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# Development of a simple method for the rapid identification of organisms causing anthrax by coagglutination test

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ABSTRACT

A protective antigen (PA) based coagglutination test was optimized in the present study for the specific and sensitive identification of bacteria causing anthrax in a cost effective and less risky manner. The test showed 100% specificity and sensitivity up to $9 \times 10^3$ formalinized vegetative cells or 11 ng of PA. The optimized test also detected anthrax toxin directly from the serum as well as blood of anthrax infected animals indicating the potential application for direct diagnosis of anthrax under field conditions.



## 1. Introduction

Anthrax, most often caused by *Bacillus anthracis* is a dreadful and fatal disease affecting warm blooded animals including humans. The disease is endemic to many parts of western, central and southern Asia [1] and is widely prevalent in southern Africa, small pockets of USA and certain regions of South America [2]. Apart from its natural occurrence, *B. anthracis* is a Tier 1 pathogen (agent that can be easily disseminated, can cause high mortality rates, public panic and social disruption, and require special actions for public health preparedness) and a potential bioterrorist weapon [3]. Therefore, it is crucial to develop a quick, less risky, sensitive and specific method for anthrax diagnosis.

Currently anthrax diagnostics are primarily based upon culturing of suspected samples and confirmation of cultures require several days with traditional methods [4]. Additionally, the challenges posed by coincidental or non-coincidental presence of pathogenic microorganisms [5] together with closely related nonpathogenic organisms of the same genus [6] necessitate rapid and specific detection techniques for the organisms that can cause anthrax. As anthrax toxin is essential for the development of anthrax and, *B. anthracis* isolates that lack plasmid encoding anthrax toxin are nonpathogenic, the toxin is the apt target for the diagnosis of disease [7]. Recently, *B cereus* strains causing anthrax by production of anthrax toxin were isolated [8], further proving the necessity of toxin-based assays for the specific identification. Although, several advanced approaches were developed for the purpose, these tend to require expensive devices or specialized technicians [9] thus, are not suitable under field conditions. Consequently, development of simpler and less expensive method is advantageous especially for the developing countries where the disease is endemic. In a previous study, we reported that detection of anthrax toxin by latex agglutination test (LAT) can be an effective technique for the diagnosis of anthrax as well as for the specific identification of cultures of organisms causing anthrax [10]. However, coagglutination test (CAT) is reported to be more sensitive, cheaper and simpler test [11]. Therefore, the present study was

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planned to evaluate whether CAT can serve as a surrogate for the specific identification of bacteria causing anthrax.

## 2. Materials and methods

### 2.1. Bacterial cultures

Two isolates of *B. anthracis* one, virulent IVRI strain (initially obtained from Bacteriological Laboratory, Mukteshwar in 1975), another uncapsulated 34F2 (Sterne) strain were obtained from Type Culture Laboratory, Division of Biological Standardization, IVRI (Indian Veterinary Research Institute) and used for optimization of CAT. *Staphylococcus aureus* Cowan I strain was used for the preparation of CAT reagent. The other bacteria used in the present study and their sources are given in Table 1.

### 2.2. Animals

Two healthy rabbits and guinea pigs were procured from Lab Animal Resource section, IVRI and maintained under standard conditions of nutrition and management. The animal ethics committee of IVRI, Deemed University approved this study.

### 2.3. Preparation of recombinant protective antigen (rPA)

rPA was prepared using heterologous bacterial expression system [10]. Briefly, amplification of *pag* encoding PA was done from pX-O1 plasmid (isolated from Sterne strain) using the gene specific primers (RE sites were underlined; PA-F: 5′-aaaggatccggttccagaccgtgacaatga-3′; PA-R: 5′-ggggctcgagtcctatctcatagccttttttagaa-3′). Subsequently, *pag* was cloned in frame into pQE Trisystem expression vector and the cloned plasmid was then transformed into *Escherichia coli* M15 competent cells. Positive clones were confirmed by PCR, double RE (restriction enzyme) digestion and sequence analysis. The expression of histidine tagged rPA was subsequently induced from the positive clones by 1 mmol/l IPTG. Afterwards, histidine tagged rPA was purified from induced culture under denaturing conditions by nickel chelating affinity chromatography. Purity, identity and immunogenicity of the rPA were confirmed by SDS-PAGE and western blotting using hyperimmune sera raised against sonicated culture of *B. anthracis*. Urea from purified rPA was subsequently removed by step-wise dialysis. Concentration of purified rPA after dialysis was estimated spectrophotometrically (Nanodrop®, USA) at 280 nm.

**Table 1**
Different bacteria used in the study

| Bacteria | Source |
|---|---|
| *Bacillus anthracis* Sterne's strain | Type Culture Laboratory, Division of Biological Standardization, IVRI, Izatnagar |
| *Bacillus anthracis* IVRI strain | Type Culture Laboratory, Division of Biological Standardization, IVRI, Izatnagar |
| *Bacillus cereus* MTCC 7409 | MTCC, Chandigarh |
| *Bacillus subtilis* MTCC 1133 | MTCC, Chandigarh |
| *Bacillus megaterium* MTCC 1684 | MTCC, Chandigarh |
| *Salmonella* Typhimurium E2393 | National Salmonella Centre, IVRI, Izatnagar |
| *Staphylococcus aureus* | Division of Biological Products, IVRI, Izatnagar |
| *Brucella abortus* Strain 19 | Division of Biological Products, IVRI, Izatnagar |
| *Pasteurella multocida* strain P52 | Division of Biological Products, IVRI, Izatnagar |
| *Escherichia coli* DH5α | Division of Biological Products, IVRI, Izatnagar |
| *Shigella flexneri* MTCC 1457 | MTCC, Chandigarh |
| *Vibrio cholerae* MTCC 3904 (Classical $O_1$) | MTCC, Chandigarh |
| *Yersinia enterocolitica* subspp. enterocolitica MTCC 4858 | MTCC, Chandigarh |
| *C. perfringens* type D (IVRI strain) | Division of Biological Products, IVRI, Izatnagar |

### 2.4. Preparation of anti PA hyperimmune sera for coating onto CAT reagent

Two healthy New Zealand White rabbits were immunized intramuscularly each with 100 μg of rPA emulsified in Freund's complete adjuvant. Animals were boosted with 100 μg of antigen at days 14, 21, and 42 with Freund's incomplete adjuvant. After 5 days of the last injection, sera antibody titre was confirmed by dot ELISA. Animals were finally bled at 7 days of the last injection; serum was separated and stored at −20 °C. The gamma globulin fractions of hyperimmune sera were isolated by 40% ammonium sulphate fractionation, and the precipitate was resuspended in phosphate-buffered saline (PBS), pH-7.4. The solution was subsequently dialysed against PBS until all ammonium sulphate had been removed. To determine the strength of hyperimmune sera, protein concentration in purified gamma globulin at 280 nm and plate ELISA of the serum using 1: 800 dilution of rPA antigen and 1: 5000 dilution of goat anti-rabbit-HRP conjugate were done. ELISA result was expressed as positive/negative (P/N) ratio [12].

### 2.5. Preparation of CAT reagent

CAT reagent was prepared from *S. aureus* Cowan I strain by previously described protocol [13] with minor modifications. Briefly, cells were grown in 10 ml of nutrient broth from the master seed and incubated at 37 °C for 24 h. After testing for purity, 3 ml of the culture was used for sowing Roux flasks each containing 125–150 ml of brain heart infusion agar. Following incubation at 37 °C for 24 h, the growth was harvested with the help of 15–20 ml of normal saline from each flask and examined for purity. Following the addition of 0.1% triphenyl tetrazolium chloride to the culture, it was kept at 37 °C for 30 min. Subsequently, 0.5% of formalin was added to the culture and kept at 80 °C for 3 h for complete inactivation. The coloured cells were then harvested by centrifugation at 2500 rpm for 10 min and washed thrice with PBS (pH 7.4). Finally, the cells were adjusted to 10% (w/v) concentration with PBS and stored at 4 °C.

### 2.6. Sensitization of coagglutination reagent with anti-PA hyperimmune serum

For sensitization, 1 ml of the above suspension of *S. aureus* was mixed with 1 ml of heat inactivated anti PA hyperimmune serum and allowed to react for two and half hours at 37 °C in shaker. The coated cells were then washed and resuspended in PBS (pH 7.4) as 10% (w/v) suspension. Sodium azide (0.1%) was added to a final concentration of 1% and the reagent was stored at 4 °C until used. Modifications to this procedure such as change in antibody concentration, incubation temperature and buffer pH were carried out and finally the above conditions were found to be optimum.

### 2.7. CAT

Both strains of *B. anthracis* were grown in 5 ml nutrient broth and incubated at 37 °C for 24 h as stationary cultures. The cultures were then inactivated by formalinization [10]. To check sterility of the obtained solutions, these were sub-cultured into 5 ml nutrient broth and incubated at 37 °C for 24 h.

For the CAT 10 μl of the inactivated culture was mixed with an equal volume of sensitized CAT reagent on a glass slide and mixed