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Original paper

The detection of antibody to *Bacillus anthracis* recombinant PA in vaccinated animal serum

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Abstract
Anthrax is a worldwide zoonosis in animals and human. In Mongolia, the confirmed case of anthrax outbreak is reported every year over the past decade. The prevention and control measure of animal anthrax is vaccination using spore of attenuated Sterne strain, but horse does not get vaccinated in Mongolia. In this study, we constructed the recombinant plasmid for over expression of anthrax protective antigen (PA)/GST fusion protein in pGEX-6P-1 vector and purified the recombinant PA (r-PA) using glutathione Sepharose column under native and denaturing conditions. Since both forms of r-PA were recognized by specific antibody against PA, ELISA system to detect antibody titer in vaccinated bovine serum was constructed. Total of 890 vaccinated cattle serum were collected from 178 cattle at 0, 3, 5, 8 and 12 months' post vaccination. As negative control, 200 cattle serum from Umnugovi aimag were selected which does not have anthrax foci. All serum was tested by rPA indirect ELISA and, antibody to PA were detected in vaccinated cattle serum but were not detected in negative serum. Therefore, rPA should be used in as monitoring of the anthrax vaccination.

Keywords: anthrax vaccine, recombinant PA protein, ELISA

1 Introduction

Anthrax is a zoonotic disease caused by *Bacillus anthracis*, gram positive bacteria which forms highly resistant and long-lasting spores in the environment [19]. It was existed in all continents in Asia including Mongolia, Thailand, India, China [2,11,12,17, 25,26]. Human anthrax subsequently occurred after the animals had died and meat was supplied to the people so that some cases of gastric and oropharyngeal anthrax was observed [8,18]. It is a highly contagious disease, though no incidence of human to human transmission was found and most of infections were cutaneous form. (5, 20). Virulence of anthrax is mediated by toxins PA, LF and EF released from plasmid pXO1 and capsule of a poly-D-γ-glutamic acid from plasmid pXO2. PA is the most immunogenic of those toxin, therefore PA seems to be an essential component of all vaccine for anthrax including Anthrax vaccine adsorbed (AVA) for human and live spores of Sterne strain vaccine for animals [5,10,19]. In the affected area, vaccine is a major tool to control the outbreak and should be continued annually at least 3 years for the disease prevention [26]. As well as in Mongolia, the animals in which area the disease occurred had to be get vaccinated. Although vaccination was proved to be very effective in minimizing losses during outbreak, determination of antibody raising against anthrax should be tested [20,26]. The measurement of anti-PA antibody is essential to evaluate the vaccination program [10].

The production of recombinant protective antigen was reported from many groups of researchers mainly for the development of new anthrax vaccines and therapeutics.

Most of those work procedure is operated using many equipment and steps to achieve large quantities of PA (3, 4, 9, 14, 16). For our scale, we needed only one step for protein purification by constructing the pGEX-6P-1 containing PA gene to express GST fusion protein. Using only small column of GSTrap fast flow, we were able to apply the sample with a disposable syringe. The purpose of rPA production is to establish the ELISA system to determine the anti-PA antibody in the naturally infected horse in Mongolia.

2 Materials and Methods

Production and purification of rPA protein

DNA of Bacillus anthracis pXO1+ strain was used as a template for amplification of PA gene by PCR. The sequences of specific primers were 5′-ATT GGA TCC GAA GTT AAA CAG GAG AAC CGG-3′ for the sense strand and 5′-AGA GTC GAC TTA TCC TAT CTC ATA GCC TTT-3′ for the anti-sense strand. The underlined bases were restriction sites of BamHI and SalI, respectively. The sSize of PCR product was about 2.2 Kb of the PA gene without the signal peptide sequences. The PCR productwas first inserted into TOPO TA cloning® vector (Invitrogen) and transformed into E. coli TOP 10 cells according to the protocol of the manufacturer. The selected positive clone was multiplied and the plasmids were purified. The directional ligation of the PA gene to the pGEX-6P-1 vector (Amersham Biosciences) was performed after cut both plasmid and vector by BamHI and SalI at their restriction sites. This product was transformed into DH5∝ cells for maintenance of the positive plasmid vector. The constructed plasmid was confirmed by restriction enzyme mapping. Finally, the pGEX-6P-1 vector containing PA gene was transformed into competent E. coli BL21 for expression of the PA protein.

One fresh colony of BL21 harboring constructed plasmid was transferred into 2.5 ml of 2xYTA broth and incubated at 37°C, 200 rpm overnight. The culture was added into 1 liter flask containing 250 of 2xYTA and incubated in 37°C, 200 rpm to reach A600 of 0.8-1. To induce the GST/PA fusion protein expression, 1 mM of IPTG was added and the culture was continued to incubate for 6 hours. After centrifugation of the culture at 8,000 rpm 4°C for 10 min. The pellet was keept at -30°C overnight. The following day, the pellet was resuspended with 12.5 ml of binding buffer containing 1 mg/ml of lysozyme and incubated on ice for 30 min. The suspension was sonicated twice for 30 sec in ice cold water and then supplemented with 1% Triton-X 100, 24U/ml DNase and 60 mM MgCl2. After incubated at 4°C for 10 min, the suspension was centrifuged at 13,000 rpm 4°C for 30 min. The pellet was collected and resuspended with 2 M Urea, 5 mM DTT in PBS 10 ml, then the suspension was again sonicated and centrifuged as described above. The pellet was resuspended in 10 ml of 8 M Urea, 5 mM DTT, 130 mM NaCl, 20 mM Tris pH 7.4 and the supernatant was collected after the suspension, then was centrifuged at 13,000 rpm 4°C for 30 min. To refold the GST/PA protein in the solution, 150 ml of binding buffer was adding to the protein solution. This solution was ready to purify for the refolded rPA by GSTrap FF using ÄKTAprime plus system (GE HealthCare Life Sciences). The samples collected from various steps in the purification process were examined by SDS-PAGE and Western blotting analysis. The rPA eluates were concentrated and PBS was exchanged as a protein solvent by using Vivaspin6 concentrator (Vivascience). The protein concentrations were determined by Dc Protein assay (Bio-rad) according to the manufacturer's instruction manual.

ELISA assay

Microtiter plates 96 well, flat bottom (Maxisorp, Nunc) were coated with 50 µl of the 5 µg/ml denaturing PA domain protein in coating buffer (0.16 g of Na2Co3, 0.29 g of NaHCO3, 100 ml distilled water, pH 9.6) and incubated overnight at 4°C. Unbound sites were blocked by adding to each well with 3% skim milk and 0.1% Tween 20 (0.01% PBS-T) and the plates were incubated at 37°C for 1 hour. The plates were then washed three times with 0.01% PBS-T. Normal horse serum were purchased from Sigma to avoid confusing result from naturally infected animal serum. The nNormal serum and the test serum were diluted at 1:1000 using 0.3% skim milk in 0.1% PBS-T as a diluent. After sera were added, the plates were incubated at 37°C for 1 hour and subsequently washed for three times. 100 µl of rabbit anti- sheep, goat and bovine IgG peroxidase conjugate (Sigma) was added to each well and the plates were incubated at 37°C for 1 hour. After three times washing, 50 µl of TMB substrate (BD Biosciences) were added to the plates and kept at

room temperature for 30 min and 50 µl of 1M H2SO4 was added to stop the reaction. The absorbance at 405 nm was determined by microplate reader. The endpoint titers were defined as the highest serum dilutions that resulted in an absorbance value twice as high as that for the normal sera.

3 Results and Discussion

Purification of recombinant PA protein (rPA)

We prepared the bacterial culture in 1-liter scale for high concentration of recombinant protein. The steps of bacterial lysate preparation were followed by the handbook of GST Gene Fusion system (GE Healthcare Life Sciences). From 1-liter culture, we obtained lysate in 50 ml volume and it was subjected to apply into GSTrap FF using ÄKTAprime plus system (GE HealthCare Life Sciences). The GST tag was removed by PreScission protease cleavage while the GST fusion protein was bound to the column. The purified rPA was examined by SDS-PAGE (Fig. 1).

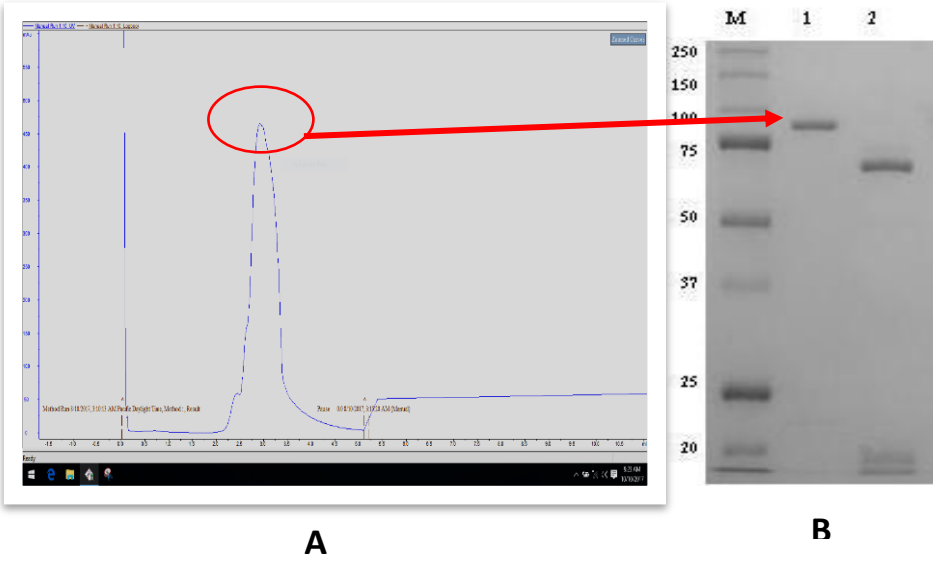

Fig. 1. A. Chart of chromatography-GST trap fast flow column (ÄKTAprime plus) **B.** SDS page analysis of rPA purification. Lane M, molecular weight marker; Lane 1, purified rPA 83 kDa; Lane 2, furin treated rPA separated by 63kDa and 20 kDa respectively.

Determination of In-house ELISA condition

To establish the indirect ELISA condition, we coated the plate with different concentration of rPA, diluted with coating buffer as an antigen (1 ug/ml-5 ug/ml protein-50 ul per well) A preliminary to it the cattle sera from Sigma and Umnugovi cattle serum were used as negative controls (n=200) and the naturally infected cattle serum as positive controls (n=2). The serum diluted from 1:100 to 1:100000 to detect for best condition of specificity and high sensitivity. The dDetermination of antigen rPA at 5ug/ml- 50ul/well and the serum dilution of 1:1000 showed significant difference in negative control. This condition was chosen In-House ELISA condition to detect antibody to B. anthracis PA. (Fig. 2.)

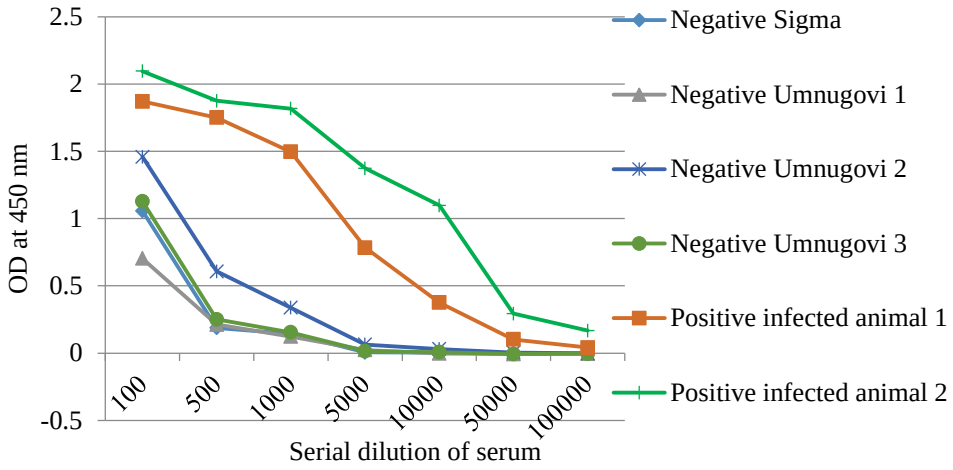

Fig 2. In-House ELISA condition

Vaccine monitoring

For the anthrax vaccine monitoring, total of 890 vaccinated cattle serum from 178 cattles were collected at 0, 3, 5, 8 and 12 months' post vaccinated from anthrax foci of Songino Khairkhan district. For the negative control 200 cattle serum from Umnugovi aimag. The sampling location of the anthrax foci are described in Fig. 3.

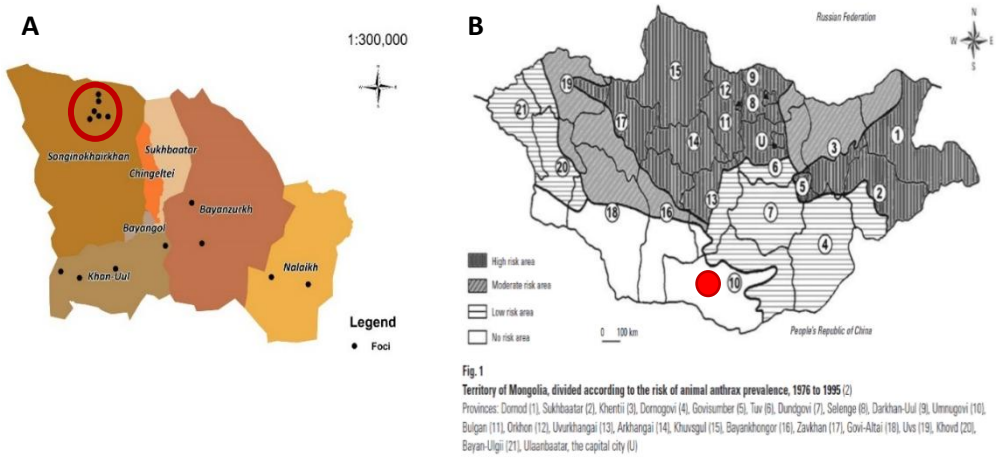

Fig 3. Sampling area for vaccine monitoring. **A.** Anthrax foci of Songino Khairkhan district for vaccinated cattle serum sample **B.** Non-anthrax foci of Umnugovi aimag for the negative control

In-House ELISA, the serum samples were diluted at 1:100-1:100000 and cut of value were calculated at OD 0.2< positive. The ELISA result shows, the antibody titer at 3 months' post vaccination was significantly increased comparing with pre-vaccination serum. The 5 months' post vaccination was the highest antibody titer and the antibody titer decreased from 8 months' post vaccination gradually to 12 months' post vaccination (Fig.4.)

Fig.4. In-House ELISA result at serial dilution of serum and time indication of vaccination

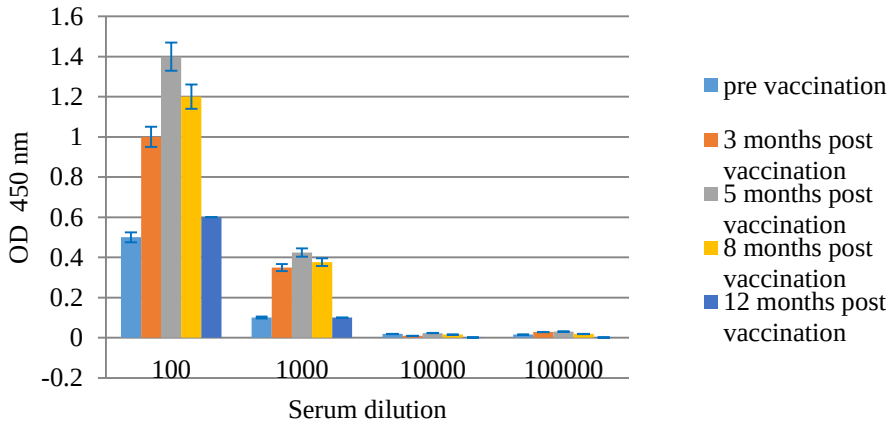

Table 1. In-House ELISA result in vaccinated animal serum

Positive		**Negative**	
Post vaccination /5 months/	Pre vaccination /0 months/	Post vaccination /5 months/	Umnugovi serum
150/178	3/178	25/178	0/200
84.2%	2.8%	14%	0%

For the evaluation of anthrax vaccine monitoring, cattle serum samples should be pre-vaccination and 5 months' post vaccination was better to detect antibody for rPA based In-House ELISA. In 5 months' post vaccination, antibody to PA were detected in 84.2% of total 178 vaccinated cattle serum and the antibody to PA were not detected in 97.2 % of pre-vaccinated cattle serum. In the negative control of Umnugovi cattle serum, the specific antibody was not detected in all of 200 samples. In result, 2.8% of pre-vaccinated cattle serum had antibody titer, it might be naturally induced antibody to *B. anthracis* PA, not to anthrax vaccine (Table 1.)

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