Bulletin UASVM Agriculture, 67(1)/2010
Print ISSN 1843-5246; Electronic ISSN 1843-5386

The Relevance of Some Physiological Parameters in Evaluating the Safety of Two Vaccine Formulation Against Contagious Agalaxia in Goats

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Abstract. The safety assessment of the experimental vaccine formula *Agalaxin forte* containing a mineral adjuvant (aluminum hydroxide) as well as an organic adjuvant (anaculture of Staphylococcus aureus), was based on haematologic, biochemical, and clinical milk cell population tests performed on goats (n = 44), as a target species, following a specific protocol. The oscillations of the determined physiological indicators were characterized by a general position between the reference values and unimportant deviations, without any clinical, biological or pathological connotation. In evaluating the safety of the *Agalaxin* vaccine formula, the potential risks have been identified as related to experimental dose (double dose or three successive doses) and the post-vaccine adverse reactions which consisted of ephemeral local reaction (30.1%), of reduced intensity. Ultimately, the safety and innocuity were appreciated as being good, with a high level of safety for all experimental variables and doses of the investigated *Agalaxin forte* vaccine series.

Keywords: goats, physiological parameters, A*galaxin forte*, safe, innocuity.

INTRODUCTION

Immunoprophylaxis offers superior advantages to curative antibiotic therapy or chemotherapy, but may be limited to the risk of potential side effects, of vaccination, which recommendation may be based on economic criteria (restrictions consecutive to treatments with antibiotics and chemotherapy, and their costs) or socio-economic criteria (prevention of infectious and contagious diseases). Data on bio-medical research abounds regarding immunological products; in this context the investigations of the present study, consisting of preclinical and clinical evaluation of general and local reactions, when testing two vaccine formulations on goats as a target species is situated.

MATERIALS AND METHODS

To assess the safety and security of the experimental vaccine formulations (*Agalaxin forte*) containing a complex adjuvant with mineral (aluminum hydroxide) and organic (*Staphylococcus aureus* anaculture) components, tests have been performed, using a control *Agalaxin* vaccine, existing market containing only mineral adjuvant (aluminum hydroxide).

According to the experimental protocol, presented in table 1, we resorted to testing a group of goats, as the target species, grouped into five experimental groups and one control, differentiated based on innocuity, safety and efficacy, and dose-related variables (single dose, overdose and repeated dose). Research has begun with the preliminary testing of 55 goats, of

which 44 were selected as clinically healthy, with basic physiological parameters within the physiological limits. Before and at the end of each test the main hematological biochemical, clinical and milk cell population parameters were necessary for assessing the health of the animals.

Tab. 1.

The experimental protocol used to test the *Agalaxin forte* vaccine on goats

TEST PRODUCT	***Agalaxin forte*** (S.C. ROMVAC COMPANY S.A.)
ACTIVE SUBSTANCES	Specific Antigen - inactivated *Mycoplasma agalactiae* (D.M. or obtained from strains isolated in Romania by Romvac S Co-SA) with minimum titre 10^6UFC/ml; Organic Adjuvant - anaculture of *Staphylococcus aureus.*
DESIGN OF THE RESEARCH STUDIES	Two vaccine formulations (Agalaxin and Agalaxin forte), two periods, single dose, overdose, repeated, testing the innocuity, safety and efficacy in healthy goats
TEST GROUP	At least 44 goats
STUDY PROCEDURE AND DISTRIBUTION IN GROUPS	Selecting animals based on exclusion and inclusion criteria *, confirming their health status through clinical examination, hematological and biochemical, formation of five experimental groups and one control, totaling 44 goats. Development of the experiment for the groups: • I (n = 10) - vaccination with one dose on day 1 + Agalaxin Forte, rappel on day 15 and repeat the initial tests at the end (21 days after rappel), day 37; • II (n = 5) - vaccination with two doses (overdose) Agalaxin forte on day 1 + rappel on day 15 and repeat the initial tests at the end (21 days after rappel), day 37; • III (n = 5) - vaccination with one dose on day 1 + Agalaxin forte a dose the dose on day 15 a 30, and repeating the initial tests at the end (21 days after rappel), day 37; • IV (n = 7) - vaccination with one dose on day 1 + Agalaxin forte rappel on day 15, before the rappel immunological tests, on 15 and 21 days after rappel on day 37; • V (n = 10) - unvaccinated, immunological tests on days 15 and 30, and the day 30 vaccination security forces Agalaxin, five animals; • VI (n = 7) - Agalaxin 1 dose vaccination with rappel on day 1 + day 15, before the rappel immunological tests, on 15 and 21 days after the day 37 rappel. Throughout the study animal health will be monitored for possible side effects or adverse reactions
ADMINISTRATION	Injection: 1 ml/dose/animal, subcutaneously (base of tail), same for double dose.
DURATION	2-3 vaccinations at an interval of 14 days.
PRIMARY PARAMETERS	Efficiency rating based on vaccine antibody titres.
SECONDARY PARAMETERS	Clinical evaluation, hematological, biochemical and mammary health status.
SAFETY PARAMETERS	Monitoring by clinical and laboratory examinations of possible side effects, both systemic manifestations (general changes intolerance, hypersensitivity, allergy) and local (innocuity character changes).
ASSAYS	Hematological (complete blood count), biochemical (metabolic profile) and mammary health determinations, determination of antibody titres by ELISA.
STATISTICS	ANOVA system, including models: treatment, sequence, subjects and periods of administration.

The recorded values of these determinations were correlatively linked, serving firstly for selection of the eligible animals and then monitoring the corresponding developments in health, pre-and post-vaccination, and especially to detect and characterize any general or local side effects.

Animal selection was based mainly on the inclusion and exclusion criteria given by the degree variation from the physiological limits of the test parameters, and respectively the contraindications of the prospectus.

Blood samples taken on EDTA were used to determine blood counts and the biochemical investigations were performed on blood serum samples collected on clot stabilizer. Blood tests (*Ht, Hb, no. erythrocytes, mean erythrocyte constants, no. leucocytes, and leukocyte subpopulations*) were performed using an automatic analyzer type Abacus Junior Vet, the biochemical parameters *(total protein, urea, creatinine, GOT, AST , GPT, ALT, GGT, albumin, calcium, magnesium*) were determined with a semiautomatic biochemistry analyzer type Screen point and the cell population parameters on milk sediment smears colored panoptically (MGG and Dia Quic Panoptic Color staining methods) *Ognean* et *al. (2007).*

Individual and average data were statistically analyzed and interpreted using biostatistics software (Graph Pad Instat V3.0, Microsoft Excel), which allowed for calculating *the mean, standard deviation and index of probability p.*

RESULTS AND DISCUSSIONS

Regarding clinical, hematological, biochemical and milk cell population examinations performed pre-and post-vaccination, we found most physiological parameters the within the physiologic limits, showing that the test was introduced only healthy animals and their health indicators have remained constant throughout the experimental period.

The evolution of hematological and biochemical parameters was characterized by large oscillations, falling within physiological limits, or with minor deviations from them. From the following graphics showing the dynamic evolution of the erythrocytes, leukocyte and biochemical parameters pre-and pos-vaccination, can be determined dominant characteristic of each investigated parameter.

As outlined in the first graph (fig.1), mean hematocrit and hemoglobin values were considered as within the physiological limits (22-38% and 8-12 g/dl), with variations between 21.58% and 28.49 % for hematocrit and between 7.55 g/dl and 9.16 g/dl for hemoglobin, not related to the experimental variable.

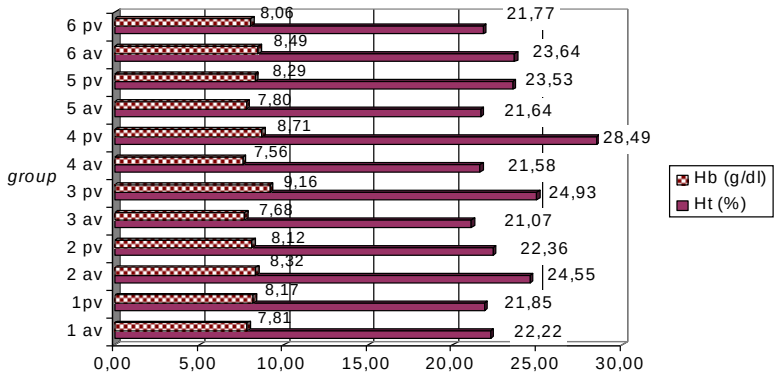

Fig. 1. Evolution of the average values of hematocrit (%) and hemoglobin concentration (g/dl) recorded pre-vaccination (av) and post-vaccination (pv) for the groups of investigated goats

In case of the total number of erythrocytes, mean values were also situated between the physiological limits (8-18 T/l), both minimum 13.05 T/l and maximum of 15.42 T/l recorded pre-vaccine. For the total number of leukocytes an upward trend was the dominant feature (10.13-13.15 G/l), but not significantly exceeding the physiological limits (4-13 G/l) (fig. 2).

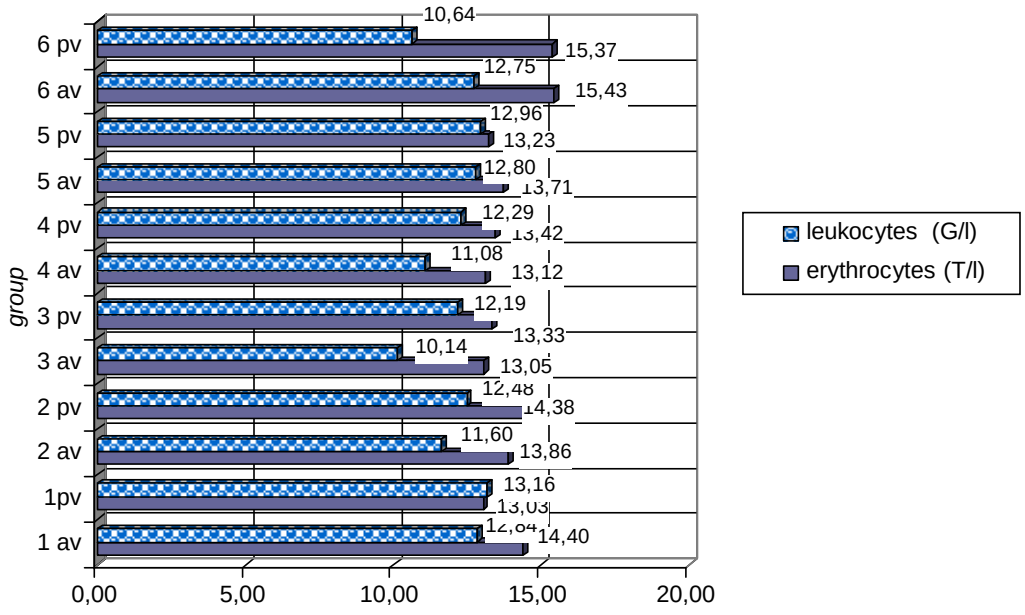

Fig. 2. The dynamics of mean values of the total number of erythrocytes (T/l) and total number of leukocytes (G/l) recorded pre-vaccination (av) and post-vaccination (pv) for the groups of investigated goats

Average values of mean erythrocyte constants showed fluctuations, but falling between

physiological limits for VEM (16.0 to 25.0 fl) and HEM (5.2 to 8.0 pg), CHEM (30.0 -36.0 g/dl) was slightly increased for two of the groups (36.18 and 36.66) (fig. 3).

Data on the distribution of leukocyte subpopulations showed in figures 4 and 5 highlights the mean proportion of neutrophils from 27.0% to 37.0%, oscillating between the physiological limits (30-61%). The same can be said about the population of eosinophils, which did not exceed the upper limit of 8%, the highest value being of 5.60% recorded post-vaccination. Lymphocyte population dynamics was likewise marked by minor variations, from 52.0% to 59.57%, falling within the physiological limits (50-70%). The proportion of monocytes exceeded the physiological limits for each of the six investigated groups both pre- and post-vaccination, the minimum value of 5.20% recorded at group 1 pre-vaccination and the maximum 11 40% in group 2 post-vaccination.

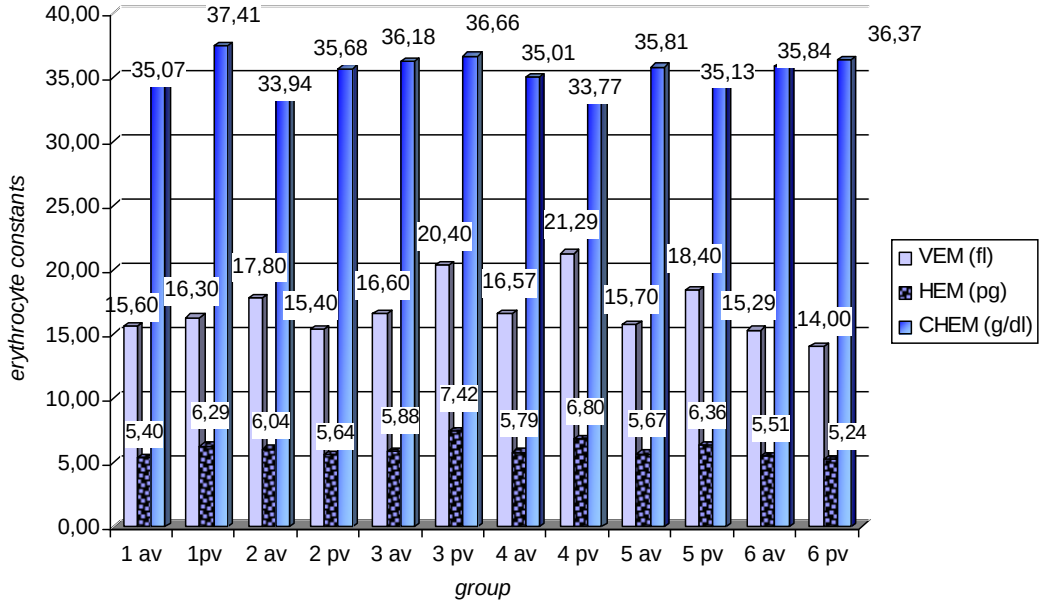

Fig. 3. The dynamics of the average values of mean erythrocyte constants recorded pre-vaccination (av) and post-vaccination (pv) for the groups of investigated goats

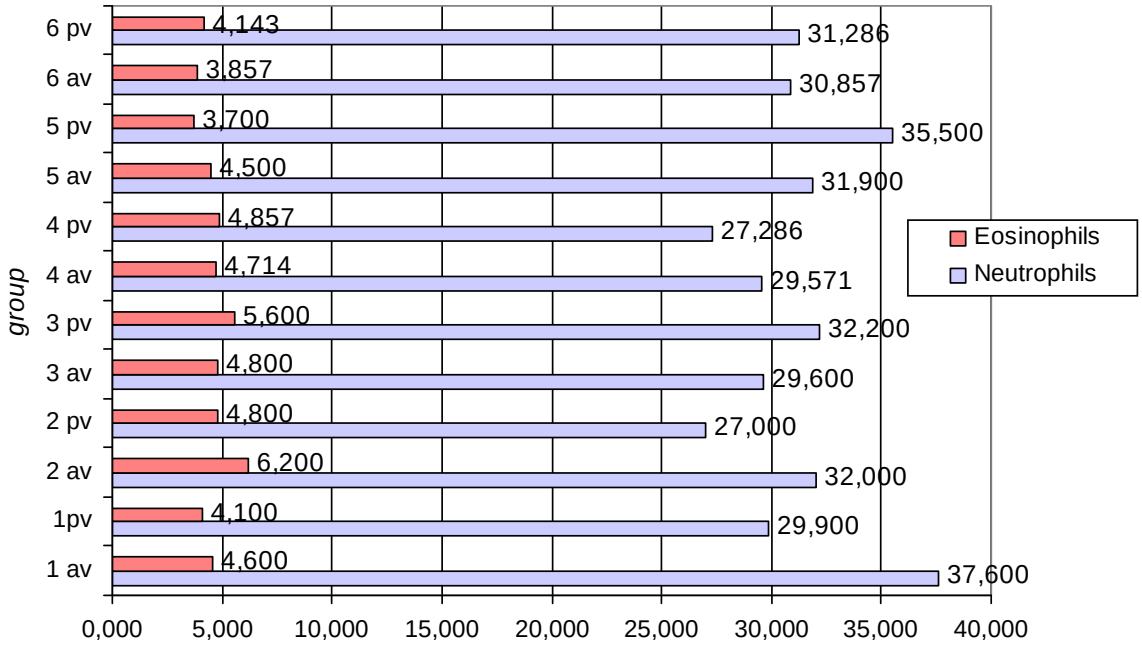

Fig. 4. Mean proportions of leukocyte subpopulations of neutrophils and eosinophils recorded pre-vaccination (av) and post-vaccination (pv) for the groups of investigated goats

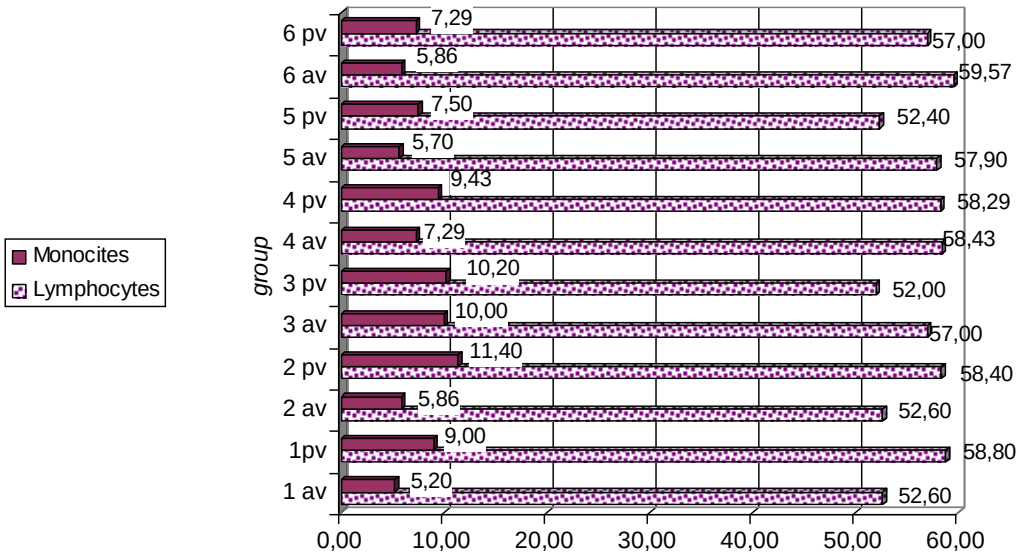

Fig. 5. Mean proportions of leucocytes subpopulations (lymphocytes and monocytes) recorded pre-vaccination (av) and post-vaccination (pv) for the groups of investigated goats

AST levels usually did not exceed the maximum physiological value of 220 IU averages ranging between minimum and maximum of 200.49 IU 130.41 IU recorded post-vaccination (fig. 6). In contrast, ALT mean values ranging between 22,814 IU and 33,129 IU, were included in the physiological limits for goats both pre-and post-vaccination in all experimental groups (fig. 7).

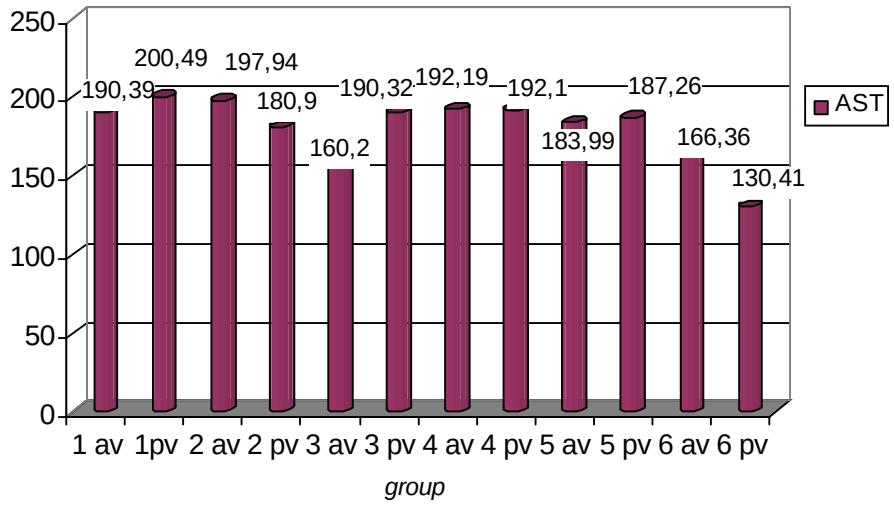

Fig. 6. Mean AST levels recorded pre-vaccination (av) and post-vaccination (pv) for the groups of investigated goats

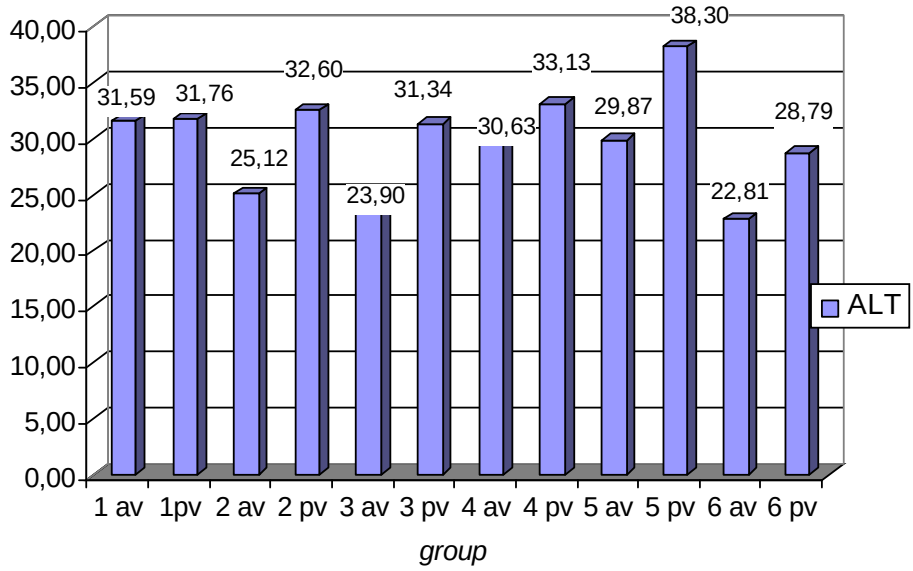

Fig. 7. Mean ALT levels recorded pre-vaccination (pv) and post-vaccination (pv) for the groups of investigated goats

Regarding the evolution of the mean values of GGT the values exceeded the maximum physiological limit of 50 IU post-vaccination for the four groups, ranging from 50.02 to 54.54 IU (fig. 8).

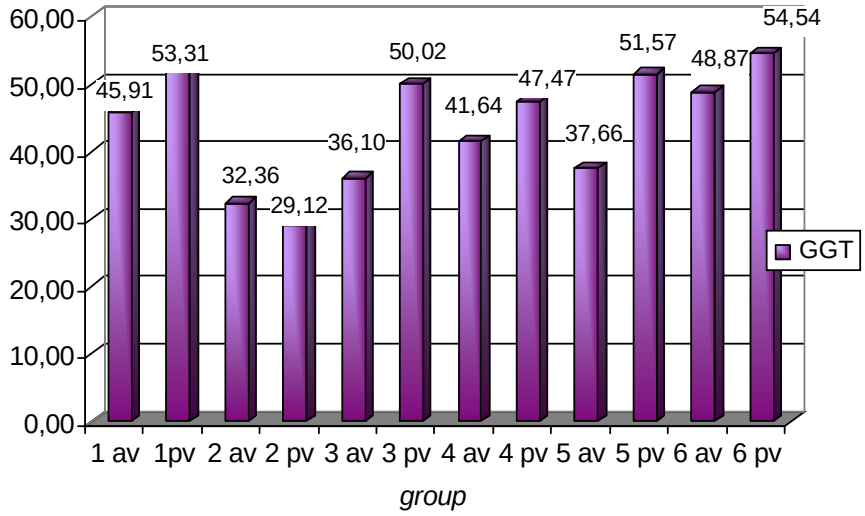

Fig. 8. Mean GGT levels recorded pre-vaccination (av) and post-vaccination (pv) for the groups of investigated goats

Urea values were characterized by slightly higher than the physiological limits (10-20 mg/dl) mean values, situated between 26.11 and 34.00 mg/dl, post-vaccination increases being more frequent. In contrast, the creatinine levels were situated between physiological limits (0.7 to 1.5 mg/dl), with mean values ranging from 0.51 mg/dl and 1.04 mg/dl (fig. 9)

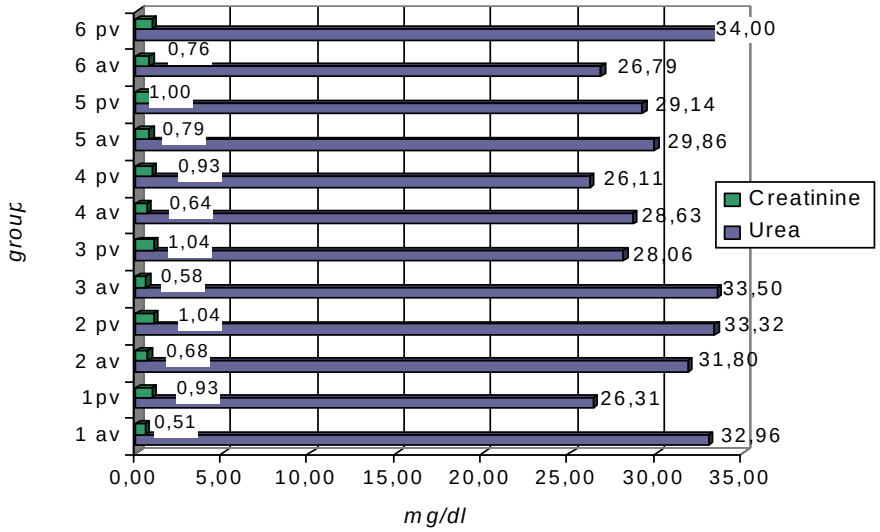

Fig. 9. Mean urea and creatinine levels recorded pre-vaccination (av) and post-vaccination (pv) for the groups of investigated goats

Regarding total serum protein, the development showed a characteristic increasing tendency in the mean values, as well as significant fluctuations from 53.36 to 79.09 g/l, some values being situated beyond the upper physiologic limit of 64-70 g/l (fig. 10).

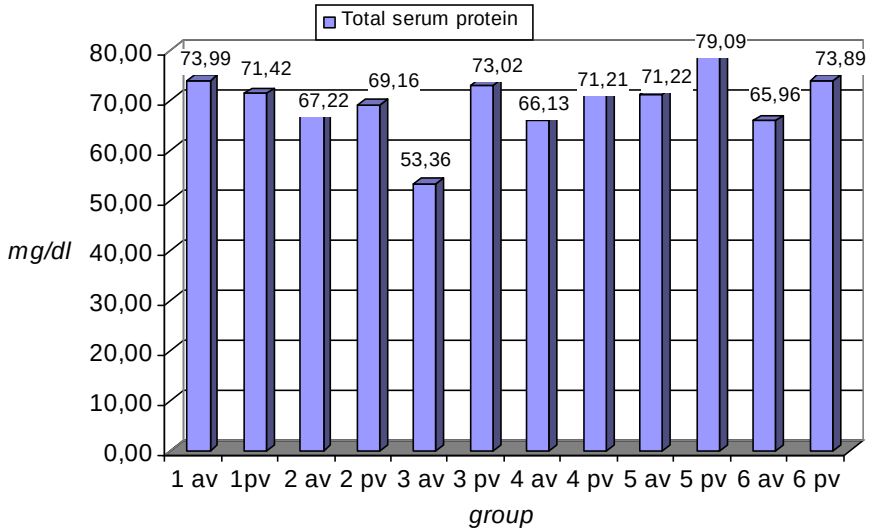

Fig. 10. Total serum protein mean values recorded pre-vaccination (av) and post-vaccination (pv) for the groups of investigated goats

The dynamics of Albumin showed also slight exceedances (3.3 to 4.7 g/l), of the physiological limits (2.3 to 4.0 g/l) pre-vaccination for five of the six groups, which returned between physiological limits of post-vaccination.

Blood calcium values, ranging between 7.57 and 9.82 mg/dl were between physiological limits (9.0 to 12.0 mg/dl) in each of the six groups, regardless of the experimental moment. Development was different for magnesemia, in which the mean values (1.20 to 3.84 mg/dl), showed an increasing trend post-vaccination, returning within physiological limits, compared with pre-vaccination values, situated below (2.8 - 3.6 mg/dl) (fig.11).

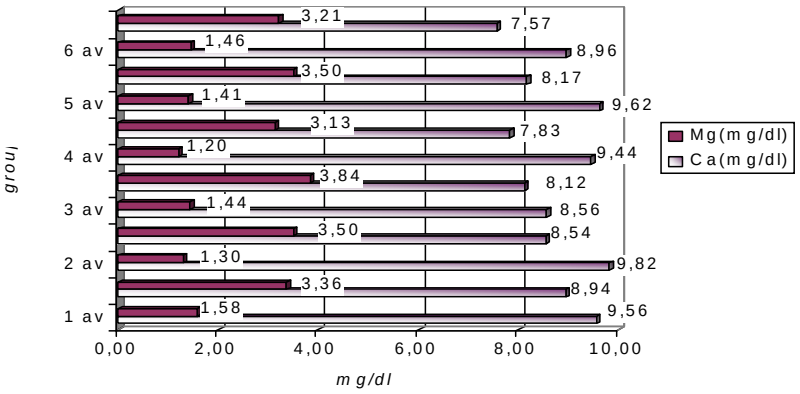

Fig. 11. Mean serum calcium levels and recorded magnesemia pre-vaccination (av) and post-vaccination (pv) for the groups of investigated goats

Regarding milk cell population the configuration resulting from all individual data from the groups of investigated goats were characterized by a predominance of macrophages (55%), followed by lymphocytes (25%) and PMN leukocytes (16.4%), epithelial cells having the lowest representation (2,6%). However the relevance milk cell population in goat mammary health assessment was given by the correlative interpretation of the corresponding normal cell population with the high frequency of atypical cellular structures, cells and cellular debris and cell from outside the mammary gland (represented by different microbes), aspects characteristic of goat milk.

The results regarding the nature and evolution of post-vaccination side effects have confirmed the absence of any form of general reaction of systemic nature, in the investigated goats, being correlated with the development of the main physiological parameters indicators of heath, monitored by clinical examination, hematological, biochemical and milk cell population, within physiological limits. Regarding the detection of any general systemic reactions, with or without humoral component, we considered the following dominant relevant clinical and laboratory parameters: maintaining internal temperature within physiological limits, maintaining the normal general condition, the presence and a maintaining of appetite; maintaining the haematological and biochemical parameters between the physiologic limits of the species, maintaining the milk microflora and milk cell population parameters within physiological limits, the normal development of milk production, lack of sensitivity to milking, the absence of limping, or other adverse reaction or clinically evident symptoms reported by handlers or owners.

Local reactions were reported subsequent to inoculation in some vaccinated goats, originally represented by deposit nodes given by the vaccine, sometimes these nodes were surrounded by discrete local alterations, of induration character or even congestion. Considered from an intensity and evolution point of view, these local reactions are typical for the formation of specific vaccine deposits, usually encountered with aluminum hydroxide adjuvants.

Tab. 2.

Evolution of the averaged data quantify the frequency and intensity of reported local reactions in vaccinated goats

Group-experimental model	**Vaccination (dose)**	**Reported local reactions postvaccine**								
		1st vaccination			**Rappel +3rd vaccination**			**Reactions/group**		
		Nr.	**%**	**Int.**	**Nr.**	**%**	**Int.**	**Nr.**	**%**	**Int.**
1-I./1dose (n=10)	20	3	15	+	2	10	+/++	5	25	+
2-I./2 doses (n=5)	10	3	30	+	2	20	++	5	50	+/++
3- I./3 doses (n=5)	15	-		-	2	13,3	+/++	2	13,3	+/++
4- E./1 dose (n=7)	14	4	28,5	+	3	21,4	++	7	50	+/++
5- S./1 dose (n=5)	10	1	10	+	1	10	++	2	20	+/++
6-A./1 dose (n=7)	14	2	14,2	++	2	14,2	+	4	28,4	+/++
Reactions/doses	**83**	**13**	**15,6**	**+**	**12**	**14,4**	**++**	**25**	**30,1**	**+/++**

+ circumscribed nodule, often rough, in form size of a cherry, with fading and total regression in 2 to 3 weeks; ++ hard or pasty lump, in shape and size of a plum, non- erythematosus or without local hypersensitivity and abcess formation tendency, having a good regression after 4-5 weeks;+++ major node, with pasty character and over infection tendency, but with a purely local development without special complications.

Statistical analysis of the frequency of local nodular reactions, originally formed around vaccine deposits, required reporting all vaccinated animals and as well the total number of vaccinations. Thus, of the total of 44 goats introduced in the experiment, 39 were vaccinated with varying number of doses, which summed the number of inocula to a total number of 83 vaccinations. It was reported weak reactions in a number of 20 of vaccinated goats, 15 of which showing a single reaction, and 5 of them two. The number of total local nodular reactions recorded after the completion of the 83 of vaccination was 25 (30.1%). Their proportion was increased after the first vaccination, when 13 were reported (15.6%), while the remaining 12 (14.4%) occurred after the rappel vaccination and the 3rd vaccination. The intensity score of the local reaction, determined as the average value was low (+/++), individual developments falling within three levels of intensity, differentiated on the basis of the characteristics of the nodular formations: + - circumscribed nodule, often rough, in form size of a cherry, with fading and total regression in 2 to 3 weeks + + - hard or pasty lump, in shape and size of a plum, non- erythematosus or without local hypersensitivity and abcess formation tendency, having a good regression after 4-5 weeks; + + + - major node, with pasty character and over infection tendency, but with a purely local development without special complications.

In assessing the persistence of the reported nodules, it was found fading after 3-4 weeks, most of them disappearing after 5-6 weeks, with no need for biopsy and histopathological investigations.

The analysis of the results from clinical trials, haematological, biochemical and milk cell populations showed *Agalaxin forte*, given in single dose, overdose and repeated doses, produced no systemic reactions, local reactions only sporadically been reported discrete and ephemeral reaction, consisting of vaccine deposit nodules, without pathological implication and of complications.

Based on the results of the pre-vaccination analysis of the recorded values of the investigated parameters, we consider six groups of animals as healthy and showed no significant deviations from normal limits.

Regarding the development of the main enzyme indicators, urea, creatinine, total serum protein and ion components in the investigated goats, it is noteworthy to mention that oscillations recorded in the average values were unimportant physiological and biological without pathological connotation.

Regarding post-vaccination local reactions such as those reported by us, existing data attributes benefits to their presence due to increasing immunization, dew to the local inflammatory focus determines the attraction of antigen presenting macrophages and other immunocompetent cells, thereby facilitating the installation of the immune response. Thus, deposits forming adjuvants (based on aluminum, calcium or oil emulsion - Freund adjuvant) typically produce a temporary inflammatory site that attracts phagocytes and antigen presenting cells for the phagocytosis of the inoculated substance (WHO). However, such reactions produced mainly by the adjuvant must be less obvious, an "ideal adjuvant" is one that contributes significantly to the effectiveness of the vaccine, without being reagenic (WHO). Furthermore, in veterinary medicine adjuvants are differentiated based on the target species, for farm animals (edible) and pets (inedible). The compromise in the use of strong vaccine adjuvants, can be justified by the need for a high level of immunization even toleration of serious side effects, such as the use of therapeutic vaccines for serious illnesses, untreatable or treated with medicinal products that generate severe adverse effects.

The adverse effects of adjuvants may be due to either aggressiveness (for reactogenic-

cytokine adjuvants) or their overdose (for classical adjuvants), the produced local reactions may vary from local mild pain and redness, to granulomas, cysts and even ulcers. Preparation of vaccines at physiological pH and osmolarity or with analgesic excipients, reduce local pain. Local post-vaccination granulomas containing activated macrophages, lymphocytes or fibroblasts, can be produced by poorly degradable particles contained in the injected adjuvants. In case of aluminum hydroxide based adjuvants, accurate subcutaneous injection is also important.

Local reactivity is influenced by route of injection, subcutaneous immunization can easily generate irritation, itching and local pain the appearance of transient swelling, if the vaccine was inoculated in areas with many sensory neurons as a result of inflammatory reaction.

Conduct in assessing the severity of local reactions provides that they may be negligible if the inoculum is rapidly dispersed; the persistence of the deposit can cause transient inflammatory response, accompanied by swelling, irritation and erythema. A series of post-vaccination reactions should not be attributed to the adjuvant itself, these can be caused by vaccine preservatives (formaldehyde, β-propiolactone) or toxins, resulting antigen preparation.

Aluminum-based adjuvant formulations are used for over five decades in human and veterinary medicine. In human medicine are already established standards on the use of aluminum adjuvant, 1.25 mg/dose in Europe (*Pharmacopeia Europa*) and the U.S. 0.85 mg/dose (*Code of Federal Regulations*), in veterinary medicine has been established that pigs tolerated well 12 mg of aluminum / dose in vaccine formulations while 40 mg of aluminum induces granulomas *(Valtulini et al, 2005)*. Various types of local reactions were reported for aluminum-based adjuvants (swelling, induration, erythema and persistent skin nodules up to 8 weeks or more, contact hypersensitivity), but not pyrogenicity, carcinogenesis and teratogenicity. According to *Butler* (*1969*), aluminum hydroxide (Alhydrogel) significantly reduces side effects of vaccines (antidifterios and tetanus), toxicity being reduced by slow releasing of the toxin and peptidoglicans or lipopolisugars from the vaccine or inoculation in situ. In this context, *Norimatsu* (1995) shows that adsorption on aluminum hydroxide before injection of lipopolisugars inhibits some systemic effects (tremor, transient leukopenia).

Group reactivity in *Agalaxin forte* vaccinated animals consisted mainly in general manifestations consisted not of a systemic nature, identifying only local reactions, ephemeral and of low intensity, with a frequency of 30.1%. Moreover, the same type of side effects have been reported in animals vaccinated with overdose (double dose) or repeated doses (3 doses), without increasing the intensity or importance. Furthermore, we expanded clinical evaluation to detect possible side effects and the following four months.

CONCLUSIONS

- Developments in erythrocyte parameters was characterized by slight changes, without significant importance or particular significance of vaccination induced influences on erythropoiesis and erythrocyte values goat.
- The summary of leukocyte parameters evolution showed only oscillations without pathologic significance, with distributions pre-and post-vaccination appropriate to stimulation of the leucocyte line involved in generating specific humoral response.
- Post-vaccination statistically ensured induced monocitosis was observed correlated with the role of antigen presenting monocytes-macrophages in antigen acquisition and

processing, this development can be boosted by the employed adjuvant.

- Developments in enzyme indicators, urea, creatinine, total protein and inorganic components of the investigated goats included wide oscillations from the physiological limits, but no biological significance and especially no pathological connotation.
- During the safety assessing of the *Agalaxin forte* vaccine, through monitoring of the physiological indices of health variable experimental dose (double dose or three successive doses) have not been identified as potential risks
- Milk cell population configuration highlighted the absence of any form of mastitis in the investigated goats, citomorfologic monitoring representing an effective examination in order to determine the mammary state health and thus the hygienic quality of milk.
- The adverse post-vaccination reactions monitored by hematological, biochemical, clinical and milk cell population assays in the *Agalaxin forte* vaccinated group, were exclusively, ephemeral, low intensity and frequency of 30.1%, local reactions corresponding to a good level innocuity for all variables of dose and vaccine series introduced in the test.

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