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Combined Anthrax and Sheep Pox Vaccine, Production and Immunization Trial in Sudan

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Abstract: An anthrax and sheep pox combined vaccine was made in a lyophilized form by mixing concentrated live spores of *Bacillus anthracis* (Sterne strain) of known count with sheep pox virus (0240 vaccine strain) of a known titer. It induced protection against anthrax in guinea pigs and sheep pox diseases in sheep after challenge with virulent *Bacillus anthracis* and sheep pox virus, respectively. The vaccine was found stable after 15 month of preservation at -20°C.

Key words: Anthrax, sheep, vaccine, immunization, production, Sudan

INTRODUCTION

Both anthrax and sheep pox diseases are endemic in the Sudan and both of them are notifiable diseases, sheep pox is included in list A and anthrax in list B (OIE, 2004). The use of single vaccine for each is practiced in the Sudan for many years but their combination as a combined vaccine has not yet been tried.

A vaccine is a preparation of weakened or killed pathogen or portion of the pathogen structure that upon administration stimulates immunity but it is incapable of causing severe infection. It may be prepared as live or inactivated product. Some live vaccines are prepared from low virulence, mild field isolates of disease-causing agents that have been found to be safe and effective when administrated by an unusual route or under conditions where exposure to the microorganism will immunize rather than to cause disease (Charles *et al*., 2002). Other live vaccines are prepared from isolates of disease-causing agents that have been modified by passage through laboratory animals, culture media, or avian embryo to select the isolate of reduced virulence. The killed vaccines may contain culture of microorganisms (that have been inactivated by chemical or other means), inactivated toxins or subunits.

Combined vaccines are products intended for protection against a single infectious disease complex caused by different strains or serotypes of organism e.g., bivalent haemorhagic septicaemia vaccine or protection against multiple infectious diseases such as acellular Pertussis, Diphteria, Tetanus toxoid and *Haemophilus influenzae* type-b (Lennart *et al*., 1996; Chin *et al*., 1999) or the combination of the previously mentioned types.

As described by the Committee for Proprietary Medicinal Products (CPMP, 1998) the following products are considered as combined vaccines: the mixture of two separates vaccines in vial before administration, the use of by-pass or dual chamber syringes containing different vaccine in each chamber and vaccines for which the components are combined into one formulation of the final bulk stages.

MATERIALS AND METHODS

Master seed for anthrax vaccine: The master seed was prepared from *Bacillus anthracis*, Sterne strain 34F. It was aliquoted and preserved as live spores at -20°C till use.

Preparation and testing of the working seed: The master seed stock was reconstituted and inoculated into several slants of casein (Oxoid) sporultation agar then incubated at 37°C for 72 h. The slants were tested for purity in nutrient agar (Oxoid) and nutrient broth (Oxoid).

Method of anthrax vaccine production: After dissolving the ingredients (OIE, 2004) and adjusting the pH to 7.4, the medium was dispensed into Roux flasks, sterilized by autoclaving and then cooled in horizontal position. After agar was solidified, excess liquid was removed aseptically and the bottles incubated at 37°C to dry and to check sterility. Volume of 2 mL of the working volume seed was inoculated into each flask and then incubated at 37°C for 96 h to let the organism to grow and sporulate on the solid medium. When more than 90% of the organism undergoes sporulation, the growth was harvested using 10 mL of sterile water per bottle.

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Viable spores count: The number of the culturable spores in the product was calculated by adding ten-fold dilution in tubes containing semisolid agar, then incubated at 37°C over night.

Sterility test: Samples of harvest from each flask were examined microscopically in wet smear and on blood agar plates incubated overnight at 37°C.

Glycerination: Twice the volume of sterile, pure neutral glycerol was added to the bulk pool. Saponin (0.1% final concentration) was also added and mixed thoroughly.

Determination of titer and dilution for use: The concentrated vaccine was diluted so that the final lot has the recommended vaccine dose (1.0×10^7 mL^{-1}). The diluent used was composed of equal volume of physiological saline and glycerol. Filling of bottles with 100 mL capacity was carried out.

Safety test: This was carried out in two sheep by inoculating subcutaneously double (1.0 mL) the required dose for each and observed for 10 days.

Sheep pox vaccine production: The batches were produced on fresh monolayer of Lamb Testicles (LT). The master seed was reconstituted with Glasgow Minimal Essential Medium (GMEM) (Oxoid) and then inoculated onto LT monolayer that has been previously washed with warm Phosphate Buffer Saline (PBS) and allowed to absorb for 30 min at 37°C before being overlaid with additional GMEM. It was left incubated for 9 days to develop Cytopathic Effect (CPE). The culture was frozen and thawed three times, the suspension removed and centrifuged at 600 g for 20 min. Harvest from each flask was mixed separately with equal volume of sterile and chilled 5% lactoalbumin hydrolysate and 10% sucrose and transferred to individually numbered bottle then stored at -20°C. Lyophilization was carried out in Edward freeze drying machine.

Sterility test: Before storage, 0.2 mL from each flask was removed, added to thioglycolate and incubated at 37°C and at room temperature.

Virus titration: It was carried out in microtiter plates as described by Kitching and Taylor (1985). It was also done by inoculating 0.2 mL in shaved flank of two sheep using dilutions from 10° to 10^6. The vaccine harvest has a minimum titer of 5 $TCID_{50}$.

Safety and efficacy: Two known susceptible sheep were inoculated Sub Cutaneously (S/C) with the double required dose (2.8 $TCID_{50}$), they were clinically examined daily and rectal temperature were recorded for two weeks. No signs of the disease were seen on the control.

Preparation of the combined vaccine: Fifty mililitre of live spore anthrax vaccine containing 5×10^9 spores mL^{-1} was mixed with 450 mL of sheep pox vaccine with 5 $TCID_{50}$ then mechanically homogenized so that one ml of the final dose for the combined vaccine meet the required ones for the individual vaccine. The mixture was dispensed in small vials then lyophilized. Each vial contains 100 doses. Reconstitution of the vaccine was made by physiological saline with 0.1% saponine.

Titration of the combined vaccine: The live spore moiety was determined by viable tube count, while the titer of sheep pox part was carried out on shaved flank of 2 sheep using Karber (1931) method.

Sterility and safety test of the combined vaccine: Five vials were selected at random. Sterility was conducted by reconstituting the vaccine with sterile physiological saline; it was cultured on blood agar then incubated aerobically and non-aerobically at 37°C for 7 days. Wet smear from the vaccine was also checked microscopically. Two sheep were inoculated S/C with double the required dose then kept observed for 10 days.

Experimental animals

Local breed sheep: Eighteen local breed sheep purchased from the market were kept in close fence paddock, given anthelmentic and anticoccidial drugs and observed for two weeks. The animals were divided into three groups; one vaccinated with 1 mL (S/C) sheep pox vaccine, the second had 1 mL of the combined vaccine (S/C) and the third remained as non-vaccinated control. Nitrocellulose dot blot was used for screening antibodies of both sheep pox virus and *Bacillus anthracis*.

Guinea pigs: Ten animals were divided into two groups, the first group vaccinated with 1 mL of the combined vaccine and the second remained non-vaccinated.

Nitrocellulose dot blot: It was used as a screening test; the method was carried out according to (Leslie and Frank, 1991).

Challenge test: All groups of sheep were challenged intradermally with 0.2 mL (2 $\log_{10}$) of sheep pox (wt) at the

clipped flank according to Kitching and Taylor (1985). Each animal of the two groups of Guinea pigs received 300 spore mL^{-1} by intramuscular route.

Stability test of the combined vaccine: The combined vaccine was preserved at -20°C and after 15 month it was checked for stability by counting the number of the viable spores and determines the titer of the virus on flank of sheep.

RESULTS

The vaccine was prepared in lyophilized form in vials containing 100 mL doses and preserved at -20°C. No adverse effect due any of the three vaccines was observed after inoculation S/C for safety test.

Potency test of the vaccines: The batch of the live spore anthrax vaccine that has been chosen has a viable spore's count of 5.0×10^9 spores mL^{-1}. The titer of the virus on the vaccinated sheep with both sheep pox and the combined vaccine was found 1.0 $TCID_{50}$ in both of them while it was found to be 3.4 $TCID_{50}$ in the non vaccinated control. On tissue culture plate the titer of the sheep pox vaccine was found 5.0 $TCID_{50}$.

Challenge test in guinea pigs: The number of the vaccinated group with the combined vaccine survived the challenge with live spores of local virulen *Bacillus anthracis* strain for the whole observation period (2 month), they develop no signs of the anthrax disease except a pregnant female aborted 24 h after challenge. The entire non-vaccinated group died following challenge. The time of death for the individuals was33, 53, 75, 94 and 99 h post challenge.

Challenge test in sheep: The temperature of the non-vaccinated control group slightly increased in day 5 and subsided in day 7. One of the animal showed slightly increased prescapular lymph node and another animal showed slight increase in precrural lymph node 6 days after challenge. No nodules were detected at the site of the vaccine inoculation except in one animal and the lesions resolved after 10 days. In day 10, the entire control group revealed small vesicles at the hairless part of the tail. The vesicle was about 0.1 cm in diameter. The vesicles were filled with clear fluid the ruptured after 2 days. He appetite of the control group was not affected, the animals continued to eat up to the end of the experiment.

The group vaccinated with sheep pox and the combined vaccine showed no increase in their normal temperature. One animal vaccinated with sheep pox vaccine revealed local nodules at the sit of inoculation of the vaccine which disappeared after 8 days, another one vaccinated with also sheep pox vaccine showed small vesicles in the hairless part of the tail, they ruptured after 2 days. No other clinical manifestations were detected in the vaccinated group.

Stability test of the combined vaccine: When the vaccine stored at -20°C for about 15 month, the number of the viable spores and the titer of the virus on flank of sheep were found almost unchanged (Table 1-4).

Table 1: End point titer of sheep pox virus titrated on flank of non-vaccinated sheep

Virus concentration	Effect on flank region +ve	-ve	Total +ve	Cumulative total effect +ve	-ve	Ratio +ve/total	% of +ve
10^{-1}	4	0	4/4	11	0	11/11	100
10^{-2}	4	0	4/4	7	0	7/7	100
10^{-3}	2	2	2/4	3	2	3/5	60
10^{-4}	1	3	1/4	1	5	1/6	17
10^{-5}	0	4	0/4	0	9	0/9	0
10^{-6}	0	4	0/4	0	13	0/13	0
Total							277

-ve = Negative, +ve = Positive

Table 2: End point titer of sheep pox virus titrated on flank of non-vaccinated control sheep

Virus concentration	Effect on flank region +ve	-ve	Total +ve	Cumulative total of effects +ve	-ve	Ratio +ve/total	% of +ve
10^{-1}	4	0	4/4	12	0	12/12	100
10^{-2}	3	1	3/4	8	1	8/9	89
10^{-3}	3	1	3/4	5	2	5/7	71
10^{-4}	1	3	1/4	2	5	2/7	30
10^{-5}	1	3	1/4	1	8	1/9	11
10^{-6}	0	4	0/4	0	12	0/12	0
Total							301

-ve = Negative, +ve = Positive

Table 3: End point titer of sheep pox virus titrated on flank of sheep vaccinated with combined sheep pox and anthrax vaccine

Virus concentration	Effect on flank region +ve	Effect on flank region -ve	Total +ve	Cumulative total effect +ve	Cumulative total effect -ve	Ratio +ve/total	% of +ve
10^{-1}	1	3	1/4	2	3	2/5	40
10^{-2}	1	3	1/4	1	6	1/7	14
10^{-3}	0	4	0/4	0	10	0/10	0
10^{-4}	0	4	0/4	0	14	0/14	0
10^{-5}	0	4	0/4	0	18	0/18	0
10^{-6}	0	4	0/4	0	22	0/22	0
Total							54

-ve = Negative, +ve = Positve

Table 4: End point titer of sheep pox virus titrated on flank of sheep vaccinated with combined sheep pox and anthrax vaccine

Virus concentration	Effect on flank region +ve	Effect on flank region -ve	Total +ve	Cumulative total effect +ve	Cumulative total effect -ve	Ratio +ve/total	% of +ve
10^{-1}	2	2	2/4	2	2	2/4	50
10^{-2}	0	4	0/4	0	6	0/6	0
10^{-3}	0	4	0/4	0	10	0/10	0
10^{-4}	0	4	0/4	0	14	0/14	0
10^{-5}	0	4	0/4	0	18	0/18	0
10^{-6}	0	4	0/4	0	22	0/22	0
Total							50

-ve = Negative, +ve = Positive

DISCUSSION

The concept of the combined vaccine is not novel, since vaccines comprising combination of inactivated diphtheria, tetanus and pertussis whole cell components (Ramon, 1949) or live measles, mumps and rubella viruses have been available for at least two decades. In veterinary medicine, there were successful trials of different vaccine combinations such as combination of vaccines against foot and mouth, haemorhagic septicaemia and black quarter diseases (Reddy *et al*., 1997). The aim of combining these two vaccines is to counter the endemic anthrax and sheep pox diseases in Sudan. In this study, live spore anthrax vaccine alone was subjected to all criteria described by the International Office of Epizootic Diseases (OIE, 2004) it was prepared in liquid form and it showed no adverse reaction when inoculated for safety test. Sheep pox vaccine was prepared in lyophilized form and also found to be safe. The combined vaccine prepared from these two vaccines was made in lyophilized form; it revealed no side effects when administrated into sheep.

In combined vaccines, the presence of more than one component often cause an interaction, leading to either diminish or an increase response to an individual component, compared to when the specific component(s) is administered alone. Such interactions are often immunological in nature, but problem may also be caused by chemical or physical interactions between the components of the vaccine so we have not added phenol to anthrax spores as routinely done in live spore anthrax vaccine production. Despite the fact that *Bacillus anthracis* is sensitive to many antibiotics (Terry *et al*., 1999) but those added during the preparation of sheep pox vaccine did not inhibit the germination of *Bacillus anthracis* when cultured on blood agar this might be due to the small concentration of those antibiotics. According to Ramyar and Bahrsefat (1970) saponin was used as adjuvants for the combined vaccine, it is highly surface active and form stable complexes with protein released from the envelopes of viruses such as influenza, measles and rabies, it is also used to be incorporated with live spore anthrax vaccine (OIE, 2004).

The efficacy of anthrax vaccine moiety was carried out in Guinea pigs since these animals are the best model used by many workers (Fellows *et al*., 2001). The survival of the vaccinated group indicated that anthrax vaccine part was effective to protect animals from the disease. The challenge test was not done in sheep due lack of suitable confinement. In non-vaccinated sheep, no frank clinical signs after challenge with virulent sheep pox virus were detected, titration on animals revealed also low titer this because the animals that have been used were purchased from the local market and all of them showed antibodies against sheep pox and *Bacillus anthracis* (Fig. 1) they were expected to be exposed to natural infection from the field because Sudan is endemic with both of the diseases. We were not able to use foreign sheep breed to carry out the experiment.

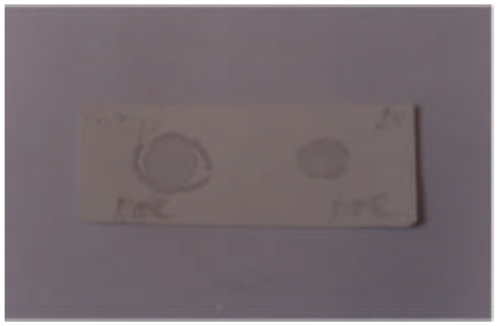

Fig. 1: Immunostaining dot blot due reaction of Bacillus anthracis spore antigen (AX) and Sheep Pox antigen (SP) with serum antibodies before (pre) vaccination with the combined vaccine

Beside anthrax and sheep pox diseases are endemic in Sudan (ELNasry, 1966; Carn, 1993; Esposito and Fener, 2001) other justifications of combining these two different vaccines was their similar duration of immunity which is one year (OIE, 2004) and their longer stability. *Bacillus anthracis* spores are resistant to drastic environmental condition comparable to the vegetative cells (Watson, 1994) and the spore vaccine can be stable for one year at room temperature (Abbas and Babiker, 2000). However, pox viruses are known to be resistant to drastic conditions, capripox vaccine particularly those include protectant, such as sucrose, lactoalbumine hydrolysate are stable at -20°C for 25 years (OIE, 2004). Since this vaccine was found safe and with considerable efficacy, we recommend to be applied in the field because it will reduce the cost and effort of vaccination and it will increase convenience and efficacy concerning logistics of prophylactic project in the field (Provost and Perreau, 1978).

REFERENCES

Abbas, M. and S. Babiker, 2000. Stability of anthrax live spore vaccine at different temperatures. Albuhuth, 8: 123-131.

Carn, M., 1993. Control of capripox infection. Vaccine, 11: 1275-1279.

Charles, A., T. Paul, W. Mark and S. Mark, 2002. Immunobiology. (5th Edn.), Manipulation of the immune response to fight infection. Garland Publishing Churchil Livingstone. New York.

Chin-Yun, L., T. John, H. Li, L. Ping, C. Hsiu, L. Wend, D. Heneri, H. Dana, X. Fang and B. Luis, 1999. An evaluation of the safety and immunogenicity od a five-component acellular pertussis, diphtheria and tetanus toxoid vaccine (DTaP) when combined with *Haemophilus infleunzae* type-B tetanus toxoid conjugate vaccine (PRP-T) in Taiwanese infants. Pediatrics, 103: 25-30.

Committee for Proprietary Medicinal Product (CPMP), 1998. Pharmaceutical and biological aspects of combined vaccines, pp: 1-14.

ELNasry, M., 1966. Present status of animal diseases and disease control. Sudan. J. Vet. Sci. Anim. Husb., 7: 34-4.

Esposito, J. and F. Fener, 2001. Poxviruses. In: Field, B.N., D.M. Knipe, P.M. Howley, R.M. Chanock, J.L. Melnick, T.P. Monathy, B. Roizman and E. Straus (Eds.), Field of Virology. (4th Edn.), Lippincott, Williams and Wilkins. Philadelphia, PA., pp: 2885-2921.

Fellows, P., B. Linscorr, B. Ivins, M. Pitt, I. Rossi, P. Gibbs and A. Friedlander, 2001. Efficacy of human anthrax vaccine in guinea pigs, rabbit and Rhesus macaques challenge by *Bacillus anthracis* isolates of diverse geographical origin. Vccine, 17: 3241-3247.

Karber, G., 1931. Beting zur Kellektiven behandlung pharmakologishe reihenversuche. Arch. Exp. Path. Pharmacol., 162: 480-483.

Kitching, R. and W. Taylor, 1985. Transmission of capripox virus. Res. Vet. Sci., 39: 196-199.

Lennart, G., O. Hans, O. Patrick, R. Elisabet and S. Jan, 1996. A controled trial of a two component-acellular, a five-component acellular and a whole cell pertussis vaccine. New Eng. J. Med., 334: 349-356.

Leslie, H. and H. Frank, 1991. Practical Immunology. (3rdEdn.), Blackwell Scientific Publication, pp: 12-13.

Office International Des Epizootique (OIE), 2004. Manual of diagnostic test and vaccine for terrestrial animals.

Provost, A. and P. Perreau, 1978. Combined vaccine in veterinary medicine in the developing countries. Dev. Biol. Stand., 41: 349-360.

Ramon, G., 1949. Rev. Imm. Cited by Raymar and Bahrsefat, 1970. Arc. Inst. Razi, 22: 123-128.

Ramyar, H. and M. Bahrsefat, 1970. A new approach to active immunization of sheep by a combined sheep pox and anthrax vaccine. Arc. Inst. Razi, 22: 123-128.

Reddy, G., P. Antony and V. Srinivasan, 1997. Serological response to combined vaccine of cattle against foot-and-mouth disease, haemorhagic septicaemia and black quarter. Indian. J. Anim. Sci., 67: 585-586.

Terry, C., B. Dixon, M. Matthew, G. Jeanne and C. Hanna, 1999. Anthrax. The New Eng. J. Med., 3: 815-826.

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