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Simultaneous Serodetection of 10 Highly Prevalent Mouse Infectious Pathogens in a Single Reaction by Multiplex Analysis

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Under current practices of mouse colony maintenance, sera from mice are analyzed for antibodies against several widespread infectious pathogens by conventional immunoassays, generally enzyme-linked immunosorbent assay (ELISA). To test for multiple agents, these methods consume large volumes of mouse serum and are laborious and time-consuming. More efficient immunoassays, using small amounts of sample, are therefore needed. Accordingly, we have developed a novel multiplex diagnostic system that employs fluorescent microbeads, coated with purified antigens, for simultaneous serodetection of 10 mouse infectious agents. Individually identifiable, fluorescent microbeads were coated with antigens from Sendai virus, mouse hepatitis virus, Theiler's mouse encephalomyelitis virus/GDVII strain, mouse minute virus, mouse cytomegalovirus, respiratory enteric orphan virus (Reo-3 virus), mouse parvovirus, calf rotavirus for epizootic diarrhea virus of infant mice, vaccinia virus for ectromelia virus, and *Mycoplasma pulmonis*. Standard sera, singly positive for antibodies to individual infectious agents, were generated by inoculation of BALB/cj and C57BL/6j mice. Sera from these experimentally infected mice, as well as sera from naturally infected mice, were analyzed using a mixture of microbeads coated with antigens of the 10 infectious agents listed above. Results demonstrated that the multiplex assay was at least as sensitive and specific as ELISA for serodetection. Importantly, the multiplex assay required only 1 microliter of serum for simultaneous serodetection of the 10 mouse infectious agents in one reaction vessel. Thus, this multiplex microbead assay is a reliable, efficient, and cost-effective diagnostic modality that will impact serosurveillance of mice used in research.

The mouse is the most widely used animal in biomedical research. Availability of specific-pathogen-free (SPF) mice for use in research is essential to obtain consistently accurate data. Experimental animals exposed to, or infected with, various infectious agents may yield questionable data, thereby confounding the findings of a given study. Mice may be screened for several important infectious pathogens (1, 3, 5, 6, 9, 10, 16, 17). Routine screening of a large number of animals, with respect to a large number of infectious agents, is a time-consuming and tedious task under current practices. Serosurveillance of mouse colonies is usually performed indirectly by introducing sentinel mice to animal rooms. After allowing for exposure, these sentinel mice are sacrificed, and their sera are tested by conventional immunoassays, such as enzyme-linked immunosorbent assay (ELISA) and/or indirect fluorescent-antibody assay (IFA). Because conventional immunoassays allow detection of only one infectious agent in a serum sample, large amounts of sample are consumed for the detection of multiple agents. Additionally, a composite of multiple individual tests requires much time, materials, and labor. This in turn encourages the use of sentinel mice instead of direct testing of individual mice in colonies. Direct monitoring of animals, for accurate knowledge of prevalence of common pathogens, is not only desirable for colony maintenance but critical for health care of valuable specialized mouse strains, such as genetically engineered mice. Furthermore, the increasing demand for research mice requires more efficient serodiagnostic assays that are readily amenable to a high-throughput format.

We have selected a relatively new technology, designated "multiple analyte profiling," from Luminex Corp. (Austin, TX), which allows simultaneous detection of multiple analytes in a small amount of sample (2). Up to 100 analytes can be measured in a single reaction. In addition, the multiple analyte profiling technology has been designed for high-throughput analysis. This method has been used to determine amounts of 15 different cytokines in small amounts of individual samples (2) as well as for detection of serum antibodies to multiple peptide epitopes (8), autoantigens (4), bacterial antigens (13, 14), and viral antigens (12).

The multiplex microbead assay is performed by the use of unique, fluorescently coded sets of polystyrene microbeads (5.6-μm diameter) (7, 15). A specific ratio of an orange and a red fluorophore is embedded within the matrix of a specific microbead set that enables identification of each bead set (7, 15). Microbead sets conjugated to known biomolecules are mixed and added to the test sample. Analytes in the sample react with biomolecules applied as a coating on the microbeads. Specific interactions are detected by a common reporter fluorochrome (e.g., phycoerythrin) conjugated to a secondary detection reagent. Thus, the multiplex microbead assay has a substantial advantage over the conventional immunoassay techniques with its ability to perform simultaneous detection of antibodies to several infectious agents in one reaction container.

This study focused on the development of a multiplex mi-

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crobead assay for detection of antibodies to 10 infectious agents in mice. Two strains of mice (BALB/cj and C57BL/6j) were inoculated with several viruses and *Mycoplasma pulmonis* to develop a panel of single-positive test sera. Microbeads coated with antigens from these agents were highly specific and sensitive for multiplex serodetection. Comparisons with ELISA and IFA demonstrated that the multiplex microbead assay is as sensitive and specific as these conventional assays in mouse serodetection. Furthermore, a volume as small as 1 microliter of serum is sufficient to perform detection of antibodies to multiple infectious agents.

MATERIALS AND METHODS

Purified viruses for conjugation to microbeads. Viruses purified by sucrose density centrifugation were purchased from Advanced Biotechnologies Inc. (Columbia, MD). These preparations were supplied at a total protein concentration of 1 mg/ml in phosphate-buffered saline (PBS) (pH 7.2). The purified viruses included mouse hepatitis virus (MHV), Theiler's mouse encephalomyelitis virus/GDVII strain (GD7), mouse minute virus (MMV), mouse cytomegalovirus (MCMV), Sendai virus, vaccinia virus, and Nebraska calf diarrhea virus (NCDV). Vaccinia virus and NCDV are cross-reactive to, and allow detection of, antibodies against ectromelia virus and epizootic diarrhea virus of infant mice (EDIM), respectively.

Cultures of viruses and *M. pulmonis*. GD7, MMV, and respiratory enteric orphan virus (Reo-3) were cultured in baby hamster kidney 21 (BHK-21) cells grown to confluency in Dulbecco's modified Eagle's medium containing 2 mM glutamine, penicillin/streptomycin, and 10% fetal bovine serum. Virus preparations were added to the confluent BHK-21 cells and allowed to adsorb to the cells at 37°C in the presence of 5% CO_2, for 1 h on a rocking shaker. Cells were cultivated in Dulbecco's modified Eagle's medium, without antibiotics, and containing 2% heat-inactivated fetal bovine serum and 2 mM glutamine. Infected cell cultures were maintained until cytopathic effects were evident (48 to 120 h). Tissue culture medium containing virus was collected, clarified by centrifugation at 1,000 × *g* for 10 min, and passed through a 0.45-μm-pore-size filter. Virus was stored at −80°C. For isolation of MMV, infected cells were collected by centrifugation at 1,000 × *g* for 10 min. Cells were harvested by treatment with 0.1% trypsin in 2 mM EDTA and resuspended in 5 ml of medium per T75 flask. To release the virus, infected cells were subjected to three sequential freeze-and-thaw cycles. Cell debris was removed by centrifugation at 1,000 × *g* for 10 min. The supernatant was clarified and stored frozen at −80°C. Virus titers were determined by endpoint dilutions. Titers of GD7 and Reo-3 were measured on BHK-21 cells, and 324K cells were used to establish the titer of MMV.

M. pulmonis, obtained from the American Type Culture Collection (Manassas, VA), was cultured and isolated per supplier's instructions. Cells were collected by centrifugation of culture media at 3,700 × *g* and washed three times with PBS. Antigen was extracted by adding lysis buffer (1% Triton X-100 in PBS, containing protease inhibitor cocktail [Roche Diagnostics GmbH, Indianapolis, IN]) to the cell pellet. The contents were mixed by vortexing and incubated at 4°C for 20 min, and lysate was filtered through an 0.45-μm-pore-size filter. Total protein concentration was measured by the Bradford method (Bio-Rad, Hercules, CA). The lysate was aliquoted and stored at −80°C until used for coating microbeads.

Production of positive sera. Mice of two different strains (BALB/cj and C57BL/6j) were inoculated with different infectious agents (24 mice per agent, per mouse strain). All mice were SPF and were a gift from Jax West Division of the Jackson Laboratory (Bar Harbor, ME). Two sources of virus preparation were used for inoculations. One was a known amount (measured as total protein) of sucrose-gradient-purified virus from Advanced Biotechnologies Inc. These included Sendai virus, vaccinia virus, and NCDV. Alternatively, infectious agents were grown and titer was determined as described above. These included GD7, MMV, Reo-3, and *M. pulmonis*.

A few animals (4 to 12) from each mouse strain were initially test inoculated with various dilutions of different infectious agents prepared in Hanks' minimal essential medium to estimate the minimal infectious dose. After the minimum dose was estimated, viruses were diluted to inoculate mice for the production of standard sera. The inoculation schedule is summarized in Table 1 for BALB/cj and in Table 2 for C57BL/6j mice. The natural route of infection was tested first. GD7, Reo-3, MMV, and Sendai virus were inoculated by intranasal route. For MHV, a cage of SPF mice was exposed to dirty bedding from MHV-infected mice. The MHV-infected mice that were used as a source of exposure were confirmed by ELISA to be seronegative for the other infectious agents including GD7, EDIM, ectromelia virus, MMV, mouse parvovirus (MPV), Sendai virus, *M. pulmonis*, and Reo-3. NCDV was inoculated by gastric lavage.

Mice were bled every 2 weeks from the retro-orbital sinus under anesthesia. Sera were collected and tested by ELISA kits from Charles River Laboratories (Wilmington, MA) for serodetection of individual infectious agents. If in 2 weeks the natural route of infection failed to yield an antibody response, mice were reinoculated by the natural route. If there was still no seroconversion after another 2 weeks, the mice were inoculated by intraperitoneal (i.p.) injection. For MMV, mice were initially inoculated twice by the intranasal route. Because these mice did not seroconvert, they were inoculated twice by the i.p. route. Two other infectious agents (e.g., *M. pulmonis* and vaccinia virus) were inoculated by the i.p. route alone. Mouse sera positive for antibodies to MPV, generated in C3H/HeN and BALB/c mice, inoculated with MPV-1b virus by the oronasal route, were a gift from David Besselsen at the University of Arizona. These sera were positive by ELISA for antibodies to MPV by the donor laboratory. Standard serum positive for MCMV was purchased from Charles River Laboratories (Wilmington, MA).

After all mice seroconverted, as determined by commercial ELISA, they were euthanized with carbon dioxide and exsanguinated by cardiocentesis. Serum from all animals was stored at −80°C.

Sera from naturally infected mice positive for antibodies to multiple infectious agents. Mouse sera from various sources are routinely submitted to the Comparative Pathology Laboratory, University of California, Davis, for antibody analysis. As tested by commercial ELISA kits, serum samples from seven mice in a naturally infected mouse population were found to be positive for antibodies to 6 of the 10 prevalent infectious agents.

Antigen preparation for coating microbeads and ELISA plates. Viral and *M. pulmonis* antigens, for use in immunoassays, were prepared by 1:1 mixing of the infectious agent preparation with 0.5% Triton X-100 (Fisher Scientific, Fairlawn, NJ) and protease inhibitor cocktail (Roche) in PBS (pH 7.2). NCDV was further treated with 1 mM EDTA and sonication at low setting (Virtis Virsonic 60; Gardiner, NY). Vaccinia virus preparations were subjected to inactivation by psoralen treatment as previously described (18). Briefly, psoralen (Sigma Chemicals, St. Louis, MO) was added at a final concentration of 10 μg/ml to the vaccinia virus antigen prepared above (0.5 mg/ml) and subjected to UV irradiation for 10 min at 254 nm (Chromato Vue Transilluminator, model T5-15; UVP, Inc., San Gabriel, CA). All antigen preparations were stored frozen at −80°C. Prior to use, preparations were thawed and centrifuged in a microcentrifuge at 10,000 × *g* for 10 min to remove debris.

Coupling antigens to microbeads. Microbeads were purchased from Luminex Corp. (Austin, TX). Various antigen preparations were chemically cross-linked to the microbeads according to the manufacturer's instructions. Bead stock was resuspended by vortexing and treatment in a sonicator bath (15 to 30 s) (Branson 1510; Danbury, CT). An aliquot of 2.5×10^6 beads was removed and centrifuged at 21,000 × *g* for 2 min. Beads were resuspended in 80 μl of activation buffer (100 mM monobasic sodium phosphate; pH 6.3) by vortexing and sonication (15 to 30 s).

To activate the beads for cross-linking to proteins, 10 μl of 50-mg/ml sulfo-*N*-hydroxysulfosuccinamide (Pierce, Rockford, IL) was added, and beads were mixed by vortexing. Then 10 μl of 50-mg/ml 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC; Pierce, Rockford, IL) was added, and beads were mixed again by vortexing. All incubations of beads were performed in the dark. The bead mixture was shaken on a rotary shaker at room temperature for 20 min and centrifuged at 21,000 × *g* for 2 min. Beads were washed twice with 250 μl of 50 mM morpholineethanesulfonic acid (MES; pH 6.0) buffer. To coat them with antigens, pelleted beads were resuspended in the relevant antigen preparation diluted in 50 mM MES (pH 6.0) buffer. Optimization of antigen concentration was performed by coating different microbead sets with a range of proteins between 10 and 200 μg/ml for each antigen. These microbead sets were then used to test against standard mouse sera, which are positive for antibodies to the relevant infectious agent. Microbead sets that provided the strongest specific signal for each antigen against the positive mouse sera were selected. The optimized protein concentration for each antigen was as follows: 25 μg/ml of vaccinia virus, 100 μg/ml of NCDV, 25 μg/ml of *M. pulmonis*, 100 μg/ml of MHV, 25 μg/ml of Sendai virus, 100 μg/ml of GD7, 50 μg/ml of CMV, and 100 μg/ml of MMV.

Recombinant protein rVP2, a specific antigen for MPV serodetection (9), was obtained from the Research Animal Diagnostic Laboratory (University of Missouri, Columbia, MO). Optimal concentration of rVP2 for coating on the beads was determined to be 3.3 μg/ml. For Reo-3, infected cell lysate was used at 330 μg/ml, and a separate bead set was coated with uninfected cell lysate (Reo-3 sham) at the same concentration.

Bead sets were also coated with biotin-conjugated goat immunoglobulin G

TABLE 1. Production of standard sera in BALB/cj mice

Pathogen	*n*	Inoculation route-dose[a]			Seroconversion[b] (% positive)	
		Primary	Secondary	Final	ELISA	Multiplex
Reo-3	24	i.n.−1.0 $TCID_{50}$	i.n.−1.0 $TCID_{50}$	None	100	100
Sendal virus	4	i.n.−1 μg/10 μl	None	None	100	100
	18	i.n.−1 μg/10 μl	i.n.−1 μg/10 μl	None	100	100
GD7	3	i.n.−2 × 10^6 PFU/10 μl	None	None	100	100
	4	i.n.−6 × 10^6 PFU/10 μl	None	None	100	100
	4	i.n.−3 × 10^6 PFU/10 μl	i.n.−6 × 10^6 PFU/10 μl	None	100	100
	4	i.n.−6 × 10^6 PFU/10 μl	i.n.−6 × 10^6 PFU/10 μl	None	100	100
	4	i.n.−2 × 10^6 PFU/10 μl	i.n.−2 × 10^6 PFU/10 μl	i.n.−6 × 10^6 PFU/10 μl	100	100
MHV	10	d.b.	None	None	60	50
	12	d.b.	d.b.	None	100	100
	4	5 d.b. inoculations at 2-wk intervals			100	100
	15	6 d.b. inoculations at 2-wk intervals			100	100
EDIM	11	g.l.−1 μg/100 μl	i.p.−1 μg/100 μl	i.p.−1 μg/100 μl	100	100
	4	g.l.−3 μg/100 μl	g.l.−3 μg/100 μl	i.p.−3 μg/100 μl	100	100
	4	g.l.−6 μg/100 μl	g.l.−6 μg/100 μl	i.p.−6 μg/100 μl	100	100
	3	g.l.−9 μg/100 μl	g.l.−9 μg/100 μl	i.p.−9 μg/100 μl	100	100
M. pulmonis	4	i.n.−40 μg/10 μl	i.n.−40 μg/10 μl	i.p.−40 μg/10 μl	100	100
	4	i.n.−77 μg/10 μl	i.n.−77 μg/10 μl	i.p.−77 μg/10 μl	100	100
	4	i.n.−150 μg/10 μl	i.n.−150 μg/10 μl	i.p.−150 μg/10 μl	100	100
	12	i.p.−150 μg/10 μl	i.p.−150 μg/10 μl	None	92	100
Vaccinia virus	24	i.p.−1 μg/100 μl	i.p.−1 μg/100 μl	i.p.−1 μg/100 μl	91	91
MMV	12	i.n.−1.0 $TCID_{50}$	i.n.−2.0 $TCID_{50}$	i.p.−2.0 $TCID_{50}$	41	41
	12	i.n.−1.0 $TCID_{50}$	i.p.−2.0 $TCID_{50}$	i.p.−2.0 $TCID_{50}$	75	75
	24	i.p.−3.0 $TCID_{50}$	i.p.−3.0 $TCID_{50}$	i.p.−3.0 $TCID_{50}$	100	100

[a] i.n., intranasal; d.b., dirty bedding; g.l., gastric lavage; $TCID_{50}$, 50% tissue culture infectious dose.
[b] For simplicity, only the final seroconversion results are presented.

(IgG; 100 μg/ml), a positive control protein for reaction with streptavidin conjugated to R-phycoerythrin (streptavidin-phycoerythrin), and bovine serum albumin (BSA;100 μg/ml) as a negative control protein (Pierce, Rockford, IL). For coupling, mixtures of activated beads and proteins were incubated by shaking on a rocker for 2 h at room temperature. After coating with proteins, beads were washed twice with 250 μl of wash buffer (0.1% Tween 20 in PBS, pH 7.4), resuspended in 250 μl of blocking buffer (1% BSA; 0.1% Tween 20 in PBS, pH 7.4; 0.05% sodium azide), and shaken on a rocker at room temperature for 30 min. After blocking, beads were resuspended in 1 ml of blocking buffer and stored at 4°C.

Multiplex detection of antibodies in mouse serum. Immunoreactions were done in 96-well, filter-bottomed plates designed for high-throughput separations (1.2-μm-pore-size MultiScreen; Millipore Corporation, Bedford, MA). Typically, 2,000 beads for each individual bead set, coated with a specific antigen, were added per well. For example, for a 13-plex assay, 2,000 beads from each set coated with an antigen were mixed to provide a total of 26,000 beads per well. Mouse serum was diluted 1:250 in 5% BLOTTO (Pierce, Rockford, IL), and 50 μl of this diluted serum was mixed with the 13-plex bead mixture per well. The mixture of beads and serum was then incubated on a shaker for 1 h at room temperature. After incubation, liquid was drained from the bottom of the plate in a vacuum manifold designed to hold 96-well plates (Millipore Corporation, Bedford, MA). The beads were washed two times by adding 100 μl of wash buffer per well and draining under vacuum. For detection of mouse IgG, biotinylated horse anti-mouse IgG was used (Vector Laboratories, Burlingame, CA) at a 1:1,000 dilution in BLOTTO, and 100 μl was added per well. Beads were mixed as before and incubated at room temperature for 30 min. Following incubation with the secondary antibody, beads were washed three times. To detect biotinylated IgG, 100 μl of streptavidin-phycoerythrin (CalTag, Burlingame, CA) was added at a dilution of 1:500 in wash buffer. The content of each well was mixed and incubated at room temperature for 15 min. Beads were washed once with wash buffer, resuspended in 100 μl of wash buffer per well, and analyzed in the Luminex-100 instrument equipped with XY-Platform for automatic reading of a 96-well plate.

Luminex-100 instrument operation and data analysis. The Luminex-100 instrument was manufactured by Luminex Corp. and purchased from Upstate USA Inc. (Lake Placid, NY). The instrument was used at default settings, set by the manufacturer for routine applications, as directed by the user's manual. Data were acquired by Luminex Data Collection software (version 1.7). This software package allowed routine operation of the instrument and data acquisition. Calibration beads, supplied by the manufacturer, were used to adjust instrument settings for bead set identification and for the detection of reporter (phycoerythrin). Events were gated to exclude doublets and other aggregates. A minimum of 100 independent, gated events were acquired for each bead set. A ratio of median fluorescence intensity (MFI) of antigen-coated bead sets to MFI of BSA-coated beads in each reaction was calculated. This ratio, designated as "signal," was used as a measure of antibody detection. After acquisition by Luminex software, the data were further processed by Microsoft Excel software. Background or normal serum reactivity for the antigen-coated bead set was determined. For each bead set, an average of signals from quadruplicate wells of pooled normal mouse sera was calculated, and three times the standard deviation value was added to the average. For routine analysis, each experiment also contained a positive-control serum for all the infectious agents. Samples were analyzed in duplicate, and a minimum of two separate multiplex immunoassay experiments was performed. Sera were designated positive for antibodies to an infectious agent if the signal from the relevant antigen-coated bead set was greater than the background.

IFA. IFA analysis of serum samples was performed by Charles River Laboratories (Wilmington, MA).

TABLE 2. Production of standard sera in C57BL/6j mice

Pathogen	*n*	Inoculation route-dose[a]			Seroconversion[b] (% positive)	
		Primary	Secondary	Final	ELISA	Multiplex
Reo-3	23	i.n.−1.0 $TCID_{50}$	i.n.−1.0 $TCID_{50}$	None	100	100
Sendai	23	i.n.−1 μg/10 μl	None	None	100	100
GD7	2	i.n.−2×10^6 PFU/10 μl	None	None	100	100
	20	i.n.−6×10^6 PFU/10 μl	None	None	73	77
	2	i.n.−2×10^6 PFU/10 μl	i.n.−2×10^6 PFU/10 μl	i.n.−6×10^6 PFU/10 μl	0	0
MHV	28	d.b.	None	None	46	46
	4	4 d.b. inoculations at 2-wk intervals			100	100
	20	6 d.b. inoculations at 2-wk intervals			100	100
EDIM	12	g.l.−1 μg/100 μl	i.p.−1 μg/100 μl	i.p.−1 μg/100 μl	100	100
	12	i.p.−1 μg/100 μl	i.p.−1 μg/100 μl	None	100	100
M. pulmonis	4	i.n.−40 μg/10 μl	i.n.−40 μg/10 μl	i.p.−40 μg/10 μl	100	100
	4	i.n.−77 μg/10 μl	i.n.−77 μg/10 μl	i.p.−77 μg/10 μl	100	100
	4	i.n.−150 μg/10 μl	i.n.−150 μg/10 μl	i.p.−150 μg/10 μl	100	100
	12	i.p.−150 μg/10 μl	i.p.−150 μg/10 μl	None	92	100
Vaccinia virus	21	i.p.−1 μg/100 μl	i.p.−1 μg/100 μl	i.p.−1 μg/100 μl	90	100
MMV	12	i.n.−1.0 $TCID_{50}$	i.p.−1.0 $TCID_{50}$	i.p.−1.0 $TCID_{50}$	100	100
	12	i.n.−1.0 $TCID_{50}$	i.n.−2.0 $TCID_{50}$	i.p.−1.0 $TCID_{50}$	83	91

[a] i.n., intranasal; d.b., dirty bedding; g.l., gastric lavage; $TCID_{50}$, 50% tissue culture infectious dose.
[b] For simplicity, only the final seroconversion results are presented.

ELISA. Two types of ELISA systems were used. One system consisted of preconfigured commercial kits for the serodetection of different mouse infectious agents. These ELISA kits, purchased from Charles River Laboratories (Wilmington, MA), were used to test mouse sera according to the manufacturer's instructions. The other ELISA system was developed with the purified antigens that were also used in the development of the multiplex microbead assay. This ELISA was used for routine testing of the antigens prior to coating on the microbeads. Immulon 4 HBX, flat-bottomed microtiter plates (ThermoLabsystems, Franklin, MA) were coated with viral preparations in the following optimized protein concentrations: Sendai virus was used at 0.1 μg/ml, MHV at 0.01 μg/ml, GD7 at 1.0 μg/ml, MMV at 3.0 μg/ml, MCMV at 1.0 μg/ml, NCDV at 0.01 μg/ml, and vaccinia virus at 0.3 μg/ml. MPV recombinant antigen was used at 0.1 μg/ml. Reo-3-infected and uninfected cell lysates were used at 50.0 μg/ml each, and *M. pulmonis* lysate was used at a 10.0-μg/ml total protein concentration. Antigens were diluted to a final optimized concentration in coating buffer: Hanks balanced salt solution containing 0.375% sodium bicarbonate. Antigens were added to plates (100 μl/well) and incubated overnight at 4°C. Plates were washed with wash buffer (0.1% Tween 20 in PBS, pH 7.4) and blocked with BLOTTO (5% nonfat powdered milk in wash buffer) for 2 h at room temperature. To each well, 100 μl of mouse serum, diluted 1:250 in BLOTTO, was added. Plates were allowed to incubate at room temperature for 1 h and washed again with wash buffer. Detection antibody, biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA), was diluted 1:10,000 in wash buffer and added to the ELISA plate at 100 μl/well. Plates were incubated with the detection antibody for 30 min at room temperature. After washing, 100 μl of Vectastain ABC detection reagent, containing horseradish peroxidase H (Vector Laboratories), was added to each well. Plates were washed and developed by adding 100 μl/well of TMB color development reagent (Sigma Chemicals, St. Louis, MO). Plates were developed at room temperature for 10 to 12 min, and the reaction was stopped by adding 50 μl/well of 1 M sulfuric acid. Color development was measured at 450 nm in the ELISA plate reader (Bio-Rad, Hercules, CA).

RESULTS

Production of standard, positive sera in BALB/cj and C57BL/6j mice. To generate standard sera, 24 BALB/cj and 24 C57BL/6j mice were inoculated with individual infectious agents. Inoculations were initially attempted by various routes of exposure to mimic transmission under colony conditions. If this route failed to produce a serological immune response, as detected by commercially purchased ELISA, then the i.p. route of inoculation was used. Inoculation routes, viral doses, and the final seroconversion results are presented for both mouse strains in Tables 1 and 2.

Standard serum production in BALB/cj mice (Table 1). Five viruses, Sendai virus, GD7, Reo-3, MHV and NCDV, produced infections in BALB/cj mice inoculated by the natural route. However, more than one dose was usually required to induce seroconversion in most of the animals. For NCDV, a majority of the mice were inoculated by the i.p. route, which resulted in 100% seroconversion (Table 1). For MVM, *M. pulmonis*, and vaccinia virus, mice were inoculated by the i.p. route alone. These three agents failed to generate antibodies by other routes of exposure (Table 1).

Standard serum production in C57BL/6j mice (Table 2). The course of infection and antibody generation in the C57BL/6j mice was similar to that described above for BALB/cj mice. However, it appears that inoculations in C57BL/6j mice by Sendai virus or GD7 virus were more successful in generating antibody responses than in BALB/cj mice. For example, 23 mice inoculated with Sendai virus and 22 mice inoculated with GD7 by the intranasal route became seropositive after only one inoculation. In contrast, most BALB/cj exposed to Sendai virus or GD7 virus by routes mimicking transmission under colony conditions required reinoculation for the induction of antibody responses.

Analysis of single-positive mouse sera by multiplex microbead assay. Serum samples, representative of individual groups of mice inoculated with specific infectious agents, were analyzed by multiplex microbead assay. Individual, antigen-coated and control microbeads were mixed to produce the

TABLE 3. Antibody detection in known singly positive sera by multiplex microbead assay[a]

Serum sample	Dilution	MFI ratio of antigen beads to BSA beads for antigen-coated microbead set:									
		Vaccinia virus	NCDV	Reo-3	MP	MHV	Sendai virus	GD7	MPV	CMV	MMV
Background		0.28	0.23	0.31	0.13	0.26	0.58	0.24	0.17	0.25	0.50
Ectro	1:2 × 10^4[b]	**167.0**	0.03	0.00	0.03	0.00	0.05	0.00	0.00	0.00	0.00
EDIM	1:250	0.19	**63.0**	0.29	0.09	0.08	0.43	0.03	0.11	0.11	0.42
Reo-3	1:250	0.14	0.16	**189.0**	0.16	0.16	0.27	0.00	0.01	0.15	0.34
MP	1:250	0.13	0.13	0.29	**19.0**	0.23	0.10	0.00	0.00	0.34	0.73
MHV	1:250	0.08	0.04	0.30	0.11	**12.0**	0.10	0.00	0.10	0.27	0.33
Sendai virus	1:250	0.17	0.24	0.35	0.14	0.18	**153.0**	0.00	0.00	0.06	0.44
GD7	1:250	0.07	0.15	0.26	0.15	0.11	0.37	**12.0**	0.02	0.09	0.35
MPV	1:250	0.09	0.11	0.25	0.08	0.08	0.17	0.00	**11.0**	0.01	0.31
CMV	AD	0.05	0.05	0.18	0.08	0.64	0.08	0.00	0.04	**27.0**	0.06
MMV	1:250	0.22	0.20	0.62	0.14	0.30	0.17	0.35	0.20	0.21	**5.00**

[a] Results are averages of two independent experiments. Boldface indicates the most specific results. Abbreviations: MP, *M. pulmonis*; Ectro, ectromelia virus; AD, as directed by the manufacturer (Charles River, MA).
[b] Higher dilution needed due to high antibody titer.

13-plex bead mixture (see Materials and Methods). Sera were diluted 1:250, unless stated otherwise, and incubated with the 13-plex microbead mixture. The results of the multiplex assay, presented in Table 3, show that specific antibodies to different infectious agents were detected with high sensitivity by the antigen-coated bead sets. Importantly, these beads did not display a significant nonspecific reaction to mouse sera, compared to background signal from normal mouse sera. Thus, these findings demonstrate that the multiplex microbead assay is highly specific and sensitive for the detection of antibodies to the panel of 10 mouse infectious agents.

Analysis of mixed, single-positive mouse sera by multiplex microbead assay. The ability of the multiplex microbead assay to detect antibodies against several infectious agents in single serum samples was tested. Because mouse sera positive for antibodies to all nine viruses and *M. pulmonis* are rare, the sera in Table 3 were mixed in a 1:1 ratio to simulate multiple infections. In addition, several combinations of the mixed positive sera, where one positive serum was omitted at a time, were also prepared. The final dilution of the mixed sera was the same as in Table 3. Various mixtures of positive sera were incubated with the mixture of antigen-coated microbeads. The results demonstrate that in both the mixture containing all of the positive sera and the mixtures where one positive serum sample was omitted, the multiplex microbead assay detected specific antibodies (Table 4). Two observations are noteworthy. First, for 7 of 10 antigen-coated microbead sets (vaccinia virus, NCDV, *M. pulmonis*, MHV, Sendai virus, CMV, and MMV), the nonspecific reactivity was slightly higher than the background. This increase may be the result of interserum interactions of sera from different animals. Second, the values of positive serum reactivity to each microbead set were about half of those observed in analysis of the single-positive sera in Table 3. The simplest explanation for this reduction in specific signal is that there may be negative interference for antibody detection when sera from multiple animals are mixed. Interestingly, in the absence of *M. pulmonis*-positive serum, a higher reactivity in the serum mixture towards all the microbead sets was observed.

Multiplex microbead assay for serodetection in mice infected with multiple infectious agents. To evaluate the multiplex microbead assay under field conditions, sera from mice naturally infected with multiple infectious agents were tested. Seven serum samples, positive for antibodies to multiple infec-

TABLE 4. Multiplex microbead assay on mixed, singly positive mouse sera from Table 3[a]

Serum sample	Dilution[b]	MFI ratio of antigen beads to BSA beads for antigen-coated microbead set:									
		Vaccinia virus	NCDV	Reo-3	MP	MHV	Sendai virus	GD7	MPV	CMV	MMV
Background		0.28	0.23	0.31	0.13	0.26	0.58	0.24	0.17	0.25	0.50
All sera	1:250	41.8	25.3	97.6	10.9	7.40	70.3	5.65	4.45	13.1	3.48
Ectro (−)	1:250	**0.33**	23.1	93.0	12.5	6.00	60.8	5.60	4.30	12.8	3.30
EDIM (−)	1:250	42.1	**0.39**	93.9	12.0	6.70	65.23	5.60	4.00	13.2	3.10
Reo-3 (−)	1:250	43.1	23.92	**0.29**	12.8	7.34	69.6	6.06	4.62	13.4	3.37
MP (−)	1:250	55.3	32.7	135	**0.24**	9.70	89.0	8.11	6.00	17.1	3.50
MHV (−)	1:250	41.0	23.7	96.8	12.1	**0.82**	65.9	5.67	4.21	12.6	3.52
Sendai virus (−)	1:250	45.8	24.2	100	12.7	7.14	**0.65**	6.20	4.60	13.0	3.10
GD7 (−)	1:250	43.6	24.1	96.0	12.0	6.80	66.4	**0.04**	4.30	12.8	3.27
MPV (−)	1:250	43.5	22.8	96.5	11.6	6.81	62.4	5.64	**0.00**	12.7	3.20
CMV (−)	1:250	45.6	23.8	93.7	10.8	6.11	68.0	5.50	4.22	**0.48**	2.90
MMV (−)	1:250	41.0	22.2	100	11.8	7.01	66.7	7.70	4.13	12.4	**1.56**

[a] Results are averages of two independent experiments. MP, *M. pulmonis;* Ectro, ectromelia virus. Boldface indicates the least specific results.
[b] Final dilution of each serum sample in the mixture.

TABLE 5. Multiplex assay for serodetection in field samples of mice[a]

Serum sample	Dilution	MFI ratio of antigen beads to BSA beads for antigen-coated microbead set:									
		Vaccinia virus	NCDV	Reo-3	MP	MHV	Sendai virus	GD7	MPV	CMV	MMV
Background		0.13	0.33	0.51	0.26	0.63	0.87	0.31	0.08	0.85	0.71
4029	1:250	0.13	34.5[b,c]	0.68[e]	0.14	23.3[b,c]	0.09	13.8[b,c]	0.00	0.55	0.31
4030	1:250	0.05	14.3[b,c]	0.15	0.08	19.6[b,c]	0.03	11.1[b,c]	0.00	0.53	0.16
4031	1:250	0.34	13.0[b,c]	0.28	0.20	25.5[b,c]	0.43	11.7[b,c]	31.0[b,c]	0.81	0.52
4032	1:250	0.14	34.7[b,c]	0.00	0.15	24.5[b,c]	0.18	9.11[b,c]	48.4[b,c]	0.45	3.43[b,c]
4033	1:250	0.16	9.52[b,c]	0.55[e]	0.30	21.7[b,c]	0.12	0.05	0.00	0.38	2.30[b]
4034	1:250	0.14	7.60[b,c]	0.15	0.16	29.2[b,c]	0.10	9.60[b,c]	151[b,c]	0.75	1.67[b,c]
4035	1:250	0.09	3.74[b,c]	2.13[b,d]	0.17	14.1[b,c]	0.09	2.72[b,c]	46.8[b,c]	0.26	0.44

[a] Results are averages of two independent experiments. MP, *M. pulmonis.* Serum samples are from individual mice.
[b] Positive by the multiplex microbead assay.
[c] Confirmed as positive by ELISA.
[d] Negative by ELISA but confirmed positive by IFA.
[e] Negative by ELISA and indeterminate by multiplex assay and IFA.

tious agents, as determined by commercial ELISA kits, were obtained from mice in an enzootically infected colony. Multiplex microbead analysis was performed in a blinded experiment. Diluted sera (1:250) were incubated with the 13-plex microbead mixture. A strong correlation was observed between the results obtained by commercial ELISA kits and the multiplex microbead assay (Table 5). All animals that were positive by ELISA for antibodies to EDIM ($n = 7$), MHV ($n = 7$), GD7 ($n = 6$), MPV ($n = 3$), MMV ($n = 2$), and Reo-3 ($n = 1$) were also positive for the same agents by the multiplex assay. Additionally, multiplex analysis detected antibodies to Reo-3 in one animal (4035) that tested negative by ELISA; this animal was subsequently confirmed to be positive for antibodies to Reo-3 by IFA. Two other mice, 4029 and 4033, were indeterminate for Reo-3 antibodies by the multiplex microbead assay; sera from these mice displayed values slightly above the background level (Table 5). Both of these mice were indeterminate for Reo-3 antibodies by IFA as well. One serum sample that was positive for antibodies to MMV (4033) by the multiplex microbead assay was negative for MMV not only by ELISA but also by IFA. It is possible that the analysis of this animal represents a false-positive result by the multiplex microbead assay. Alternatively, the multiplex assay may be more sensitive than ELISA and IFA for the detection of antibodies to MMV.

DISCUSSION

This report describes the development of a multiplex microbead assay to detect antibodies to 10 infectious agents, using only 1 microliter of mouse serum. A major advantage of the multiplex format is its ability to detect antibodies to several antigens in a small amount of sample; this feature substantially saves sample, time, and labor compared to conventional immunoassays. The multiplex microbead technology is very flexible, because microbead sets conjugated with different antigens can be mixed to include only those that are needed; this feature avoids waste of important antigens. The flexibility of microbead multiplex system also allows incorporation of internal controls directly into the test sample (11). This built-in system of controls enables the direct and simultaneous measurement of specificity and sensitivity in detection of immune responses to multiple antigens. Additionally, in comparison to ELISA, the multiplex microbead assay provides a much larger dynamic range, which allows accurate analysis of antibody levels without additional dilution of sera with high antibody titers. Furthermore, the multiplex microbead assay is readily adaptable to high-throughput format. This adaptability is important for handling a large number of mouse samples for serodetection.

To develop the multiplex microbead assay system, we produced mouse sera positive for antibodies to individual infectious agents (Tables 1 and 2). Attempts were made to inoculate these mice by routes mimicking natural transmission. Because our goal was to develop multiplex serodetection with wide applicability to mouse strains that range in magnitude of antibody responses, the BALB/cj and C57BL/6j strains were used to produce monospecific sera. Sera from these experimentally inoculated mice were critical for unambiguous evaluation of sensitivity and specificity of the multiplex microbead assay. Mouse sera obtained from commercial sources are generated by inoculating mice with a mixture of several antigens. These sera may be positive for antibodies to multiple agents but are provided as positive for only one agent; antibodies to any other agents, if present, are not specified. Such sera may be useful to test the performance of the conventional immunoassays such as ELISA, IFA, and Western blotting. However, these commercial sera are unsuitable for use in the multiplex microbead assay. This complication was further exacerbated by lot-to-lot variation of the sera, resulting in high titers against a different set of antigens in each lot. Accordingly, the monospecific sera produced in this project will be valuable reference material for future assay development and validation, particularly as the multiplex microbead assay is expanded for serodiagnosis of additional infectious agents.

The multiplex serodiagnostic assay was tested by using sera from animals containing antibodies to single infectious agents. As shown in Table 3, microbeads coated with individual antigens reacted to mouse sera in a specific manner. The data also demonstrated that the microbeads did not display nonspecific reactivity to sera containing irrelevant antibodies. The specificity of the multiplex microbead assay was further tested in simultaneous serodetection of multiple agents in a single reaction. Two experiments were performed: (i) standard positive sera were mixed and reacted against the antigen-coated bead

sets (Table 4) and (ii) field sera from mice infected with several agents were obtained from a mouse facility and tested (Table 5). Both experiments clearly demonstrated a high degree of sensitivity and specificity for the multiplex microbead assay. In Table 4, where positive sera from several mice were mixed, minor nonspecific reactivity of the antigen-coated beads was observed, e.g., in the serum mixture lacking MMV-positive serum, resulting in a decrease in the signal-to-noise ratio. These results may reflect potential interactions and interference among sera from different animals. However, any such inconsistencies were resolved in sera from animals infected with multiple agents, yielding results that were clearly interpretable (Table 5). Similar types of minor interferences have also been observed in multiplex immunoassay in a mixture of individual sera from rhesus macaques positive for antibodies to a single infectious agent. Again, such interferences were not observed in individual rhesus macaque sera positive for antibodies to multiple infectious agents (I. Khan and P. Luciw, unpublished data).

Clinical validation of the multiplex microbead assay will require analysis of a large number of field serum samples that are also tested by conventional immunoassays, which detect antibodies to one infectious agent at one time. Accordingly, several thousand samples will be analyzed to establish statistically significant levels of sensitivity and specificity. In addition, a high-throughput format of the multiplex microbead assay is currently under evaluation to facilitate validation.

The increased efficiency of the multiplex microbead immunoassay and the requirement for only one microliter of serum will promote direct testing of individual mice. In current practice for health maintenance of SPF colonies, indirect testing by the use of sentinel mice may have certain disadvantages. For example, a recent report showed that some sentinel mice used in serosurveillance were infected with MPV at the time of arrival from the rodent vendor and were the source of MPV infection in the colony animals (16). Thus, direct testing of mice may improve the accuracy of health monitoring in mouse colonies. Because the multiplex microbead assay requires only 1 microliter of sample, this method is ideally suited for serosurveillance of mouse colonies in general, and in particular of more valuable mice, such as genetically engineered lines. In addition, the ability to detect and measure multiple analytes in a small volume of serum (or plasma) allows for analysis of samples collected in the live phase of a research protocol.

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