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Immunization response of rabbits and Guinea pigs to different antigens of *Bacillus anthracis* vaccine strain

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ABSTRACT

A crude protective antigen (CPA), anthrax live spore vaccine (LS) and formaldehyde inactivated spores (FIS) of *Bacillus anthracis*, were prepared to evaluate their immune response in rabbits. Also these antigens were inoculated into guinea pigs to study their protective efficacy. In rabbits vaccinated with LS anthrax vaccine, antibodies were raised against the LS, FIS and CPA, antibodies induced against FIS and CPA has similar optical densities (ODs) but it is increased in LS. However, rabbits inoculated with CPA, again produced antibodies against the three antigens this is due to anti- PA neutralizing and anti-spore activities. Protective efficacy of LS, FIS and CPA in guinea pigs varied, it was 100%, 40%, and 20% respectively. The non-vaccinated control animals died in 48 and 72 hours but most of guinea pigs inoculated with FIS and CPA died after 72 hours this might be due partial immunity acquired by the FIS and CPA.

INTRODUCTION:

The Gram positive *Bacillus anthracis* is a spore forming bacteria that causes anthrax. Dormant spores are highly resistant to stressful conditions and they are able to survive for decades in the environment, (Mock and Fouet, 2001). Pathogenesis of anthrax by inhalation, ingestion and cutaneous anthrax includes the entry of *Bacillus anthracis* spores into the host, followed by germination, bacterial multiplication, dissemination and toxin production (Jargalsaikhan *et al.*, 2006). Although many species of bacteria form spores, *Bacillus anthracis* is of particular interest because the vegetative form of this organism has the ability to cause the fatal

disease anthrax. Recent studies have shown that *Bacillus anthracis* is genetically similar to other spore - forming bacteria including *Bacillus cereus and Bacillus thuringiensis*. These studies include conventional bacteriological assays and more recently RFLP and other molecular finger printing methods (Keim *et al.*, 2000, Hill *et al.*, 2004). Identification and classification of spore bacteria by metabolic studies and gene polymorphisms by genome analysis have focused on the vegetative form of these organisms. The nature of spore and their ability to survive harsh extraction procedures have, until recently, circumvented all but rudimentary studies on the biochemical nature and, in particular, the molecular structure of *Bacillus* spores. The primary determinants of *Bacillus anthracis* pathogenesis are lethal toxin, edema toxin and capsule, the genes for which are carried on the large virulent plasmids pXO_1 and pXO_2 (Green *et al.*, 1985, Uchida *et al.*, 1986). Both lethal toxin and edema toxin are bipartite A-B toxins with a common subunit, protective antigen that mediate entry of the active toxin components lethal factor (LF) and edema factor (EF) into the host cell cytoplasm (Banks *et al.*, 2006). The genes that encode PA, LF and EF are *pagA*, *lef*, and *cya* respectively (Robertson and Leppela, 1986, Mock *et al.*, 1988, Tippetts and Robertson, 1988, Vodkin and Leppela, 1983). The development of pXO_1^+ and pXO_2^- non-encapsulated vaccine was accomplished by Sterne (1939) and this vaccine is still used in livestock (Turnbull, 1991). A Sterne-like strain was also used in the former Soviet Union and was reported to reduce the incidence of cutaneous anthrax disease (Shlyakhov and Rubinstein, 1994).

Since pXO_1^+ and pXO_2^- strain contain functional toxin genes and cause significant disease in mice, Brossier *et al.* developed a vaccine strain that expressed catalytically inactive form of EF and LF (Brossier *et al.*, 2000). This strain is avirulent and induced a robust toxin-neutralization response that protected mice from lethal subcutaneous *Bacillus anthracis* challenge. The anthrax vaccine currently available are, anthrax vaccine adored (AVA) for human, live attenuated spore vaccines derived from encapsulated strains, for veterinary use only (Brey, 2005), and the UK anthrax vaccine precipitated, which has been used over 40 years (Williamson *et al*., 2005). The live spore ST1 vaccine has been used in Russia for many years in human (Romanov, 1980). Many researchers have demonstrated that high titers of anti-PA antibody also give passive protection in rabbits, guinea pigs and mice (Kobiler *et al.*, 2002; Beedham *et al*., 2001; Pitt *et al.*, 2001).

The aim of this study was to compare the efficacy of the crude protective antigen (CPA), anthrax live spore vaccine (LS) and formaldehyde inactivated spores (FIS) of *Bacillus anthracis* for protecting rabbits and guinea pigs.

MATERIALS and METHODS:

Bacterial strain: The Sterne strain *B. anthracis* was obtained from the Department of bacterial vaccine in Veterinary Research Institute (VRI), Soba, Sudan

Live spore (LS) anthrax vaccine: This was obtained from the department of bacterial vaccine production, Veterinary Research Institute, which prepared as described by the OIE (2004).

Formaldehyde inactivated spores (FIS) preparation: 0.25 ml of Formaldehyde inactivated spores (FIS)were prepared as described by OIE (2004). They were collected in 15 ml tube, washed three times by normal saline. 0.25 ml of formaldehyde (34%) was added to the spores then incubated overnight at 37^0C. Inactivation of the spores was confirmed by culturing them onto blood agar at 37^0C for 48 hours. Formaldehyde inactivated spores were collected and kept until used.

Crude protective antigen CPA was done as prescribed by Abbas *et al.,* (2007).

Immunization of rabbits: Twenty locally breed rabbits were divided into 4 groups (Gs) each one compose of 5 animals. The groups were kept separate from each others. Each rabbit in group 1 (G1) received 0.5 ml ($0.5X10^7$ spores) of the live spore vaccine those of G2 received also $0.5X10^7$ spores of formaldehyde treated spores (FTS) and those of G3 received 1 ml of CPA. Group 4 (G4) had 1 ml of normal saline and remained as non-vaccinated control group. The route of administering the antigens in all the above groups was subcutaneous. Sera were collected 21 days after vaccination and stored at -20^0C till used.

ELISA test: Enzyme -linked immunosorbent assay (ELISA) was used to determine the titre of antibodies specific for CPA (20 µg /ml) and spore surface antigens. Briefly, 96 well microtiter plates (Corn) were coated with 100 µL of CPA, 1/50 diluted sera was used and anti-rabbit antibodies whole molecule coupled to peroxidase was finally added with a dilution of 1/1000. Optical density (OD) was determined at 492 nm in ELISA Reader (ASYS Hitech GmbH)

Another microtiter plate coated with spores (10^7spores/ well). Spores' were fixed with paraformaldehyde (37%) at 37^0C. Anti - rabbit antibody was used, antibodies response was determined as previously mentioned. Sera from G1 were tested by CPA and spores antigens for detecting their corresponding antibodies.

Immunization and challenge of guinea pigs: Twenty guinea pigs (300 -500 g) were divided into 4 groups (Gs) ,G1 was vaccinated with live spore anthrax vaccine ($0.5X10^7$ spores /ml), G2 had $0.5X10^7$ spores inactivated spores and G3 had 1 ml of CPA while each animal in the non-vaccinated control group had 1 ml of normal saline. Challenge with a locally virulent strain was carried 21 days post vaccination; each animal received 300 live spores intramuscularly (Abbas *et al.*, 2007).

Statistics: X^2 test was used for statistical analysis.

RESULTS and DISCUSSION:

Immunity to *Bacillus anthracis* is mediated largely by humoral response, and the primary immune correlates of protection are circulating antibodies specific for protective antigen (PA) that have neutralizing activity (Little *et al.*, 2004, Phipps *et al.*, 2004). The rabbits in G1were expected to elicit antibodies for both protective antigen and spore antigens, since they had live spore vaccine.

In table (1) the titres of antibodies response to CPA and FIS spores was almost equal but it was slightly increased in the case of LS. This result agreed with that obtained by Fabien *et al.*, (2002).

Antibodies to PA may neutralize toxin activity by blocking the binding of PA to its receptor, and/or by formation of complex. The PA has been an attractive target for vaccine development.

Antibodies response to CPA was not high this might be due to impure crude protective antigen that used in this experiment. However, highly purified PA preparations and recombinant PA have been tested in various animal models, including mice, guinea pigs, rabbits and monkeys (Fellows, *et al.*,2001,Ivins *etal.*,1998,Zaucha *et al.*,1998) in combination with various adjuvants (Ivins *et al.*,1995, Ivins *et al.*,1992). Moreover, several studies illustrate the difficulty of evaluating PA vaccines and establishing a direct correlation between PA-specific antibody titres and protection (Ivins *et al.*, 1995, Ivins *et al.*, 1992, Turnbull, 1991, Turnbull, 1986, Turnbull, 1988).

In table (2), the quantitative antibodies to CPA, LS and FIS show optical densities (ODs) almost similar but it is increased in LS response. According to Cote *et al* (2005),

PA was found to be associated with spores. In rabbits, PA induces polyclonal antibodies that, in addition to their anti-toxin neutralizing activities, exhibit anti-spore activities, this agrees with the results that obtained in this study. Also, in recent investigation, *Bacillus anthracis* spore associated proteins have been identified in reactions with the sera of humans immunized with Anthrax Vaccine Adsorbed (AVA) (Kudva *et al.*, 2005)

The efficacy of LS, FIS and CPA to protect guinea pigs is shown in table (3). The live spore vaccine (LS) protected 100% of the guinea pigs for the whole observation period (30 days) while the protective efficacy for FIS and CPA was 40%, 20% respectively. However, pure protective antigen (PA) used by Fabien *et al.,* (2002) induced 22% protection of guinea pigs, FIS alone induced only 25% protection but it was 100% when they combined PA with10^7 FIS. The difference in survival between guinea pigs vaccinated with LS and those received FIS was found statistically significant ($P< 0.05$), the difference was also significant between guinea pigs vaccinated with LS and those had PA (P=0.01). All non-vaccinated control animals died within 72 hours. Time of death for animals vaccinated with FIS was 96- 144 hours and that of CPA was 72-120, it is a little bit longer than that of non-vaccinated animals due to partial immunity induced by the CPA and FIS.

It is concluded from this study that live spore vaccine of *Bacillus anthracis* (Sterne strain) was 100% protecting guinea pigs and it induced high antibody titre in rabbits.

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Table 1: Mean antibodies response of rabbits vaccinated with LS vaccine

Antigen	Serological response (OD)
CPA	1.465
LS	1.926
FIS	1.467
Control	0.560

Table 2: Antibodies response of rabbits inoculated with CPA

Antigen	Serological response (OD)
CPA	0.967
LS	1.049
FIS	0.847
Control	0.165

Table 3: Protective immunity induced in guinea pigs by LS, FIS and CPA

Immunization	protection	Time of death/hour
LS	5/5 (100%)	-
FIS	2/5 (40%)	96,120,144
CPA	1/5 (20%)	72,96,120,120
Control	0/5 (0%)	48,48,48,72,72