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THE INFLUENCE OF THE ANTI- *Mycoplasma agalactiae* VACCINE STRAIN ON THE HUMORAL IMMUNITY IN SHEEP

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Abstract

Vaccination against contagious agalactia of sheep, an OIE declarable, highly economically impacting disease, is the most widespread method to reduce the disease prevalence. This research monitored the influence of M. agalactiae S/94 and AG6, two commercial vaccines for sheep, on systemic humoral immunity in two flocks of ewes (n1=95, n2=220, respectively). Blood was sampled from both flocks before and one month after the booster vaccination and subjected to total Ig (24%o zinc sulphate precipitation test) and circulating immune complexes' (4.2% polyethylene glycol precipitation test) evaluation. There were significant differences (p<0.001-0.004) in total Ig levels for both strains (7.68±2.63 and 23.68±5.7 for S/94 and 11.1±3.58 and 26.11±3.4 Vernes degrees for AG6, before and after the vaccination, respectively), but no differences in CIC concentrations (p=0.08 and p=0.59, for S/94 and AG6) between the samplings or the strains (p=0.342) were found. A strong positive correlation (r=0.889, p<0.05) was established between total Ig and CIC levels for the AG6 but not the S/94 strain, therefore other influential factors (individual, adjuvant, frequency of vaccination) should be investigated.

Key words*: vaccination, M. agalactiae, S/94/AG6 strain, humoral immunity.*

INTRODUCTION

Mycoplasmic mastitis (contagious agalactia) in sheep and goats represent one of the most challenging infectious pathologies in traditional, but also intensive raising of small ruminants, due to its health, welfare and also economic impact (decreased milk production, movement and thus, feeding difficulties, arthritis and ocular lesions in all other categories besides lactating ewes) (Almeida et al., 1992; Cottew and Yeats,1982; DaMassa et al., 1992). The etiology involves mainly *Mycoplasma agalactiae* in most of the flocks either ovine or caprine. Still, *M. capricolum capricolum* and *M. putrefaciens* along with *M. mycoides capri* could be also isolated in animals with either agalactia or arthritis (Fox et al., 2003).

The use of non-specific humoral response as indicator for the general immunity in vaccinated animals, besides providing standardization means concerning the immune status of the sheep and further, its efficacy to protect against mycoplasma disease, could bring considerable support to defining some peculiarities linked to the profile of mycoplasmosis in this species (Avramidis et al., 2002). Assessment of the functional level of different non-specific effectors during the evolution of the disease allows the interpretation of some features and phases connected to the post vaccination response, probably linked to the re-emergence of the disease in spite of the anti-agalactia constant vaccination in the areas of high epidemiological risk (Rodriguez et al., 2002; Szeredi et al., 2003).

The experiment aimed at clarifying and better understanding the humoral reactivity, which may also contribute to the establishment of some enhanced measures towards the control of the disease. The study also sought to evaluate the efficacy of different mycoplasma antigens in immunizing against contagious agalactia, by use of two commercial vaccines.

MATERIALS AND METHODS

Biological material and the experimental protocol. The investigations were conducted on two sheep flocks of 95 (n1) and 220 (n2) animals each, including different age categories of animals, such as ewes but also lambs, and rams to avoid orchitis and arthritis in the latter.

The animals were Merino crosses with the local Turcana breed, raised in a semi-intensive technology, periodically following the transhumance routes in NW Romania.

The animals were vaccinated with two types of vaccines, as a primary vaccination, following the producer's protocol.

Vaccines. This research monitored the influence of *M. agalactiae* S/94 and AG6, two strains included in two types of commercial vaccines for sheep, by looking at the systemic humoral immunity developed post-vaccination.

Mycoplasma agalactiae, strain S/94 induces an increase in agglutinating antibodies of at least 2 ln, evidenced by the slow tube agglutination reaction (RSAL), min 5 UE. This inactivated vaccine is recommended for the active immunization against the contagious agalactia of clinically healthy sheep and goats, both in the free herds and in those in which the disease progresses. The vaccine was administered in a dose of 1 ml/animal, regardless of the size of the animals, subcutaneously, at the base of the tail or in the fold. In ewes, the first vaccination was performed in the second part of the pregnancy (month 3), and the booster after 21 days. In youngsters and rams, the vaccination was carried out along with the other animals. Immunity is complete 21 days after the booster vaccination and lasts for 6 month post-vaccination, according to the manufacturer's instructions.

The AG 6 strain vaccine is a Mycoplasma agalactiae suspension inactivated by formalin and heat, adsorbed with aluminum hydroxide gel (min 7-10 UE/dose). This vaccine was used in the second flock following the same instructions as for S/94.

Blood samples were taken twice, before the vaccination and one month after the second dose, in both flocks. The blood was collected on a clotting gel for sera, which were separated after 2 hours by centrifugation (at 1308·g for 10·min) before processing by 24‰ zinc sulphate precipitation test (total Ig) and 4.2% polyethylene glycol precipitation test (circulating immune complexes, CIC) (Ghergariu et al., 2000).

Methods. Immunoglobulin measurements (Khokhlova et al., 2004). Total immune globulins, known as opsonins, play an important role in the 'first line of defence', that is innate immunity, against aggressors. At a pH·7.4, the electric charge and colloidal stability of gamma globulins are lower than those of serum albumins. Thus, concentrations as low as 24·mg/l/ of metal salts precipitate the immunoglobulin. A volume of 6.6·ml of serum was mixed with 193.4·ml of a 0.024% barbital buffer zinc sulphate solution and allowed to precipitate for 30·min at room temperature (22-23°C). Optical density (ODU) then was read spectrophotometrically (l = 475·nm, d = 0.5·cm) and transformed in Vernes degrees, by x 100 multiplication.

Circulating immune complexes' measurements (CIC) (Khokhlova et al., 2004). Quantifying the level of circulating immune complexes (CIC) allows evaluation of the molecular clearance capacity at a particular moment. A 4.2% polyethylene glycol (PEG) solution in borate buffer was used as the precipitating agent, while buffer-treated samples served as controls for borate-induced precipitation. The reaction was performed in a 96-well-plate to enhance spectophotometrical readings. Volumes of 196.7·ml of borate buffer and PEG solution, respectively, were mixed with 3.3·ml samples of the serum, for each sample, in parallel wells. The samples were allowed to precipitate at room temperature (22-23°C) for 60·min, then read spectrophotometrically at a wavelength of 450·nm in the test plate (d = 0.5·cm) (multichannel spectrophotometer SUMAL PE2, Karl Zeiss, Jena, Germany). CIC concentrations, expressed in optical density units (ODU) were calculated by subtracting the value of the control (serum + buffer) from that of the PEG precipitate and further expressed in units (U) by multiplication 10^3 times.

RESULTS AND DISCUSSIONS

The use of the zinc sulphate precipitation assay for the dosage of gammaglobulins provides a

supplement to the immunological profile in vaccinated animals, even without the identification of the classes (IgG versus IgM) of immunoglobulins involved. Following the results obtained, it is observed that the mean values of serum concentrations of gammaglobulins in animals vaccinated against contagious agalactia with S/96 strain are higher than the values of the concentrations recorded before vaccination. Nevertheless, in the flock vaccinated with the AG 6 strains, the increase did not differ significantly from the flock vaccinated with S/96, but the increase was lesser than in the first flock (164.47% versus 107.85%). The statistical significance of the increase in total Ig levels was high in both flocks ($p<0.01$-0.001).

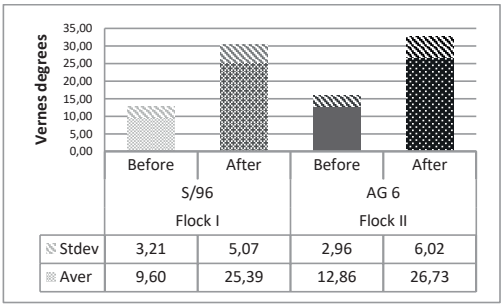

Figure 1. Changes in total Ig during the immunisation (Vernes degrees, x ± s)

Immune complexes arise continuously in the case of Ag-Ac coupling and are efficiently removed by the reticuloendothelial system. In some cases, the formation of immune complexes leads to hypersensitivity reactions.

The formation of immune complexes occurs in different cases: long-term infections, autoimmune diseases, on surfaces, for example in the lung, after repeated inhalation of antigens of different nature, and their presence indicates either an effective way to remove the antigen, or especially in the case of their chronic presence in the body, the ineffectiveness of removing antigens (Moraru 1984). The structure of immune complexes depends on the nature of the antigen and antibody that are involved in their synthesis, the antibodies involved are usually IgG-type (Roitt, 2001).

Frequently, CIC levels provide a measure of the body's reactivity, on one hand, and the severeness of the disease, on the other hand; in microbial diseases, excessive formation of CIC can lead to their deposition in various organs, trigerring the membrane attack complex of the complement and the consequent destruction of the tissue, release of new antigens and the induction of antibodies against the modified self.

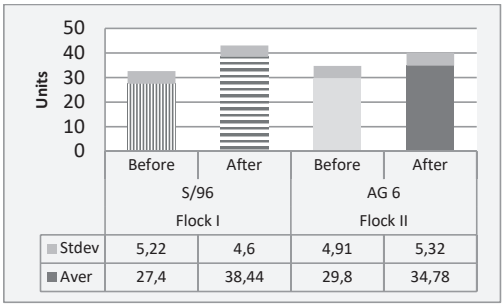

Figure 2. Changes in CIC during the immunisation (units, x ± s)

In Figure 2, the levels of immune complexes registered higher average values in the case of vaccinated sheep, while in unvaccinated sheep the serum concentration of immune complexes was lower.

The values obtained show that the total Ig and CIC levels are little influenced by AGAVAC vaccination, the differences not being statistically significant.

The role of vaccines lies in stimulating the immune system against pathogens, because it guarantees that a new contact of the body with the pathogen does not cause the disease. In order to improve the effectiveness of vaccines, it is important to preserve the characteristics of the pathogen without causing any harm to the animals. It is important that the inactivated substances in the vaccine do not greatly alter the properties of the antigenic proteins. Adjuvants are added to the preparation of inactivated vaccines to indirectly enhance the immunogenic properties of the antigen (Stewart-Tull, 1996).

The literature mentions that the high temperature used to inactivate the *M. agalactiae* vaccine antigen has a destructive effect on its surface proteins (Tola et al., 1999).

It should be noted that AG6 vaccine was inactivated with formalin and heat, and S/96 anti-galactic vaccine was only inactivated with formalin. Inactivation with sodium hypochlorite has the same altering effects on immunoproteins. By switching from the use of

formalin to the use of phenol, then from phenol to saponins as adjuvants, the surface proteins remain with a structure as close as possible to the intact mycoplasma. Tola et al. (1999) show that vaccines inactivated with saponins and phenol induce the formation of a high level of antibodies. The effectiveness of saponins is also noted by other authors (Rurangirwa et al., 1987). Saponin acts as an adjuvant and inactivator at the same time. This eliminates the problems of dose mixing and toxicity caused by various adjuvants.

The use of inactivated phenol and saponin vaccines limits economic losses, especially in areas that are economically dependent on sheep farming.

In regions where contagious agalaxia develops endemic, economic losses result from decreased milk production and shortened lactation, and the prophylaxis of this disease is not fully elucidated due to incomplete knowledge of the pathogenesis of the infection and the fact that highly effective vaccines have limited accessibility.

Table 1. Statistical significance of the total Ig and CIC variations

Antigen	Statistical significance		T test	
	Total Ig	CIC	Total Ig	CIC
S/96	p<0.01	NS	3.7	0.5
AG 6	p<0.001	NS	5.95	1.98

In previous studies, immunoprophylaxis of contagious agalactia was based on udder preparations from infected animals. These vaccines have not been particularly effective and at the same time have been vectors of other pathogens, such as scrapie agent (Caramelli et al., 2001). Recently, a number of studies have been conducted on the development of new anti-galactic vaccines with higher efficacy and safety (Leon et al., 1995; Buonavoglia et al., 1998; Tola et al., 1999; Greco et al., 2002). Experimental vaccines combined with aluminum hydroxide (Al(OH)3) or mineral oils as adjuvants have been shown to be effective (Buonavoglia et al., 1998; Tola et al., 1999; Greco et al., 2002), but the study their safety and immunogenicity require further investigation. Although aluminum hydroxide adjuvanted vaccines have been shown to be safe, they do induce the synthesis of a short-lived antibody titer that persists for a short period of time. In contrast, mineral oil adjuvant vaccines have a high immunogenic capacity, with high levels of antibodies, which persist for a long time and cause only a mild inflammatory reaction around the point of inoculation without being associated with systematic adverse reactions. Vaccines with mineral oil adjuvants are able to prevent the clinical manifestations of contagious agalaxis of animals affected by *M. agalactiae,* but sometimes there is a small increase in the retro-mammary lymph nodes (Buonavoglia et al., 2008).

Both tested strains, S/96 and AG6 induced a significant post-vaccination increases in total immunoglobulin titers (Table 1). No such differences were observed in the case of circulating immune complexes, where the differences between pre- and post-vaccine levels were non-significant for both flocks.

CONCLUSIONS

Comparing the two anti-galactic vaccines, one (S/96) containing formalin-inactivated antigen and the other (AG6) inactivated by heat and formalin, it can be seen that the latter enhances a more pronounced immunoglobulin increase (higher statistical significance of differences) than first, which may lead us to the conclusion that this booster with this vaccine is the cause of more active immune synthesis, because the antigen is practically more distorted than in the first vaccine.

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