

THE OVERALL EFFECT OF ANTI ANTHRAX VACCINATION ON THE IMMUNITY IN EXTENSIVELY RAISED BOVINE

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Abstract

Anti anthrax immune prophylaxis is a stressful operation, with a positive protective outcome but also impacting negatively on the immune system. The research aimed to investigate the post vaccination changes in humoral and cell mediated immunity, other than the specific anti anthrax ones, and quantify the restorative potential of the bilberry and marigold extracts in vaccinated animals.

The experiment was carried out on extensively raised lactating cows ($n=34$) and young cattle ($n=21$), blood being sampled before and two weeks after the injection of live attenuated R 1190 Stamatin vaccine and processed by blood picture, calculation of N/L index as a stress indicator, total Ig and circulating immune complexes as well as the *in vitro* blast transformation of leukocytes.

The results indicated that the N/L ratio allows an estimate of the stress level subsequent to vaccination, in relation to the diminished adaptive cell mediated response in the young cattle. The lower level of total Ig connected with a significantly ($p<0.05$) lower rate of complexation in young animals versus older ones stresses the beneficial influence of repeated vaccinations in the latter category. Similarly, there was an increased adaptive cell mediated response in older animals.

The stimulating activity of extracts from *Vaccinium myrtillus* and *Calendula officinalis* was better expressed in cell cultures from younger animals, supporting the possible therapeutic use of these in restoring the diminished overall immune response in this category.

Key words: anthrax vaccination, milking cows, young cattle, overall immunity, vegetal extracts.

INTRODUCTION

Anthrax, caused by *Bacillus anthracis*, still represents one of the major diseases of domestic ruminants in some regions of the world, while being exotic but a permanent threat to others (Peiso et al., 2011). The presence and spread of the disease is conditioned by the existence of at least 20°C temperatures, required for the sporulation and thus for creating persistent sources of infection. The disease is more common in warm and temperate climates, and less frequent in cold climates. It is also an important zoonosis known since antiquity, evolving sometimes as an epidemic. Persistence of anthrax spores for a long period in soil, even over 200 years (De Vos V et al.,

2004), leads to the statement that no country can claim to eradicate the disease. The incidence decreased spectacularly in the last 150 years, since the bacteria and its resistant form were identified. The application of sanitary measures along with antibiotic treatments, and especially the development and widespread use of immunological products in both humans and animals, managed to decrease the incidence even more (Peiso et al., 2011). Nevertheless, endemic disease outbreaks occur in different geographical regions of the world, especially in developing countries of Asia, Africa and South America (Dutz, 1981), where injectable vaccination procedures are difficult to apply to all sensitive individuals. The disease is also present in the European countries, as a re-

emerging pathogen, most notably, it appeared after 27 years in Sweden (2008) (S. Stenberg et al., 2010) and Italy (2011) (Palazzo Lucia et al., 2012). Thus, the problem of protective vaccination against this disease has not yet been solved.

Most livestock vaccines in use throughout the world today for immunization against anthrax are derivatives of the live spore vaccine formulated in 1937 (Turnbull, 1991). Protective antigen vaccines, capsule based vaccines and dual protection vaccines were also designed using various adjuvants from aluminium hydroxide to monophosphoryl lipid A, squalene lecithin/Tween 80 emulsion and saponin QS-21 and (Ivins et al., 1995, Scorpio et al., 2006).

B. anthracis, in its vegetative form, acts within the host by expressing virulence factors (Scorpio et al., 2006). These, along with the potential adjuvants' side effects (ie, irritative effect of saponin) could further direct inactivation and evasion of elements of the host innate immune response

Mainly Ascoli-Valenti or AGID tests were used to serologically identify the agent. Cellular immunity measurements were neglected, as *B. anthracis* exerts its pathogenic activity as an extracellular agent (Scorpio et al., 2006). However, a better insight into the protective capacity of a vaccine could include tests that also measure the *in vitro* responses of the immune cells, along with the total opsonins and their complexation potential.

This research aimed to investigate the post vaccination changes in humoral and cell-mediated immunity, other than the specific anti anthrax antibodies, including the stress index (N/L ratio) and quantify the restorative potential of the bilberry and marigold extracts in vaccinated animals.

MATERIALS AND METHODS

The experiment was carried out on extensively raised lactating cows (n=34, 4 to 10 years) and young cattle (n=21, 4 month to 1.5 years), from three different breeds: Romanian Spotted, Simmenthal and Red Holstein. The vaccination was done using Romanian vaccine containing R 1190

Stamatin strain.

Blood samples were collected on heparine (50 IU/ml) and for serum in sterile containers and smears were performed ahead of the anti-anthrax vaccination and two weeks later.

Neutrophil/lymphocyte ratios. The smears were stained by Dia-Quick Panoptic method and white blood cells were counted. The neutrophil/lymphocyte ratio (N/L) was calculated to estimate stress levels (O'Loughlin et al., 2011).

Circulating immune complex measurements (Khokhlova et al, 2004). Measurement of the level of circulating immune complexes (CIC) allows evaluation of the molecular clearance capacity at a particular moment. Part of the collected blood was allowed to clot for 30 min at 37°C and then centrifuged at 1308 g for 10 min. Sera were removed and kept at -20°C until tested. 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 spectrophotometrical 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. The precipitation took place 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 conventional units (CU) were calculated by subtracting the value of the control (serum + buffer) from that of the PEG precipitate and multiplying it with 10³.

Immunoglobulin measurements. Total immunoglobulin, known as opsonins, play an important role in the 'first line of defense', of the innate immunity. 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⁻¹ of Zn salts precipitate the immunoglobulins. 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. Optical density (ODU) then was read spectrophotometrically ($\lambda=475$ nm, $d=0.5$ cm)(Khokhlova et al., 2004) and final values were calculated in Vernes degrees, as $ODU \times 10^2$.

In vitro blast transformation of leukocytes. The blast transformation test, that measures the level of cell-mediated immunity was assessed using whole blood. The heparinized blood samples were subjected to the *in vitro* blast transformation test in maximum four hours after sampling. Each blood sample was diluted 1:4 with RPMI 1640 lymphocyte culture medium, supplemented with 5% fetal calf serum (FCS), antibiotics (1000 IU penicillin and 1000 μ g streptomycin/ml) and buffered to pH 7.2-7.4 with a sterile 5% sodium bicarbonate solution. As mitogens PHA M (Difco) and Con A (Calbiochem) were used (1 μ l of each/well) to which two culture variants with blackberry and marygold extracts were added (1.5 μ l vegetal extract/200 μ l of the blood suspended in RPMI). Cell growth was measured by the glucose consumption technique. The transformation index (TI) was calculated as follows: $TI\% = [(MG-SG)/MG] \times 100$, where TI=blast transformation index, MG=glucose concentration in the initial culture medium and SG=glucose concentration in the sample after incubation (Khokhlova et al, 2004).

Statistical analysis was done by use of the Excel program, and the significance of the differences was estimated by the t Student test.

RESULTS AND DISCUSSIONS

Vaccine protection studies indicate residual virulence of the attenuated live spore vaccines, therefore vaccinating certain species such as goats, which show a higher susceptibility to post vaccination secondary reactions when compared to other species, requires more attention (Turnbull, 1991). Immunological studies focused on protective level of antibodies, due to extracellular character of the bacteria. Nevertheless, the role of other immunological “players” in eliciting the protective effect against the pathogenic strains of *B. anthracis* still remains of interest.

The negative impact of stress on functioning of the immune system has been well documented in bovine, especially in connection with technological malfunctions or certain veterinary practices (O’Loughlin et al., 2011).

Neutrophil/lymphocyte ratios represent an accessible way to estimate stress levels, since it was predicted that they would increase along with increasing stress (Davies et al., 2008). Some studies indicate that N/L ratio was used to monitor the stress alleviation while changing the moment of clostridial or respiratory vaccine administration to calves (Richeson et al., 2009) or to evaluate the changes in stress levels of goats, when vaccinated against foot and mouth disease (Jo et al., 2014). There is no information on neutrophile/lymphocyte ratio (N/L) as a measure of stress in cattle after vaccination against anthrax. The results obtained in this study indicated that N/L ratio could be used to evaluate stress levels in young bovine as opposed to older ones, a statistically significant increase ($p<0.05$) being encountered two weeks after vaccination (Fig. 1). The N/L ratio correlated with the cellular immune response in case of the marigold treated variant both before and after vaccination in cows ($p<0.05$, with $r=0.523$ before and $r=-0.349$ after the vaccination). The sense of the correlation, reversed after vaccination, indicated that an decreased N/L ratio increased the specific *in vitro* cell-mediated response. In young animals, the significant ($p<0.05$) correlations between N/L ratios and leukocyte stimulation indices were present only for the control and alcohol treated variants. Subsequent to vaccination, the increased N/L ratio was strongly negatively correlated with the leukocyte stimulation indices ($p<0.001$, $r= -0.626$), suggesting a decrease in the specific cell mediated immunity in spite of the antigenic stimulation. Thus, in the case of vaccination against anthrax, the N/L ratio could have a predictive value on the extent of the specific cell mediated immunity two weeks after the vaccination in young bovine.

The total circulating immunoglobulins (Ig) concentrations could improve the overall picture of nonspecific antimicrobial defense

capacity (Bellido et al., 1981). The values obtained were statistically significantly ($p < 0.05-0.01$) increased for both groups

(Table 1) by 73.4% and 65.5% for cows and young animals, respectively.

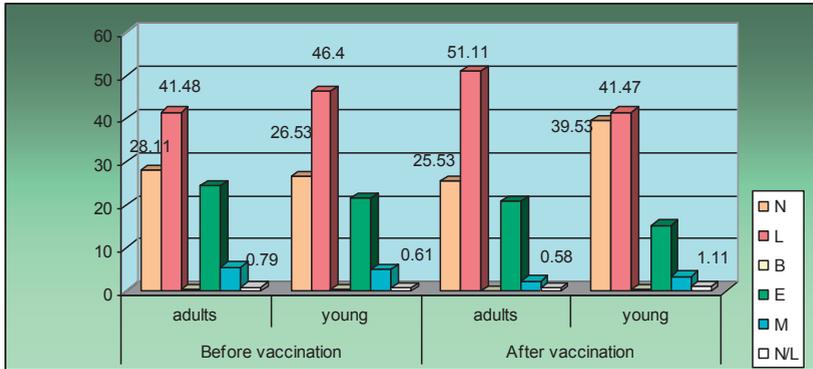


Figure 1. Neutrophil/lymphocyte ratios as stress indicators prior to and after anti-anthrax vaccination

The lower values obtained for young cattle could be attributed to the stimulating effect of repeated vaccinations in adult animals which were vaccinated every year, thus stressing the impact of numbers of antigen primings on protection in these animals.

Circulating immune complexes (CIC) consecutive to the coupling between antibody and antigen molecules will accumulate, when in excess, in the kidney (Hebert, 1988). Increased CIC levels usually indicate an autoimmune process and also could offer an estimate of the clearance capacity of the body versus immune complexes, and thus of protection. The antigenic stimulation by anti-anthrax vaccination highly increased the clearance of CIC, thus diminishing the

circulating levels of the complexes in adults by 50% ($p < 0.05$), supposedly due to a more active and faster anamnestic response in these animals. On the contrary, in young animals, these levels were significantly ($p < 0.001$, 317.6%) increased (Table 1). One possible explanation for this may be the gradual adaptation of clearance mechanisms in young animals to the increased opsonin levels, this causing slower elimination.

The *in vitro* proliferative response of lymphocytes to mitogens or antigens can be assessed by blast transformation test, allowing the interpretation of a potential specific cell-mediated response during microbial aggression (Chase, 2013, Carr et al., 2012).

Table 1. Total Ig and CIC levels before and after anti-anthrax vaccination ($X \pm s$)

Parameter	Adult animals		Young animals	
	Before vaccination	After vaccination	Before vaccination	After vaccination
Total opsonins ¹	7.31±4.59	12.10±6.68	3.94±1.29	6.95±2.09
CIC ²	2.00±0.9	1.00±0.1	1.7±0.1	5.4±2.57

¹- Values expressed in Vernes degrees

²- Values expressed in conventional units

Table 2. *In vitro* blast transformation indices prior and after the anti-anthrax vaccination in bovine (X±s)

Cows											
Before vaccination						After vaccination					
Ctrl	Alc	Con	PHA	Bb	Marig	Ctrl	Alc	Con	PHA	Bb	Marig
58.04± 8.59	55.15± 9.99	53.52± 8.40	56.36± 7.64	48.57± 13.2	56.4±1 2.57	63.64± 7.38	60.18± 7.14	64.95± 5.66	61.46± 6.06	55.6±7 .95	63.95± 7.74
Young bovine											
Before vaccination						After vaccination					
Ctrl	Alc	Con	PHA	Bb	Marig	Ctrl	Alc	Con	PHA	Bb	Marig
61.42± 7.48	63.31± 7.89	65.16± 8.81	63.33± 4.42	52.37 ±9.60	57.94 ±11.94	60.2±1 2.20	60.85± 6.55	61.73± 7.01	57.23± 6.53	55.77± 4.27	62.39± 8.88

Legend: Ctrl=control variant, Alc=alcohol, Con= ConA, PHA= phytohemagglutinin, Bb= blueberry alcoholic extract, Maryg= English marigold alcoholic extract

There were no significant changes in the stimulation indices neither in cows nor in young animals, after the vaccination, although some fluctuations were visible for different variants (Table 2). Nevertheless, the effect of vegetal extracts was visible, with an immune enhancing activity for the marigold extract in both categories. This results suggested that the alcoholic extract of marigold could provide the augmentation of cell-mediated immunity during the specific response to anti anthrax vaccine in bovine.

CONCLUSIONS

The experimental results of this study indicated that the N/L ratio could be used as an indicator of the stress levels in cattle vaccinated against anthrax, emphasizing the effects of stress in young animals.

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