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HUMORAL IMMUNE RESPONSE TO DIFFERENT ROUTES OF MYXOMATOSIS VACCINE APPLICATION

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Abstract: The aim of our study was to monitor the dynamics of the serological response to different application routes of live attenuated myxomatosis vaccine. The study included 42 Californian breed rabbits, aged 3 mo, of both sexes. They were separated into 7 groups: 6 experimental and 1 control. All experimental groups were vaccinated on day 0 with a single dose of myxomatosis vaccine (min $10^{3.3}$ tissue culture infective dose 50 [$TCID_{50}$], max $10^{5.8}$ $TCID_{50}$). Three of the groups were injected with monovalent attenuated myxomatosis vaccine using different types of application: intradermal (i.d.), intramuscular (i.m.) and subcutaneous (s.c.). The other 3 groups were injected with bivalent attenuated vaccine against myxomatosis and rabbit haemorrhagic disease; again the routes of administration were i.d., i.m. and s.c.. There were no clinical signs or serious side effects after vaccination. The serological response was evaluated on days 7, 15 and 30 with a monoclonal antibody based-competition enzyme-linked immunosorbent assay (cELISA). More rapid and potent humoral response was detected in groups with i.d. inoculation in comparison to i.m. and s.c. routes. Vaccination with monovalent vaccine against myxomatosis induced higher antibody titre in comparison to bivalent vaccine. Our study showed that the vaccine application route and the type of vaccine used influence the speed and intensity of antibody response.

Key Words: myxoma virus, myxomatosis vaccination, ELISA, antibody response.

INTRODUCTION

Myxomatosis is a generalised virus infection which affects mainly *Oryctolagus cuniculus*. The virus is a part of the *Leporipox* genus, subfamily *Chordopoxvirinae*, family *Poxviridae* and is a large DNA-virus (King *et al.,* 2012). The natural hosts of the virus are both *Sylvilagus brasiliensis* in Central and South America and *Sylvilagus bachmani* in Mexico and California which develop mild illness with local lesions (Marshall and Regnery, 1960; Fenner and Ratcliffe, 1965). Nowadays the disease is distributed worldwide and often fatally affects wild, farmed and pet rabbits.

Myxomatosis shows 2 clinical forms: nodular myxomatosis, which is characterised by the formation of multiple mucoid tumours in different parts of the body, purulent rhinitis and conjunctivitis; and the amyxomatous form, with mainly respiratory symptoms (Best and Kerr, 2000). Myxoma virus infection causes severe immunosuppression (Jeklova *et al.,* 2007). Lethality in non-immune rabbits affected with high virulent strains is more than 90% (Fenner and Ratcliffe, 1965). Mortality is more often caused by secondary bacterial infection when the disease is caused by low-mild virulent strains.

The main transmission vectors of viral strains responsible for the classical "nodular" form of the disease are mosquitos or other blood-sucking insects, which inoculate the myxoma virus intradermally (Day *et al.,* 1956). Direct contact between infected animals may also play a role in transmission of the virus, especially for virulent strains responsible for the "amyxomatous" form of the disease (Farsang *et al.,* 2003).

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Vaccination can be a successful way of preventing myxomatosis in farmed rabbits (Calvete *et al.,* 2004; Ferreira *et al.,* 2009; Dalton *et al.,* 2012). However, vaccination failures have to be taken into account, either due to the use of non-conventional administration routes, as in wild rabbits, or because of interference of the vaccine with the natural antibodies (Rouco *et al.,* 2016). There are 2 general types of myxomatosis vaccines – heterologous and homologous. The former contains live Shope fibroma virus and induces successful but short-term cross protection, although there are data that it cannot prevent clinical signs and naso-conjunctival shedding of the myxoma virus (Marlier *et al.,* 2000). The antigen in the homologous vaccine is an attenuated myxoma virus strain that induces stable protective immunity (Marlier, 2010).

The homologous vaccine can be monovalent (against myxoma virus only) or bivalent (against myxoma virus and rabbit haemorrhagic disease virus - RHDV) (Bertagnoli *et al.,* 1996, Lemiere, 2000). The bivalent vaccine could be obtained by associating 2 separate preparations, one attenuated for myxomatosis and one inactivated for RHD, or a recombinant virus in which the myxoma virus is genetically modified in order to express the main structural protein of RHDV (Barcena *et al.,* 2000; Spibey *et al.,* 2012). The attenuated pox vaccines can be administered by intradermal and subcutaneous route, and some of them also intramuscularly (Bhanuprakash, 2012). However, different routes of Shope fibroma virus vaccine administration lead to variations in immune response (Alfonso and Pagès-Manté, 2003). It is not clear if and how much the intrinsic valence and properties of the vaccine are significant factors in conditioning the quality and entity of the immune response. It was also observed that the application route of virulent myxoma virus can lead to differences in the course of the disease (Genova, Manev, personal data).

Although cell-mediated immune response has a pivotal role in protection against poxviruses, immunoglobulins possess significant importance in resolving poxvirus infection (Panchanathan *et al.,* 2008). Antibodies are used as biomarkers for successful vaccination (OIE, 2014). The gold standard for the serological evaluation post infection or post vaccination is enzyme-linked immunosorbent assay (ELISA) (Kerr, 1997; Lavazza *et al.,* 2004; Dan, 2014).

The aim of our study was to evaluate the dynamics of the humoral immune response post vaccination of naïve rabbits using monovalent and bivalent vaccines. The importance of inoculation route was observed and the post injection side effects and local reactions were detected and discussed.

MATERIALS AND METHODS

Animals

The trial was carried out in the rabbit breeding farm of the Institute of Animal Science, Kostinbrod - Bulgaria. The study included 42 clinically healthy rabbits (3 mo old) of Californian breed from both sexes in ratio 1:1. The rabbits were regularly dehelminthed and were all ascertained as seronegative for myxoma virus and RHDV by testing them before vaccination. During the period, the animals were kept individually in separate cages, maintained under the same conditions and fed with nourishing fodder, hay and root crops. Food and water were given *ad libitum*. All procedures followed good clinical practice and were under the agreement of the Animal Health and Welfare Commission of the Bulgarian Food Safety Agency.

Vaccines

Two types of licensed vaccines were used, produced by Bioveta, Czech Republic. The first was a monovalent, homologue, lyophilised, attenuated myxomatosis vaccine, which includes myxoma virus in a single dose – min $10^{3.3}$ tissue culture infective dose 50 ($TCID_{50}$) max $10^{5.8}$ $TCID_{50}$. The second one was bivalent homologue, lyophilised, attenuated vaccine against myxomatosis and RHD which contains the same myxoma virus strain min $10^{3.3}$ $TCID_{50}$ max $10^{5.8}$ $TCID_{50}$ and inactivated RHDV- CAMP V-351: min 128 - max 1024 HAU. Vaccines were stored according to the manufacturer requirements. Before use, they were brought to room temperature and diluted respectively with 10 mL injection solution for subcutaneous (s.c.) and intramuscular (i.m.) administration and with 1 mL for intradermal (i.d.) application. At this rate, the single dose for s.c. and i.m. application route was 1 mL and for i.d. route - 0.1 mL.

Study design

The animals were divided into 7 groups, each composed of 6 rabbits. Three groups (A, B, C) were vaccinated with monovalent vaccine, and 3 other groups (D, E, and F) with a bivalent vaccine, whereas the "control group" rabbits were injected with a physiologic saline solution. Regarding the administration method, 2 groups were employed for each route: A and D groups were vaccinated by i.d. route, B and E groups by i.m. route, and C and F by s.c. route.

The experimental groups were vaccinated with single dose at day 0 (D0). Intradermal application was performed on the inner part of the ear at one point with a 26 G injection needle. Intramuscular injections were performed in the caudal thigh muscles with 20 G injection needles, and the subcutaneous in the neck region with 20 G injection needles. Control rabbits were injected with 1 mL of physiologic saline solution at D0. Blood samples were taken using the saphenous vein at D7, D15 and D30 post vaccination (p.v.) to evaluate the antibody titre against myxoma virus. The same day the sera were centrifuged - 2000 rpm for 10 min using Gemmy, Model: PLC-025, separated in sterile tubes and frozen at –40°C till the moment of evaluation.

Serological examination

We used standard serological tests for the detection of postvaccinal antibodies - competitive enzyme-linked immunosorbent assay (cELISA) developed in Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini" (Lavazza *et al.,* 2004). This method is centred on the use of a monoclonal antiboby (mAb) (1E5) that respectively recognises the myxoma virus immunodominant envelope protein (IMV –open reading frame M071L). The test antigen was myxoma virus strain California grown on RK13 cell culture. The sample is positive if the optical density (OD) value at the 1/40 dilution is more than 0.2 OD units above the value of the negative serum used as a control. According to the previous observation from the field use of cELISA assay for assessing post vaccination immunity (Le Normand *et al.,* 2015), antibody titres ranging from 1/160 to 1/640 were considered a sign of successful immune response.

Statistical analysis

Statistical analysis of the results was performed using one-way analysis of variance (ANOVA) with Student's t-test with the level of significance set at *P*-values of less than 0.05. The results are presented as means.

RESULTS AND DISCUSSION

The formation of a nodule at the i.d. injection site was considered a necessary requirement for successful vaccine application. Alfonso and Pagès-Manté (2003) demonstrated the positive correlation between nodule size post i.d. vaccination with Dermojet to immune response with Shope fibroma virus, and the nodules were presented till 28 d p.v. Such correlation cannot be seen after i.d. needle application of vaccinal myxoma virus. The nodule progressively disappeared up to 24 h post vaccination. Possible explanations for differences may be the inoculation technique (Dermojet *vs.* needle), type of vaccine (heterologous *vs.* homologous), or breed distinctions (crossbred New Zealand White/Californian *vs.* Californian).

Two of the rabbits from group D demonstrated transient local reaction with hyperaemia, pruritus and swelling at the inoculation site. One animal from group A demonstrated pain and swelling at the injection site in the day following vaccination. The body temperature of all animals was monitored for the first 7 d p.v. and showed no alteration above the normal reference range (38-40°C) (Morimoto, 2009). There were no episodes of anorexia or illness throughout the experimental period. We confirmed the conclusion of Alfonso and Pagès-Manté (2003) that the myxomatosis vaccination did not affect negatively the general health status of the rabbits. On the other hand, we cannot exclude that the vaccinal myxoma virus strain could have some immunosuppressive effects –as suggested by Jeklova *et al.* (2007).

The serological results (mean values) of the different groups at D7, D15 and D30 are summarised in Figure 1.

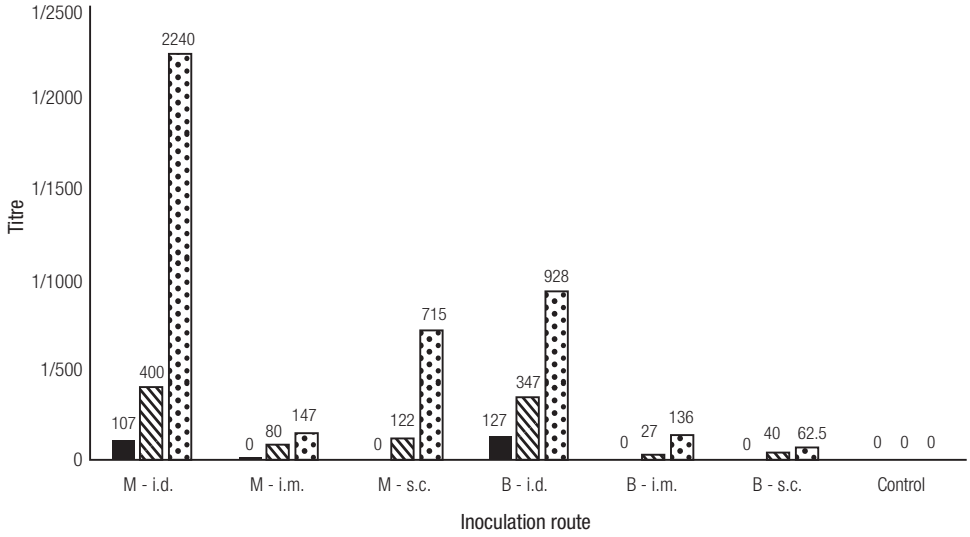

Figure 1: Serological response to myxomatosis vaccination at D7, D15 and D30 according to different route of inoculation. Average titre is reported as dilution (1/….). M: monovalent vaccine; B: bivalent vaccine; i.d.: intradermal route; i.m.: intramuscular route s.c.: subcutaneous route. ■ D7; ▨ D15; ▩: D30.

Seroconversion was already present at D7 in i.d. groups for both types of vaccines (M and B) with similar average titres (1/107 *vs.* 1/127 respectively), whereas all the other groups vaccinated by s.c. or i.m. did not demonstrate antibodies at the same day point with the sole exception of a low value in animals vaccinated with M by i.m. (titre 1/10). Intradermal route of vaccine application produced rapid immune response. Thus, in regions with higher epidemiological risk for myxomatosis infection, the intradermal route may be the preferred way of vaccine inoculation.

Results from D15 show that the antibody titres of the i.d. vaccinated animals were again not affected by the type of vaccines (M=1/400 and B=1/347). Indeed, they were higher in comparison to the other groups which showed titre values between 1/27 and 1/122. In the s.c. and i.m. groups, slightly higher titres were obtained with M vaccine (1/80-1/122) than with B vaccine (1/27-1/40).

At D30, the highest antibody titres were even more increased in i.d. groups with statistical differences (t=0.483, d.f.=12, $P<0.05$) between animals vaccinated i.d. (1/2240) and those vaccinated s.c. (1/715) and i.m. (1/147) using the monovalent vaccine. Comparison between the groups inoculated with the bivalent vaccine established that the antibody titres in i.d.(1/928) were higher than in the s.c. (1/62.5) and i.m.(1/136) groups, but without statistical differences (t=0.383, d.f.=12, $P<0.05$). The vaccination success after the i.d. route can be explained with the longer contact between the antigen and the antigen-presenting cells and the high number of dendritic cells in the derma (Levin *et al.,* 2015). In addition, antigen may be taken up directly by lymphatic vessels for transport to antigen-presenting cells in the lymph nodes (Kim *et al.,* 2012) and thus, considering that myxoma virus has a specific target in epithelial cells, the delivery of vaccine to the epidermis or dermis may result in superior and quick immune responses when compared to i.m. or s.c. injections.

High antibody titres were found in the group that was vaccinated s.c. with the monovalent vaccine. The i.m. application showed similar results in both vaccine types, but considerably lower in comparison to the i.d. and s.c. routes. Therefore, it seems that the result hinges more on the administration route than the valence of the vaccine. Our results are in agreement with those of Dalton *et al.* (2015), who found that vaccine administration methods influence the immune response and can be a factor leading to vaccination failure.

Overall, the results from the combined myxoma virus + RHD vaccination demonstrated an apparently lower level of humoral response to myxoma virus, which is partially in contrast with the conclusions of Lemiere (2000). However it is quite difficult, without performing challenge experiments, to determine whether such titres are indicative or not of a complete protection against high virulent myxoma virus. In fact, it is well known that antibody titres are not the only parameter correlated to protection against myxomatosis. As with other poxvirus infections, cell mediated immunity and neutralising antibodies are probably essential for clearing the virus and providing long term protection from reinfection and disease (Kerr, 2012).

CONCLUSIONS

Our study showed that the route of application and the type of vaccine used can influence the postvaccinal immune response for myxoma virus. The same antigen dose can provoke serological response with different speed and effectiveness depending on the application route. Intramuscular injection was found to be inappropriate for myxomatosis vaccination, whereas intradermal and subcutaneous routes proved preferable.

The use of monovalent vaccines against myxomatosis and RHD could be recommended at least when targeting prophylaxis in zootechnical rabbits, as the vaccination programmes for these 2 diseases are usually different and may vary in relation to the risk of infection. Vice versa, when vaccination campaigns are carried out in the field to protect wild animals, or when vaccination is performed in pet rabbits, it could be more efficient to administer bivalent vaccines, especially where both diseases are endemic and if handling stress to animals would be reduced. Moreover, in these last 2 cases, instead of a bivalent vaccine obtained by mixing together 2 types of vaccines, a single bivalent vaccine based on a recombinant myxoma virus strain expressing RHDV VP60, which is registered and commercially available, could also be used.

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