THE APPLICATION OF ELISA TO MONITOR THE VACCINAL RESPONSE OF ANTHRAX VACCINATED RUMINANTS

S. HARDJOUTOMO, M.B. POERWADIKARTA, B.E. PATTEN and K. BARKAH
Research Institute for Veterinary Science, Bogor, West Java, Indonesia

ABSTRACT

Hardjoutomo,S., M.B. Poerwadikarta, B.E. Patten and K. Barkah. 1993. The application of ELISA to monitor the vaccinal response of *Anthrax* vaccinated ruminants. *Penyakit Hewan* 25 (46A): 7-10.

Groups of beef cattle, sheep and goats raised on a confined open grazing land at the Institute Pertanian Bogor research farm were vaccinated with anthrax spore vaccine, made by the Pusat Veterinaria Farma (Surabaya, East Java) at a dose of 1 mL per head of cattle and 0.5 mL per head of sheep or goats, subcutaneously. A second dose of vaccine was given at 8 weeks after the initial vaccination to one group of the cattle and sheep, but not to the goats. All animals were bled regularly at 2-4 week intervals and sera were tested by an anthrax antibody ELISA. The optical density was converted to ELISA units (EU) and used to express the antibody level in the vaccinated animals. The results of the antibody ELISA indicated that vaccinated ruminants have higher EU values than unvaccinated animals. All experimental animals responded to the initial vaccination, with antibodies lasting for 12 weeks post-vaccination. Animals that received the booster injection showed a marked rise in antibody response within 2 weeks of vaccination, the antibody level then decreased gradually thereafter. Further studies are required to assess the duration of the antibody response after the second vaccination.

Ke words: ELISA, vaccine, *Anthrax*, ruminants

ABSTRAK

Hardjoutomo,S., M.B. Poerwadikarta, B.E. Patten dan K. Barkah. 1993. Penggunaan ELISA untuk memantau respon vaksinasi Anthrax pada ruminansia. *Penyakit Hewan* 25 (46A): 7-10.

Sekelompok sapi potong, domba dan kambing yang dipelihara pada padang gembalaan di Jonggol, Kabupaten Bogor digunakan sebagai hewan-hewan percobaan. Ternak tersebut divaksin dengan vaksin antraks spora buatan Pusvetma, Surabaya, dengan dosis 1 mL per ekor sapi dan 0.5 mL per ekor domba atau kambing dengan aplikasi subkutan. Suntikan ke-2, sebagai booster, diberikan pada 8 minggu setelah vaksinasi ke-1 diberlakukan bagi sekelompok sapi dan domba saja. Secara berkala, dengan selang waktu 2-4 minggu, serum dari ternak-ternak percobaan tersebut diperiksa secara ELISA antibodi antraks. *Optical density* (diutarakan dalam ELISA Unit, EU) digunakan untuk mengukur kandungan antibodinya. Hasil menunjukkan bahwa ternak yang divaksin memberikan nilai EU yang lebih tinggi daripada ternak yang tak divaksin. Bagi ternak yang hanya 1x divaksin, titer antibodi bertahan hanya sampai 12 minggu saja. Sedangkan bagi ternak yang mendapatkan suntikan *booster*, kenaikan EU-nya yang dicapai dalam 2 minggu pasca booster berjalan sampai beberapa minggu berikutnya. Penelitian lanjutan dirasa perlu dilakukan untuk mengetahui sampai berapa lama titer antibodi pasca booster tadi dapat bertahan.

Kata kunci: ELISA, vaksin, *Anthrax*, ruminansia

INTRODUCTION

Anthrax was first reported in Indonesia in 1885 (Soemanagara, 1958) and is an enzootic disease in several provinces of the country. Between 1978 and 1985 anthrax was reported in nine of 27 provinces of Indonesia involving the deaths of 4,310 animals including 2,902 cattle and buffalo, 419 sheep and goats, 891 horses and 98 pigs. Anthrax is classed as a notifiable animal diseases in Indonesia and is also an important zoonotic disease with human infections being reported in a number of endemic anthrax infected areas (Hardjoutomo, 1990).

Anthrax vaccination using a Weybridge strain 34F2 spore vaccine produced according to the requirements of WHO (1967) by Pusat Veteriner Farmia (Pusvetma), Surabaya Indonesia, has been used for the prevention of anthrax infection by the Indonesian government District Livestock Services (DLSs). However, limited funds have resulted in only a small quantity of vaccine being produced and distributed for use throughout the country (Hardjoutomo, 1990).

A number of the more challenging problems in respect of anthrax in Indonesia are to evaluate the effectiveness of the vaccination program conducted in the enzootic areas, to supply laboratory confirmation of field diagnoses of anthrax outbreaks and to control the persistent outbreaks anthrax in enzootic areas (Hardjoutomo *et al.*, 1990). Work has been undertaken at the Research Institute for Veterinary Science (Balitvet), Bogor Indonesia in association with an Australian Centre for International Agricultural Research (ACIAR) funded project to implement an ELISA to determine the antibody

response of large and small ruminants to vaccination with a commercial *B. anthracis* spore vaccine. This paper presents results of the application of an ELISA to evaluate the antibody response of anthrax vaccinated ruminants in West Java.

MATERIALS AND METHODS

Animals

A total of 32 sheep, 16 goats and 32 cattle, reared on the Institut Pertanian Bogor (IPB) research farm, Jonggol, West Java, were used for this study. The animals were allowed access to open grazing throughout the course of the study and were randomly divided into groups of eight, namely: sheep - groups A, B, C and D; goats - groups E and F; cattle - groups G, H, I and J.

Vaccination

Animals were vaccinated with a live anthrax spore vaccine *B. anthracis* (strain 34F2, Pus vetma, Surabaya, East Java) which contained 5 x 10^8 spores per mL. An anthrax spore vaccine made by Balitvet, Bogor and containing approximately the same number of spores of *B. anthracis* Sterne strain, was also included for comparison in the study. The vaccines were administered subcutaneously at a dose of 1.0 mL for cattle and 0.5 mL for sheep or goats, according to the recommendation of the manufacturers of the vaccines and in accordance with the British Veterinary Codex.

Cattle group G, sheep group B and goat group E received one dose of PusVetma vaccine at week 0. Cattle group H and sheep group A received one dose of PusVetma vaccine at week 0 and a second vaccination using the same dose at week 8. Cattle group J and sheep group D received one dose of Balitvet vaccine at week 0. Groups of cattle, sheep and goats were left unvaccinated as controls.

Animals were bled biweekly for the first 14 weeks and then every four weeks thereafter for the period of the study. Blood was collected in sterile silicon coated vacuum evacuated tubes (Vacutainers, Becton-Dickinson USA). The blood was allowed to clot at room temperature and the serum collected and then stored at -20 °C until required for testing.

Bacterial culture

B. anthracis was cultured in nutrient broth by grown overnight at 37 °C. The seed culture was then inoculated into Syn N broth medium which was incubated overnight at 37 °C Syn N broth was made by mixing 500 mL of solution A and 55 mL of solution B. Each 500 mL of solution A contained 6.0 g Casamino acids (Oxoid), 16 mg $CaCl_2.2H_2O$, 25 mg $MgSO_4.7H_2O$, 0.51 g KOH and 51 mg DL-serine. After adjusting the pH to 6.9 and autoclaving at 121 °C for 15 minutes, small volumes of sterile filtered L-cystine (20 mg) and thiamine-HCl (150 µg) were added. Solution B contained 30 g $NaHCO_3$, 10 g glucose and 5 mg $MnSO_4$ per 500 mL. This solution was sterilised by filtration.

ELISA

The ELISA antigen was prepared from a culture supernatant of *B. anthracis* bekasi strain, a capsulated strain isolated from buffalo at Balitvet, Bogor. The culture was centrifuged at 8,000 *g* for 20 minutes and the supernatant collected. The crude supernatant was titrated in a checkerboard manner against a known positive and negative serum to determine the optimum working dilution of the antigen.

Anthrax ELISA antigen was diluted 1:500 in 0.1 M carbonate/bicarbonate buffer pH 9.2 and 100 µL was added to each well of a 96-well U bottom microtitre plate (Nunc, Denmark). The plate was incubated overnight at 4 °C. Unbound antigen was removed by washing the plates with 0.1 M phosphate buffered saline pH 7.4 with 0.05% tween 20 (PBS-Tween). One hundred µL of serum, diluted 1:200 in PBS-Tween, was added in duplicate to the plate. Known negative and positive cattle or sheep sera were included on each plate for quality control purposes and to calculate a standard curve for each plate. After incubation at room temperature for 1 hour, the plates were washed with PBS-Tween and 100 µL of conjugate, diluted to working strength, was added to each well. An anti-cow Ig horseradish peroxidase labelled (affinity purified) conjugate (Silenus Laboratories, Australia) was used for testing of the cattle serum and an anti-sheep/goat Ig horseradish peroxidase labelled (affinity purified) conjugate (Silenus Laboratories, Australia) was used for testing of sheep and goat sample. The conjugate was diluted to working concentration in PBS-Tween with 0.02% casein (Sigma Chemicals, USA). After incubation for 1 hour at room temperature the plates were washed with PBS-tween and 100 µL of a freshly prepared substrate solution prepared from 1 mM 2,2' azino-bis[3-ethylbenthiazolin 6-sulphonate] (ABTS) in 0.1 M citric acid buffer pH 4.2 with 2.5 mM H_2O_2 (Urea-peroxide, Fluka Chemicals, Switzerland). The plates were incubated at room temperature for 1 hour and the optical density of the

substrate reaction was measured using a Titertek Multiskan MCC (Flow Laboratories, UK) at a wavelength of 414 nm.

The optical density readings were processed using a computer program (Platereader Program V3.2, Regional Veterinary Laboratory, Benalla Australia) to convert the optical density readings into ELISA units (EU) by comparing the optical density readings of the test serum to the standard curve of the control positive serum. The positive/negative discrimination point was taken as the mean plus 2 standard deviations (SD) of the pre vaccination readings of the test group.

RESULTS AND DISCUSSION

The ELISA antigen prepared from Syn-N culture supernatant of *B. anthracis* cells appeared to contain protein material on PAGE in the 80,000-90,000 molecular weight region as expected for the toxin components of anthrax (data not presented). The antigen contained proteins with apparent molecular weight (MW) of 80,000 to 90,000 and did not contain a large amount of other cellular antigens. *B. anthracis* is known to produce a plasmid mediated tripartite toxin consisting of oedema factor (EF, factor I MW 89,000), protective antigen (PA, factor II MW 85,000) and lethal factor (LF, factor III MW 83,000) (Mikesell *et al.*, 1983; Ezzel *et al.*, 1984).

An ELISA was developed as there are few suitable diagnostic tests available to measure the response of animals to *B. anthracis* vaccination or as a suitable measure of protection to infection. Vaccines are tested according to their ability to protect guinea pigs against challenge 21 days after vaccination (British Veterinary Codex) and protection tests have been developed for use in rats. The ascoli test, which relies on the development of a macroscopic antigen-antibody precipitin is the only serological test described for use with *B. anthracis* that does not rely on animal challenge.

The tripartite toxin components of *B. anthracis* were chosen as a possible ELISA antigen to detect antibody against the organism because the toxin components as well as the plasmid mediated capsule are known to play a role in the virulence of the organism (Mikesell *et al.*, 1983; Turnbull *et al.*, 1988). Surface antigens of *B. anthracis* were not considered as an ELISA antigen due to their similarity to surface antigens of other Bacilli. The similarity of the antigens with other organisms is likely to lead to non-specific reactions being detected by the ELISA test, so reducing the specificity and sensitivity of the assay. While the poly-D-glutamic acid capsule of *B. anthracis* is considered to provide protection to phagocytosis and lysis of the bacteria after it has invaded the host, its use as an ELISA antigen was ruled out due to the difficulty of visualising the capsule on electrophoresis, the difficulty in attaching the material to a solid phase and because of its similarity with the capsular material of other bacilli (*B. subtilis, B. licheniformis*).

The pre-vaccination mean anti-B. *anthracis* antibody levels, as measured by ELISA, were 54 ± 40 EU, 17 ± 1 EU and 18 ± 3 Eu for cattle, sheep and goats, respectively. The positive/negative discrimination point therefore 134 EU for cattle, 19 EU for sheep and 24 for goats.

Cattle vaccinated with both the Pusvetma and Balitvet vaccines demonstrated an increased antibody level following the first vaccination (week 0) (Figure 1.). The antibody response peaked at 212 ± 86 EU 2 weeks after the initial vaccination and then dropped graddually until it was at the level of the control animals after week 14. The cattle in group H, which received a second vaccinationat 8 weeks after the first vaccination, showed a marked anamnestic rise in antibody level within two weeks post injection. The animals showed a peak titre of 748 ± 208 EU which then gradually decreased over the following 16 weeks to a level of 211 ± 86 EU (Figure 1.). The response of cattle to the administration of the PusVetma vaccine was variable within the groups but the antibody response was still greater than 134 EU and so the animals were still regarded as having a significant anti-B. *anthracis* antibody level. A similar result has also been observed in a previous study by Hardjoutomo *et al.* (1990).

The sheep and goats had a lower pre-vaccination antibody level than the cattle, 18 EU versus 54 EU. Sheep group B and goat group E showed a similar rise in antibody to the cattle groups following vaccination (Figure 2.). The antibody response reached a peak after week 2,65 ± 20 EU for sheep group A and 77 ± 91 EU for goat group E. The antibody response then decreased such that at 8 weeks post-vaccination they were approximately the same as the control groups (result not shown). Sheep group A, which received a second dose of vaccine at week 8, showed a similar anamnestic antibody response as the cattle group H. This group reached a peak antibody level of 283 EU 4 weeks after the second vaccination and still had an antibody level of 179 EU at week 26. A small but non-significant difference was evident in the response to the PusVetma and Balitvet anthrax vaccines in both the cattle and sheep.

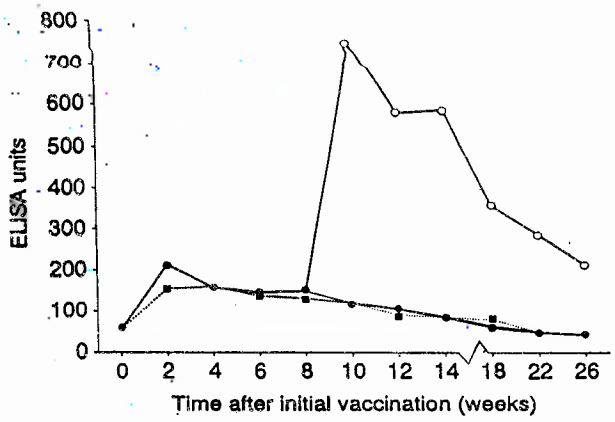

Figure 1. Antibody response of cattle to anthrax spore vaccine. • Pusvetma vaccine (one dose); • Pusvetma vaccine (two doses); ■ Balitvet vaccine (Sterne strain, one dose). The first dose was given at week zero, the second at week 8

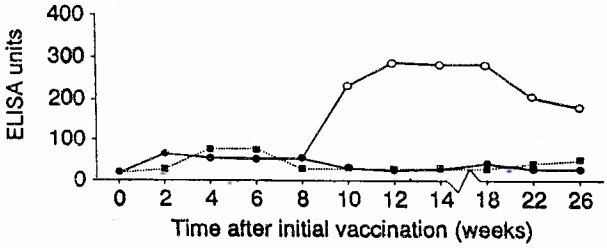

Figure 2. Antibody response of sheep and goats to anthrax spore vaccine. • sheep (one dose); • sheep (two doses); ■ goats (one dose). All animals received Pusvetma vaccine. The first dose was given at week zero, the second at week 8

This study indicated that a second B. *anthracis* vaccination produces an increase in antibody level which has a longer decay period than the antibody response of animals that received only one dose of vaccine. The antibody decay shown by both cattle group G, sheep group B and goat group E indicates that the antibody level has returned to a negative level within 6 to 8 weeks post-vaccination. This suggests the possibility that the animals are again susceptible to challenge by B. *anthracis*, although this study has no evidence to prove this point. It was also notable that at the termination of the present study (26 weeks post-primary vaccination) the antibody level of the two-dose vacinated groups were significantly greater, 211 ± 86 EU for cattle and 179 ± 90 EU for sheep, than the control groups of animals, 54 ± 40 EU for cattle and 16 ± 0 EU for sheep. A second B. *anthracis* vaccination, 4 to 6 weeks after the initial vaccination, may result in a more significant and prolonged protection to infection. Further study is required to assess the protective significance of the antibody response following one and two doses of B. *anthracis* live spore vaccine.

ACKNOWLEDGMENTS

The support of the Director, Research Institute for Veterinary Science, Bogor through Balitvet Research Project, and of ACIAR PN8907 in the conduct of this study is gratefully acknowledged.

REFERENCES

EZZEL J.W., B.W. IVINS and S.H. LEPPLA. 1984. Immunoelectrophoresis analysis, toxicity and kinetics of in-vitro production of the protective antigen and lethal factor components of *Bacillus anthracis* toxin. *Infect. Immun.* 45: 761-767.

HARDJOUTOMO, S. 1990. Anthrax in Indonesia: A continuing problem for a developing country. Salisbury Medical Bulletin, Special Supplement, 88: 14-15.

HARDJOUTOMO, S., B.E. PATTEN, K. BARKAH and J. SUBIARJAT. 1990. The response of animals to vaccination against anthrax as monitored by ELISA. Proceeding of the 7th Congress of Federation of Asian Veterinary Associations (supplement) Pattaya, Thailand: 30-41.

MIKESELL, P., B.E. IVINS, J.D. RISTROPH and T.M. DREIER. 1983. Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. *Infect. Immun.* 39: 371-376.

SOEMANAGARA, R.M.T. 1958. Ichtisar singkat dari penyakit radang limpa, penyakit ngorok dan radang paha di Indonesia. Bagian I. Anthrax, Radang limpa. *Hemera Zoa* 65: 95-100.

TURNBULL, P.C.B., S.H. LEPPLA, M.G BROSTER, C.P. QUINN and J. MELLING. 1988. Antibodies to anthrax toxin in humans and guinea pigs and their relevance to protective immunity. *Med. Microbiol. Immunol.* 177: 293-303.

WHO (WORLD HEALTH ORGANISATION). 1967. Requirements for anthrax spore vaccine (Live for Veterinary Use). WHO Expert Committee on Biological Standardisation 19th Report.