Veterinary Immunology and Immunopathology 211 (2019) 38–43

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

# Natural antibodies and their relationship with total immunoglobulins and acquired antibody response in goat kid (*Capra hircus*, L. 1758) serum

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ARTICLE INFO

*Keywords:*
Antibody response
Indirect-ELISA
Natural antibodies
Total immunoglobulins

ABSTRACT

Natural antibodies (NAb) are antibodies that can bind to a particular antigen without any apparent antigenic stimulation. In this paper, a careful analysis has been carried out on NAb levels in goat kid serum; possible correlations with the total immunoglobulin (tot-Ig) levels and specific antibody (SpAb) response were considered. Twenty randomly chosen kids were submitted to a first blood sampling (day 0). After 60 and 100 days, new blood samplings were carried out in the same animals. On day 0, after blood collection, all animals were immunized with a commercial vaccine; the immunization was repeated 30 days apart. Some exogenous antigens were tested to verify their immunoreactivity to NAb. Among them, the synthetic hapten 2,4,6-trinitrophenyl (TNP) conjugated with bovine serum albumin, resulted as the antigen with the higher immunoreactivity to NAb. Tot-Ig levels increased over time ($p < 0.001$). On the contrary, NAb levels, both IgG- and IgM-isotypes, significantly decreased during the experimental period ($p < 0.001$ and $< 0.05$, respectively). Linear regression analyses showed a high correlation between IgM-NAb and tot-IgM levels ($p < 0.001$) at all the evaluated sampling times. However, a significant correlation between IgG-NAb and IgM-NAb was found only at the 1st ($p < 0.01$) and at the 2nd sampling ($p < 0.05$). No significant correlations were found between SpAb response and the other assessed humoral immune parameters. The obtained results are discussed in the light of the possible use of NAb assessment for the evaluation of the immune system activity in goat.

## 1. Introduction

Natural antibodies (NAb) are termed as antibodies (Ab) that can bind to a particular antigen or pathogen without any apparent antigenic stimulation (Boes, 2000). Unlike antigen-induced Ab, NAb are characterized by a broad specificity repertoire, including self and non-self structures such as carbohydrates and glycolipids, usually with low binding affinity (Casali and Schettino, 1996; Boes, 2000; Cecchini et al., 2016). Curiously, because of their broad specificity repertoire, NAb reactivity usually has been mistaken as "background" in immunoassays (Oschenbein and Zinkernagel, 2000). NAb are believed to play a pivotal role in the first line of defence against pathogens once they passed physical barriers by directly neutralizing the pathogen and activating the complement system (Oschenbein and Zinkernagel, 2000). NAb synthesis in man and mice is guaranteed by a small subset of B cells, termed B-1 cells, generated during foetal or neonatal development, whereas specific Ab production is guaranteed by the B2 subset of B-cells, poorly present in neonates, which consequently trigger a very poor acquired immune response (Boes, 2000). Even if IgM-isotype plays a predominant role in mice and humans (Berland and Wortis, 2002; Dono et al., 2004), IgG- and IgA-isotypes have been found in blood and secretions of many animal species (Casali and Schettino, 1996; Haghighi et al., 2006; Ploegaert et al., 2011; Cecchini et al., 2016).

NAb levels are usually determined via immunoenzymatic assay (indirect-ELISA). In the analytical procedure, microtitre plates are coated with antigens against which the animal is immunologically naïve. Thus, the chosen antigens are assumed not to be present in the common environment of the target species, and, therefore, can be assumed as antigens to which the species is not naturally exposed. The selection of the antigen of choise is crucial in the NAb assessment. In this regard, several antigens have been proposed in different species and most of them were of natural origin (Casali and Schettino, 1996; Boes, 2000). However, the choice can also involve the use of artificial (not-naturally) antigens, in respect of which the animals are certainly naïve. In this regard, the haptens 2,4,6-trinitrophenyl- (TNP-) and 2,4-dinitrophenyl- (DNP-), usually conjugated to albumin, are widely used

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https://doi.org/10.1016/j.vetimm.2019.04.004
Received 19 December 2018; Received in revised form 15 April 2019; Accepted 19 April 2019

because of their high immunoreactivities (Klinman and Press, 1975; Gonzalez et al., 1988; Cecchini et al., 2016; Cecchini and Caputo, 2017).

In animal husbandry, since NAb act as a first line of defence against pathogens, their assessment represents a potential marker of health status of the individuals or, even, of the animal group (van Knegsel et al., 2012; Thompson-Crispi et al., 2013; Machado et al., 2014; Mayasari et al., 2016a; Van Altena et al., 2016).

To our knowledge, little information are available about NAb in small ruminants. Dugovich et al. (2017) have studied IgM-isotype in the wild desert bighorn sheep (*Ovis canadensis nelsoni*, Merriam 1897), describing a procedure for measuring natural antibodies reactive to cell envelope proteins of *Vibrio coralliilyticus*, a marine bacterial pathogen that infects corals and oysters to which the wild sheep is unlikely to have been exposed. These authors demonstrated that IgM-NAb bind to proteins, and, in particular, to bacterial epitopes, and NAb levels are correlated with the ability of plasma to kill strains of *E. coli*. Another wild sheep, the Soay sheep, living unmanaged on a remote Scottish island, was studied for the presence of natural auto-antibodies (NAAB) binding nuclear and cytoplasmic antigens by the use a modified ELISA kit (Graham et al., 2010). High levels of antinuclear NAAB were found and they were positively associated with adult female survival during harsh winters but negatively associated to reproductive activity. Moreover, the same authors pointed out a positive correlation between antinuclear NAAB and total immunoglobulin G (tot-IgG) levels as well as between antinuclear NAAB levels and the specific Ab (SpAb) titres against *Teladorsagia circumcincta*, a parasitic nematode infecting the gastric glands (Graham et al., 2010). In addition, some authors have focused their studies on the possible relationship between NAb levels and the acquired immune response in animals but the results are controversial. In fact, positive correlations between NAb levels and SpAb response were demonstrated by some authors (Parmentier et al., 2004; Thompson-Crispi et al., 2013; Cecchini et al., 2016; Van Altena et al., 2016). In contrast, Sinyakov et al. (2006) showed that the SpAb response can be negatively correlated with NAb levels when NAb binding the specific antigen are present. Thus, the vaccine failure can be due to the use of certain antigens that should stimulate a SpAb response already existing as NAb.

The aim of the present study was to develop in goat kid serum samples an immunoenzymatic method (indirect-ELISA) that is able to detect among some non-self antigens, the substance with the highest immunoreactivity to NAb, both IgG- and IgM- isotypes, in goat kid serum samples. Subsequently, the relation among NAb levels, tot-Ig levels and SpAb response to a commercial vaccine was examined.

## 2. Materials and methods

### 2.1. Experimental design, immunization and sampling

This study was conducted on a private farm located at 650 m above sea level in Campania (Petina, Salerno, Italy) from December 2017 to March 2018. All procedures were carried out in strict accordance with European legislation regarding the protection of animals used for scientific purposes (European Directive 2010/63), as recognized and adopted by the Italian law (DL 2014/26). No animal has suffered as a consequence of the conducted experiment. Twenty kids of Camosciata delle Alpi goat breed aged three months and an average weight of 15.0 ± 1.0 kg were randomly chosen from a population of 60 female kids and their 50 dams and enrolled in this study. In the breeding season the females were naturally inseminated by five males, raising the breeders all together in promiscuity. The animals were bred all together and housed indoors, but the dams were taken to graze at 10:00 in the morning and to shelter at 17:00 while the kids were left in the stable. The kids were naturally suckled and further fed with pasture hay ad libitum and with 300 g die$^{-1}$ of a commercial concentrate, containing 17% of crude protein, 3% fat, 7.30% crude fiber, 9.90% ash. All animals were clinically healthy and free from internal and external parasites. The evaluation of health status was based on rectal temperature, heart rate, respiratory rate, appetite and faecal consistency (data not shown).

Kids were submitted to the first blood sampling the first day of the experimental period (day-0), that was repeated after 60 (day-60) and 100 (day-100) days from the beginning of the experiment. Blood samples were collected in the morning from the external jugular vein using Vacutainer tubes without anticoagulant. Serum was obtained after clotting by centrifugation (3000 rpm for 10 min at 4 °C) and stored at −20 °C until immunological analyses were performed. At day-0 and after blood sampling, all animals were immunized by subcutaneous injection on the side of the neck with 1 mL of the commercial vaccine Bravoxin 10 (MSD Animal Health), containing inactivated clostridial antigens with aluminium potassium sulphate as adjuvant. A second dose of vaccine was repeated 30 days apart.

### 2.2. Natural antibody levels, immunoglobulin levels and specific antibody response: procedure optimization and analysis by indirect-ELISA

An indirect-ELISA was developed to evaluate the reactivity of NAb, both IgG- and IgM-isotypes, to the following eight non-self antigens in order to verify among them the substance with the highest immunoreactivity: 2,4,6-trinitrophenyl bovine serum albumin (TNP-BSA, Bioresearch Technologies, USA), lipopolysaccharides (LPS) from *Escherichia coli* (serotype 055:B5, Sigma-Aldrich, Italy), keyhole limpet hemocyanin (KLH, Sigma-Aldrich), chitosan from shrimp shells (Sigma-Aldrich) and alginic acid from brown algae (Sigma-Aldrich), bovine serum albumin (BSA, Sigma-Aldrich), dinitrophenyl human serum albumin (DNP-HuSA, Sigma-Aldrich) and human serum albumin (HuSA, Sigma-Aldrich). During the optimization phase of the methodology, 10 sera obtained from the first sampling (day-0) were randomly chosen and used to develop the immunoenzymatic assay. Flat-bottomed 96-well microtitre plates (Maxisorp, Nunc, Denmark) were coated with the different antigen solutions (30 μg mL$^{-1}$, concentrations that gave no or limited background reaction with the secondary conjugated antibodies) in coating buffer (50 mM carbonate buffer, pH 9.6) and incubated overnight at 4 °C. For each washing step, every well was washed 3 times with 300 μl of 0.01 M phosphate buffer, 0.05 M NaCl (PBS), containing 0.5% Tween 20, pH 7.2 (PBS-T) and 3 times with 300 μl of PBS. Uncoated sites were blocked with blocking buffer (0.5% fish gelatine in PBS-T) for 1 h at 37 °C. After blocking, 100 μl of kid sera were added in duplicate in twofold serial dilutions from l/400 to l/25,600 in blocking buffer and incubated for 1 h at 37 °C. After incubation (1 h, 37 °C) with 100 μl of the polyclonal Ab rabbit anti-goat IgG-HRP (A50-100 P, Bethyl Laboratories, INC) or rabbit anti-goat IgM-HRP (A50-105 P, Bethyl Laboratories, INC), both diluted 1: 10,000 in blocking buffer, the enzymatic reaction was obtained with 100 μl 0.2 mM o-phenylenediamine (OPD) plus 0.03% hydrogen peroxide ($H_2O_2$) in 50 mM citrate phosphate buffer, pH 5. Plates were incubated for 20 min in the dark and the reaction was stopped by adding 50 μl of 2 M $H_2SO_4$. Optical densities (ODs) were read at 492 nm on a microplate reader (model 550, BioRad). The incubation with blocking buffer in place of serum and with blocking buffer in place of both serum and conjugates represented the negative controls. The upper negative limit with a 99.9% confidence was obtained by positive value (cut-off) set at the mean optical density value plus three times standard deviation of the mean of wells in which blocking buffer replaced the antigen. The indirect-ELISA, following the same procedure described above, was repeated on the same sera only using TNP-BSA in the coating phase, the antigen showing the highest reactivity to NAb in serum (see results and Supplementary Fig. 1), at the concentration between 1 and 30 μg mL$^{-1}$, in order to show the optimal antigen concentration for NAb assessment. Once achieved the optimal conditions for optimized antigen concentration and serum dilution (5 μg ml$^{-1}$ and 1/1,500, respectively), the immunoenzymatic assay was performed on all the sera. Analytical

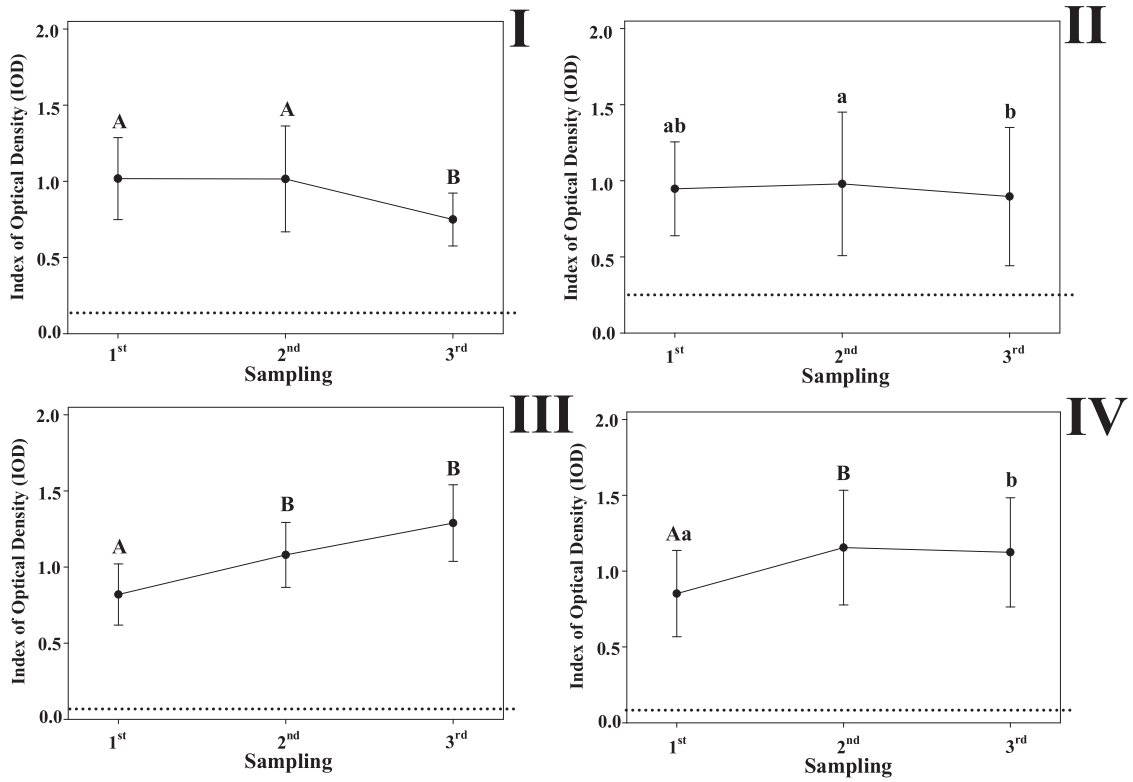


**Fig. 1.** TNP-binding IgG-NAb (I), TNP-binding IgM-NAb (II), tot-IgG (III) and tot-IgM (IV) levels in kid serum on the three sampling times. The results are expressed as mean ± standard deviation of index of optical density (IOD) obtained after dividing the mean of optical density (OD) of each sample by the mean OD of a pool of randomly chosen ten kid sera of the first sampling. Different letters indicate statistically significant differences ($p < 0.01$, capital letters or $p < 0.05$, lowercase letters). The dotted lines represent the upper limit of negativity with a 99.9% confidence limit (cut-off value).

data were expressed as a quotient of the mean of OD of each serum and the mean of OD of the positive control sample (a pool of the randomly chosen ten kid sera of the 1st sampling) in each plate.

The immunoenzymatic assay to evaluate the tot-Ig levels, both IgG- and IgM-isotypes, was performed essentially as previously reported (Cecchini and Caputo, 2017). Microtitre plate wells were adsorbed with 100 µL of kid sera, at the optimized dilution of 1/2,000,000 (for IgG-isotype) and 1/100,000 (for IgM-isotype) in coating buffer, and incubated overnight at 4 °C. After blocking, the plates were subjected to the same procedure as above, applying the two polyclonal Ab rabbit anti-goat IgG- and IgM-HRP, before plate development and OD reading. Analytical data were expressed as a quotient of the mean of OD of each serum and the mean of OD of the positive control sample (the same pool of kid sera as above) in each plate.

The indirect-ELISA for the specific Ab response (SpAb) evaluation against clostridial antigens was performed adsorbing the microtitre plate with the vaccine suspension at the optimized antigen concentration of 5 µg mL$^{-1}$ in coating buffer. Protein concentration of the vaccine suspension, equal to 3 mg mL$^{-1}$, was analysed with Bradford reagent (Sigma-Aldrich), using BSA as standard. After blocking, kid sera at the optimized dilution of 1/10,000 in blocking buffer were added to wells. Subsequently, the polyclonal Ab rabbit anti-goat IgG-HRP was added and finally the plate development and OD reading were obtained as described above. The upper negative limit with a 99.9% confidence was obtained by positive value (cut-off) set at the mean optical density value of five negative (pre-immune) controls plus three times standard deviation of the mean, as reported by Crowther (2001). Analytical data were expressed as a quotient of the mean of OD of each serum and the mean of OD of the positive control sample (a pool of randomly chosen ten kid sera of the 2nd sampling) in each plate.

### 2.3. Statistical analysis

As regards the specificity of NAb against different non-self antigens, in order to choice the non-self antigen with higher immunoreactivity, two-way analysis of variance (ANOVA) was used with antigens and serum dilutions as independent variables. When a significant overall difference was detected, differences among means were determined by Tukey's pairwise comparisons. A probability level of $p < 0.05$ was considered significant. Once the antigen with the highest immunoreactivity was identified (TNP-hapten, see results), the same analysis was repeated on data obtained with different antigen concentrations and serum dilutions as independent variables.

As for the data on immunological constants, once their normal distribution was confirmed using the Kolmogorov-Smirnov test ($p > 0.05$), they were analysed by one-way analysis of variance (ANOVA) for repeated measures, using the following statistical model:

$$Y_{ij} = \mu_{ij} + \pi_{ij} + e_{ij}$$

Where: $Y_{ij}$ is the continuous response of subject *i* at time *j*, $\mu_{ij}$ the fixed mean at time *j* for individuals like individual *i*, $\pi_{ij}$ random effect for subject *i* at time *j* and $e_{ij}$ the normally distributed random error for subject *i* at time *j*. Differences among means were determined by Tukey's pairwise comparisons ($p < 0.05$).

Moreover, linear regression analyses were performed to verify any possible correlations among the different assessed parameters at the same sampling time, according to the equation for a straight line:

$$Y = b_0 + b_1 x$$

Where: Y is the dependent variable, x the independent variable, $b_0$ the intercept and $b_1$ the slope. Results were considered statistically significant when the p-value was less than 0.05.

All statistical analyses were performed using SigmaPlot for

Windows Version 11.0 statistical software (Systat Software Inc., San Jose, CA, USA).

## 3. Results

The ten sera randomly selected for the development of the assay showed significantly higher levels for TNP-binding NAb ($p < 0.01$), both as NAb IgG- and IgM-isotypes, if compared with the levels obtained with the other non-self antigens in the range of $\log_2$ serum dilution between 8.64–12.64 and 8.64–10.64 for NAb IgG- and IgM-isotypes, respectively. Lower immunoreactivities were shown when the other non-self antigens were adsorbed in the microtitre plates, although most of them were significantly higher in comparison to the background reactivity obtained when the blocking buffer replaced the antigen (supplementary Fig.1). In fact, lower reactions to LPS, chitosan and alginic acid were observed when the rabbit anti-goat IgG antibody was applied, showing that these substances are not properly recognized as non-self antigens by goat IgG-NAb, in comparison to TNP-BSA and, to a lesser extent, DNP-HuSA and KLH. Higher binding to alginic acid, DNP-HuSA, LPS and chitosan were shown after applying the rabbit anti-goat IgM conjugate, but the levels of IgM-NAb binding these antigens were lower in comparison to TNP-binding IgM-NAb ($p < 0.05$). Further, a weak reactivity, not significantly different ($p > 0.05$) from the background reactivity, was obtained to KLH when the rabbit anti-goat IgM conjugate was applied. This demonstrated that KLH is not properly recognized as non-self antigen by goat IgM-NAb. As regards BSA and HuSA, there was not any reactivity neither when the anti-goat IgG nor the anti-goat IgM conjugates were applied, with absorbance values similar to those obtained by replacing the antigens with the blocking buffer (data not shown). TNP-binding NAb in kid sera were also analysed by using different concentrations of the selected non-self antigen in the coating step of the indirect-ELISA procedure. Similar results were obtained by using the selected non-self antigen at concentrations ranging from 5 to 30 $\mu g\,mL^{-1}$ (supplementary Fig. 2). A significantly reduced binding was observed when wells were coated with lower antigen concentrations at 1 $\mu g\,mL^{-1}$ and 0.5 $\mu g\,mL^{-1}$ for NAb IgG- and IgM-isotypes, respectively. As regards the OD obtained by applying different sera dilutions, our results showed a linear response when the dilution ranged from 1:800 to 1:3200, both for IgG- and IgM-NAb. Based on the above results, NAb levels were then evaluated by using TNP-BSA as non-self antigen at the concentration of 5 $\mu g\,mL^{-1}$, with kid serum dilution set at 1:1500.

Tot-IgG and tot-IgM levels progressively increased throughout the experimental period (Fig. 1). Tot-IgG levels significantly increased ($p < 0.01$) both in the 2nd sampling (day-60,) and the 3rd sampling (day-100) compared to the first sampling (day-0), whereas no difference between the 2nd and the 3rd sampling was found ($p = 0.09$). At the same time, tot-IgM level was significantly higher at the 2nd and at the 3rd samplings in comparison to the 1st sampling ($p < 0.01$ and $p < 0.05$, respectively), but no difference between the 2nd and the 3rd sampling was shown ($p = 0.32$). Contrary to the trend of the tot-Ig levels, both IgG-NAb and IgM-NAb levels did not differ between the 1st and the 2nd sampling after which they underwent a significant reduction ($p < 0.01$ and $p < 0.05$, respectively) on the 3rd sampling (Fig. 1), although no significant difference was found in IgM-NAb level between the 1st and the 3rd sampling ($p = 0.08$). As regards the SpAb response, statistical analysis showed a significant reduction ($p < 0.001$) of Ab titre between the 2nd and the 3rd sampling, obtained after 30 and 70 days from the second administration of the vaccine (Fig. 2).

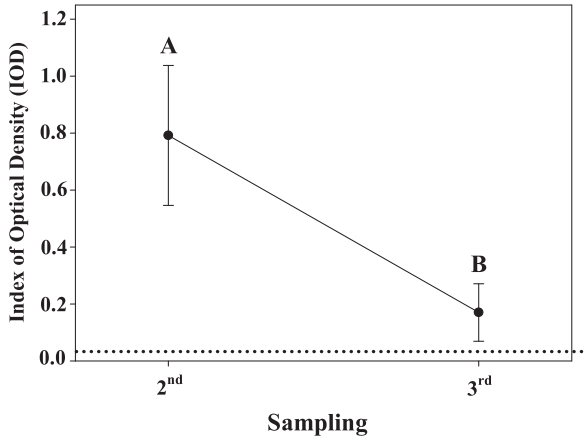


**Fig. 2.** Specific antibody response (SpAb) against BRAVOXIN 10 vaccine in kid serum on the second and third sampling times. The results are expressed as mean ± standard deviation of index of optical density (IOD) obtained after dividing the mean of optical density (OD) of each sample by the mean OD of a pool of randomly chosen ten kid sera of the second sampling. For the complete legend see Fig. 1.

Linear regression analyses were performed to search possible correlation among the evaluated immune parameters within each sampling time. SpAb response was not significantly correlated with the other immune parameters ($p > 0.05$) on neither the 2nd nor the 3rd sampling. TNP-binding IgM-NAb and tot-IgM were highly correlated ($p < 0.001$) in all the three sampling times (Pearson's coefficient $r = 0.896$, 0.672 and 0.928, respectively), whereas the correlation between TNP-binding IgG- and IgM-NAb was found only on the 1st ($r = 0.573$, $p < 0.01$) and the 2nd sampling ($r = 0.534$, $p < 0.05$), whereas it resulted as a tendency on the 3rd one ($r = 0.474$, $p < 0.10$).

## 4. Discussion

In this paper NAb levels in goat kid serum and their possible correlations with tot-Ig levels and SpAb response were shown. Since the choice of the right antigen is fundamental for the NAb evaluation, we first tested some foreign antigens assumed not to be present in the common environment of goat. Therefore, it can be considered that the animals were immunologically naïve to them. Among the tested antigens, the synthetic hapten TNP, conjugated with BSA, gave the highest immunoreactivity to NAb, both IgG- and IgM-isotypes. In fact, we demonstrated that the NAb binding was directed to TNP-hapten and not against BSA, as shown by the weak reaction obtained when BSA was adsorbed as antigen in the microtitre plates. Because the TNP-hapten is an artificial molecule not found in nature, it seems to be a proper antigen to measure NAb, being unlikely that it can be recognized by SpAb. TNP-hapten was previously used as foreign antigen for NAb assessment in other animal species precisely because of its high immunoreactivity (Klinman and Press, 1975; Gonzalez et al., 1988; Magnadottir et al., 2009; Cecchini et al., 2016; Cecchini and Caputo, 2017). The reason for its immunoreactivity seems to lie in its three dimensional structure, similar to common, pathogenic molecular patterns possibly proline-rich motifs (Gonzalez et al., 1988; Tchernychev et al., 1997).

According to literature, several tests have been developed to study NAb binding to exogenous proteins. Where NAb are evaluated by the use of a single foreign protein, KLH is the most widely used because KLH-binding NAb were shown to be correlated to some commercial or physiological traits in livestock (Sun et al., 2011; van Knegsel et al., 2012; Machado et al., 2014; van der Klein et al., 2015; Mayasari et al., 2016a). Furthermore, the relatively high heritability of KLH-binding NAb is considered as a promising selection method to improve general disease resistance both in poultry and cattle industry (Thompson-Crispi et al., 2013; Berghof et al., 2015; Van Altena et al., 2016; de Klerk et al., 2018). On the contrary, our results suggest that KLH is not the proper antigen for NAb evaluation in goat. In fact, although a relevant immunoreactivity to IgG-isotype was shown, albeit significantly lower than by using TNP-hapten ($p < 0.05$), a weak reactivity was demonstrated to IgM-isotype. At the same time, also the other tested foreign antigens gave discordant results, depending on whether IgG- or Ig-M

isotypes were considered (Supplementary Fig. 1). While the synthetic DNP-hapten showed immunoreactivity both to IgG- and IgM-isotypes, chitosan and alginic acid, respectively from shrimp shells and brown algae, and LPS showed variable immunoreactivity depending on the applied anti-goat Ig antibody.

Although the use of a foreign substance provides a cheap and repeatable tool to determine the levels of NAb in animals, their levels are not always predictive of an innate immune protection in a naïve individual before an external antigenic stimulation induces a specific immune response. Our data showed that goat NAb levels are not related with the SpAb response against the applied vaccine. In this regard, literature presents conflicting results. In a previous paper (Cecchini et al., 2016), we showed, in laying hen serum samples, a positive correlation between IgM-NAb levels binding the TNP-hapten and the SpAb response against human-γ-globulins (HγG), a foreign protein having a great immunogenic power, inducing a T-dependent Ab response (Cecchini and Saroglia, 2002). In contrast, Sinyakov et al. (2006) showed a negative correlation between NAb levels and acquired Ab titres in vaccination trials. According to these authors, high NAb levels can cause a reduced specific Ab response due to their aspecific binding to specific antigens.

Conversely, other papers pointed out the positive correlation between NAb levels and health status traits. Banos et al. (2013) hypothesized that high KLH-binding IgG-NAb levels are associated with an improved responsiveness of the innate immune system to pathogens, although the same high NAb levels were also related to a poor nutritional status. High natural auto-antibodies (NAAB) levels are associated with an increased survival in the wild soay sheep (Graham et al., 2010), as well as high specific Ab responsiveness against sheep red blood cells (SRBC) and high IgG-NAb levels in chickens (Parmentier et al., 2004). Moreover, milk NAb levels were shown to be positively associated with resistance to mastitis in cows (Ploegaert et al., 2011). This topic has been deepened by Van Altena et al. (2016) who demonstrated that cow sera with high KLH-binding NAb levels had more Ab against common microbial structures and intact *Escherichia coli* and *Salmonella typhimurium*. According to Nussey et al. (2014), female soay sheep with high NAAB binding to antinuclear antigens and high tot-IgM levels, assessed in summertime, are more likely to survive the subsequent winter. In the same paper, the authors observed a reduced survival rate in animals with low IgM-NAb levels binding to KLH. Therefore, the assessment of the relation between the NAb level and the SpAb response seems to depend on the species, the foreign antigen applied for NAb evaluation, the nature of the antigen used to induce the specific Ab responsiveness and some environmental factors (Haghighi et al., 2006; Star et al.,2007).

In the present study, the significant correlations among the assessed humoral innate parameters (tot-Ig and NAb) reinforce the idea that NAb might be a valid tool to evaluate the innate immune activity also in goat, as shown in other species. In particular, IgM-NAb and tot-IgM levels are significantly correlated in all sampling times ($p < 0.001$). However, we observed an increase of tot-Ig levels together with a simultaneous decrease of NAb levels over time; this does not agree with previous findings in poultry and dairy cows (Srinivasan et al., 1999; Parmentier et al., 2004; Berghof et al., 2010). It should be yet highlighted that the short-term period of our experiment, in comparison with longer-term experiments performed by other authors, may have masked the effective trend of NAb levels. In fact, the increasing trend of NAb levels over time is usually attributed to the cumulative result of antigenic stimulation of the poly-specific receptors of B1-cells, responsible for NAb synthesis (Tomer and Shoenfeld, 1988). Thus, the higher levels of NAb in old individuals corresponds with the idea that exogenous stimuli enhance the NAb synthesis (Prokesova et al., 1996; Martin et al., 2001). Furthermore, a cross-reactivity between NAb and antigens with which the animals may have come into contact cannot be excluded (Geyer et al., 2005; de Klerk et al., 2018). This seems to suggest that a caution is needed in NAb assessment due to the possibility of confusing a specific immune responsiveness for a NAb-mediated reactivity. It should also be highlighted that the study of NAb in livestock animals involves undoubted difficulties compared to those carried out in laboratory animals, involving specific B cell populations, germ-free or transgenic animals, clonal B cells and experimentally infected animals. Therefore, we cannot exclude that sometimes NAb may be mistaken for a cross-reactivity to antigens against which the animal has previously developed a specific immune response. Accordingly, the use of newborns before colostrum intake to study NAb biology to self and not-self antigens in livestock animals would be more correct, at least in those species in which the placenta does not permit the transfer of maternal immunoglobulins to the foetus, as studied by Mayasari et al. (2015 and 2016b). In fact, only NAb binding self antigens were found in plasma of calves before colostrum intake (Mayasari et al., 2016b), whereas the presence of NAb binding the non-self antigens KLH and HuSA was subsequent to colostrum intake (Mayasari et al., 2015). These findings demonstrate that the presence of foreign antigen-binding NAb depends on maternal transfer via colostrum and further sensitizations to environmental antigens.

## 5. Conclusions

A better knowledge of NAb occurrence in goat could be crucial to deeper understand the innate immune activity and its relation with the SpAb response. Our results demonstrate that TNP-binding NAb are not correlated to the SpAb response obtained against a commercial vaccine containing clostridial antigens. Elucidating the role of NAb in goat immune system activity and their relation with the SpAb response requires further investigation, mainly exploring self antigens and other foreign substances which can be recognized by NAb in newborns before colostrum intake.

## Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

## Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## 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.2019.04.004.

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