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Flow-cytometric Analysis of *Bacillus anthracis* Spores

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ABSTRACT

Flow-cytometric technique has been established as a powerful tool for detection and identification of microbiological agents. Unambiguous and rapid detection of *Bacillus anthracis* spores has been reported using immunoglobulin G-fluorescein isothiocyanate conjugate against live spores. In addition to the high sensitivity, the present technique could differentiate between spores of closely related species, eg, *Bacillus cereus* and *Bacillus subtilis* using fluorescence intensity. The technique can be used for detection of live as well as inactivated spores making it more congenial for screening of suspected samples of bioterrorism.

Keywords: Flow cytometry, *Bacillus anthracis*, bacterial spores, FITC, immunofluorescence, bioterrorism, fluorescein isothiocyanate-labelled antibodies, fluorescein isothiocyanate

1. INTRODUCTION

Bacillus anthracis, the causative agent of anthrax, has been in the limelight for the past several years because of its importance in public health and military sectors. Vegetative cells of *Bacillus anthracis* do not survive in the natural environment due to the prevalence of harsh conditions that are uncongenial for their growth. So these transform themselves into resting or dormant structures that can withstand the extremes of temperature, p*H*, ultraviolet radiations, etc. These dormant structures, called spores, remain viable in the environment for decades[1]. Most cases of anthrax are caused by these spores, which can be taken up by the susceptible animal or human population through three different routes. It can be through skin (called cutaneous anthrax); through oral route, resulting in gastrointestinal anthrax; or through inhalation, causing pulmonary anthrax. Among these three types of anthrax, it is the pulmonary anthrax that is the most lethal and mortality rate can go up to 90 per cent. Although if diagnosed timely, the anthrax of any type can be treated effectively using appropriate antibiotics, but if goes undiagnosed and the bacteria start synthesising toxin inside the living system, then there is no cure. Furthermore, LD_{50} of the anthrax is as low as 4000 spores[2]. So these properties of *Bacillus anthracis* have prompted the scientists to develop methods for its rapid and sensitive detection.

A large number of culture methods for isolation and identification of *Bacillus anthracis* have been reported[3]. However, these methods are ineffective in inhibiting the background microflora, particularly *Bacillus cereus* and *Bacillus subtilis*, that are closely associated with *Bacillus anthracis*. Moreover, culture methods take 18-24 h and characterisation is done through a series of biochemical tests[4]. The development of polymerase chain reaction (PCR)

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and antibody-based systems like immunoradiometric assay (IRMA), enzyme-linked immunosorbent assay (ELISA), immuno-fluorescence, and immuno-chemiluminescence for the detection of *Bacillus anthracis* have been investigated by several workers[5-10]. Most of these methods are very sensitive but the two major drawbacks are the slow speed of detection, and cross-reactivity with other related *Bacillus* species. Therefore, a simple automated technique resulting in unambiguous identification within minutes is highly desirable.

In the recent past, use of flow cytometry for bacterial characterisation has relied upon immuno-fluorescence-based methods[11-14,16]. Flow cytometry provides a rapid and precise method for the detection, enumeration, and identification of the bacteria. In flow-cytometric measurements, individual particles/organisms are passed through a sensor using a hydrodynamically focused linear stream, where the particles intercept light from a laser or a high-pressure arc lamp. The scatter as well as the fluorescence emitted from the particles is converted to an electrical pulse, which is analysed after suitable amplification and digitisation. This study is an attempt to use fluorescein isothiocyanate (FITC) labelled antibodies to analyse the surface antigens related to *Bacillus anthracis* and its close relatives, viz., *Bacillus cereus* and *Bacillus subtilis*.

2. MATERIALS REQUIRED

2.1 Spore Procurement and Preparation

Non-pathogenic *Bacillus anthracis sterne* strain procured from the Institute of Veterinary and Preventive Medicine, Vellore. Spores of *Bacillus cereus* and *Bacillus subtilis* were procured from Difco, USA. *Bacillus anthracis* was grown on brain-heart infusion (BHI) agar for 18-24 h at 37 ^{o}C. The cell mass on the plate was scrapped and inoculated into G-medium broth[15]. The culture was allowed to sporulate by incubating the broth at 37 ^{o}C on an orbital shaker at 200 rpm for two days, followed by incubation for two more days at 25 ^{o}C. Spores were harvested by centrifugation and resuspended in distilled water. The suspension was heated at 60 ^{o}C for 90 min to inactivate vegetative cells. Spores were further purified by giving 4-5 washings with phosphate buffer saline (PBS, p*H*: 7.2).

2.2 Antibody Preparation and Labelling

Bacillus anthracis live spores (5×10^{5} CFU/ml) in phosphate buffer saline were used for immunisation of New Zealand white rabbits. Immunisation was carried out for two months at an interval of 15 days after which the serum was collected. Serum proteins were concentrated by sodium sulphate precipitation at a final concentration of 18 per cent. Precipitated proteins were dissolved in phosphate buffer saline and dialysed before loading onto gel filtration column packed with Bio-gel $A_{1.5}$ (Bio-Rad, USA) pre-equilibrated with phosphate buffer (0.15 M, p*H*: 7.2). The immunoglobin G (IgG) containing fractions were pooled and dialysed against 0.02 M potassium dihydrogen phosphate (p*H*: 8.0). Five milliliters of IgG (20 mg/ml) was incubated with equal volume of *Bacillus cereus* and *Bacillus subtilis* (10^{8} CFU/ml) for 2 h at 37 ^{o}C, followed by overnight incubation at 4 ^{o}C. The process was repeated until the cross reactivity was eliminated as revealed by immunofluorescence microscopy.

Purified immunoglobulin G (IgG) antibodies were conjugated with fluorescein isothiocyanate (FITC) as described earlier[9]. Immunoglobulin G diluted in 0.15 M disodium hydrogen phosphate (p*H*: 9.0) was allowed to react with FITC (100 µg/10 mg IgG) at room temperature for 1 h. Free dye was removed by passing through Sephadex G-25 column and the F/P ratio was determined by calculating the optical density of the conjugate at 495 nm and 280 nm.

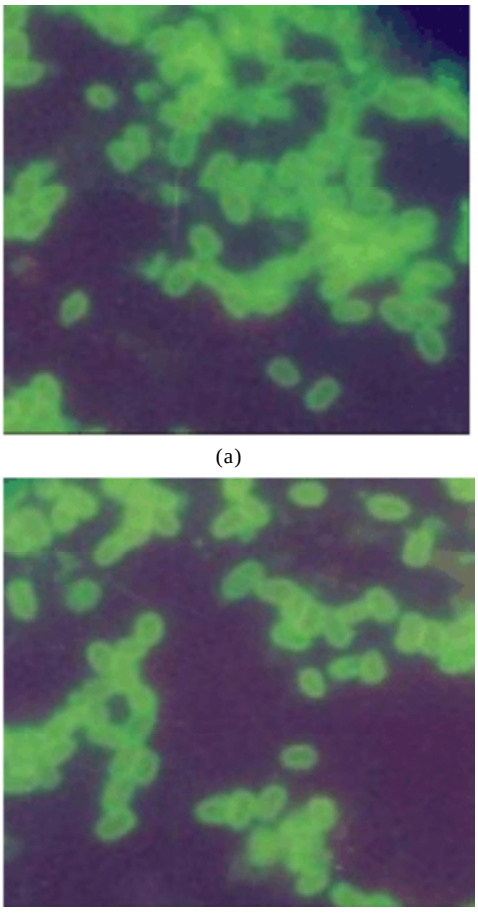
3. METHODOLOGY

3.1 Immunofluorescence Microscopy

Twenty microliters of spore suspension (live or formalin-fixed) was spotted on the slide (Flow Lab, USA), dried at 60 ^{o}C and fixed with methanol. Twenty microliters of FITC conjugate (1:20 diluted) was poured onto the fixed spores and the slide was incubated for 30 min at room temperature in a moist dark chamber. The slide was washed with 5-6 changes of phosphate buffer saline for 30-40 min and observed under a fluorescence microscope.

3.2 Immuno-flow Cytometry

Spores (10^6 CFU/ml) of *Bacillus anthracis*, *Bacillus cereus* and *Bacillus subtilis* were suspended in 980 µl of PBS (p*H*: 7.4), and 20 µl of anti-*Bacillus anthracis* spore IgG-FITC conjugate (1:50 dilution) was added. After incubation for 30 min at room temperature, spores were washed once and resuspended in phosphate buffer saline (1 ml) and analysed by a flow cytometer equipped with blue line laser (FACS Calibur, Becton Dickinson, USA). The sample was excited at 488 nm. Forward angle and side scatter, as well as green fluorescence from the spores bound to FITC-labelled anti-*Bacillus anthrasis* antibodies were acquired using list mode using Cell Quest software provided by the manufacturer. Data were analysed using appropriate offline gates with unstained spores as cutoff.



(a)

(b)

Figure 1. ***Bacillus anthracis*** **live spores stained with antibodies raised against: (a) live spores and (b) formalin-fixed spores as seen under fluorescence microscope.**

4. RESULTS AND DISCUSSION

Spores of *Bacillus anthracis* (live and formalin-fixed), *Bacillus cereus*, and *Bacillus subtilis* were stained with IgG-FITC conjugate and observed under fluorescent microscope. The FITC conjugated antibodies raised against live as well as formalin-fixed spores were able to stain live spores of *Bacillus anthracis* with apparently equal intensities (Fig. 1). On the other hand, when the conjugate was used to stain spores of *Bacillus cereus* and *Bacillus subtilis*, none of the spores could be observed under fluorescence microscope.

Formalin fixation of spores did not significantly alter the light scatter properties of spores as revealed by forward angle scatter (FSC) and side scatter (SSC) signals (Fig. 2). The forward scatter intensity correlates with the spore size while the intensity of side scatter indicates the granularity of spores, ie, the presence of internal structures. So the two kinds of spores, ie, live and formalin-fixed spores cannot be differentiated on the basis of light scatter properties. This led to the study of immunofluorescence of live and formalin-fixed spores. Immunoreactivity of live and formalin-inactivated spores was clearly

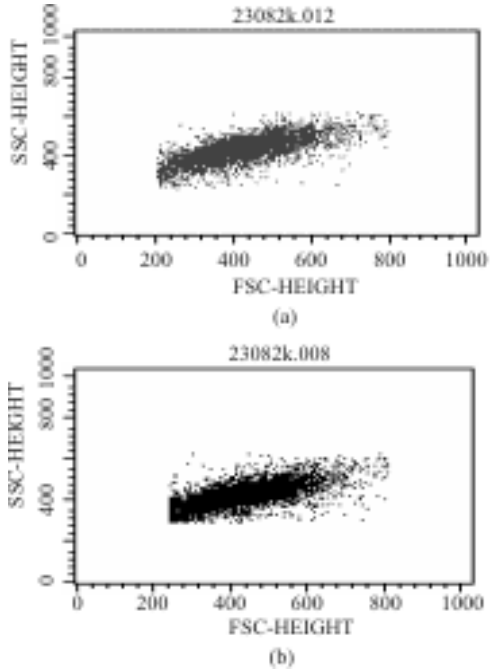


Figure 2. Bivariate plots of forward scatter versus side: Scatter of (a) live (unfixed) *Bacillus anthracis* spores and (b) formalin-fixed *Bacillus anthracis* spores.

evident by the large increase in the intensity of green fluorescence light as compared to unstained spores (Fig. 3). Fluorescence intensity of fixed spores was higher as compared to live spores when these were stained with antibodies raised against live spores [Fig. 3(b) and (d)]. Although in formalin-fixed spores, a heterogenous population of green fluorescence was observed [Fig. 3(d)], the predominant population showed higher fluorescence intensity as compared to unfixed spores.

It becomes further clear by histograms overlay of live and formalin-fixed spores showing variable fluorescence intensities of formalin-fixed spores (Fig. 4). Detection and identification of *Bacillus anthracis* spores using flow cytometry has been demonstrated earlier using formalin-fixed spores[5,7,13]. Stopa[14] has reported the use of live as well as gamma-irradiated spores for detection and identification of *Bacillus anthracis* spores. But he did not make any observation with regard to difference in fluorescence intensity between the two types of spores.

In the present study, the cross-reactivity of anti-*Bacillus anthracis* antibodies with *Bacillus-cereus* and *Bacillus subtilis* spores, which are antigenically closer to *Bacillus anthracis* have been investigated. The results showed that only 1.5 per cent spores of *Bacillus subtilis* were cross-reactive with antibodies raised against *Bacillus anthracis* live spores, as compared to 20 per cent in case of *Bacillus cereus* spores, which are considered to be the closest relative of *Bacillus anthracis* [(Fig. 5 (e)-(h)]. This cross-reactivity was attributed to the use of polyclonal antibodies and was significantly lower, an observation also made by Stopa[14]. It is intriguing that such cross-reactivity was not observed under fluorescence microscope. This can be explained by the presence of small number of cross-reactive epitopes on the surface of *Bacillus cereus* and *Bacillus subtilis* spores, which bind only to the limited number of FITC conjugated antibodies, thus emitted fluorescence is not enough to be perceived by the eyes through microscope. On the other hand, flow cytometry, being more sensitive, could detect fluorescence of even minor quantum.

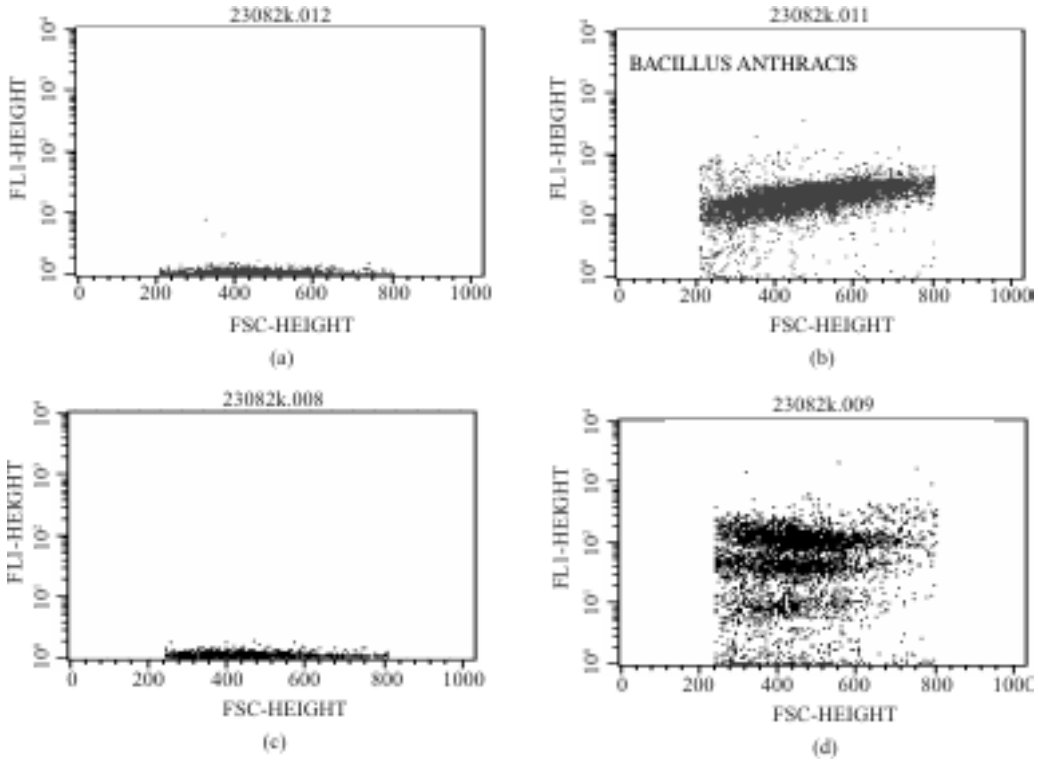


Figure 3. Bivariate plots of forward scatter and green fluorescence from FITC-labelled anti-*Bacillus anthracis* (live spores) antibodies of unfixed (a and b) and formalin-fixed (c and d) spores; (a) and (c): Unstained spores; (b) and (d): Stained spores.

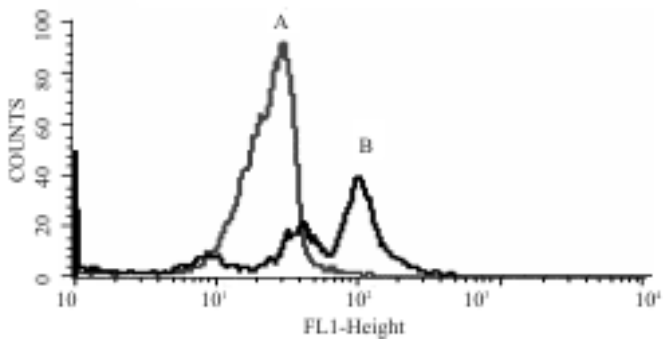


Figure 4. Overlay of histograms obtained from the distribution of green fluorescence (FITC-labelled anti-*Bacillus anthracis* antibody) from: (graph A) live (unfixed) *Bacillus anthracis* spores and (graph B) formalin-fixed spores.

It is also evident from Fig. (5) that fluorescence intensity is affected by formalin-fixation of spores. When anti-*Bacillus anthracis* antibodies were raised against formalin-fixed spores, the fluorescence intensity was significantly increased [(Fig. 5 (a)-(d) as compared to antibodies raised against live spores. However, these antibodies were found to be cross-reactive with *Bacillus cereus*. Therefore, antibodies raised against formalin-fixed spores are not good for specific detection of *Bacillus anthracis* spores. On the other hand, antibodies raised against live spores are better for detection of live as well as formalin-fixed spores.

The results of this study have two useful implications, firstly that different types of *Bacillus* spores can be easily differentiated by flow-cytometric analysis using immunofluorescence. Secondly, inactivation of pathogenic spores of *Bacillus anthracis*, which is a desirable safety feature, prior to sample processing, during the act of sabotage or terrorist activities, will not affect the flow-cytometric analysis for their detection and identification. Unlike the other techniques of *Bacillus anthracis* detection, ie, fluorescence microscopy[9], PCR[10], and ELISA[7],

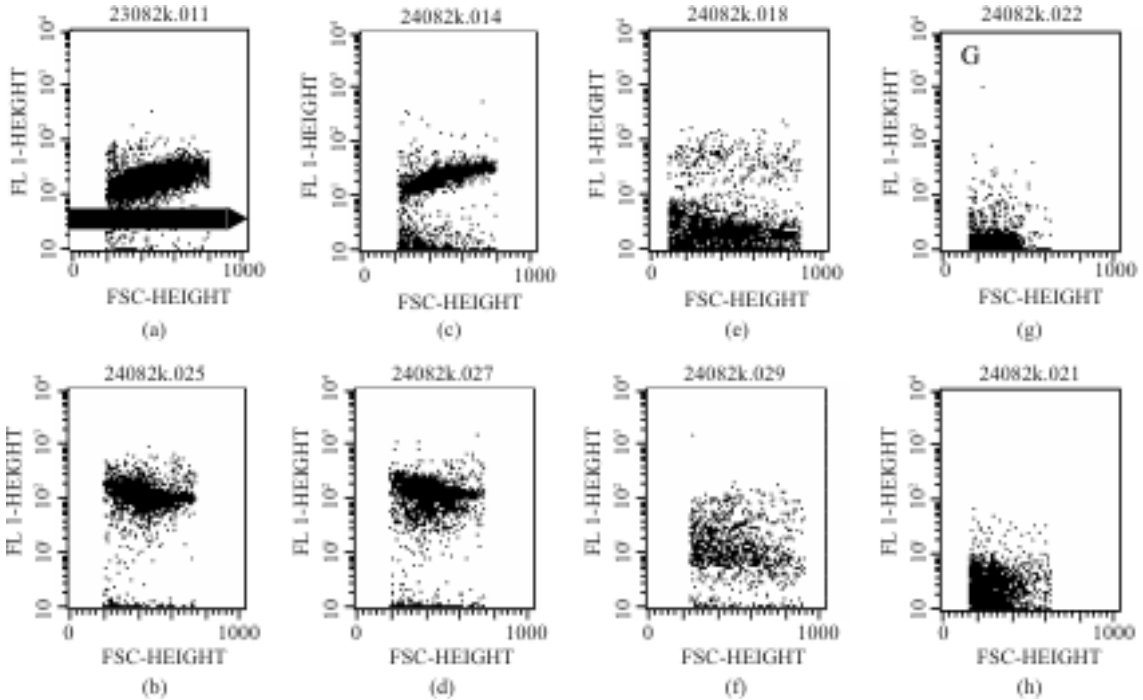


Figure 5. Bivariate plots of forward scatter and green fluorescence from FITC-labelled anti-*Bacillus anthracis* antibodies: (a) live (unfixed) ***Bacillus anthracis*** **spores stained with antibodies raised against *Bacillus anthracis* live spores (b) live (unfixed) *Bacillus anthracis* spores stained with antibodies raised against formalin-fixed spores, (c) formlin-fixed *Bacillus anthracis* spores stained with antibodies raised against live spores, (d) formalin-fixed *Bacillus anthracis* spores stained with antibodies raised against formalin-fixed spores, (e) live *Bacillus cereus* spores stained with antibodies raised against *Bacillus anthracis* live spores, (f) live *Bacillus cereus* spores stained with antibodies raised against formalin-fixed spores, (g) live *Bacillus subtilis* spores stained with antibodies raised against *Bacillus anthracis* live spores, and (h) live *Bacillus subtilis* spores stained with antibodies raised against formalin-fixed spores.**

where detection is made complicated by the behaviour of whole population, flow-cytometric detection based on the individual spore in the population is advantageous. The method, in principle, can also be used for the detection of *Bacillus anthracis* spores from the environmental samples, using aqueous polymer two-phase system, which is a simple purification strategy[9], prior to flow-cytometric analysis.

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