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Rapid detection of *Bacillus anthracis* spores using a super-paramagnetic lateral-flow immunological detection system ☆

Dian-Bing Wang [a,1], Bo Tian [a,b,1], Zhi-Ping Zhang [a], Jiao-Yu Deng [a], Zong-Qiang Cui [a], Rui-Fu Yang [c], Xu-Ying Wang [d], Hong-Ping Wei [a,*], Xian-En Zhang [a,*]

[a] State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China
[c] State Key Laboratory of Pathogen and Biosecurity, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, Beijing 100071, China
[d] State Key Laboratory of Agromicrobiology, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

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ABSTRACT

There is an urgent need for convenient, sensitive, and specific methods to detect the spores of *Bacillus anthracis*, the causative agent of anthrax, because of the bioterrorism threat posed by this bacterium. In this study, we firstly develop a super-paramagnetic lateral-flow immunological detection system for *B. anthracis* spores. This system involves the use of a portable magnetic assay reader, super-paramagnetic iron oxide particles, lateral-flow strips and two different monoclonal antibodies directed against *B. anthracis* spores. This detection system specifically recognises as few as 400 pure *B. anthracis* spores in 30 min. This system has a linear range of 4×10^3–10^6 CFU ml^{-1} and reproducible detection limits of 200 spores mg^{-1} milk powder and 130 spores mg^{-1} soil for simulated samples. In addition, this approach shows no obvious cross-reaction with other related *Bacillus* spores, even at high concentrations, and has no significant dependence on the duration of the storage of the immunological strips. Therefore, this super-paramagnetic lateral-flow immunological detection system is a promising tool for the rapid and sensitive detection of *Bacillus anthracis* spores under field conditions.

1. Introduction

Bacillus anthracis, the causative agent of anthrax, is a Gram-positive spore-forming bacterium. In response to nutrient deprivation, *B. anthracis* produces spores that can survive for a long period in harsh environments, including temperature extremes, high levels of radiation, chemical assault and even the vacuum of outer space (Nicholson et al., 2000); thus, *B. anthracis* is listed as a "Category A" biological weapon by the Centers for Disease Control and Prevention of the United States. The bioterrorism event in the fall of 2001 was one of the latest demonstrations of the threat of *B. anthracis* to public health. Recently, researchers around the world have shown increased interest in *B. anthracis* and have begun efforts to develop many types of methods for the detection of *B. anthracis* spores due to safety concerns.

Various preparative techniques are capable of identifying *B. anthracis* spores, including immunological assays (Kleine-Albers and Bohm, 1989; Mechaly et al., 2008; Wang et al., 2006), PCR-based methods (Beyer et al., 1995; Qi et al., 2001; Skottman et al., 2007), biosensor detection systems (Campbell and Mutharasan, 2006; Garcia-Aljaro et al., 2010; Gatto-Menking et al., 1995; Hao et al., 2009; Pal and Alocilja, 2010; Tims and Lim, 2004; Wang et al., 2009a) and other advanced approaches (Bruno and Kiel, 1999; Boyer et al., 2007; Kaman et al., 2010; Huan et al., 2011; Oh et al., 2011; Tan et al., 2011). These techniques tend to require expensive devices or complicated protocols.

In our previous work, we succeeded in producing high-quality monoclonal antibodies against the surface of *B. anthracis*. These antibodies can easily differentiate *B. anthracis* from *B. cereus*, *B. subtilis*, *B. thuringiensis* and other close relatives (Wang et al., 2009b). Using these mAbs, we established rapid, sensitive, label-free detection platforms based on a surface plasmon resonance biosensor and a quartz crystal microbalance biosensor (Hao et al., 2009; Wang et al., 2009a).

Since 1990s, the lateral-flow immunoassay has been widely used in the detection of pathogens, drugs, and other analytes in resource-poor or non-laboratory environments due to its short detection time, low cost and simplicity. Generally, lateral-flow immunoassays involved the use of labels in the form of coloured or fluorescent nanoparticles, such as colloidal gold, latex, liposomes, quantum dots and upconverting phosphors, among which colloidal gold is the most commonly used, followed by coloured latex particles (Posthuma-Trumpie et al., 2009). Recently, several

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* Corresponding author. Tel.: +86 27 87199115; fax: +86 27 87199492.
E-mail addresses: hpwei@wh.iov.cn (H.-P. Wei), x.zhang@wh.iov.cn (X.-E. Zhang).

[1] These authors contributed equally to this work.

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studies have demonstrated that super-paramagnetic particles have the potential to replace traditional labels because these particles can produce a steady magnetic signal that can be entirely captured by devices, thus improving the sensitivity of the lateral-flow assay and allowing the labelled analyte to be quantified (LaBorde and O'Farrell, 2002; Peck et al., 2006; Workman et al., 2009; Granade et al., 2010; Handali et al., 2010; Liu et al., 2011).

The aim of the current study is to provide a convenient method for the detection of *B. anthracis* spores under field conditions. To that end, we developed a super-paramagnetic lateral-flow immunological detection system that involves the use of a portable magnetic assay reader (MagnaBioSciences, USA), super-paramagnetic iron oxide particles, lateral-flow strips and two different monoclonal antibodies directed against *B. anthracis* spores. The system was firstly used to detect the pure spores and spores-containing artificial samples, and the results are reported herein.

2. Materials and methods

2.1. Specimens

B. anthracis A16 (pXO1$^+$, pXO2$^+$), *B. thuringiensis* BMB171, *B. thuringiensis* GBJ001, *B. cereus* ATCC33018, *B. cereus* IS195 and *B. mycoides* were used in this study. The spores were prepared by growing the bacteria at 37 °C on modified Difco sporulation medium (DSM) (Sonenshein et al., 1974) containing 6 g tryptone, 3 g yeast extract, 10 g NaCl, 1 g KCl, 0.25 g $Mg_2SO_4 \cdot 7H_2O$, 0.23 g $Ca(NO_3)_2$, 0.197 g $MnCl_2 \cdot 4\,H_2O$, 0.0002 g $FeSO_4$ and 15 g agar per litre.

When more than 95% free spores appeared, the spores were collected with cold sterile ultrapure water, centrifuged and washed extensively three times. The washed spores were then resuspended in sterile saline and stored at 4 °C. The spores were quantified by serially diluting the spore stock and plating 100 µl aliquots on Luria broth (LB) plates in triplicate. The number of spores (CFU ml^{-1}) was counted following overnight culture at 37 °C.

2.2. Preparation of mAbs

Two mouse monoclonal antibodies, designated 8 G3 and 12F6, were raised against intact *B. anthracis* A16 spores as described in our previous work (Wang et al., 2009b). Briefly, preparations containing 10^6 spores of *B. anthracis* strain A16 inactivated with 1.5% formaldehyde were injected subcutaneously into 6-week-old BALB/c mice. The immunisation was repeated 3× at 2-week intervals before boosting by intraperitoneal injection. The spleen cells were removed 3 days later and fused with myeloma cells according to the procedures of Kohler and Milstein (1975). The hybridomas were cloned by limiting dilution and screened using an enzyme-linked immunosorbent assay (ELISA). The mAbs were purified by caprylic acid–ammonium sulphate precipitation of ascites (Perosa et al., 1990) and analysed by SDS-PAGE to determine their purity.

2.3. Conjugation of super-paramagnetic particles with mAbs

The mAb designated 12F6 was conjugated to super-paramagnetic iron oxide particles (100 nm, 200 nm and 300 nm, Ademtech, Pessac, France) using cross-linking chemistry. The carboxylated beads were mixed with N-hydroxy-sulfosuccinimide (sulfo-NHS) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) in MES-buffered saline (pH 4.7) at room temperature for 120 min to form an amine-reactive sulfo-NHS ester. After the addition of the antibody, the solution was incubated for 2 h at 37 °C, resulting in a stable amide bond between the antibody and the particles. Before the final wash and storage at 4 °C in 0.01 M PBS (storage buffer), the residual active coupling sites on the particles were blocked with 5% BSA for 2 h at 37 °C.

2.4. Preparation of super-paramagnetic lateral-flow strips

Using a Dispensing Platform (BioDot, Irvine, CA, USA), 2 mg ml^{-1} mAb 8 G3 in 10 mM PBS was loaded onto a nitrocellulose membrane to serve as a test line (HiFlow Plus HFB13502; Millipore Corporation, Bedford, MA), and 1 mg ml^{-1} goat anti-mice IgG (Boster, China) in 10 mM PBS was loaded onto a nitrocellulose membrane to serve as a control line. The membrane was dried overnight at room temperature before use.

The super-paramagnetic lateral-flow strip was assembled using a piece of nitrocellulose membrane with antibodies, a narrow glass fibre conjugate pad (Millipore Corp.) containing super-paramagnetic particles conjugated with mAb, a wide glass fibre sample pad (Millipore Corp.) and a cellulose fibre absorbent pad (Millipore Corp.). Both the conjugate pad and the absorbent

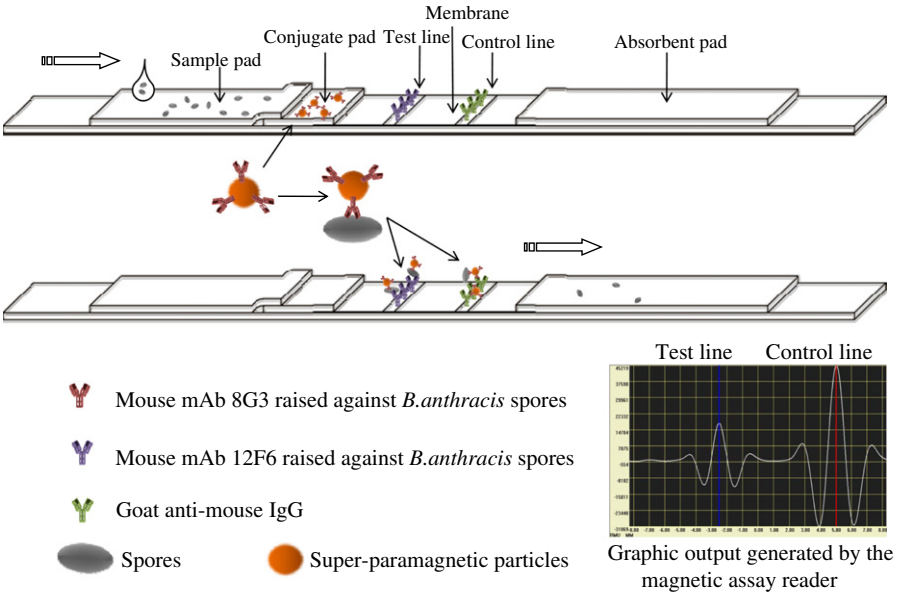

Fig. 1. Schematic of the magnetic lateral-flow immunological detection system for *B. anthracis* spore detection.