Journal of Medical Laboratory and Diagnosis Vol. 2(5), pp. 51-53, October 2011
Available online at http://www.academicjournals.org/JMLD
ISSN 2141-2618 ©2011 Academic Journals

Short communication

Combination of cultivation and lateral flow assay for easy, highly sensitive and presumptive detection of *Bacillus anthracis* spores

Valérie Guglielmo-Viret[1], Nicholas J. Vietri[2], Françoise Desor[1]and Philippe Thullier[1]*

[1]Unité de biotechnologie des anticorps et des toxines, Département de microbiologie, Institut de recherche biomédicale des armées (IRBA-CRSSA), 24 avenue des Maquis du Grésivaudan, 38702 La Tronche, France.
[2]Bacteriology Division, United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Frederick MD, USA.

Accepted 21 April, 2011

A combination of cultivation and lateral flow assay detecting protective antigen (PA) for easy and presumptive detection of *Bacillus anthracis* spores is proposed. This combination is similar to most approaches relying on PCR for detection of spores, as it begins with a 12 h cultivation step. After this culture however, this test is more rapid and far easier to perform than available molecular techniques. With a sensitivity of one spore per test (10 spores/ml), this combined test is amongst the most sensitive techniques while being very cost-effective. In addition, the principles behind this test may be applicable to the detection of other organisms.

Key words: Lateral flow assays (LFA), protective antigen (PA), membrane test platform (MTP),decomplementedfetal calf serum (FCS).

INTRODUCTION

Lateral flow assays (LFA) tests are convenient, cost effective, point-of-care diagnostics, (Ngom et al., 2010; Splettstoesser et al., 2010; Attree et al., 2007; Thullier et al., 2003) which have been used for the detection of bio-threat agents such as ricin (Thullier and Griffiths, 2009; Guglielmo-Viret et al., 2007), and have been evaluated as bench top tools for the rapid, presumptive identification of bacteria such as *Yersinapestis*(Tomaso et al., 2007). Although, the detection of *Bacillus anthracis*spores by genetic amplification is more rapid than standard bacterial culture methods, the low efficacy of DNA extraction directly from spore samples frequently necessitates enrichment by culturebefore nucleic acid detection (Kumar and Tuteja, 2009; Gulledge et al., 2010). The goal of the present study was to develop a highly sensitive, easy-to-use screening tool for the systematic, presumptive identification of *B. anthracis* spores, which could take less time than “culture plus PCR” approaches, and be useful in a situation where large numbers of unknown samples are encountered and threaten to overwhelm a laboratory’s capacity. For this strategy, an optimized culture step and a LFA, which detects the protective antigen (PA) component of the *B. anthracis*toxin, was developed and tested independently in two laboratories.

*Correspondingauthor. E-mail: pthullier@yahoo.com.

MATERIALS AND METHODS

The optimised LFA is similar to one previously reported (Guglielmo-Viret et al., 2007) except as detailedsubsequently.The capture antibody used was a rabbit polyclonal (CRSSA/704776), obtained in-house by immunisation against PA with standard techniques and purified (Hi-trap protein A HP columns, GE Healthcare, Orsay, France). This antibody (2 mg/ml) was coated at a rate of 1µl/cm (Isoflow reagent dispenser, Biodot, Chichester, U.K.), in the form of a test line, 1 cm above the bottom end of a Membrane Test Platform (MTP, Whatman, Maidstone, G.B.). Another reactant (Goldline, British Biocell International, Cardiff, U.K.) was coated in the same manner, but 1 cm higher, to form a control line, used to verify the quality of the sample migration when no signal is readable on the test line. The detection antibody was a commercially available murine monoclonal anti-PA antibody, called BAP0105 (AbCys S.A., Paris, France). It was incubated with gold nanobeads conjugated to anti-mouse IgG (British Biocell International, Cardiff, U.K.), at a rate of 10 ng antibody per 5 µl nanobeads for one hourat 37°C, resulting in gold nanobeads indirectly conjugated to

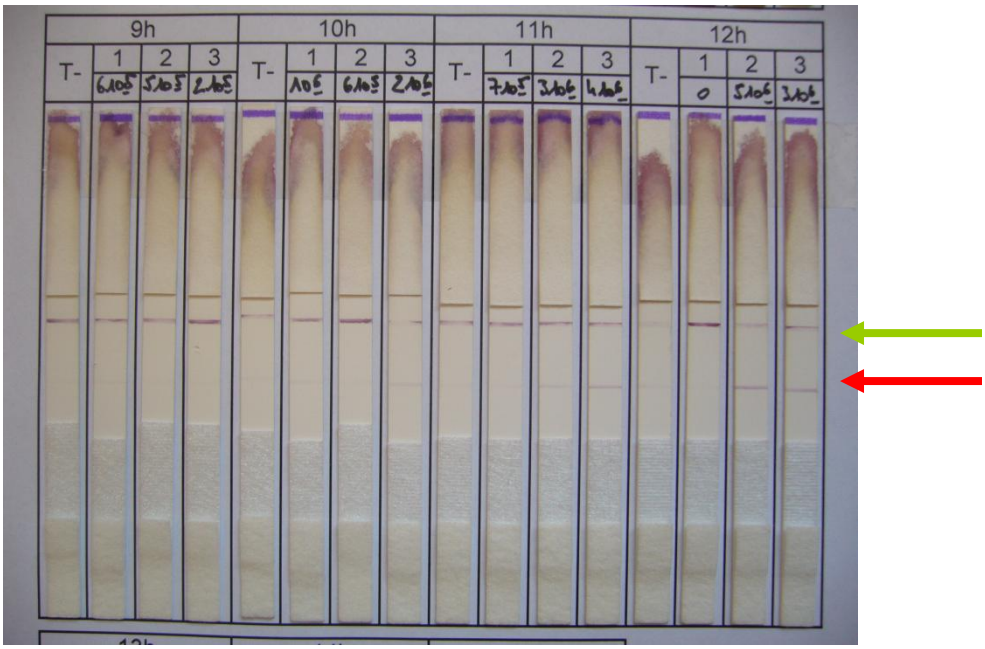

Figure 1. Triplicate cultures inoculated with an estimated one *B. anthracis* spore, and tested by LFA after 9, 10, 11 or 12 h (from left panel to right panel) culture for the presence of protective antigen. The test line of the LFA is indicated by the red arrow and the control line by the green arrow. A negative sample (left part of each panel) is cultivated and tested in each condition. Ten hours after inoculation, no signal could be distinguished from the negative control. After 11 h, a weak positive signal can be seen in two of the three replicates. After 12 hours, positive results (strips no. 2 and 3 only, culture no. 1 was not inoculated as indicated in the text) are clearly visible. Strips were taken out of the culture tubes to be photographed.

BAP0105. The limit of sensitivity of the test was 5 ng PA/ml, as evaluated using a commercial source of PA (List laboratories, Campbell, CA).

RESULTS AND DISCUSSION

Although *B. anthracis* germination and toxin secretion have multiple *in vivo* determinants (Sirard et al.,1994), it was hypothesized that culture in decomplementedfetal calf serum (FCS) at 35°C with 10% CO_2 would be a compatible *in vitro* environment. In fact, these culture conditions allowed the earlier detection of PA compared to culture in SB, TS or RM medium, even though RM medium was specifically designed to enhance anthrax toxin production (Leppla, 1988). Another advantage of the use of serum is that un-inoculated FCS, incubated overnight under these culture conditions,does not require the prior addition of buffer or detergent to migrate properly on this LFA.

The limit of sensitivity for spore detection was initially evaluated by inoculating 100 µl of *B. anthracis* (Sterne strain) spore suspension in water, into 400 µl of pure FCS (Gibco, Carlsbad, USA), in 5ml glass tubes to avoid PA adsorption. Just prior to culture, the spore suspension was heated at 75°C for 10 min to kill any vegetative bacilli, and thisstep largely suppresses the risk of contamination that could,otherwise, have overgrown *B. anthracis* spores and caused false negative results. Thirty minutes after culture, the strip was simply dropped into the culture tube and read through its wall. Using the convention of overnight bacterial culture for amplification prior to DNA extraction, we tested performance of the test strip by progressively lowering the spore concentration. Spore suspensions of ten spores per ml, corresponding to one spore per culture tube, gave unequivocal positive signals at the test line after 12 h of culture.

Using fully virulent *B. anthracis* (strain 9602) (the Sterne and 9602 strains were from IRBA-CRSSA collection) in a BSL 3 laboratory, the sensitivity of this assay was evaluated in three separate experiments giving equivalent results, one of which is presented on Figure 1. Parallel cultures were inoculated with 100 µl of a spore suspension (12 spores/ml) and tested in triplicate at 9, 10, 11 and 12 h after inoculation. At twelve hours after inoculation, as seen on the right panel, the positive signals became much stronger than the negative control and are easily observed on strips number 2 and 3. This corresponds to a culture concentration of approximately $5x10^6$ bacteria per ml. The lack of a positive signal in replicate number 1 was due to the difficulty of trying to inoculate culture tubes with a single spore, as this culture was confirmed to be sterile thus represented in fact a negative control. Unequivocal positive signals were also

observed, in a BSL 3 laboratory, after a 12 hculture of a 100 µl spore suspensions of the *B. anthracis*STI-1 (34 spores/ml) and Ames (22 spores/ml) strains, while the toxin negative strain Δ-Ames (14 spores/ml)was utilizedas a negative control (STI-1, Ames, Δ-Ames strains werefrom the USAMRIID collection).

When conducting this test, a positive control consisting of a culture treated exactly the same as the sample, but inoculated with 1 or 2 Sterne strain spores, should be performed in parallel. If this positive control remains negative after 12 h, the sample contains an inhibitor and should be diluted, but sensitivity is lowered accordingly.

According to a recent publication, direct DNA extraction followed by RT-PCR has been developed for the detection of *B. anthracis* spores without a culture enrichment step (Dauphin and Bowen, 2009). This method, which involved an extraction kit and has a reported sensitivity of 5 CFU, requires a minimum starting concentration of 10^3 spores per ml which may be problematic when solid surfaces, for instances, are swabbed. The method we describe here is less expensive, more sensitive (one spore per test corresponding to a concentration of only 10 spores/ml) and significantly easier to use. Another rapid method, the FDA-approved RedlineAlert® test, has been recently developed(http://www.tetracore.com/pdfs/Tetracore_ RedLine_Alert_Test.pdf).It is based on the detection of a surface protein found on vegetative *B. anthracis* using an immunochromatographic strip. However, this test requires growth on solid media for 15 to 24 h (until colonies reach 2 to 5 mm in diameter) before it can be utilized. Our test is thus 3 to 12 h more rapid, and since it does not require the prior isolation and visual identification of a suspected *B. anthracis,* may be less prone to generating a false negative result. Our test should be evaluated on additional *B. anthracis* strains and in various spiked or naturally contaminated matrices, to further assess its sensitivity. To further assess its specificity, it could also be tested on other bacilli than *B. anthracis*e specially of the *Bacillus cereus* group. This test could also be improved by the parallel detection of additional anthrax toxin components but, as presented in the present short communication, this “culture plus LFA detecting PA” strategy represents an efficient approach for *B. anthracis* spore detection, whose principle may be of more general interest.

ACKNOWLEDGEMENTSAND DISCLAIMER

This study was funded by EMA/LR 602-C2.The opinions, interpretations, conclusions and recommendations present here are those of the authors and are not necessarily endorsed by the U.S. nor the French Armed Forces.

REFERENCES

Attree O, Guglielmo-Viret V, Gros V, Thullier P (2007). Development and comparison of two immunoassay formats for rapid detection of botulinum neurotoxin type A. J.Immunol. Methods, 31: 325(1-2):78-87.

Dauphin LA, Bowen MD (2009 Feb). A simple method for the rapid removal of *Bacillus anthracis* spores from DNA preparations. J.Microbiol. Methods, 76(2):212-214.

Guglielmo-Viret V, Splettstoesser W, Thullier P (2007). An immunochromatographic test for the diagnosis of ricin inhalational poisoning. Clin.Toxicol. (Phila), 45(5):505-511.

Gulledge JS, Luna VA, Luna AJ, Zartman R, Cannons AC (2010). Detection of low numbers of *Bacillus anthracis* spores in three soils using five commercial DNA extraction methods with and without an enrichment step. J. Appl.Microbiol.,p. 19.

Kumar S, Tuteja U (2009). Detection of virulence-associated genes in clinical isolates of *Bacillus anthracis* by multiplex PCR and DNA probes. J.Microbiol.Biotechnol., 19(11):1475-1481.

Leppla SH (1988). Production and purification of anthrax toxin. Methods Enzymol.,165:103-116.

Ngom B, Guo Y, Wang X, Bi D (2010). Development and application of lateral flow test strip technology for detection of infectious agents and chemical contaminants: A review. Anal. Bioanal. Chem., 397(3):1113-1135.

Sirard JC, Mock M, Fouet A (1994). The three *Bacillus anthracis* toxin genes are coordinately regulated by bicarbonate and temperature. J Bacteriol.,176(16):5188-5192.

Splettstoesser W, Guglielmo-Viret V, Seibold E, Thullier P (2010). Evaluation of an immunochromatographic test for rapid and reliable serodiagnosis of human tularemia and detection of *Francisellatularensis*-specific antibodies in sera from different mammalian species. J.Clin.Microbiol., 48(5):1629-1634.

Thullier P, Griffiths G (2009). Broad recognition of ricin toxins prepared from a range of Ricinus cultivars using immunochromatographic tests. Clin.Toxicol. (Phila), 47(7):643-650.

Thullier P, Guglielmo V, Rajerison M, Chanteau S (2003). Short report: Serodiagnosis of plague in humans and rats using a rapid test. Am. J. Trop. Med.Hyg., 69(4):450-451.

Tomaso H, Thullier P, Seibold E, Guglielmo V, Buckendahl A, Rahalison L (2007). Comparison of hand-held test kits, immunofluorescence microscopy, enzyme-linked immunosorbent assay, and flow cytometric analysis for rapid presumptive identification of *Yersinia pestis*. J.Clin.Microbiol.,45(10):3404-3407.