Fast and Sensitive Detection of *Bacillus anthracis* Spores by Immunoassay

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Bacillus anthracis is one of the most dangerous potential biological weapons, and it is essential to develop a rapid and simple method to detect *B. anthracis* spores in environmental samples. The immunoassay is a rapid and easy-to-use method for the detection of *B. anthracis* by means of antibodies directed against surface spore antigens. With this objective in view, we have produced a panel of monoclonal antibodies against *B. anthracis* and developed colorimetric and electrochemiluminescence (ECL) immunoassays. Using Meso Scale Discovery ECL technology, which is based on electrochemiluminescence (ECL) detection utilizing a sulfo-Tag label that emits light upon electrochemical stimulation (using a dedicated ECL plate reader, an electrical current is placed across the microplate with electrodes integrated into the bottom of the plate, resulting in a series of electrically induced reactions leading to a luminescent signal), a detection limit ranging between 0.3×10^3 and 10^3 CFU/ml (i.e., 30 to 100 spores per test), depending on the *B. anthracis* strain assayed, was achieved. In complex matrices (5 mg/ml of soil or simulated powder), the detection level (without any sample purification or concentration) was never altered more than 3-fold compared with the results obtained in phosphate-buffered saline.

Bacillus anthracis, the etiological agent of anthrax, is a Gram-positive bacterium capable of forming endospores when vegetative cells are deprived of an essential nutrient. The core of the *B. anthracis* spore is surrounded by several integuments, the outermost of which is the exosporium (12). Spores are highly resistant to temperature, pressure, and UV radiation and to a wide variety of chemical toxins (2, 25). These properties allow the spores to survive in soil for many years until an appropriate environment allows the spore to germinate and grow as vegetative cells (17). *B. anthracis* has long been recognized as a potential bioterrorism weapon, and since its use in the 2001 attack in the United States, there has been a growing need for a rapid and accurate test to detect *B. anthracis* spores.

Most current rapid tests are based on nucleic acid detection, which has the advantage of being specific and highly sensitive, with a detection limit of between 1 and 30 spores per reaction (1, 3, 11, 15). However, the main drawback of these methods is the need for a clean starting sample concentrated in a small volume. In addition, these technologies generally use sophisticated equipment reserved for laboratory analysis, although small hand-held PCR assays are now becoming available for field testing. Immunoassays based on detection of surface spore antigens can provide a first-line, easy-to-use, and rapid method for detection of *B. anthracis* spores. Specific immunodetection of *B. anthracis* spores is challenging because of possible cross-reactivity of the antibodies (Abs) with near-neighbor species such as *Bacillus cereus* and *Bacillus thuringiensis*. Antibodies against the exosporium (28), the vegetative cells (19), oligosaccharide epitopes of the BclA spore surface protein (16, 32, 33), and extracellular antigen I (EAI) have been raised and used in immunoassays for *B. anthracis* detection (35). *B. anthracis* spores were successfully detected by immunofluorescence and cytometry techniques (20, 21, 27), but not with high specificity, because polyclonal antibodies were used in both cases and these methods are not suitable for samples containing a small quantity of target spores overwhelmed by other organisms in a complex matrix such as soil. Few immunoassays have been

evaluated for detection of *B. anthracis* spores in environmental samples (4, 10, 34). Using immunomagnetic beads, a detection limit of between 10^2 and 10^5 spores, depending on the strain, was achieved by Bruno et al., but assay sensitivity was compromised in the soil matrix (4). Sensitive detection of *B. anthracis* was reported for assays using an evanescent wave fiber-optic biosensor (34) and the integrating waveguide Biosensor (10), with detection limits of 4×10^4 and 10^4 spores/ml, respectively. For all these immunoassays, sensitivity and specificity are highly dependent on the antibodies used. Here we describe the production and characterization of new monoclonal antibodies (MAbs) raised against surface epitopes of the *B. anthracis* spore. The resulting sandwich immunoassay allowed sensitive and specific detection of *B. anthracis* spores. Using the A1 monoclonal antibody as the capture antibody and R93 MAb as the tracer antibody, colorimetric detection and electrochemiluminescence (ECL) detection were compared. Furthermore, the effect of different white powder matrices and soils on the detection of *B. anthracis* spores was evaluated.

MATERIALS AND METHODS

Monoclonal antibody production. Three Biozzi mice were immunized by intraplantary injection of 10^7 formaldehyde-inactivated *B. anthracis* spores (incubated in 4% formaldehyde for 4 h at 37°C) from two strains (7702 Sterne and RA3R) in complete Freund adjuvant. At 4-week intervals, three subsequent injections were done with the same dose of spores. Two weeks after each injection, the immune response, i.e., the levels of anti-*B. anthracis* spore antibodies, was evaluated by enzyme-linked immunosorbent assay (ELISA) (see below). Mice with the highest ELISA titer were selected for preparation of monoclonal antibodies. Three days

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before fusion, selected mice received an intravenous injection of 10^7 spores. Spleen cells from mice were fused with myeloma NS1 cells as previously described (7). After fusion, in the first screening by ELISA using spore-coated plates, 80 of 870 (9.2%) hybridomas and 110 of 812 (13.5%) hybridomas appeared to react with 7702 and RA3R spores, respectively. Following subcloning by limiting dilution, 28 and 20 clones expressing anti-7702 spore MABs and anti-RA3R spore MABs, respectively, were stabilized.

Spore preparation. *B. anthracis* strains (7702, RA3R, and 9602 strains), recombinant strain PF09 (7702 Δ bclA), *B. cereus* strains (*B. cereus* 569, *B. cereus* 9241, and *B. cereus* 10987), *B. thuringiensis* strains (*B. thuringiensis* 407 and *B. thuringiensis* 9727), and *B. subtilis* strain 168 were obtained from M. Mock (Institut Pasteur). The *B. anthracis* Vollum strain was from the Health Protection Agency Culture Collection.

Spores were prepared from NBY (nutrient broth yeast extract) agar incubated for 7 days at 30°C. After three washes in distilled water, spores were purified by differential centrifugation for 30 min at $6,000 \times g$ at 4°C through layers of 45% to 55% Riosellectan (Schering) (76% renografin) prepared in distilled water and washed three times in cold distilled water (18).

Screening of hybridoma culture supernatants and spore ELISA. The presence of antibody in the hybridoma culture supernatant was detected using ELISA. Briefly, 50 μ l of 10^7 spores per ml in water was added to each well of a poly-L-lysine-pretreated 96-well Maxisorb (Nunc) plate and incubated overnight at 37°C. The plates were then saturated with enzyme immunoassay (EIA) buffer (0.1 M phosphate buffer [pH 7.4], 0.1% bovine serum albumin [BSA], 0.15 M NaCl, 0.001% sodium azide) for 4 h at room temperature. Then, 50 μ l/well of hybridoma culture supernatant was added for reaction overnight at 4°C. After three washes, 50 μ l/well of goat anti-mouse antibody covalently coupled to acetylcholinesterase (AChE) was added for 4 h at room temperature. After 6 washes, AChE activity was revealed by the colorimetric method of Ellman et al. (5).

Determination of binding complementarity for each pair of MABs. The binding of the different MABs to *B. anthracis* spores was analyzed using immunometric tests, with one capture MAB immobilized on the solid phase and the other biotin-labeled MAB used as a tracer. Briefly, 100 μ l of spore suspension (2×10^5 inactivated 7702 spores in duplicate) was added to a microtiter plate coated with one of the MABs. After an 18-h incubation at 4°C, the plates were washed before addition of 100 μ l of biotin-labeled MAB (0.5 μ g/ml). After a 3-h reaction at 20°C, plates were washed and reacted with 100 μ l of AChE-labeled streptavidin (2 Ellman units/ml) for 1 h at 20°C. Plates were then extensively washed, and solid-phase bound AChE was revealed by addition of 200 μ l of Ellman's reagent.

Immunometric assays using MAB-biotin or MAB-AChE conjugates. Immunometric assays were performed in 96-well Maxisorb immunoplates (Nunc) coated with purified antibody. Simultaneously, 100 μ l of spore suspension and biotin-labeled or AChE-labeled MAB was loaded into the wells; plates were centrifuged at $1,500 \times g$ for 5 min and reacted for 18 h or 2 h, respectively, at room temperature. In the case of biotin-labeled MAB, plates were washed and further reacted with 100 μ l of AChE-labeled streptavidin (2 Ellman units/ml) for 1 h at 20°C. After several washes, plate results were revealed by addition of 200 μ l of Ellman's reagent.

Electrochemiluminescence assays. Standard 96-well Sector PR 100 plates (Meso Scale Discovery) were coated with 50 μ l of MAB (80 μ g/ml)–50 mM phosphate buffer (pH 7.4). After an 18-h incubation at 20°C, plates were washed and blocked with 5% BSA–phosphate-buffered saline (PBS) for 1 h. A 100- μ l volume of spore suspension and 20 μ l of ruthenium-labeled MAB (prepared with Meso Scale Discovery Sulfo-TAG *N*-hydroxysuccinimide [NHS]-ester according to the manufacturer's instructions [Meso Scale Discovery]) were loaded into the wells, centrifuged at $1,500 \times g$ for 5 min, and reacted for 2 h at room temperature. The wells were washed 5 times in 50 mM phosphate buffer (pH 7.4), and 150 μ l of $1 \times$ T buffer (Meso Scale Discovery) was added to each well. The plates were read with a Meso Scale Discovery PR100 plate reader.

Western blot analysis of spore extracts and BclA protein. Spores (10^9) were heated for 15 min at 90°C with 50 μ l of extraction buffer (50 mM Tris HCl [pH 10], 8 M urea, 2% 2-mercaptoethanol) and centrifuged at 13,000 rpm for 10 min. The supernatant was diluted 10 times in Laemmli buffer, heated for 5 min at 100°C, and electrophoresed using 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% nonfat dry milk and then probed with 1 μ g/ml of anti-*Bacillus anthracis* MABs. Goat anti-mouse immunoglobulin coupled to horseradish peroxidase was used as a secondary antibody at a 1/500 dilution, and peroxidase activity was detected by chemiluminescence (ECL Plus Western Blotting Detection system; GE Healthcare-Amersham).

For BclA analysis, the polyhistidine-tagged recombinant BclA protein was produced in *Escherichia coli* strain M15(pREP4) and purified using a nickel-nitrilotriacetic acid (Ni-NTA) affinity column (chelating Sepharose Fast Flow; GE Healthcare).

Limit of detection of anti-*B. anthracis* spore immunoassays. The four-parameter logistic (4-PL) function was used to model the characteristic curve for the immunoassays. The limit of detection was calculated by interpolating the average background signal (measured in eight wells) plus 3 standard deviations on the standard curve.

RESULTS

Characterization of monoclonal antibodies to *B. anthracis* spores. A total of 28 and 20 hybridoma clones expressing monoclonal antibodies against *B. anthracis* 7702 spore (MABs of the A series) and *B. anthracis* RA3R spore (MABs of the R series), respectively, were stabilized, and antibodies were produced (see Materials and Methods).

MAB specificity was first characterized using ELISA to test the capacity of MABs to bind a panel of selected strains from the *Bacillus cereus* group and *Bacillus subtilis*: 3 *B. anthracis* spore strains, a BclA deletion mutant of strain 7702 (PF09), 3 *B. cereus* and 2 *B. thuringiensis* spore strains very closely related to *B. anthracis* species, and the *B. subtilis* 168 strain (see Materials and Methods and Table 1). *B. subtilis* spores differ structurally from those of the *B. cereus* group, since the spores are not surrounded by the exosporium. All the strains were formaldehyde treated before being tested with biotinylated antibodies by ELISA (see Materials and Methods).

These experiments classified the 48 MABs into 7 groups as shown in Table 1. Group I included 8 MABs that showed a complete lack of specificity, since they bound all bacillus strains tested, including *B. subtilis* spores. Group II (3 MABs) also exhibited broad recognition of the *Bacillus* genus, with the exception of *B. subtilis*. Groups III and IV comprised 7 and 3 MABs, respectively, all resulting from the immunization with strain RA3R. Group III bound strongly to the 4 *B. anthracis* strains and to *B. cereus* strain 9241. Some of these MABs may recognize *B. thuringiensis* 9727 and/or *B. cereus* 10987. Group IV recognized only RA3R from the 4 *B. anthracis* strains and also some of the *B. cereus* strains and *B. thuringiensis* strain 9727. The 27 remaining MABs (groups V to VII) bound wild-type strain 7702 but not BclA mutant strain PF09, suggesting that they recognize the BclA protein. Interestingly, all these MABs were IgG whereas IgM appeared to predominate in the 4 other groups. Western blot experiments with exosporium extracts of strains 7702 and PF09 and the BclA recombinant protein further demonstrated this clear difference in reactivity between the 21 MABs of groups I to IV and the 27 MABs of groups V to VII. As illustrated in Fig. 1 for MAB A36 (representative of group 6 and 7 MABs), only the 27 antibodies binding

TABLE 1 Immunoreactivity of anti-*Bacillus anthracis* MAbs with closely related inactivated strains from *B. cereus*, *B. thuringiensis*, and *B. subtilis*^a

Group	MAb	<i>B. anthracis</i>					<i>B. cereus</i>		<i>B. thuringiensis</i>		<i>B. subtilis</i>	WB BclA	Isotype
		Ba 9602	Ba 7702	Ba RA3R	Ba PF09	Bc 569	Bc 9241	Bc 10987	Bt 407	Bt 9727	Bs 168		
I	R12	+++	+++	+++	+++	+++	+++	++	++	+++	+	—	IgM
	R-73	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	—	IgM
	A6	+++	+++	+++	+++	+++	+++	++	+++	+++	++	—	IgG
	R94	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	—	IgM
	A20	++	++	++	+++	+++	+++	+++	+++	+++	++	—	IgG
	A23	+++	+++	+++	+++	+++	+++	++	+++	+++	++	—	IgG
	A40	+++	+++	+++	+++	+++	+++	++	+++	+++	++	—	IgG
	A77	+++	+++	+++	+++	+++	+++	++	++	+++	++	—	IgG
II	A49	++	++	+	+++	+++	+++	+	++	+++	—	—	IgM
	A65	+++	+++	+++	+++	+++	+++	+++	+++	+++	—	—	IgM
	A70	+++	+++	+++	+++	+++	+++	++	+++	+++	—	—	IgG
III	R31	+++	+++	+++	+++	—	+++	+++	—	+/-	—	—	IgM
	R44	+++	+++	+++	+++	—	+++	—	—	+/-	—	—	IgM
	R61	+++	+++	+++	+++	—	+++	—	—	+/-	—	—	IgM
	R63	+++	+++	+++	+++	—	+++	—	—	+/-	—	—	IgM
	R71	+++	+++	+++	+++	—	+++	—	—	—	—	—	IgM
	R97	+++	+++	+++	+++	—	+++	—	—	+/-	—	—	IgM
	R109	+++	+++	+++	+++	—	+++	++	—	+/-	—	—	IgM
IV	R16	—	—	+	—	—	—	—	—	—	—	—	IgG
	R45	—	—	+++	—	+	++	+++	—	+++	—	—	IgM
	R68	—	—	+++	—	—	—	+++	—	+	—	—	IgM
V	A39	+++	+++	+++	—	+++	—	+	+++	+++	—	+	IgG
	R30	+++	++	+++	—	+	++	+++	+	+	—	+	IgG
	R74	+++	+++	+++	—	+++	+++	++	+++	+++	—	+	IgG
	A1	+++	++	+++	—	+	++	+	+	++	—	+	IgG
	A12	++	+	+++	—	+	+++	+++	+	+	—	+	IgG
	A18	++	+	+	—	++	+	++	+	+	—	+	IgG
	A53	+++	++	++	—	+	++	+++	+	++	—	+	IgG
	A55	+++	+++	+++	—	+	++	+++	++	++	—	+	IgG
	A80	+++	++	+++	—	+	++	+++	+	++	—	+	IgG
VI	R54	++	++	+++	—	—	—	—	+	++	—	+	IgG
	R78	++	+++	+++	—	—	—	++	—	+++	—	+	IgG
	A15	+++	+++	+++	—	+	—	—	—	+++	—	+	IgG
VII	R13	++	++	++	—	—	—	—	—	++	—	+	IgG
	R17	+++	+++	+++	—	—	—	—	—	+++	—	+	IgG
	R93	+++	++	+++	—	—	—	—	—	+++	—	+	IgG
	A3	+++	+++	+++	—	—	—	—	—	+++	—	+	IgG
	A4	+++	+++	+++	—	—	—	—	—	+++	—	+	IgG
	A7	+++	+++	+++	—	—	—	—	—	+++	—	+	IgG
	A8	+++	+++	+++	—	—	—	—	—	+++	—	+	IgG
	A14	+++	+++	+++	—	—	—	—	—	+++	—	+	IgG
	A36	+++	+++	+++	—	—	—	—	—	+++	—	+	IgG
	A42	+++	+++	+++	—	—	—	—	—	+++	—	+	IgG
	A44	+++	+++	+++	—	—	—	—	—	+++	—	+	IgG
	A50	+++	+++	+++	—	—	—	—	—	+++	—	+	IgG
	A52	+++	+++	+++	—	—	—	—	—	+++	—	+	IgG
	A69	+++	+++	+++	—	—	—	—	—	+++	—	+	IgG
	A71	+++	++	++	—	—	—	—	—	+++	—	+	IgG

^a Reaction of 0.5 µg/ml of MAbs using ELISA (see Materials and Methods). —, negative signals; +, positive by Western blot analysis or signals > 0.1 absorbance units by ELISA; ++, signals > 0.3 absorbance units by ELISA; +++, signals > 0.5 absorbance units by ELISA. Isotype (IgM or IgG) and reactivity of monoclonal antibodies with BclA by Western blot analysis are indicated.

strain 7702 and not strain PF09 recognized both the BclA protein present at the surface of spores and the recombinant BclA (Table 1), indicating that these antibodies are directed more precisely against the protein part of BclA at the spore surface. Among these antibodies, 15 (group VII) bound all the *B. anthracis* spores tested and only one of the related *B. thuringiensis* strains, strain 9727, which is phylogenetically very close to *B. anthracis*. This restricted

specificity was not observed for group V, whose MAbs bound several *B. cereus* and *B. thuringiensis* strains, while group VI (3 MAbs) presented intermediate specificity.

Detection of *B. anthracis* spores by a two-site immunometric assay. Complementary binding studies were performed to establish a two-site immunometric site assay for *B. anthracis* spores. The 2,304 combinations resulting from the 48 MAbs obtained

FIG 1 Reactivity of anti-spore MAbs with exosporium and recombinant BclA. Proteins were separated by SDS-PAGE and analyzed by immunoblotting with MAb 77A-36. Lane 1, exosporium extract from a *B. anthracis* (strain 7702) spore; lane 2, exosporium extract from BclA mutant strain PF09; lane 3, purified recombinant BclA.

were tested using a sequential procedure and 2×10^5 formaldehyde-inactivated 7702 spores. More than 571 combinations provided good detection, and 184 pairs appeared most efficient (signal > 0.2 absorbance units [AU] and > 0.5 AU, respectively, after 1 h of staining; see Fig. 2). It is worth noting that most of these combinations involved group V to VII MAbs. The best assays, those giving a good ratio of signal to noise, were further evaluated for a dilution series using strain 7702. Figure 3 shows the results for the eight more sensitive and specific combinations (18-h reaction). All these immunoassays had similar sensitivities of close to 10^4 CFU/ml. To optimize the assay, the combination that included MAb A1 as the capture antibody and MAb R93 labeled with AChE as the tracer was selected. The best sensitivity was obtained by using a simultaneous procedure (simultaneous reaction of the sample and the AChE-R93 tracer with the capture MAb). The formation of the immune complex was accelerated by performing a 5-min centrifugation step before a 2-h immunoreaction at room temperature. Figure 4 presents the standard curves

FIG 3 Capacity of selected ELISA for the detection of *B. anthracis* spores. Different concentrations of inactivated *B. anthracis* Sterne spores were detected with various combinations of MAbs. In order to allow a direct comparison, the nonspecific binding of each pair of MAbs was subtracted. OD, optical density.

obtained for live spores from *B. anthracis*, *B. cereus*, *B. thuringiensis*, and *B. subtilis*. Very good sensitivity was obtained for all *B. anthracis* strains tested, with a limit of detection between 10^3 and 2×10^3 CFU/ml, depending on the *B. anthracis* strain. As expected, the *B. thuringiensis* 9727 strain was also well detected, with a limit of detection close to 3×10^3 CFU/ml, whereas other non-*B. anthracis* strains were not detected even with a 10^7 CFU/ml concentration. The combination using MAb R45 as the capture antibody and AChE-labeled R-93 as the tracer allows the specific detection of strain RA3R versus the *B. cereus* and *B. thuringiensis* strains tested and 5% cross-reactivity with some other *B. anthracis* strains (data not shown). The detection limit was close to 5×10^3 CFU/ml.

Comparison of ECL assay with colorimetric ELISA. To com-

FIG 2 Colorimetric signal obtained for combinations of mAbs used as capture and tracer antibodies. Tests were performed with the inactivated 7702 strain (2×10^5 spores/ml). Black squares, signals > 0.2 absorbance units; light grey squares, signals > 0.05 absorbance units; white squares, low or nonspecific signals.

FIG 4 Standard curves for various *Bacillus* strains in PBS–0.1% BSA buffer. Immunoassays were performed with A1 as the capture antibody and R93 as the AChE tracer antibody. The bacillus concentration range was 10^3 to 10^6 spores per ml. The inset shows the low-concentration part of the curve. The nonspecific binding of each MABs was subtracted. OD, optical density.

pare the two detection techniques, the same capture and tracer antibodies (A1 MAb and R93 MAb, respectively) were used. For colorimetric ELISA, the tracer antibody was coupled to AChE (one tetrameric AChE form for one antibody) (8), while for the ECL assay, the same antibody was coupled to ruthenium (see Materials and Methods). Both ELISA and ECL were optimized in terms of the concentration of capture antibody and the concentration of detection antibodies. For both assays, samples were reacted for 2 h at 20°C, with the solid-phase antibody and the tracer antibody being reacted simultaneously. After a washing step, the reaction solution was added and the plates were read 30 min afterward for the colorimetric assay and immediately for the ECL reagent. In both assays, we observed that a 5-min centrifugation step before the 2-h immunoreaction step increased the sensitivity by 1 order of magnitude (data not shown).

As shown in Fig. 5, the curves were mainly linear, allowing detection of the tested strains over a large concentration range. The slope of the ECL curve was greater. The limit of detection of the AChE-colorimetric assay ranged between 10^3 and 4×10^3 CFU/ml for the *B. anthracis* strain, while the ECL assay provided sensitivity ranging between 0.3×10^2 and 10^3 CFU/ml, thus appearing three to four times more sensitive than the colorimetric ELISA.

Detection of *B. anthracis* spores in the presence of other *Bacillus* strains as a masking agent. The possible interference of other *Bacillus* strains in the detection was evaluated by mixing *B. anthracis* strain Sterne 7702 (at a final concentration of 10^4 CFU/ml) with various *Bacillus* spores at increasing concentrations from 10^5 to 10^8 CFU/ml. As shown in Fig. 6, the presence of low or high concentrations of spore from *B. cereus*, *B. thuringiensis*, or *B. subtilis* did not significantly affect the detection of the Sterne 7702 spores. In fact, the presence of such related spores induced only a 20% variation of the signal for a concentration of *B. anthracis* of 10^4 spores/ml.

Spore detection in domestic powder and environmental samples. Samples were prepared as a suspension of 5 mg/ml powder in PBS supplemented with 0.1% BSA. Dried milk, flour, baking soda, and chalk were selected as representative

FIG 5 Comparison of ELISA (A) and ECL assay (B) for the detection of various *B. anthracis* strains and *B. thuringiensis* strain 9727. Spores were diluted in EIA buffer and assayed using simultaneous procedures with a 2-h incubation time. The same pair of antibodies was used, with A1 as the capture antibody and R93 as the tracer antibody. The nonspecific binding of each MAB was subtracted. OD, optical density.

domestic powders, and soil was chosen as an environmental sample. The effect of these suspensions on the detection of 2.5×10^4 and 2.5×10^5 *B. anthracis* spores (7702 strain) was evaluated using both the AChE colorimetric ELISA and the ECL assay. As shown in Fig. 7, taking the results obtained for EIA buffer as a reference, few differences were observed for colorimetric ELISA, with a 40% decrease of the specific signal upon adding chalk and a 5-fold increase of the nonspecific binding observed with the soil solution. The effects appeared greater for the ECL assay, with large variations in specific signals for soil and chalk (30% and 50% decreases, respectively), while for flour only 30% of the specific signal remained. ECL detection thus seems more affected by the medium than the colorimetric enzyme assay. Similar results were obtained with the Ames and the Vollum *B. anthracis* strains (data not shown).

DISCUSSION

PCR is unambiguously the most sensitive of the rapid methods used to detect pathogens such as *B. anthracis* spores, reaching a sensitivity of 1 to 10 equivalent genomes per reaction. However, the application of PCR to environmental samples has many pitfalls due to its susceptibility to inhibitors and contamination, as outlined in recent papers (22–24). In addition, due to the rigid wall of bacillus spores, additional treatments are required for DNA isolation.

FIG 6 Detection of *B. anthracis* strain Sterne 7702 (+, 10^4 spores; -, no spore) in the presence of spores (concentration range, 10^5 to 10^8 spores per ml) from *B. cereus*, *B. thuringiensis*, and *B. subtilis* strains. Data represent the results of a Meso Scale Discovery immunoassay performed with A1 MAb as the capture antibody and R93 MAb as the AChE tracer.

FIG 7 Comparison of ELISA (A) and ECL assay (B) for the detection of *B. anthracis* strain Sterne 7702 spores spiked into various suspensions of domestic powders (chalk, sugar, baking soda, flour, and milk) and soil. Black bars, signals with 2×10^5 CFU/ml of *B. anthracis* spores; gray bars, signals with 2×10^4 CFU/ml of *B. anthracis* spores; white bars, signal in the absence of spore.

Immunoassays based on the detection of surface exosporium should be considered an alternative method for detection of *B. anthracis* spores, even if performing such assays is challenging, because of the presence of cross-reactive antigens in closely related strains, such as the *B. cereus* strains. In the present study, a large panel of 48 MAbs directed against the surface of spores from two *B. anthracis* strains, i.e., the Sterne 7702 and RA3R strains, was obtained. Several of the MAbs were highly specific for *B. anthracis* spores and recognized BclA, the major surface protein of the spore. This protein has a central collagen-like region formed by polymorphic GXX repeats that are strain specific (26, 29, 30). BclA is also found in *B. cereus* and *B. thuringiensis* but not in *B. subtilis*. Very specific and sensitive immunoassays for *B. anthracis* strains were developed, exhibiting cross-reactivity only with the very closely related *B. thuringiensis* 9727 strain. It is worth noting that, unlike the other *B. thuringiensis* strains, strain 9727 is associated with severe disease, and its pathogenicity has been confirmed by infection of both immunosuppressed and immunocompetent mouse (13, 14). Swiecki et al. (28) raised monoclonal antibodies against *B. anthracis* spores that all cross-reacted with *B. thuringiensis* strain 9727, which confirms the difficulty of obtaining antibodies that specifically recognize all *B. anthracis* strains. In the present study, *B. anthracis* strain RA3R was included because it belongs to subgroup B2, which is the most common subgroup in France but extremely rare elsewhere in the world (6).

To date, few colorimetric ELISAs that detect *B. anthracis* spores

have been developed. Using antibodies directed against the anthrose tetrasaccharide, Kuehn et al. developed a specific and sensitive ELISA able to detect 1×10^4 to 5×10^4 inactivated *B. anthracis* CDC1014 spores/ml (16). The most sensitive spore detection performed by means of a two-site immunometric assay was achieved using Luminex technology with a detection limit of 10^3 to 10^4 spores per ml (31).

After optimization, the present colorimetric ELISA had a limit of detection ranging between 10^3 CFU/ml (strains Vollum, RA3R, and 9602) and 4×10^3 CFU/ml (strain 7702). Using our best pair of antibodies and the same assay conditions (time of incubation, centrifugation step), the ECL Meso Scale Discovery technology was three to four times more sensitive than ELISA, with a limit of detection of between 3×10^2 and 10^3 spores/ml (30 to 100 spores per well), making ECL the most sensitive immunoassay described so far for detection of *B. anthracis* spores. Similar differences between the two technologies in terms of sensitivity were reported for the detection of ricin B chain by Guglielmo and Thullier (9), with ECL outperforming ELISA. It is worth noting that during the present study, we consistently observed that the immunodetection of spores can be very significantly improved if the immunoreaction (2 h at room temperature) is preceded by a short centrifugation step (5 min, $1,500 \times g$). This very likely reflects the more rapid access of spores to the surface-bound antibodies. In fact, the signals measured under these conditions were equivalent to those observed after an overnight reaction (data not shown). This centrifugation step was routinely included in the procedure. Both the ELISA and the ECL assay described here are able to detect *B. anthracis* spores in different powders and in soil with, in the worst case, only a 3-fold decrease of the signal. Furthermore, even in the presence of a 1,000-fold excess of closely related strains possibly found in soil, *B. anthracis* spores were still detectable. These assays therefore appear the most sensitive immune methods for detection of *B. anthracis* spores (strains Sterne, RA3R, 9602, Ames, and Vollum) in complex matrices, with at least 2.5×10^4 CFU/5 mg of powder or soil detected without pretreatment or purification. These experiments demonstrate that these two immunometric assays, coupled to either colorimetric or ECL detection, are robust and sensitive techniques for detection of *B. anthracis* spores in the environment.

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