the antigen, its concentration, route of administration, genetics of the immunized individual and the vaccination protocol used. All these factors might have influenced the qualitative and quantitative analysis of the immune response during the observation period reflecting the dynamics of the antibody curve (Igietseme *et al.* 2004).

The live attenuated vaccine antigen used in this experiment, may have influenced the peaks and declines in the antibody production observed in similar periods in vaccinated groups, since the live attenuated bacterium can multiply itself constantly in the animal body stimulating in various forms the antibody production (Santos *et al.* 2016).

In all the tested doses, an antibody production was observed, which can lead to infer that the administration of a single dose of vaccine with lower concentration of antigen can stimulate the animals' immune system in the production of specific IgG against CP. This is an important

advantage for the industry as manifactoring might require in a smaller quantity of inputs (Barbosa *et al.* 2017). For producers, it facilitates the management by spending less on manpower and purchase of vaccine for revaccination, given the application of a single dose of the immunogen.

In groups revaccinated at 21 days (G2 and G4), a faster production of antibodies, that would be the expected pattern, was not observed. This may have occurred because when an antigen stimulates cells B, the process of somatic hypermutations of these antigen-specific cells in germinal centers begins with the purpose of potentiating a secondary response faster and more specific. However, hypermutations may also lead to reduction or inability of affinity with the antigen retarding the secondary response (Castelan *et al.* 2008, Lui *et al.* 2017).

Production of immunoglobulins was observed up to twelve months of follow up, however, the G2 group showed an increase of immunoglobulins after 364 days, unlike the other groups that had a drop in serum dosages. This can be explained by the antigens accumulation in follicular dendritic cells in the lymph nodes, that would stimulate periodically in a more extended time the memory cells for antibodies production (Binns *et al.* 2007).

Lima *et al.* (2017), using an attenuated strain T1 in the dosage of $2x10^5$ and $2x10^7$ at 60 days with revaccination, observed antibody production in sheep, but did not record a statistical significance among the vaccinated and revaccinated groups of animals, results similar to those found in this study, which also did not observed significant differences between the vaccinated and revaccinated groups of animals at 21 days. Lima *et al.* (2017) also indicated that a vaccine dosage, would stimulate the immune system in the antibodies production for at least 12 months, corroborating the results presented in this study.

Animals without a defined breed, Anglo-Nubian and cross-bred alpine breeds also had the same pattern recognition over time, inferring that breed does not influence the antigen recognition, since it is assumed that animals without defined breed are more resistant to diseases (Vale *et al.* 2003). Breed, therefore, would not be a factor that has influenced the antibodies production curve in this experiment.

The acute phase proteins can be indicators of the innate response activation which is the first sign of cells activation related to the immune system inflammatory process.

The activation of these cells prepares the immune system for a subsequent specific and more efficient response. As the vaccine under study is attenuated, it was expected that it would stimulate natural infection, stimulating the innate response, which may be verified through the concentrations of acute phase proteins, however, this concentration of proteins was not so intense (Dorella *et al.* 2006; Simplicio *et al.* 2015).

In this study, for the vaccinated animals group, the haptoglobin concentrations were higher at 7 days of measurement which may be associated with the innate response to vaccination, similar results to those of Eckersall *et al.* (2007) who observed mean values of haptoglobin levels in sheep experimentally infected with CP, significantly greater at 7 days of infection.

In the revaccinated group a higher concentration of haptoglobin was observed at 21 days, being that group 4 presented serum levels 3.2 times higher than the animals in the control group. In this group, the vaccinal stimulus provided a better production of haptoglobin, in a longer period of time. Simplicio *et al.* (2017) reported increases of 621% of concentrations of haptoglobin in goats with naturally acquired staphylococcal mastitis when compared with the control group, indicating that haptoglobin is a good inflammatory process indicator.

In goats experimentally infected with CP serum, concentrations of haptoglobin were verified significantly higher when compared to the control group (Jeber *et al.* 2016). Sheep infected with *Pasteurella multocida* showed high levels of acute phase proteins especially haptoglobin with mean value of 1.65g/dL, whereas the non-infected presented 0.0048g/dL (El-Deeb & Elmaslemany, 2016), reinforcing the importance of protein as a response to inflammatory processes.

In other animal species, and using vaccination stimulus to verify production of acute phase proteins, Riber *et al.* (2015) worked with pigs vaccinated against *Lawsonia intracellularis* with attenuated strain, and found no significant differences in the haptoglobin concentration between the vaccinated and the control group, and the vaccine did not induce detectable levels of IgG and IFN-γ.

The natural or experimental infection can stimulate the proteins production much more intense, as in the case of haptoglobin and ceruloplasmin, when compared with the use of vaccine antigen in its attenuated form as it was used in this study (Dorella *et al.* 2006).

Future investigations become necessary to investigate to what extent the innate system is stimulated with attenuated vaccine antigen and even inactivated antigens that constitute the majority of vaccine formulations.

From day 28 of the experiment on, a decrease was observed in serum concentrations of ceruloplasmin, haptoglobin, mainly in vaccinated groups, a trend

that can be explained by the increase of specific adaptive response with elevation in the antibodies production (Silva, 2001), being noted in this period, a beginning of production of IgG above the cutoff point, *i.e.*, a replacement of the innate response by the adaptive response (Castelan *et al.* 2010).

This may also indicate that revaccination does not induce an amplification of the innate response but a stimulation of the humoral response and immunologic memory (Lui *et al.* 2017).

In this study, the haptoglobin and ceruloplasmin were the proteins that may be related to the innate response; however, more studies on the dynamics of variations of these proteins should be developed to elucidate the innate response stimulation to vaccination stimulus (Rudoler *et al.* 2015).

Conclusions

The results showed that the lyophilized vaccine induced humoral immune response in goats vaccinated over twelve months, verified by the dosage of total IgG, which implies that a revaccination would be indicated after this period and that in a single dose of vaccine a smaller quantity of antigen (10^6 CFU/mL) can be used for animal vaccination, once there were no statistically significant differences between the groups.

Haptoglobin and ceruloplasmin were the proteins that may be related with the innate response to stimulation with lyophilized attenuated vaccine against CP.

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