Immunomagnetic-Electrochemiluminescent Detection of *Bacillus anthracis* Spores in Soil Matrices

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Rapid (≤ 1.5 -h) detection of anthrax spores in soil suspensions was accomplished by an immunomagnetic electrochemiluminescence method. Strain-dependent detection limits in the range of 10^2 to 10^5 spores were achieved in buffer. The rank order of sensitivity for the assay in buffer was Sterne > Ames > Vollum 1B. Detection was up to 3 orders of magnitude less sensitive in soil suspensions, and the rank order of sensitivity was altered.

Anthrax is a bacterial disease of human medical, agricultural, and military significance (3, 6, 8, 9), since it is caused by an organism that forms spores that can remain viable in soils for decades (5). Soil-borne spores may enter their victims by the oral, cutaneous, or respiratory route (1–3, 9). Respired-dosage experiments in primates have produced varied results but generally place the 50% lethal dose between 4,000 and 50,000 spores (1–3, 9). The course of respiratory anthrax is rapid, with exponential septicemia occurring within 1 day of infection (3, 9). Thus, any anthrax detection technology should be rapid, sensitivity to the level of thousands of spores, and able to detect spores in a variety of soil matrices.

Sensitive detection by immunomagnetic electrochemiluminescence (IM-ECL) of nonpathogenic Sterne strain anthrax spores (≤ 100 spores) in phosphate buffer (4) and other pathogenic bacteria in foods (10) has been previously reported. The present work extends those studies by detection of three strains of *Bacillus anthracis* spores in two different soil suspensions such as might be found under diverse military or agricultural field screening conditions.

Spores of one avirulent (Sterne) and two gamma-irradiated virulent (Ames and Vollum 1B) *B. anthracis* strains and *B. cereus* ATCC 7064 (American Type Culture Collection, Rockville, Md.) were the generous gifts of John Ezzell, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md. *B. thuringiensis* (ATCC 10792) and *B. thuringiensis* subsp. *israelensis* (ATCC 35646) were obtained from the American Type Culture Collection, Rockville, Md. Stock spore suspensions were made in phosphate-buffered saline (PBS; pH 7.2 to 7.4) and stored at 4°C for less than 2 weeks. Spore concentrations were determined by hemacytometer counts under phase-contrast microscopy.

Goat polyclonal antiserum was raised against a mixed inoculum of Ames, Sterne, and Vollum 1B *B. anthracis* spores and designated Gt 578 by Antibodies Inc., Davis, Calif. All capture antiserum (Gt 578) was biotinylated, and reporter antiserum (also Gt 578) was labeled with an *N*-hydroxysuccinimide ester

derivative of ruthenium(II) trisbipyridine [$\text{Ru}(\text{bpy})_3^{2+}$; Igen Corp., Gaithersburg, Md.] as previously described (4).

For PBS-based IM-ECL assays, streptavidin-coated paramagnetic microbeads (M280; 2.8 μm in diameter) were obtained from Dynal Corp., Lake Success, N.Y. A 20- μl volume of streptavidin-coated magnetic beads (1 mg/ml; 20 μg) was incubated with 100 ng of biotinylated capture Gt 578 antiserum in 25 μl of PBS–2% normal goat serum for 10 min at ambient temperature. Tenfold dilutions of anthrax spores (0 to 10^6 spores per assay) in 20 μl of PBS were allowed to react for 1 h with gentle (60-rpm) agitation. Subsequently, 200 ng of $\text{Ru}(\text{bpy})_3^{2+}$ -labeled Gt 578 reporter antiserum in 25 μl of PBS–2% normal goat serum were added for 15 min, and the sample tubes were vortexed on the ORIGIN analyzer (IM-ECL sensor; Igen Corp.) carousel at 110 rpm for 1 h. Finally, 200 μl of Igen ECL assay buffer, containing tripropylamine, was added per tube. Sample ECL intensities (relative photon counts) were quantitated by the ORIGIN analyzer at a photomultiplier potential of 650 V.

For spore-spiked soil IM-ECL assays, two soil suspensions from fine particulates of a moist dark brown to black soil (soil 1) and a dry light yellowish sandy soil (soil 2), taken from two different locations in the vicinity of Aberdeen Proving Ground, Md., were prepared in PBS. Both suspensions had A_{600} values of around 1.0 (approximately 1 mg of soil per ml of suspension), and the pH of both suspensions remained between 7.2 and 7.4. Spore capture and assay were essentially performed as in the PBS-based assay, except that the soil suspensions were used as diluents. In some experiments, assays were performed by introducing the spore-magnetic bead-soil suspensions directly into the sensor. In other experiments, IM separation (IMS) was performed with a permanent rare earth magnet for 15 min after 1 h of IM spore capture in the two different soil suspensions. Spores purified by IMS were resuspended in PBS and washed twice by IMS prior to ECL assay.

Figure 1A illustrates that IM-ECL detection of Sterne strain spores was highly sensitive in PBS and matched that previously reported (≤ 100 spores [4]). However, the lower detection limits for the other two strains were much higher than for the Sterne strain. The lower limit for Ames spores was between 10^3 and 10^4 spores, while the lower limit for Vollum 1B spores was between 10^4 and 10^5 spores.

Titer determination for each strain of *B. anthracis* spores by IM-ECL assay in a soil suspension (soil 1) with an optical

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FIG. 1. (A) IM-ECL detection of various strains of *B. anthracis* spores in PBS. (B) IM-ECL detection of various strains of *B. anthracis* spores in a suspension of soil 1 without a wash step. Values represent the mean ECL intensities and standard deviations of three independent assays per datum point.

density near 1.0 demonstrated a shift toward larger spore numbers (i.e., lower detectability) for each strain compared with ECL values acquired in buffer (Fig. 1A). These results (Fig. 1B) were obtained by directly introducing the spore-magnetic bead-soil suspension into the IM-ECL sensor without a prior wash step. The loss of sensitivity in soil suspension suggested that IMS should be performed prior to the ECL assay.

Another interesting aspect of the results shown in Fig. 1B was that the rank order of reactivity of the Gt 578 IM-ECL assay changed from Sterne > Ames > Vollum 1B in buffer to Ames > Sterne > Vollum 1B in soil suspension. A repeat of this experiment (results not shown) verified that the strains were not mistakenly switched and suggested that the result was due to a selective depletion of monospecific anti-Sterne spore antibodies from the polyclonal Gt 578 antiserum or antigenic masking by soil components.

On the basis of data presented in Fig. 1, it was decided to assess the potential of IMS to improve spore detection in soil suspensions. Therefore, the IM-ECL results obtained with and without IMS prior to the ECL assay were compared for two different soils as described above. The results of these experiments (Fig. 2) indicate that IMS is of value in eliminating the background ECL signal from soils. Results given in Fig. 2 also confirm the observation of altered strain rank order due to the presence of soils, as shown in Fig. 1.

The results of interference assays (not shown) performed by the Gt 578 IM-ECL assay with both spore and vegetative-cell preparations of one *B. cereus* strain (ATCC 7064) and two *B. thuringiensis* strains (ATCC 10792 and ATCC 35646) consistently showed percent cross-reactivities of <0.01% (10). The lack of cross-reactivity with other *Bacillus* species in IM-ECL assays may be partly due to mechanical forces during the vortex mixing step, which select for higher-avidity binding to Sterne, Ames, and Vollum 1B strains used in the goat inoculum. Although the assay sensitivity was compromised in soil matrices, the IM-ECL method is still attractive for detection of

FIG. 2. IM-ECL detection of 10^5 spores of various strains of *B. anthracis* in two different types of soil suspensions with (+ IMS) and without (- IMS) IMS prior to introduction of samples into the IM-ECL sensor. Values represent the mean ECL intensities and standard deviations of three independent assays per datum point.

anthrax spores in soils because of its relative speed, simplicity, and ease of use for processing large numbers of samples.

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