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Enhanced Production of Protective Antigen, a Potent Diagnostic Protein of *Bacillus anthracis*, the Causative Agent of Anthrax

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ABSTRACT

Protective antigen (PA) produced by *Bacillus anthracis* is a highly immunogenic protein. Therefore, it has significant importance in serodiagnosis as well as a vaccine candidate for anthrax. In the present study, codons for PA gene were optimised and synthesised for its expression in *Escherichia coli*. Various expression conditions were optimised for scaled up production of rPA. The final yield of affinity chromatography purified protein was 40.8 mg/l during batch fermentation. For further purification, affinity purified protein was diafiltered and subjected to anion exchange chromatography. SDS-PAGE and Western blot was used to characterise the purified rPA protein. The diagnostic potential of purified rPA was evaluated in Western blot using standards reference serum AVR 801 and cutaneous anthrax clinical sera. The results of the present study established the optimum production of rPA in *E. coli* after codon optimisation for its subsequent use in diagnosis of anthrax infection.

Keywords: Anthrax; Protective antigen; Codon optimisation; Ion exchange chromatography; Diagnosis

1. INTRODUCTION

Bacillus anthracis, the etiological agent of anthrax is a Gram positive, rod shaped and endospore forming bacteria[1]. Primarily, anthrax is a zoonotic and fatal disease which can spread from infected animals to other animals as well as human being[2]. However, chances of its spread from human to human are very scanty. Generally, human gets infected incidentally by contact with either diseased animals or their products like skin, meat or hides. In human, mainly there are three routes anthrax infection; through skin (cutaneous), by inhalation (pulmonary), and by eating (gastrointestinal)[3]. However, recently a new mode of infection known as injectional anthrax has also been known which spreads through the infected needles in drug users[4,5]. The pulmonary and gastrointestinal anthrax are considered as the most fatal, whereas cutaneous anthrax is most common.

The toxicity and pathogenicity of *B. anthracis* is due to its tripartite toxins encoded by the genes present on pXO1 plasmid and capsule encoded by the genes present on pXO2 plasmid, respectively. The tripartite toxin consists of protective antigen (PA), lethal factor (LF), and edema factor (EF), which are of 83 kDa, 90 kDa and 89 kDa, respectively[6]. PA itself is non-toxic, however on combination with LF and EF, it makes the lethal toxin and edema toxin, respectively. Hence, being the central component of both the toxins, it is one of the best choice for diagnostic markers[7-11]. Besides, PA is highly immunogenic, and therefore has been the best choice for development of vaccine candidates for anthrax[12,13].

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Hence, PA is required for development of diagnostic as well as prophylactic systems for anthrax. For development of any detection/diagnostic assay or for vaccine purpose, high yield production of recombinant protein is required. Owing to risk involved in handling the *B. anthracis* culture for production and purification of native PA, it is always desirable to produce recombinant PA (rPA) in heterologous host. However, due to differences in codon preferences between *B. anthracis* and *E. coli*, problems arises in expression of *B. anthracis* genes cloned in *E. coli*. Hence, in this study, we optimised the codons of *B. anthracis* PA gene for its expression in *E. coli* to construct a synthetic gene. Further scale up and purification were done for rPA protein. The purified protein was used for the serodiagnosis of anthrax infection.

2. MATERIALS AND METHODS

2.1 Codon Optimisation and Cloning of *pag* Gene

The sequence of *B. anthracis pag* gene was extracted from GenBank Accession No. AF306782.1 and the most favored codons of *E. coli* were selected according to the codon usage Database and graphical codon usage analyzer. The study was approved by Institutional Biosafety Committee of Defence Research and Development Establishment, DRDO, Ministry of Defence, Government of India vide protocol no. IBSC/19/BPD/AKG/3. The optimised gene with the restriction sites (*NdeI* & *XhoI*) was custom synthesised by Bio Basic Inc, Canada. The custom synthesised *pag* gene was sub-cloned into the pET30a+ vector and transformed into *E. coli* BL21 (DE3) for the expression of protein.

2.2 Inducible Expression of Codon Optimised PA in Shake Flask Cultures

The transformed *E. coli* was grown at 37 °C in LB broth (10 ml test tube) with kanamycine (30 µg/ ml) and after getting the optical density (OD) of 0.5-0.6 at 600 nm, 1.0 mM IPTG was added to induce the expression. After 4 h of induction, the cultures were centrifuged at 8000 rpm for 10 min at 4 °C and washed twice with triple distilled water. For expression screening, washed pellet (from 1 ml of 4 h induced culture) was resuspended in 100 µl cell lysis buffer by vortexing. After centrifugation, the supernatant was carefully removed and characterised on SDS-PAGE. Histidine tag at C-terminal in the recombinant protein was confirmed with anti-His antibody by Western blot.

Effect of inducer concentration, induction time and induction temperature was studied for enhanced expression of PA. For inducer concentration trials, different IPTG concentrations 0.5 mM, 0.75 mM, 1.0 mM, and 1.5 mM for fixed time (4 h) at 37 °C was checked in LB media. To optimise induction duration, the cultures were induced with 1 mM IPTG for 4 h and 16 h and for optimisation of post induction temperature, the cultures were grown as above and subjected to temperature of 25 °C and 37 °C. Cells were harvested by centrifugation and after lysis, supernatant of all the samples were analysed by SDS-PAGE for protein expression. To further increase the rPA expression level, cultivation media were also optimised. To 10 ml each of the five different media Luria martini broth (LB), super broth (SB), super optimal broth (SOB), super optimal broth with catabolite repression (SOC) and terrific broth (TB) with antibiotics in duplicate were inoculated with 1 ml of overnight grown culture. After attaining OD 600 nm of about 0.5-0.9, the culture was induced with IPTG (1.0 mM). After 4 h post induction, cultures were harvested using centrifugation.

2.3 Batch Fermentation Studies

Further, rPA was bulk produced in a 5 litre working volume bioreactor (Bioflo 3000: New Brunswick, USA). For each fermentation run, a 50 ml LB broth with antibiotics was inoculated with 1 ml of stock culture and incubated at 180 rpm for 8 h at 37 °C. Further, 400 ml sterile TB medium with antibiotic was inoculated with above grown culture (1% v/v) and incubated at 180 rpm for 14 h at 37 °C. This inoculum was used as seed (5% v/v) for 4.5 l of TB medium with antibiotic in Bioreactor vessel. Initially, the temperature, pH, agitation rate and air flow rate of bioreactor were kept as 37 °C, 7.0, 100 rpm and 2.5 LPM, respectively . After induction, the cultivation temperature was maintained at 37 °C, whereas the rate of aeration varied between 0.5 VVM to 2.0 VVM. The dissolved oxygen (DO) was kept at 20 per cent - 30 per cent of air saturation by varying agitation rate between 100 rpm and 400 rpm as well as using pure oxygen with air, if necessary. The cultivation pH was controlled between pH 6.8 and pH 7.0 using 25 per cent ammonia solution or H_3PO_4, whenever required. Antifoam was added to prevent foaming during cultivation. After 4 h of inoculation when the culture reached to mid log phase, 1 mM IPTG was added to induce the protein expression. After 4 h of induction, the cell pellet was recovered by centrifugation of culture at 8000 rpm for 30 min at 4 °C. The growth of culture in bioreactor was monitored at regular intervals by measuring OD at 600 nm.

2.4 Purification of rPA using Affinity and Ion Exchange Chromatography

For purification of rPA, cell pellet was washed twice with distilled water followed by washing with cell wash buffer[14]. The cells were further dissolved in cell lysis buffer as explained previously[14] and sonicated for 10 min with 9 s on and off using sonicator (Biologics, USA). The homogenate was clarified by centrifugation at 8,000 rpm for 45 min and the collected pellet washed with IB wash buffer-1 followed by IB wash buffer-2[14]. The solubilisation buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 300 mM NaCl, 8 M Urea, pH 8.0) was added to the washed pellet and incubated overnight at 4 °C on magnetic stirrer for purification. After filtering the supernatant through 0.45 µm membrane, immobilised metal affinity chromatography was used for purification of PA. A 5 ml pre-packed Ni-NTA affinity chromatography column was pre equilibrated using equilibration buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 300 mM Nacl, 8 M Urea, pH 8.0). The pre-packed column was obtained from Qiagen, Germany and used in AKTA explorer system (GE Healthcare Life Sciences, USA). After loading the sample, column was washed using wash buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 300 mM Nacl, 8 M Urea, pH 6.3) with 10 column volume. The protein was eluted in elution buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 300 mM Nacl, 8 M Urea, pH 4.5) and analysed by SDS-PAGE. The eluted fractions were pooled and diafiltered using decreasing concentration of Urea from 6 M to 0 M.

Ion exchange chromatography (IEX) was used after diafiltration of IMAC purified rPA for its further purification. For IEX, diafiltered sample was loaded onto the column containing pre-equilibrated Q-Sepharose anion exchange resin. Column was washed with 10 CV of wash buffer (10 mM Tris-Cl, 100 mM NaCl, pH 6.0) and bound proteins were eluted with ion exchange elution buffer (10 mM Tris-Cl, 300 mM NaCl, pH 6.0). Elutes were analysed by SDS-PAGE and quantified by BCA assay. The purified rPA was stored at – 80 °C for further use.

2.5 Evaluation for Diagnostic Potential by Western Blot Analysis

The purified rPA was evaluated for its diagnostic potential by Western blot analysis with clinical sera sample[10]. After separation of rPA on 12 per cent SDS-PAGE, it was transferred to PVDF membrane and blocked with 5 per cent skimmed milk powder (SMP) in phosphate-buffered saline (PBS) at 4 °C for 16 h. Blocked membranes were washed thrice for 5 min with PBS containing 0.1 per cent Tween-20 (v/v) and incubated at 37 °C for 1 h with anthrax infected patient sera (1:100 dilution in PBS containing1 per cent SMP). Standard reference serum AVR 801 (gifted by Dr Conrad P. Quinn, CDC, Atlanata) was also used in the study. After washing, the membranes were incubated with (Fc specific) HRP conjugated goat anti-human IgG (Sigma, USA) for 1 h at 37 °C in 1: 500 dilutions in PBS containing 1 per cent SMP. After washing, the membranes were

developed in diaminobenzidine (DAB)/H_2O_2 substrate solution for 15 min at 37 °C.

3. RESULTS AND DISCUSSIONS

3.1 Codon Optimisation of PA Gene

The transformed *E. coli* yields the best expression of genes with preferential codons that are recognised by abundant tRNA species[15]. Hence, to improve the expression of PA of *B. anthracis*, the codons of its *pag* gene were altered within the gene sequence and a synthetic gene was designed and constructed as shown in Fig. 1. The synthetic gene comprised of the optimally expressed codons of *E. coli*, without altering the amino acid sequence. Thus, codon optimised sequence was used to increase protein expression.

3.2 Expression of Codon Optimised rPA

The synthetic PA gene was cloned in vector pET30a+ vector and transformed in *E. coli* BL21 (DE3). A protein of 83 kDa was evident in induced culture, whereas no protein was observed in un-induced culture, confirming the expression of PA in induced culture as shown in Fig. 2(a). Further, the rPA was confirmed by Western blotting with anti-His antibody as shown in Fig. 2(b).

3.3 Optimisation of Culture Conditions for High Production

Various parameters including concentration of IPTG, induction time and temperature were optimised for optimum production of rPA. The SDS PAGE profile depicting the effect of inducer concentration, and induction temperature and time has been shown in Figs. 3(a) and 3(b), respectively. Varying IPTG concentrations starting from 0.5 mM to 1.5 mM didn't affect protein expression at significant level as shown in Fig. 3(a). Therefore, IPTG concentration of 1 mM was selected for further studies. Maximum protein expression was found at 4 h of induction as compared to 16 h as shown in Fig. 3(b). Further, it was concluded that expression of protein at 37 °C was higher than at 25 °C as shown in Fig. 3(b).

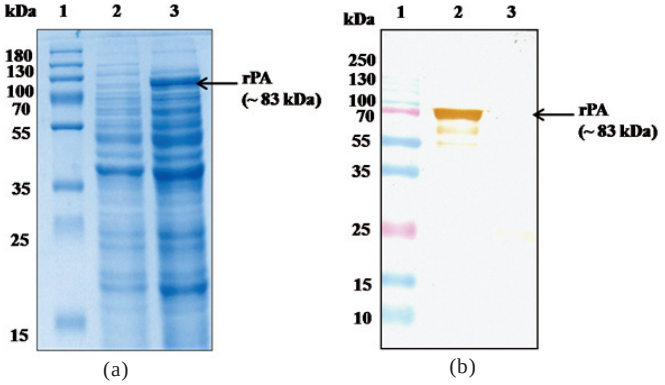

Figure 2. Expression profile of rPA. (a) SDS-PAGE profile of expressed protein. Lane 1, MW marker; lane 2 Un-induced culture; lane 3, Induced culture containing rPA. (b) Western blot analysis to confirm expression of rPA. Lane 1, MW marker; lane 2, Induced culture; lane 3, Un-induced culture.

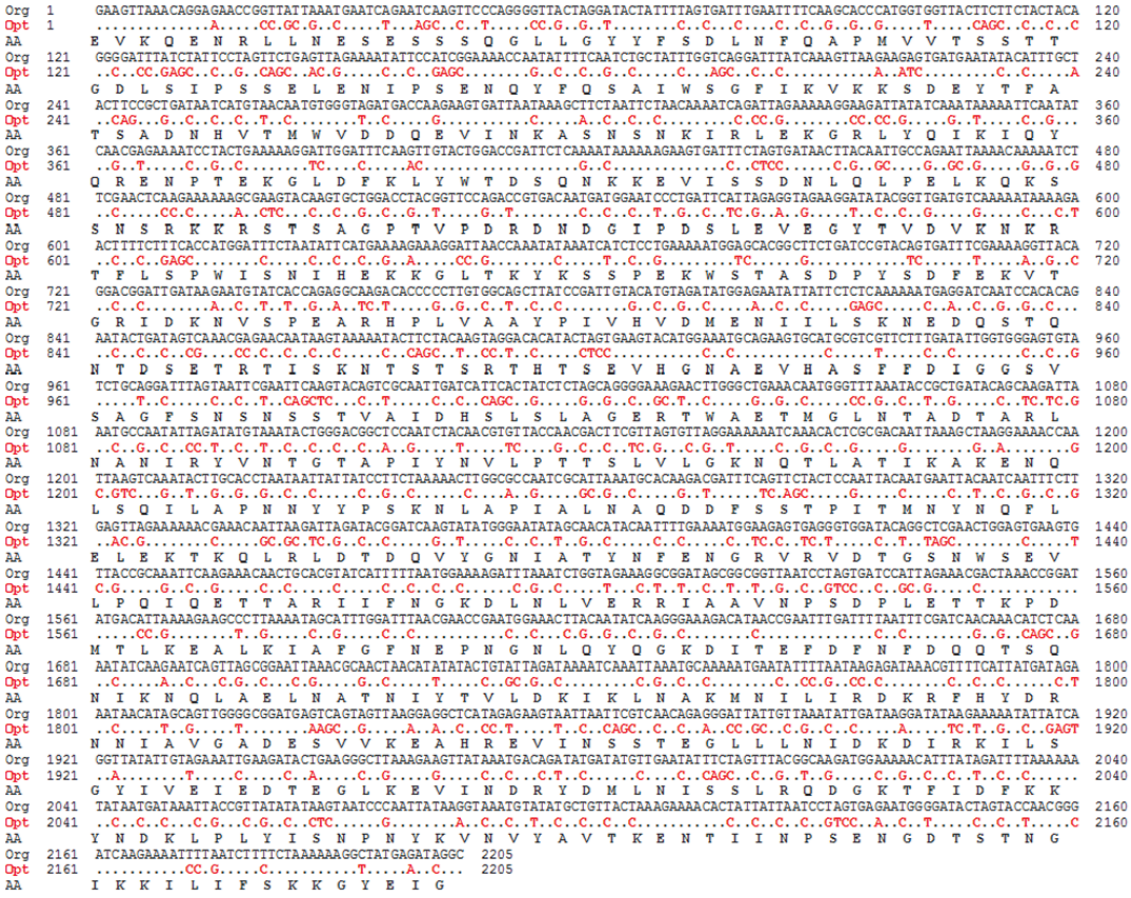

Figure 1. Sequence of the original (Org) and the optimised (Opt) PA gene. Black dots represent the identical residues.

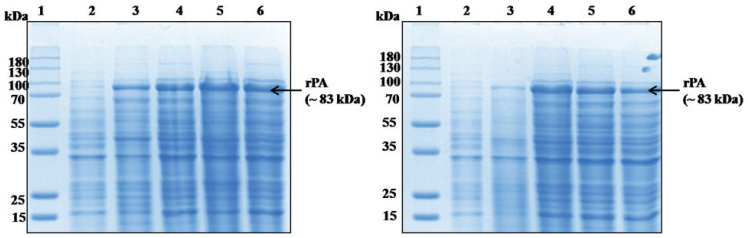

Figure 3. SDS-PAGE analysis for effect of inducr concentration and post induction time and temperature on expression of rPA. (a): Effect of inducer concentration. Lane 1, MW marker; lane 2, Un-induced culture; lane 3, 0.25 mM; lane 4, 0.75 mM; lane 5, 1.0 mM; lane 6, 1.5 mM. (b): Effect of time and temperature. Lane 1, MW marker; lane 2, Un-induced culture; lane 3, 25 °C/ 4 h; lane 4, 37 °C/ 4 h; lane 5, 25 °C/ 16 h; lane 6, 37 °C/ 16 h.

To improve the expression of rPA, five different media were tested at shake flask culture conditions. These media gave different growth and protein expression profile. However, the TB medium performed the best in the present study for enhanced expression of rPA in *E. coli* as shown in Fig. 4. The SDS-PAGE profile for localisation study of expressed rPA showed that majority of the expressed protein was in insoluble form.

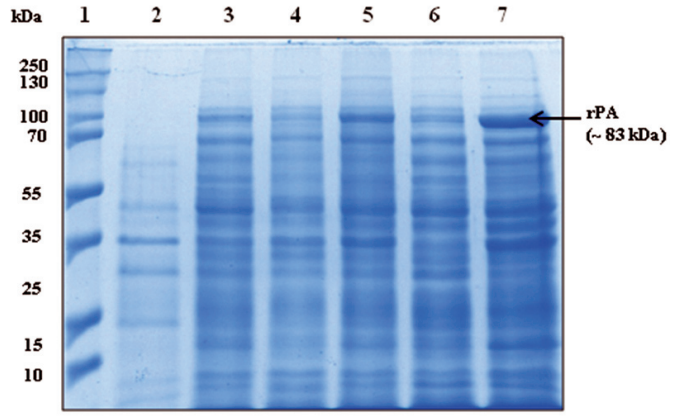

Figure 4. SDS-PAGE analysis for effect of different media on expression of rPA. Lane 1, MW marker; lane 2, Un-induced culture; lane 3, LB; lane 4, SOB; lane 5, SB; lane 6, SOC; lane 7, TB medium.

3.4 Scale up of rPA using Bioreactor

Batch fermentation was used for enhanced production of rPA. The parameters optimised at small scale were employed in 5 liter bioreactor. For scale-up, cultures were grown in TB medium followed by induction with 1 mM IPTG. Dissolved oxygen (DO) level was maintained above 20 per cent during bioreactor cultivations by agitation (100-400 rpm), aeration and/or using pure oxygen. Initially DO level was more than 30 per cent, which gradually decreased with culture growth. Finally, a wet cell weight of 14. 5 g/l was achieved after batch cultivation.

Generally, the recombinant proteins are required in higher amounts for development of detection or protection systems. For bulk production of such protein, scale-up process needs to be optimised. Batch fermentation is the ideal tool for bulk production of proteins with diagnostics, prophylactics or therapeutics potential[16]. Using TB growth media, batch fermentation yielded about three times higher wet biomass in comparison to shake flasks in this study.

3.5 Purification and Characterisation of rPA

The rPA was purified from the biomass obtained from shake flask cultures as well as batch fermentation processes. The desired protein of 83 kDa aggregated in the inclusion bodies (IBs). From the lysed cell mass, IBs were harvested, solubilised in 8 M Urea buffer and purified by IMAC. The rPA was eluted with elution buffer, pH 4.5 as shown in Fig. 5 followed by diafiltration. The diafiltered protein was further purified by ion exchange chromatography.

In TB medium, the rPA yield was 40.8 mg/l and 19.0 mg/l in batch process and shake flask culture, respectively after IMAC as shown in Table 1. A final yield of more than 2-times rPA was obtained in batch process than shake flask culture as shown in Table 1. The SDS–PAGE analyses corroborated that highly pure rPA could be recovered using chromatographic purification

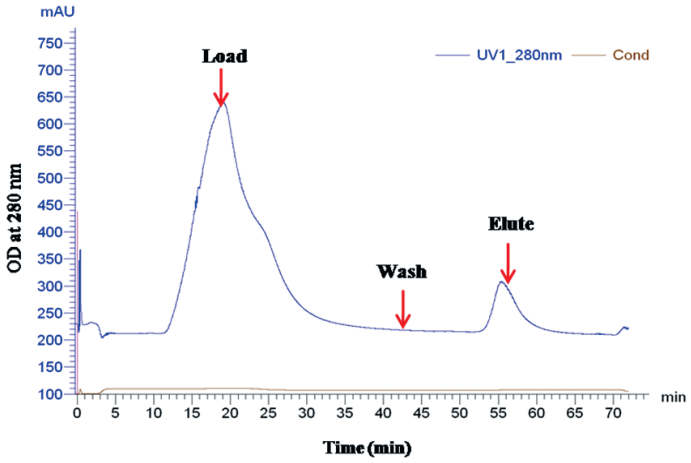

Figure 5. Chromatogram of affinity purification of rPA.

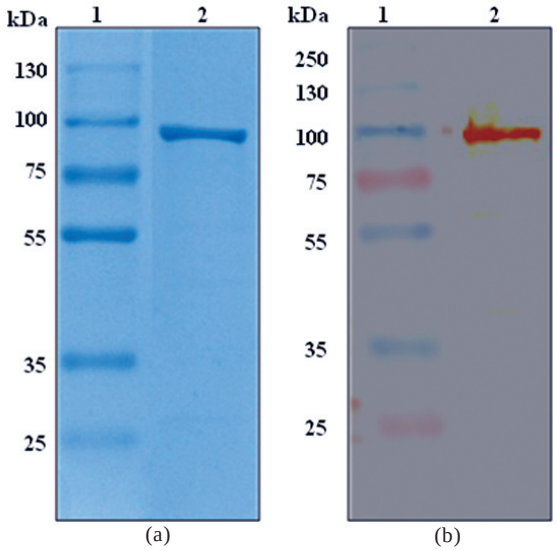

Figure 6. Purification and characterisation of rPA. (a) SDS PAGE. Lane 1, MW marker; lane 2, purified protein. (b) Western blot. Lane 1, MW marker; lane 2, Purified protein.

as shown in Fig. 6(a). The purified rPA was then subjected to western blot assay using anti-his antibody as shown in Fig. 6(b).

Table 1. Comparison of recombinant protective antigen expression levels in recombinant *E. coli* grown using different culture medium and cultivation conditions

Media type	Cultivation mode	Wet cell weight (g/l)	Final product concentration (mg/l)
LB	Shake flask	2.1	7.2
TB	Shake flask	4.96	19.0
TB	Batch fermentation	14.5	40.8

3.6 Evaluation of Diagnostic Potential of rPA Protein

The diagnostic potential of purified rPA was checked by Western blot using reference serum AVR 801 as well as clinical sera of anthrax infected patient. Human healthy serum was used as negative control while anthrax infected clinical sera was used at 1:100 dilution in Western blot. In Fig. 7, reference serum AVR 801 as well as clinical sera were found reactive with rPA antigen confirming the potential of rPA in diagnosis of anthrax infection in human.

Protective antigen is well known target antigen in detection/diagnostic systems as well as vaccine candidate for anthrax because PA is a central antigen of anthrax. For development of diagnostic and detection assay bulk production as well as purification of protein is required. In this study, the codon optimised rPA was successfully expressed, bulk produced and purified under denaturing condition. Purified rPA was diafiltered and again purified by anion exchange chromatography. The biological activity of purified rPA was confirmed by Western blot with positive clinical sera of anthrax. Thus, this purified rPA can be used for diagnosis of anthrax in patient serum samples.

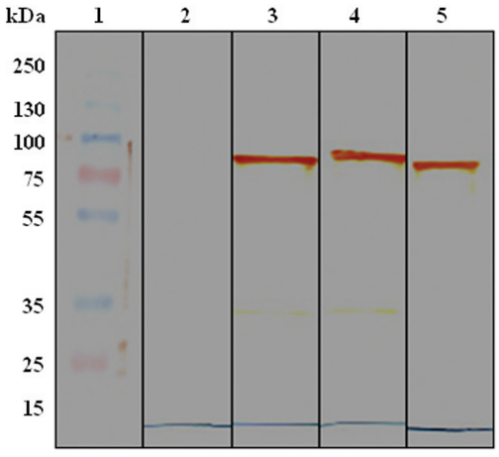

Figure 7. Evaluation of diagnostic potential of purified rPA. Western blot. Lane 1, MW marker; lane 2, Healthy serum sample; lane 3, AVR 801; lane 4-5, Clinical sera.

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He performed the experiments and wrote the manuscript in this study.

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She contributed in cloning and expression of the protein.

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In the current study, he was involved in production of recombinant proteins and manuscript writing.

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In the current study, he designed the experiments for this manuscript.

Dr Ajay Kumar Goel received his PhD (Microbiology) from CCS Haryana Agricultural University, Hisar, in 1999. Currently working as a Scientist 'F' and Head, Bioprocess Technology Division, DRDO-Defence Research and Development Establishment, Gwalior. He has more than 100 research paper, 6 patent, radio talks, books and several overseas presentations to his credit. His current research interest includes development of detection and protection systems for potential biothreat agents.
In the current study, he conceptualised the experiments for this manuscript.