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Immune Responses of Sheep Vaccinated with Combined Anthrax and Capripox Vaccine

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Abstract: The combined anthrax and sheep pox vaccine was prepared in a lyophilized form containing the recommended doses of both sheep and goat strain 0240 pox and anthrax live spore vaccine. Immunization of sheep with this vaccine revealed a considerable immune response when detected with immunocapture Enzyme Linked Immunosorbent Assay (ELISA), indirect ELISA and with (3-(4, 5-dimethylthiazolyl-2) -2, 5-diphenyltetrazolium bromide) (MTT) lymphocytes proliferation assay. The difference in titers of antibodies raised against capripox virus antigen was significant, it was also significant for antibodies raised against crude Protective Antigen (cPA) and killed spore antigen. Phytohemagglutinin (PHA) revealed high Stimulation Index (SI) before vaccination but low one after vaccination while the SPPV antigen showed slight SI before vaccination and high SI after vaccination indicating cellular immune response to this antigen. Low SI of PHA after vaccination might be due antimitogenic effect of SPV antigens.

Key words: Immune responses, sheep vaccination, capripox vaccine, MTT, PHA, ELISA

INTRODUCTION

Vaccination is the best known and the most successful application of immunological principles to human and animal health. The first vaccine was named after vaccinia, the cow pox virus. Jenner pioneers its use 200 ago, it was the first deliberate scientific attempt to prevent small pox but it was done in complete ignorance of viruses and immunology (Roitt *et al*., 2001). Different types of antigens were used as vaccines but the live vaccines are found to be better than the killed ones (Roitt *et al*., 2001).

Anthrax infections are initiated by endospores of *Bacillus anthracis*; the spores do not divide and have a measurable metabolism and are resistant to drying heat, ultraviolet, gamma radiation and many disinfectants (Watson, 1994). Their hardness and dormancy have allowed anthrax spores to be developed as biological weapon by a number of nations (Harris, 1994).

Many serological tests are used for anthrax diagnosis. In the indirect hemagglutination test, a 4- fold rise in paired sera obtained at least 2 weeks apart indicates infection or vaccination (Wilson, 1984). A single titer of 1: 8 or higher may represents past infection or immunity as a result of vaccination. ELISA assay was used to determine titers of antibodies (total immunoglobulin) specific for purified Protective Antigen (PA) as described by Pezard *et al*. (1995) or used to detect to spores surface proteins (Fabien *et al*., 2002).

Rao *et al*. (1997) have studied the sheep pox virus soluble antigens. Their study revealed proteins of 14 molecular weights, 9 of them were found to be precipitinogens and 5 were identified as structural components of virus particles. Agar gel diffusion test is usually done to detect the precipitating antigens of capripox virus but this method could not differentiate pox and parapox viruses. In neutralization test, the test sera can be either titrated against constant dose of capripox virus or standard virus strain can be titrated against constant dilution of test sera in order to calculate neutralization index. Virus specific antibody responses of structural proteins of some pox viruses can be analyzed by western blot. The antibody response to the 32 Kda and 26 Kda protein of capripox virus provide a firm basis for differentiation (Chand *et al*., 1994). Immunosorbent An ELISA has been developed using express structural Proteins 32 (P32) of capripox and monoclonal antibodies raised against the P32 (Carn *et al*., 1994). The aim of this work is to study the immune response of the combined anthrax and cappripox vaccine in sheep.

MATERIALS AND METHODS

Combined anthrax and capripox vaccine: It was prepared by mixture and homogenization of volumes of 1.0×0^{10} mL^{-1} viable spores of *Bacillus anthracis* (Sterne strain) to capripox vaccines of 5 $TCID_{50}$ so that a

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single dose of this vaccine equal to the recommended doses of both anthrax and sheep pox vaccine. The vaccine was prepared in lyophilized form.

Experimental animals: Ten sheep of local breed were divided into two groups, each of 5 animals. Group 1 vaccinated with the combined vaccine and group 2 remained as non-vaccinated control. The recommended dose of the combined vaccine was administered Subcutaneously (S/C).

Sera and lymphocytes collection: Peripherals blood of sheep was collected by jugular vein puncture into 10 mL heparinized vacutainers before vaccination and 21 days after. Lymphocytes were collected on Ficoll Histopaque (Sigma) then used as fresh cells. The sera samples were aliquoted and then preserved at -20°C till used.

Protein determination: It was carried out according to Biuret method (Randox, 1997).

Preparation of hyperimmune sera to capripox virus: A rabbit was injected S/C with 1.0 mL (2.5 $TCID_{50}$) of capripox vaccine at days 0, 14, 21 and 30 days interval. Serum was collected 10 days after the last injection; and preserved at -20°C until use.

Anthrax spores preparation: The live spores prepared for anthrax vaccine (OIE, 2004) were washed 3 time with PBS then adjusted to 10^8 spore mL^{-1} (56 μg mL^{-1})

Preparation of crude protective antigen (cPA): One mililitre containing about 1000 spores was inoculated into 250 mL sterile Brain Heart Infusion broth (BHI) (Oxoid), incubated overnight at 37°C to grow, the pH of the culture was adjusted to 8.0 then the culture was filtered through sintered glass filter to remove most of Lethal Factor (LF) and Edema Factor (EF) toxins (Milton and George, 1954, Stephen and Gregory, 1986). The filtrate was aliquoted and preserved at -20°C for use.

Indirect ELISA: This test was carried out to determine antibodies titers (total immunoglobulin) raised against *Bacillus anthracis* (Sterne strain) killed spores antigens (56 μg mL^{-1}) and the crude Protective Antigen (cPA) (20 μg mL^{-1}). Titers of antibodies to spore surface proteins were determined by indirect ELISA. Wells of 96-well microtiter plates (Corn) were coated with formaldehyde-treated spores (10^7 spores/well) overnight at 37°C (Fabien *et al*., 2002). Spores were then fixed with paraformaldehyde (3.4%), diluted test sera (1/50) were transferred to the wells. Antisheep antibodies (Sigma) coupled to peroxidase were used at dilution 1/1000. An arbitrary A_{492} value of 0.5 was used to calculate the end point titers in Multiscan ELISA reader (labsystem multiscan MS, Version 3, 0, Helsinki, Finland).Antibodies for crude PA (cPA) were determined according to Pezard *et al*. (1995).

Immunocapture ELISA (Ic-ELISA): This test was carried out to measure antibodies raised against capripox virus. The method done by Rao *et al*. (1997) was followed with mild modification where the dilution of the conjugate was 1/2500 instead of 1/5000 and casein (Oxoid) was used instead of bovine serum albumin in blocking buffer, other procedures were the same.

(3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (MTT) Lymphocytes proliferation assay: The test was carried out according to Mosmann (1983). In brief. One hundred microlitre of freshly prepared lymphocytes (6×10^5 mL^{-1}) from each animal were transferred into 3 columns each in triplicate in wells of microtiter plates (Corn).The first column from each animal received 40 μL (40 μg mL^{-1}) Phytohemagglutinin (PHA) (Sigma),the second 40 μL of concentrated capripox virus and the third column remained as non stimulating control. The plates were incubated at 37°C in CO_2 chamber for 48 h. Twenty microlitre (5 mg mL^{-1}) of sterilized MTT was added to each well including control, the plates were incubated again overnight in the same previously mentioned condition.One hundred microlitre of acidified Isopropanol (0.04 NHCL) was added to each well after color development to dissolve the purple formazan crystal. The plate was read at 492 nm absorbance in ELISA reader (Cory *et al*., 1991).

RESULTS

Indirect ELISA test: In Table 1, where cPA was used as antigen, the differences between antibodies before and after vaccination were found significant ($p = 0.001$).

Antibodies raised against the spores antigen were also prominent and the difference before and after vaccination was significant ($p = 0.001$) as shown in Table 2.

Ic-ELISA test: The differences between the Optical Densities (OD) of all samples before and after vaccination with the combined vaccine (SPPV moiety), as shown in Table 3, were found significant ($p = 0.01$).

Table 1: Antibodies response of sheep vaccinated with combined vaccine detected by indirect ELISA using cPA

Sheep No.	Pre-vaccination samples (OD)*	Post-vaccination samples (OD)*
1	0.333±0.01	0.546±0.04
2	0.322±0.04	0.653±0.06
3	0.323±0.03	0.506±0.02
4	0.217±0.01	0.497±0.01
5	0.253±0.02	0.506±0.04
Mean	0.290±0.02	0.542±0.03

OD*= Optical Density at 492 nm±SD, cPA= Crude Protective Antigen

Table 2: Antibodies response of sheep vaccinated with combined vaccine detected by indirect ELISA using killed spores antigen

Sheep No.	Pre-vaccination samples (OD*)	Post-vaccination samples (OD*)
1	0.710±0.10	0.987±0.07
2	0.682±0.04	1.089±0.05
3	0.730±0.03	1.032±0.03
4	0.770±0.02	0.988±0.03
5	0.708±0.02	1.103±0.04
Mean	0.720±0.04	1.040±0.05

OD*= Optical Density at 492 nm±SD

Table 3: Antibodies response for capripox antigen of sheep vaccinated with combined vaccine using capture ELISA

Sheep No.	Pre-vaccination sample (OD*)	Post-vaccination sample (OD*)
1	0.274±0.01	0.411±0.02
2	0.299±0.01	0.570±0.04
3	0.202±0.00	0.318±0.03
4	0.289±0.00	0.431±0.03
5	0.322±0.01	0.388±0.05
Mean	0.277±0.00	0.424±0.03

OD*= Optical Density at 492 nm±Standard Deviation (SD)

Table 4: Proliferation response of non-vaccinated sheep lymphocytes stimulated with PHA and SPV antigen as measured by absorbance (OD) at 492nm

Sheep No.	Mean OD of non-stimulated lymphocytes	Mean OD of lymphocytes stimulated with CPV Ag	Mean OD of lymphocytes stimulated with PHA
1	0.443±0.00	0.568±0.01	0.854±0.05
2	0.284±0.00	0.314±0.00	0.762±0.01
3	0.274±0.00	0.333±0.04	0.695±0.10
4	0.193±0.02	0.244±0.01	0.684±0.10
5	0.245±0.04	0.299±0.06	0.668±0.09
Mean	0.288±0.01	0.353±0.03	0.727±0.07

CPV = Capripox Virus antigen, PHA = Phytohemaglutinin, OD = Optical Density±Standard Deviation (SD)

Table 5: Proliferation response of vaccinated sheep lymphocytes stimulated with PHA and CPV antigen as measured by absorbance (OD) at 492 nm

Sheep no.	OD of non-stimulated lymphocytes	OD of lymphocytes stimulated with SPV	Od of lymphocytes stimulated with PHA
1	0.402±0.00	0.850±0.04	0.470±0.00
2	0.289±0.14	0.847±0.03	0.326±0.04
3	0.385±0.00	0.854±0.04	0.313±0.02
4	0.215±0.00	0.704±0.15	0.215±0.01
5	0.267±.003	0.550±0.04	0.261±0.01
Mean	0.311±0.03	0.760±0.06	0.371±0.02

CPV = Capripox Virus antigen, PHA = Phytohemagglutinn, OD = Optical Density±Standard Deviation (SD)

Table 6: Stimulation indices of PHA and CPV- stimulated lymphocytes of sheep before vaccination with the CV

Sheep No.	SPA	PHA
1	1.3	1.9
2	1.1	1.7
3	1.4	2.7
4	1.3	3.1
5	1.2	2.7
Mean	1.3	2.5

CV = Combined Vaccine, CPV = Capripox Virus antigen, PHA = Phytohemaglutinin

Table 7: Stimulation indices of PHA and CPV-stimulated lymphocytes of sheep vaccinated with the CV

Sheep No.	SPV	PHA
1	3.6	1.1
2	1.8	1.0
3	2.1	1.2
4	2.3	1.1
5	2.0	1.0
Mean	2.9	1.1

CV = Combined Vaccine, PHA = Phytohemaglutinin, CPV = Capripox Virus antigen

MTT lymphocytes proliferation assay: Table 4 shows lymphocytes proliferation response detected with MTT assay; all stimulated samples with PHA before vaccination were found significantly higher than the non-stimulated samples ($p = 0.000$). Samples of lymphocytes collected after vaccination (Table 5) then stimulated with PHA revealed almost no stimulation and the difference of the OD was insignificant ($p = 0.830$). Lymphocytes stimulated with SPV before vaccination (Table 4) showed slight stimulation, when compared to the non-stimulated sample and the null hypothesis probability was significant ($p = 0.017$) but the samples collected after vaccination showed more significant difference ($p = 0.001$). The mean Stimulation Indices (SI) of lymphocytes collected before vaccination (Table 6) and then stimulated with the virus and PHA were 1.3 and 2.5, respectively. As for samples collected after vaccination (Table 7), the SI for the virus and PHA were 2.9 and 1.1, respectively.

DISCUSSION

The basic requirements for manufacture and quality control of combined vaccines are essentially the same as for other biological products and are given in relevant guidelines for assuring the quality of biological products e.g. Good Manufacture Practices for biological products in WHO expert Committee on Biological Standardization.(Forty second Report, Geneva, World Health Organization, 1992, Annex 1.). The aim of this subject is to study the immune response of sheep due exposure to different antigens components of the combined anthrax and capripox vaccine and to see if there

is any interference between these antigens. Two antigens were prepared from *Bacillus anthracis*, these were the crude Protective Antigen (cPA) and the spore antigen while the capripox virus was used as whole antigen. The PA was prepared in crude form (Boor, 1955; Stephen and Gregory, 1986) because we do not have the facilities to produce a purified one.

Although ELISA is widely used to detect antibodies and antigens in a variety of test systems and it is more sensitive than neutralization test (Carn *et al*., 1994) but the problem of background reaction (Sharma *et al*., 1988) and the requirement of recombinant antigens that we could not be able to obtain, (Carn, 1995) limit their use for routine diagnosis of SPPV in our laboratory. To avoid these obstacles immunocapture ELISA was used as an alternative test as described by Rao *et al.* (1997). As shown in Table 2, the difference between antibodies titer raised due SPV antigen before and after vaccination was significant ($p = 0.012$). On the other hand the indirect ELISA for the live spore moiety of the combined vaccine revealed a considerable antibodies response and the reaction towards the spore antigens and the cPA was prominent, the difference in titer for both of them was significant ($p = 0.001$).

In the non-vaccinated sheep, lymphocytes blastogenesis was significant ($p = 0.017$) when stimulated with the SPV antigen showing 1.3 mean Stimulation Index (SI) however, lymphocytes proliferation was higher when they were stimulated with PHA ($p = 0.00$) and the mean SI was 2.5. The significant proliferation due to the virus might be attributed to previous natural infection of sheep from the field. Lymphocytes collected from the vaccinated group revealed proliferation response ($p = 0.001$) with 2.9 SI. Interestingly, blastogenesis of lymphocytes from this group was insignificant ($p = 0.830$) and the SI was 1.1 indicating the poor proliferation response.

Bacillus anthracis spores germinate inside the animal body and produce three toxins namely Protective Antigen (PA), Lethal Factor (LF) and Edema Factor (EF) (Leppla, 1991) each of these toxin has a role in anthrax pathogenicity. From the results neither the spores nor the toxins had any inhibitory effect on the virus since adequate humoral and cell mediated response were induced after vaccination. However, capripox virus also appeared not to interfere with immune response to *Bacillus anthracis* antigens. Cell mediated immune response has not been tested for the different antigens of *Bacillus anthracis* because information in this concern was not available. We concluded from this study that, this combined vaccine induced adequate immune response for both sheep pox virus and the different antigens of *Bacillus anthracis*, Sterne strain.

REFERENCES

Boor, A.K., 1955. An antigen preparation *in vitro* for immunization against anthrax. Preparation and evaluation of crude protective antigen. J. Infect. Dis., pp: 194-22.

Carn, V.M., 1995. An antigen trapping ELISA for the detection of capripoxvirus in tissue culture supernatant and biopsy samples. J. Virol. Methods. 51: 95-102.

Carn, V.M., R.P. Kitching, J.M. Hammond, P. Chand, J. Anderson and D.N. Black, 1994. Use of recombinant antigen in indirect ELISA for detecting bovine antibody tocapripoxvirus. J. Virol. Methods. 49: 285-294.

Chand, P., R.P. Kitching and D.N. Black, 1994. Western blot analysis of virus specific antibody responses of capripox and contagious pustular dermatitis viral infection in sheep. Epidemiol. Infect., 113: 377-38.

Harris, H.S., 1994. Factors of death: Japaneese secret biological war, 1932-1945 and the American cover-up. London: Routledge.

Leppla, H.S., 1991. The Anthrax Toxin Complex. In: Alouf, J. and J.H. Freer, (Eds.), Sourcebook of Bacterial Protein Toxins. London: Academic Press, pp: 277-302.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assay. J. Immunol. Methods, 65: 55-63.

Pezard, C., M. Weber, J. Sirard and P. Berche, 1995. Protective immunity induced by *Bacillus anthracis* toxin-deficient strains. Infect. Immun., 63: 1369-1372.

Randox, Mmanual, 1997. Total protein determination.

Rao, T.V., P. Malik, S. Nandy and B.S. Negi, 1997. Evaluation of immunocapture ELISA for diagnosis of goat pox. Acta Virol., 41: 345-348.

Roit, I., J. Brostoff and D. Male, 2001. Immunology (6th Edn.), Mobsly. London. England.

Stephen, F. and B. Gregory, 1986. Comparative efficacy of *Bacillus anthracis* live spore vaccine and protective antigen vaccine against anthrax in Guinea pig. Infct. Immun., 52: 509-512.

Watson, A.K., 1994. Information on which to base assessments of risk from environments contaminated with anthrax spores. Epidemiol. Infect.,113: 479-490.

Wilson, G., 1984. Bacilli, aerobic spre-bearin bacill. In: Topley and Wlison. Principles of bacteriology, virology and immunity. (7th Edn.), Systemis bacteriology. Edward, Arnold. London, pp: 432-433.

World Health Organization, 1992. Forty second report. Geneva. Annex 1.