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Anthrax Spore Detection by a Luminex Assay Based on Monoclonal Antibodies That Recognize Anthrose-Containing Oligosaccharides▽

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The similarity of endospore surface antigens between bacteria of the *Bacillus cereus* group complicates the development of selective antibody-based anthrax detection systems. The surface of *B. anthracis* endospores exposes a tetrasaccharide containing the monosaccharide anthrose. Anti-tetrasaccharide monoclonal antibodies (MAbs) and anti-anthrose-rhamnose disaccharide MAbs were produced and tested for their fine specificities in a direct spore enzyme-linked immunosorbent assay (ELISA) with inactivated spores of a broad spectrum of *B. anthracis* strains and related species of the *Bacillus* genus. Although the two sets of MAbs had different fine specificities, all of them recognized the tested *B. anthracis* strains and showed only a limited cross-reactivity with two *B. cereus* strains. The MAbs were further tested for their ability to be implemented in a highly sensitive and specific bead-based Luminex assay. This assay detected spores from different *B. anthracis* strains and two cross-reactive *B. cereus* strains, correlating with the results obtained in direct spore ELISA. The Luminex assay (detection limit 10^3 to 10^4 spores per ml) was much more sensitive than the corresponding sandwich ELISA. Although not strictly specific for *B. anthracis* spores, the developed Luminex assay represents a useful first-line screening tool for the detection of *B. anthracis* spores.

Anthrax is an acute zoonotic disease caused by the spore-forming bacterium *Bacillus anthracis*. It affects primarily herbivores in many countries of Southern Europe, South America, Asia, and Africa (24). Endospores are the infecting agent and remarkably resistant to extreme heat, dryness, chemicals, or irradiation, thus ensuring long-term survival. The principal virulence factors are a capsule and two exotoxins produced by the growing vegetative form. The major sources of human anthrax infection are direct or indirect contact with infected animals. A reliable identification of *B. anthracis* is challenging, due to the monomorphic nature of the *B. cereus* group, which comprises *B. cereus*, *B. anthracis*, *B. thuringiensis*, and *B. mycoides* (10). The similarity of spore cell surface antigens of the bacteria of this group makes it difficult to create selective, reliable, antibody-based detection systems. DNA-based assays and traditional phenotyping of bacteria are the most accurate detection systems but are also complex, expensive, or slow. The use of *B. anthracis* spores as a biological weapon has stressed the need to learn more about spore components that can be used for efficient vaccines and rapid detection systems.

The *B. anthracis* endospore comprises a genome-containing core compartment and three protective layers called the cortex, coat, and exosporium (8). The glycoprotein *Bacillus* collagen-like protein of *anthracis* (BclA) is an immunodominant structural component of the exosporium that is extensively glycosylated with two *O*-linked carbohydrates, a 715-Da tetrasaccharide and a 324-Da disaccharide (6). The tetrasaccharide contains three rhamnose residues and an unusual terminal sugar, 2-*O*-methyl-4-(3-hydroxy-3-methylbutanamido)-4,6-dideoxy-D-glucopyranose, named anthrose. As anthrose was not found in spores of related strains of bacteria, it has been considered a potential biomarker for the detection of anthrax (6). In this study, the detection of *B. anthracis* spores was achieved by an assay based on the Luminex technology with monoclonal antibodies (MAbs) derived from mice immunized with anthrose-containing synthetic oligosaccharides.

MATERIALS AND METHODS

Generation of anti-anthrose-rhamnose disaccharide MAbs. The anthrose-containing synthetic carbohydrates were prepared as described previously (20, 22, 23). Mice carrying human immunoglobulin Cγ1 heavy and Cκ light chain gene segments (16) were immunized with an anthrose-rhamnose disaccharide conjugated to keyhole limpet hemocyanin (KLH) and formulated in ImmunEasy adjuvant (Qiagen AG, Hombrechtikon, Switzerland). Mice received 3 doses of 40 μg conjugate at 3-week intervals. Three days before cell fusion, a mouse received an intravenous booster injection with 40 μg of conjugate in phosphate-buffered saline (PBS). From the sacrificed mouse, the spleen was aseptically removed, and a spleen cell suspension in Iscove's modified Dulbecco's medium (IMDM) was mixed with PAI mouse myeloma cells as a fusion partner. Spleen and myeloma cells in a ratio of 1:1 were centrifuged; after the supernatant was discarded, the pellet was mixed with 1 ml prewarmed polyethylene glycol 1500 sterile solution. After 60 s, 10 ml of culture medium was added. After 10 min, cells were suspended in IMDM containing hypoxanthine, aminopterin, thymidine, and 20% fetal bovine serum and cultured in 96-well plates. Cells secreting disaccharide-specific IgG were selected using disaccharide-bovine serum albu-

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TABLE 1. Cross-reactivity of MAbs raised against anthrose-containing synthetic carbohydrates with spores of *Bacillus* spp.[a]

Bacillus sp.	Strain	Code	Plasmid content	OD from spore ELISA of:		Mean fluorescence intensity (Luminex assay)
				MTA1	MTD6	
B. anthracis	Ames	Ba0	pX01+ pX02+	**0.44**	**1.41**	**19,398**
	Böhm A58	Ba1	pX01− pX02−	**0.33**	**1.54**	**18,719**
	Böhm A1	Ba2	pX01+ pX02−	**1.01**	**1.66**	**7,229**
	Böhm A73202.2000	Ba3	pX01− pX02+	**1.31**	**2.16**	**21,591**
	NCTC 8234 Sterne	Ba4	pX01+ pX02−	**1.76**	**2.36**	**11,120**
	NCTC 02620	Ba7	pX01+ pX02+	**0.23**	**0.77**	**24,262**
B. cereus	ATCC 10876	Bc1		**1.52**	**2.09**	**23,710**
	ATCC 13061	Bc2		−0.09	−0.05	141
	ATCC 14579	Bc3		−0.04	−0.05	300
	ATCC 33019	Bc4		**0.24**	**1.15**	**5,964**
	ATCC 11778	Bc5		0.01	0.07	470
B. subtilis	ATCC 6051	Bs1		−0.10	−0.13	28
	ATCC 6633	Bs2		−0.10	−0.14	26
	ATCC 11774	Bs3		−0.11	−0.13	23
	Biocontrol AG	Bs4		ND	ND	38
B. thuringiensis	Kurstaki SP09	Bt1		−0.02	−0.03	47
	ATCC 10792	Bt2		−0.09	−0.13	33
B. atrophaeus	ATCC 9372	Bat		−0.10	−0.14	27
B. circulans	ATCC 61	Bcir		ND	ND	33
B. licheniformis	ATCC 12759	Bl1		−0.10	−0.11	73
	ATCC 14580	Bl2		−0.08	−0.12	27
B. megaterium	ATCC 9885	Bm1		−0.10	−0.13	23
	ATCC 14581	Bm2		−0.10	−0.13	28
B. sphaericus	ATCC 4525	Bsp		−0.08	−0.13	23
B. pumilus	ATCC 14884	Bp		−0.10	−0.13	28

[a] *B. anthracis* strains were chemically inactivated by paraformaldehyde with the exception of the Ames strain (Ba0), which was gamma irradiated. All the other *Bacillus* species and strains were not inactivated. Spores were used at a concentration of 1×10^6/ml. ELISA results are expressed as test optical densities minus two times the blank (blank, 0.10). Luminex values are shown as the mean fluorescence intensities from two experiments. Bolded values indicate MAb cross-reactive samples. ND, not done.

min (BSA)-coated enzyme-linked immunosorbent assay (ELISA) plates. Six hybridoma cell lines producing disaccharide-specific MAbs were identified, cloned twice by limiting dilution, and named MTD1 to MTD6. The production of anti-tetrasaccharide MAbs is described in reference 21.

Animals were housed in temperature-controlled rooms (22°C ± 3°C). Conventional laboratory feeding and unlimited drinking water were provided to the mice. Approval for animal experimentation was obtained from the responsible authorities, and all experiments were performed in strict accordance with the Rules and Regulations for the Protection of Animal Rights laid down by the Swiss Bundesamt für Veterinärwesen. All animal manipulations were performed under controlled laboratory conditions by specifically qualified personnel in full conformity with Swiss and European regulations.

Spore production and inactivation. Strains of *Bacillus* spp. (Table 1) were cultured on tryptone soya agar (Oxoid, Basel, Switzerland) at 37°C for 1 to 2 days. Then, the culture plates were kept in the dark at room temperature for 4 weeks. Colony material was suspended in sterile water, and spores were collected by centrifugation at 5,000 × *g* for 30 min at 4°C. To remove vegetative cells, spores were treated with 65% isopropanol for 1 h at room temperature and subsequently washed with sterile water until the supernatant appeared clear. The washed spores were stored in sterile water at 4°C, and the concentrations were determined by using a Thoma counting chamber. *B. anthracis* spores were inactivated by suspending 10^8 spores in 1 ml 10% paraformaldehyde for 1 h, subsequently washed with PBS, and recounted. For *Yersinia pestis* (ICM 1/41) and *Brucella melitensis* (NCTC 10094, biotype 1), inactivation was essentially done as described above using 3% formaldehyde. For both inactivation methods, sterility was verified by cultivation. Cultivation and inactivation of risk group 3 bacteria were done in a BSL-3 laboratory.

Enzyme-linked immunosorbent assay (ELISA). For the detection of saccharide-binding antibodies, Immulon 4 microtiter plates (Dynex Technologies Inc., Chantilly, VA) were coated at 4°C overnight with 50 μl of a 10-μg/ml solution of saccharide-BSA conjugate in PBS, pH 7.2. Wells were then blocked with 5% milk powder in PBS for 1 h at room temperature followed by three washings with PBS containing 0.05% Tween 20. Plates were then incubated with serial dilutions of MAbs in PBS containing 0.05% Tween 20 and 0.5% milk powder for 2 h at room temperature. After being washed, the plates were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (γ-chain-specific) antibodies (Sigma, St. Louis, MO) for 1 h at room temperature and then washed. Phosphatase substrate (1 mg/ml *p*-nitrophenyl phosphate) in buffer (0.14% Na_2CO_3, 0.3% $NaHCO_3$, 0.02% $MgCl_2$ [pH 9.6]) was added and incubated at room temperature. The optical density (OD) of the reaction product was recorded after an appropriate time at 405 nm using a microplate reader (Sunrise [Tecan Trading AG, Switzerland]).

For the detection of spore-binding antibodies, Maxisorp microtiter plates (Nunc/Thermo Fisher Scientific, Wohlen, Switzerland) were coated at 4°C overnight with 100 μl of a spore suspension in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Wells were then blocked with 3% BSA in PBS and washed with PBS containing 0.05% Tween 20. Wells were incubated with MAbs at a concentration of 1 μg/ml for 1 h. After being washed, the plates were incubated with peroxidase-conjugated goat anti-mouse IgG (γ-chain-specific) antibodies (KPL Inc.,

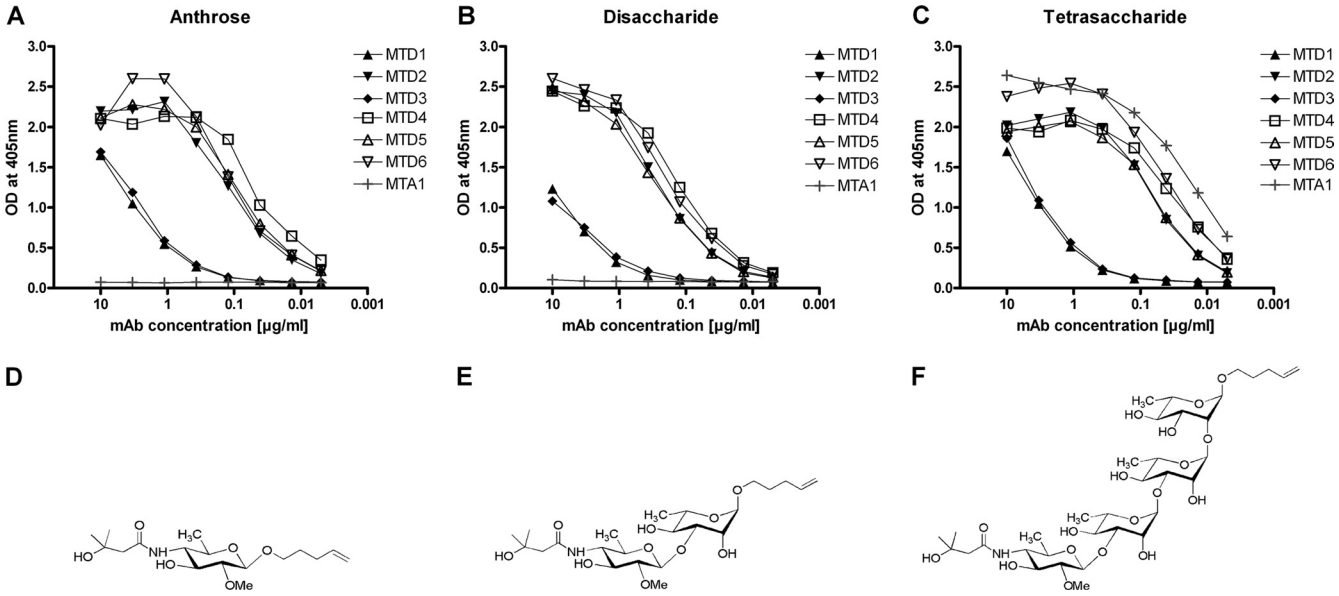

FIG. 1. Reactivity of anti-disaccharide MAbs with carbohydrate-BSA conjugates bound to ELISA microtiter plates. Shown are response patterns of individual anthrose-rhamnose disaccharide-specific MAbs (MTD1 to MTD6) and of a tetrasaccharide-specific MAb (MTA1) with anthrose-BSA (A), disaccharide-BSA (B), and tetrasaccharide-BSA (C). Structure of the synthetic anthrose (D), anthrose-rhamnose disaccharide (E), and tetrasaccharide (F).

Gaithersburg, MD) for 1 h and finally developed with the ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] substrate.

In antigen capture ELISA, microtiter plates were coated with 100 μl of a 10-μg/ml solution of unlabeled MAbs in PBS. After being blocked with 3% BSA in PBS and washed, wells were incubated with dilutions of spores in PBS. Biotinylated detection MAbs (5 μg/ml) were added and incubated for 1 h. Biotinylation was performed using the EZ-Link sulfo-NHS-biotin labeling kit (Pierce/Thermo Fisher Scientific Inc., Rockford, MA) according to the manufacturer's instructions. After repeated washing, streptavidin-peroxidase polymer conjugate (1 μg/ml) (Sigma, St. Louis, MO) was added and developed with the ABTS substrate.

Immunofluorescence assays (IFA). Inactivated spore suspensions were mixed with 2 volumes of a solution containing 4% paraformaldehyde. Droplets of 40 μl of cell suspension were added to each well of a diagnostic microscope slide (Flow Laboratories, Baar, Switzerland) and incubated for 30 min at room temperature. Spores were blocked with blocking solution containing 100 mg/ml fatty acid-free bovine serum albumin in PBS. Immunostaining was performed by incubating the wells with 25 μl of an appropriate MAb dilution in blocking solution in a humid chamber for 1 h at room temperature. After five washes with blocking solution, 25 μl of 5-μg/ml indocarbocyanine dye-conjugated affinity-pure $F(ab')_2$ fragment goat anti-mouse IgG heavy-chain antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted in blocking solution, was added to the wells and incubated for 1 h at room temperature. Finally, the wells were washed five times, mounted with ProLong gold antifade reagent (Invitrogen AG, Basel, Switzerland), and covered with a coverslip. Antibody binding was assessed by fluorescence microscopy.

Luminex assay. Antibody MTA1 (21) was coupled to MagPlex microspheres (Luminex Corporation, Austin, TX) according to the manufacturer's instructions and diluted in blocking buffer (1% BSA in PBS). In the coupling reaction, 6 μg of antibody was applied to 5×10^5 beads. For the assay, 2,000 beads in a volume of 50 μl were used per microtiter well. Fifty-microliter mixed bacterial samples were added to each bead-containing well and incubated for 2 h on a shaker at 37°C. After being washed with PBS containing 0.05% Tween 20, 50 μl of biotinylated detection antibody MTD6 diluted in blocking buffer was added to each well and incubated for 1 h, as described above. After repeated washing, 50 μl of a streptavidin-R phycoerythrin (ProZyme Inc., Hayward, CA) solution was added. Plates were incubated for 30 min as described above and washed. The beads were resuspended in 125 μl of blocking buffer, and the plate was placed on the shaker for 1 min. The assay was analyzed in a BioPlex 200 instrument (Bio-Rad Laboratories, Hercules, CA) by counting 100 beads per region. The data are reported as mean fluorescence intensities.

RESULTS

Generation and characterization of anti-anthrose-rhamnose disaccharide MAbs. Anthrose-rhamnose disaccharide-specific MAbs were generated basically in the same way as the previously described tetrasaccharide-specific MAbs MTA1 to MTA3 (20, 21). Chemically synthesized disaccharide (Fig. 1E) was covalently attached to the keyhole limpet hemocyanin (KLH) carrier protein by reductive amination. After repeated immunizations of mice with the disaccharide conjugate delivered with a CpG-based adjuvant (ImmunEasy, Qiagen), six anti-disaccharide MAbs (named MTD1 to MTD6) were generated that reacted with a synthetic disaccharide-BSA conjugate in ELISA (Fig. 1B). Analyses of the IgG subclass profiles of the induced disaccharide-specific MAbs showed a predominance of the mouse IgG1(λ) isotype; only MTD6 was of the IgG2b(λ) isotype.

While the binding patterns of disaccharide- and tetrasaccharide-specific MAbs differed, MAbs generated against the same antigen exhibited similar fine specificities. All anti-disaccharide MAbs showed cross-reactivity with the tetrasaccharide (Fig. 1C) and the anthrose monosaccharide (Fig. 1A), demonstrating that rhamnose moieties were not crucial structural elements of their epitopes. MAbs MTD1 and MTD3 showed lower affinities for the synthetic antigens than the other MAbs and were therefore not selected for the further assay development. The failure of MAbs from tetrasaccharide-immunized mice to bind to anthrose (Fig. 1A) and to the disaccharide (Fig. 1B) indicated that rhamnose sugars attached to anthrose were essential for recognition.

Cross-reactivity of the disaccharide-specific MAbs with endogenous tetrasaccharide expressed by *B. anthracis* strain Ba4 was established by indirect immunofluorescence assay (Fig. 2)

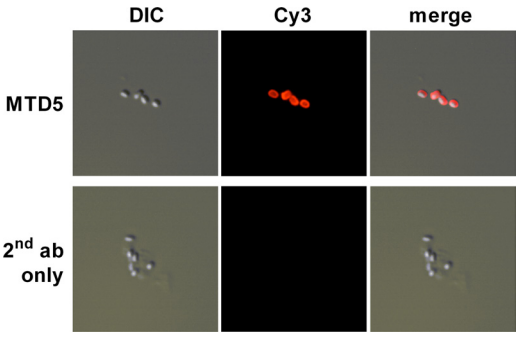

FIG. 2. Cross-reactivity of anti-disaccharide MAbs with *B. anthracis* strain Ba4 in IFA. Paraformaldehyde-inactivated endospores were stained with the anti-disaccharide MAbs and a secondary antibody conjugated to Cy3. All six MAbs yielded comparable results, and a representative staining is shown for MAb MTD5. Differential interference contrast (DIC) images and an overlay of both signals are also depicted.

and with immunoblotted *B. anthracis* endospore lysates (not shown).

Binding of the newly generated anti-disaccharide MAbs as well as of the anti-tetrasaccharide MAbs to spores of a broad spectrum of different *Bacillus* spp. was analyzed in a direct ELISA using plates coated with a spore suspension. Irrespective of their different fine specificities, both sets of MAbs recognized spores of the tested *B. anthracis* strains but also showed cross-reactivity with the *B. cereus* strains Bc1 and Bc4. Spores of none of the other *Bacillus* spp. were reactive with the MAbs. All tested MAbs showed uniform reactivity patterns, and representative results with the anti-tetrasaccharide MAb MTA1 and the anti-disaccharide MAb MTD6 are shown (Table 1).

Development of a Luminex assay for rapid detection of anthrax spores. To develop a highly sensitive and specific assay for the detection of anthrax spores from complex samples, the anti-carbohydrate MAbs were used for the development of a Luminex sandwich assay. The Luminex technology is based on fluorescent beads that are color coded. Each bead subset can be coated with a reagent specific for a particular analyte, allowing the capture and detection of this analyte from a complex sample. Within the BioPlex analyzer, lasers excite the internal dyes that identify each bead particle and also any reporter dye captured during the assay. Here, the anti-tetrasaccharide MAb MTA1 was coupled to magnetic beads and used as the capture antibody, and the biotinylated anti-disaccharide MAb MTD6 was used as the detection antibody. The Luminex assay detected the different *B. anthracis* strains and the *B. cereus* strains Bc1 and Bc4, correlating with the results obtained in direct spore ELISA (Table 1). For the *B. cereus* strains Bc2, Bc3, and Bc5, fluorescence background signals were weak at very high spore concentrations ($\geq 1 \times 10^6$ spores/ml) and were absent within one lower log stage of spores (not shown). Other antibody combinations were not adapted for the Luminex assay, since all MAbs showed similar reactivity patterns in direct spore ELISA.

The sensitivity of the developed bead-based assay was determined by analyzing a serial dilution of the *B. anthracis* spores. The limit of detection (LOD) was defined by the spore

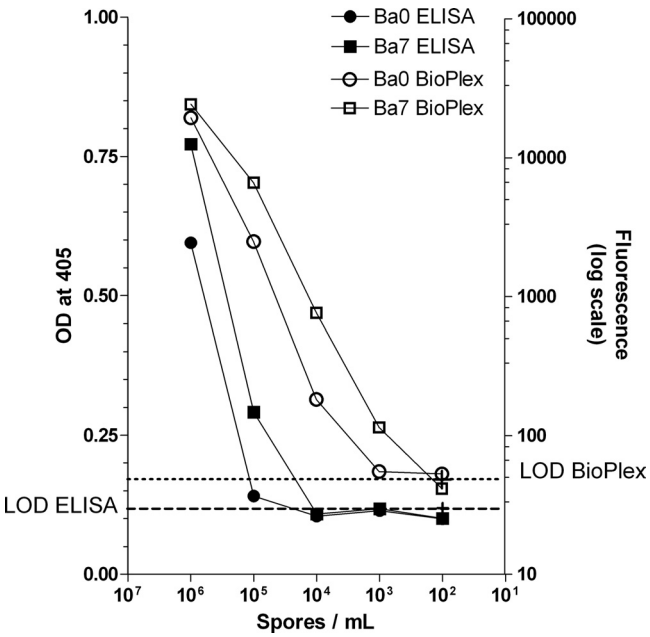

FIG. 3. Sensitivity of the Luminex assay compared to that of the corresponding antigen capture ELISA. The filled symbols represent ELISA (left *y* axis) values, and the open symbols represent the BioPlex (right *y* axis) values. Dashed lines indicate the limit of detection (LOD). BioPlex LOD was defined by two times the mean fluorescence intensity of the blank (mean blank, 26.75; standard deviation [SD], 0.35). Capture ELISA LOD was defined by the mean blank plus two times the SD and used as the threshold for positive results (mean blank, 0.105; SD, 0.007).

concentration yielding a signal two times as high as the mean fluorescence intensity of the blank (dashed lines in Fig. 3). Depending on the anthrax strain tested, the assay was able to detect 50 to 500 spores in a sample volume of 50 μl. The sensitivity of the Luminex assay was 10- to 100-fold higher than that of a corresponding antigen capture ELISA (Fig. 3), where at least 5×10^3 spores per 50 μl sample volume were required for an accurate detection. The developed Luminex assay for anthrax spore detection was further evaluated in mixed samples combining three inactivated bacterial species. In these complex samples, the anthrax spores were accurately detected, and no cross-reactivities were observed (Fig. 4).

DISCUSSION

Antibodies can provide the basis for specific and sensitive immunoassays for the diagnosis of infectious diseases (2, 3). The development of immunochemical assays specific for *B. anthracis* endospores has been hampered by the presence of cross-reactive antigens in closely related spores, in particular in *B. cereus*. Since the anthrose monosaccharide was not found in spores of the *B. cereus* T strain and a *B. thuringiensis* subsp. *kurstaki* strain (6), it has been considered a potential target antigen for the detection of *B. anthracis* spores. After the first chemical synthesis of the anthrose-containing tetrasaccharide (23), several synthetic approaches of the tetrasaccharide or corresponding sequences have been reported (1, 4, 9, 15, 17–19). Covalent attachment of the synthetic tetrasaccharide to a

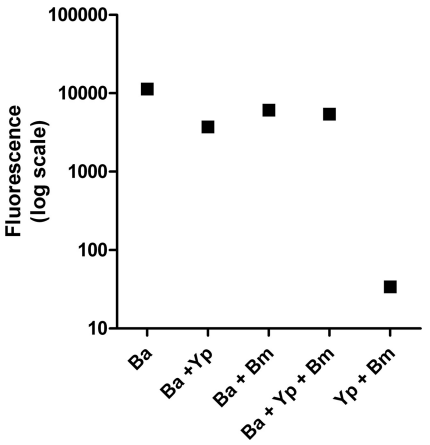

FIG. 4. Performance of a bead-based Luminex assay for the detection of anthrax spores in complex biological samples. Test samples contained *B. anthracis* Ba0 (Ba), *Y. pestis* (Yp), or *B. melitensis* (Bm) bacteria either alone or in combination. Shown are reporter dye fluorescence intensities measured for MTA1 beads. Bacteria were used at a concentration of 1×10^6 cells/ml.

carrier protein produced a carbohydrate-protein conjugate that was immunogenic in mice (21). Cross-reactivity of anti-tetrasaccharide MAbs with native *B. anthracis* endospores in immunofluorescence analysis confirmed the structural analysis of the tetrasaccharide and its expression on the endospore surface (21). Screening of anti-tetrasaccharide MAbs for cross-reactivity with immunoblotted spore lysates of panels of *B. anthracis* and *B. cereus* strains demonstrated the presence of anthrose in all *B. anthracis* strains tested and in some *B. cereus* strains (20). A recent genomic analysis demonstrated the presence of the anthrose biosynthetic operon in *B. cereus* strains (7). Additionally, structures similar to anthrose were found in the capsular polysaccharide of *Shewanella* spp. MR-4 and on flagella of *Pseudomonas syringae* (13).

Anti-tetrasaccharide MAbs (21) and anti-anthrose-rhamnose disaccharide MAbs described in this study were tested for their specificities in a direct ELISA using plates coated with spores of *B. anthracis* strains and related species of the *Bacillus* genus. Both types of MAbs recognized the tested *B. anthracis* strains but also showed cross-reactivity with two *B. cereus* strains, confirming the previously observed cross-reactivity with immunoblotted spore lysates of the same *B. cereus* strains (20). Kuehn and colleagues (14) generated one polyclonal antibody against the anthrose-containing tetrasaccharide that showed no cross-reactivities with other *Bacillus* strains in capture ELISA. Interestingly, the synthetic anthrose monosaccharide included the already-essential structural motifs required for binding of the anti-disaccharide MAbs. In contrast, the anti-tetrasaccharide MAbs recognized more complex epitopes that probably comprised all four sugar residues of the tetrasaccharide. Therefore, the target epitope might be better accessible for the anti-disaccharide MAbs. Biosafety containment requires an inactivation of probes containing *B. anthracis* spores. Inactivation by paraformaldehyde treatment did not destruct the epitopes recognized by the carbohydrate-specific antibodies, as also observed previously (14). Moreover, the sugar was also conserved on irradiated spores. Antigen conservation is not self-evident, since it was demonstrated that inactivation methods can affect the sensitivity of nucleic acid- and antibody-based assays for the detection of *B. anthracis* endospores (5).

Even though the MAbs generated against anthrose-containing structures were not strictly specific for *B. anthracis*, they still may represent a basis for the development of useful first-line screening platforms. Therefore, MAbs MTA1 and MTD6 were further tested as components of a highly sensitive immunodetection assay based on the Luminex technology platform. Use of the assay to detect individual spores of a broad range of *Bacillus* spp. strains correlated with the results obtained in direct spore ELISA, demonstrating the suitability of the bead-based platform to capture and detect spore particles. The sensitivity of spore detection by the Luminex assay was substantially increased compared to the corresponding capture ELISA, with the detection of 10^3 to 10^4 spores per ml. Currently available immunological detection systems offer higher detection limits (11, 12, 14). The platform can be also used in multiplex assays, and we are currently evaluating the simultaneous detection of different biothreat bacteria in mixed samples. In addition, we are testing the generated anti-carbohydrate monoclonal antibodies with field samples from different countries where anthrax is endemic to incorporate a broader genetic diversity of strains within the *B. cereus* group.

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