AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology®

A Bead-Based Flow Cytometric Assay for Monitoring *Yersinia pestis* Exposure in Wildlife

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ABSTRACT *Yersinia pestis* is the causative agent of plague and is considered a category A priority pathogen due to its potential for high transmissibility and the significant morbidity and mortality it causes in humans. *Y. pestis* is endemic to the western United States and much of the world, necessitating programs to monitor for this pathogen on the landscape. Elevated human risk of plague infection has been spatially correlated with spikes in seropositive wildlife numbers, particularly rodent-eating carnivores, which are frequently in contact with the enzootic hosts and the associated arthropod vectors of *Y. pestis*. In this study, we describe a semiautomated bead-based flow cytometric assay developed for plague monitoring in wildlife called the F1 Luminex plague assay (F1-LPA). Based upon Luminex/Bio-Plex technology, the F1-LPA targets serological responses to the F1 capsular antigen of *Y. pestis* and was optimized to analyze antibodies eluted from wildlife blood samples preserved on Nobuto filter paper strips. In comparative evaluations with passive hemagglutination, the gold standard tool for wildlife plague serodiagnosis, the F1-LPA demonstrated as much as 64× improvement in analytical sensitivity for F1-specific IgG detection and allowed for unambiguous classification of IgG status. The functionality of the F1-LPA was demonstrated for coyotes and other canids, which are the primary sentinels in wildlife plague monitoring, as well as felids and raccoons. Additionally, assay formats that do not require species-specific immunological reagents, which are not routinely available for several wildlife species used in plague monitoring, were determined to be functional in the F1-LPA.

KEYWORDS Bio-Plex, Luminex, *Yersinia pestis*, plague, serodiagnosis, wildlife

Plague is a potentially severe zoonosis caused by the gammaproteobacterium *Yersinia pestis*. In areas where it is endemic, which includes the western United States, *Y. pestis* is generally considered to be maintained within certain rodent and flea species (1). Spillover into susceptible epizootic hosts can occur, with most mammals, including humans, susceptible to *Y. pestis* infection/colonization (1). During such spillover events, spikes in the number of *Y. pestis*-seropositive wildlife from the corresponding area are thought to be indicative of increased human risk (2–5). In addition, *Y. pestis* infects domestic pets such as dogs and cats, is a primary threat to the endangered black-footed ferret (*Mustela nigripes*), and infects, sometimes fatally, a large number of other wildlife species (6–8).

Landscape-level serological monitoring of rodent-eating carnivores is considered an important tool for assessing plague risk (2). The rationale behind this strategy is that these carnivores routinely contact the enzootic hosts and the associated arthropod vectors of *Y. pestis* through predation and scavenging, subsequently exposing them to the agent. Therefore, sampling of one carnivore can be equivalent to sampling hun-

Received 20 February 2018 **Returned for modification** 20 March 2018 **Accepted** 19 April 2018

Accepted manuscript posted online 25 April 2018

Citation Chandler JC, Baeten LA, Griffin DL, Gidlewski T, DeLiberto TJ, Petersen JM, Pappert R, Young JW, Bevins SN. 2018. A bead-based flow cytometric assay for monitoring *Yersinia pestis* exposure in wildlife. J Clin Microbiol 56:e00273-18. https://doi.org/10.1128/JCM.00273-18.

Editor Brad Fenwick, University of Tennessee at Knoxville

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dreds of rodents (3), ultimately lowering the cost and burden of plague monitoring compared to the testing of rodents and fleas. Monitoring of wildlife for *Y. pestis* exposure is partially accomplished through the Wildlife Service's National Wildlife Disease Program (NWDP) within the United States Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS), in collaboration with the United States Centers for Disease Control and Prevention (CDC), with over 69,000 samples collected from 2000 to 2017 (9). Coyotes (*Canis latrans*) are the main sentinel species used in NWDP's plague monitoring program, as they can develop a strong anti-plague antibody response but are generally resistant to clinical disease (2, 6, 10). Blood samples from these and other animals are collected on Nobuto strips during the course of standard wildlife damage management operations in targeted areas and then transported to a reference laboratory for serological analysis (9, 11).

NWDP's plague monitoring program has historically used a passive hemagglutination assay (PHA) and passive hemagglutination inhibition (PHI) assay that measures total antibody directed against the *Y. pestis* F1 capsular antigen (F1), the most specific and widely used serodiagnostic plague antigen (12–16). However, interpretation of this test is subjective, and interpretation of titer may be biased to antibodies produced early in the immune response (IgM), which are widely considered to be more effective in agglutination assays (17, 18). These limitations, along with the availability of platforms reported to provide unambiguous data interpretation and the ability to differentiate between antibody isotypes, suggests that a new generation of tools for wildlife plague monitoring can be developed.

Splettstoesser et al. previously described a novel plague serodiagnostic that utilized paramagnetic polystyrene beads conjugated to F1 which could be reacted against human sera (19). Following the addition of fluorescein-labeled secondary antibody to the reacted beads, F1 seroreactivity could be semiquantitatively assessed via flow cytometric analyses. The performance of this assay was comparable, if not more sensitive than, to traditional plague serodiagnostics, including enzyme-linked immunosorbent assay and immunoblotting. Multiplexable bead-based flow cytometric serodiagnostic assays are now routinely performed on commercially available systems (20, 21) and have been coupled to semiautomated bead handling to improve reproducibility and throughput while reducing technician effort (22, 23). However, in many instances, the assay must be designed and optimized by the user. The objective of the present study was to develop a bead-based flow cytometric serodiagnostic assay, named the F1 Luminex plague assay (F1-LPA), for detecting antibodies in wildlife exposed to *Y. pestis*. Comparative testing with PHA and F1 immunoblotting showed that the F1-LPA was functional with canid, felid, and raccoon samples, with high analytical sensitivity for F1-specific IgG detection.

MATERIALS AND METHODS

Serum and blood samples. Serum samples were obtained from experimentally infected coyotes from a previous study (10). Whole-blood samples from coyotes and other wildlife were collected on Nobuto strips as previously described by the NWDP canid plague monitoring program (11).

For the F1-LPA assay, the elution of antibodies from Nobuto strips was accomplished by incubating the Nobuto strips, contained within a 1.5-ml tube, overnight at 4°C with 1 ml BupH Tris-buffered saline (pH 7.2) (Thermo Fisher, Waltham, MA) (TBS) containing 3% nonfat dried milk (Sigma-Aldrich, Saint Louis, MO) and 0.1% Tween 20 (Sigma-Aldrich) (TBSNT). Following incubation, the eluates were mixed by vortexing and then centrifuged at 5,000 $\times$ *g* for 10 min at ambient temperature. Supernatants were removed by pipetting, transferred to a new Eppendorf tube, and frozen at −80°C until further analyses could be performed. For calculation of serum titer, the following was used. Assuming correct blood saturation of the Nobuto strip, which is equivalent to 100 μl of whole blood, serum accounts for approximately half of the blood volume (dilution factor of 2). Thus, the resulting dilution of the Nobuto serum sample was 1:20.

Serological analyses using passive hemagglutination and passive hemagglutination inhibition assays (PHA/PHI) were performed as described previously (12). Briefly, antibodies from Nobuto strips were eluted by cutting the narrow portion of a single strip into equal halves and incubating overnight at 4°C with 0.4 ml borate buffer. Eluate from strips was heat inactivated and blood absorbed for 30 min at room temperature with sheep red blood cells, followed by centrifugation at 2,300 $\times$ *g* for 5 min. The resulting supernatant was transferred to a new tube and utilized for testing. Assuming each Nobuto strip contained 50 μl serum, the resulting dilution of the Nobuto serum sample was 1:8. For passive

hemagglutination testing, dilution of the serum in hemagglutination diluent and addition of F1 antigen-sensitized sheep red blood cells results in a starting dilution of 1:32.

SDS-PAGE and immunoblotting. The recombinant F1 antigen (0.125 or 0.25 μg) (BEI Resources, Manassas, VA) was separated by SDS-PAGE using NuPAGE 4 to 12% Bis-Tris SDS-polyacrylamide gels (Thermo Fisher) and transferred to nitrocellulose membranes by electroblotting (24). Nitrocellulose membranes were blocked in 10 ml TBSNT for 1 h prior to incubation with 10 ml coyote serum or Nobuto serum samples (1:2,000) prepared in TBSNT at ambient temperature with gentle shaking for 3 h, followed by three washes in TBS supplemented with 0.1% Tween 20 (TBST). The secondary antibody, alkaline phosphatase-conjugated anti-dog IgG (whole molecule) produced in rabbit (product number A0793-1ML; Sigma-Aldrich) or alkaline phosphatase-conjugated anti-cat IgG (H+L) IgG (product number 15-20-06; SeraCare Life Sciences, Milford, MA) (1:4,000), was applied for 1 h at ambient temperature with gentle shaking. A final series of TBST and TBS washes were performed, and antibody-reactive proteins were detected using a solution of 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium made with SigmaFAST tablets (Sigma-Aldrich) suspended in TBS.

Conjugation of F1 to Bio-Plex Pro magnetic COOH beads. Bio-Plex Pro magnetic COOH beads (region 76) (Bio-Rad, Hercules, CA) were labeled with recombinant *Y. pestis* F1 capsular antigen (BEI Resources) in accordance with the manufacturers' instructions. Specifically, 0, 3, 6, 9, or 12 μg of F1 was used in conjugation reaction mixtures with 1.25×10^6 of the carboxylated magnetic beads.

Semiautomated bead handling. A total of 2,500 F1-conjugated Bio-Plex Pro magnetic COOH beads (region 76) were diluted in TBSNT to a final volume of 50 μl for each test within a KingFisher 96 KF microplate (Thermo Fisher) and protected from light. An equivalent volume of serum or Nobuto serum sample was added to the respective beads and mixed for 90 min at 1,400 rpm at ambient temperature. Following this incubation, the serum-bead combination was processed using a KingFisher Flex (Thermo Fisher) operated with BindIT software, ver. 3.3. Briefly, the serum and bead suspension were mixed before the beads were transferred (via magnetic separation) to new microtiter plates containing 150 μl of TBST per well, where they were washed twice. Following washing, beads were transferred to a microplate containing 100 μl of TBST with 4 μg/ml anti-dog IgG (Fc specific)-biotin antibody (product number SAB3700104-2MG; Sigma-Aldrich) per well and incubated for 30 min. For felids, a biotinylated anti-cat secondary antibody was used (product number SAB3700061-2MG; Sigma-Aldrich) at 4 μg/ml. For raccoons, anti-dog IgG-biotin antibody was used as described above, or raccoon IgG heavy- and light-chain antibodies (product number A140-123A; Bethyl Laboratories, Montgomery, TX) were biotinylated using a Pierce antibody biotinylation kit (Thermo) and used at 4 μg/ml. Alternatively, biotinylated conjugates of protein A and/or protein G (Rockland Immunochemicals, Limerick, PA) were used in lieu of the secondary antibody, with working concentrations from 1:1,000 to 1:200,000 tested (1:1,000 used for all experiments shown here). Beads were again washed as described above and then in 150 μl TBS before being transferred to a microtiter plate containing 100 μl of TBS per well supplemented with 1× Bio-Plex streptavidin-phycoerythrin (Bio-Rad). Finally, beads were deposited in 100 μl of TBS in preparation for analysis on the Bio-Plex 200 system.

Bead-based immunoassay and interpretation of results. All assays were performed at ambient temperature using the Bio-Plex 200 (Bio-Rad) operating with Bio-Plex manager 6.0, build 617, in accordance with procedures established by the manufacturer, analyzing a total of 50 Bio-Plex Pro magnetic COOH beads per sample. Prior to the analysis of samples, the Bio-Plex 200 was validated and calibrated using the Bio-Plex validation kit 4.0 and Bio-Plex calibration kit, respectively.

Reporting of a Nobuto elution sample as positive, indeterminate, or negative by the F1-LPA was accomplished by combining the outputs of two interpretative strategies to analyze mean fluorescence intensity (MFI) data. In the first of these, Nobuto elution samples (1:20) with a signal-to-noise (S/N) ratio of ≥10 (using an MFI of 250 as baseline background noise) were considered to have signal above background, which was considered a putative positive. In the second interpretative strategy, raw MFI data for each sample (1:20) were normalized to standards (S1 and S2). Standards consisted of canid Nobuto serum samples that were characterized by PHA and F1 immunoblotting, which produced a dynamic signal range on the F1-LPA. Specifically, S1 produced an average MFI of <100, and S2 had an average MFI of about 10,500 using the F1-LPA. Normalization of sample signal intensity was achieved using the following equation: (sample MFI − S1 MFI)/(S2 MFI − S1 MFI). Using this interpretative format, samples were considered positive when they produced an MFI of ≥1.6× of S2 minus S1. This threshold was established through immunoblot analysis, where only sporadic detection of F1 was achievable when sample MFIs were ≤1.5× of S2 minus S1 (see Table 4; see also Table S4 in the supplemental material). A third standard, S3, which produced a saturating MFI, was also included to ensure consistent performance of the assay. For reporting when both F1-LPA data interpretation formats were in agreement of a positive signal, the sample was considered to be a true positive. When only one F1-LPA data interpretation format yielded a positive signal, the sample was considered to be indeterminate. When both F1-LPA data interpretation formats were in agreement of a negative signal, the sample was considered to be a true negative. Due to the small number of felid and raccoon samples available, immunoblot-based detection thresholds could not be determined. For these samples, S/N ratios between 10 and 49.9 were considered to be indeterminate, and those of ≥50 were considered to be positive.

RESULTS

Development and optimization of the F1-LPA. The first step in F1-LPA development was to assess F1 coupling to the functionalized beads used as the solid-phase antigen support in Luminex assays. Beads conjugated with *N*-hydroxysuccinimide (NHS)

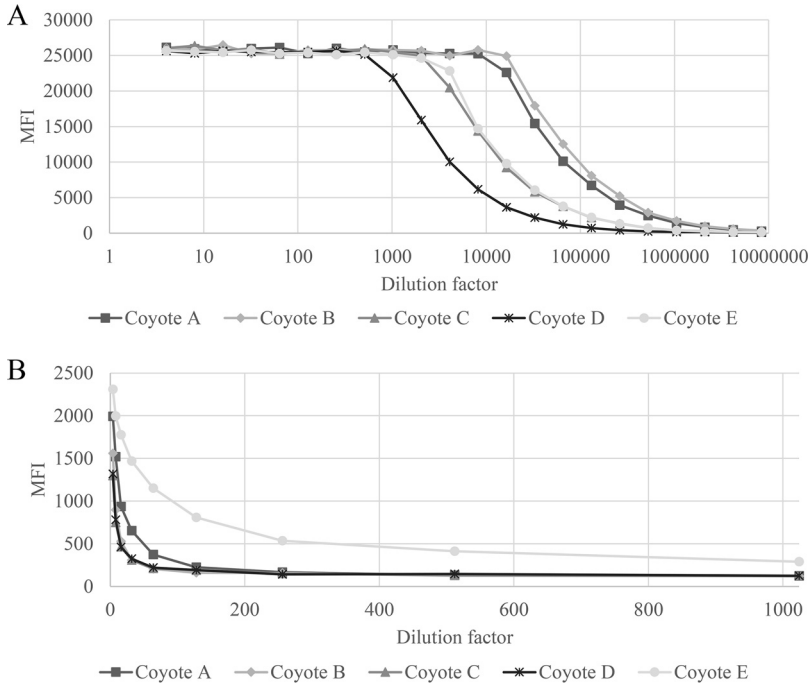

FIG 1 Dilution-dependent signal response of sera from five coyotes (coyotes A to E) experimentally infected with *Y. pestis* and profiled using the F1-LPA. (A) Coyote sera at either 140 or 156 dpi. PHA-determined titers for these coyotes (A to E) were 1:8,192, 1:16,384, 1:1,024, 1:256, and 1:1,024, respectively. (B) Coyote sera at 0 dpi; samples were all PHA negative.

esters were reacted with 3, 5, 9, or 12 μg of F1. Bead preparations were subsequently screened using eight coyote Nobuto serum samples (1:400) with F1 reactivity determined by PHA and immunoblotting (4 positive/4 negative). Little variation in signal intensity was apparent between bead preparations, where positive and negative samples produced an average mean fluorescence intensity (MFI) of 14,777 and 115, respectively. The same eight Nobuto samples were also tested with unlabeled beads to evaluate potential cross-reactivity with unreacted carboxyl moieties used in carbodiimide cross-linking to NHS. There were minimal differences in the signal intensities between positive and negative samples, with an average MFI of 1,285 and 1,128, respectively. This indicated the potential for limited cross-reactivity to beads not properly functionalized. To minimize reagent requirements, beads derived from coupling reactions using 3 μg of F1 were used for all subsequent experiments. In our laboratories the F1-labeled beads were stable for at least 120 days when stored at 4°C.

Experiments were performed to optimize parameters for semiautomated bead handling, including blocking, secondary antibody concentrations and incubation times, and streptavidin-phycoerythrin incubation times, as well as washing conditions for the F1-LPA. Of note, little to no interference was observed when nonfat dried milk was used as a blocking reagent, despite its known inhibitory effect on streptavidin-biotin reactions, which are used in the F1-LPA (25), indicating adequate washing occurred throughout bead handling.

Background MFI, dilution-dependent signal decay, and signal saturation of the F1-LPA were next evaluated using sera from coyotes ($n = 5$) experimentally infected with *Y. pestis* (Fig. 1). F1 titers for these samples were previously established using the PHA, with all preinfection samples being negative and the titer for serum samples drawn 140 or 156 days postinfection (dpi) being 1:8,192, 1:16,384, 1:1,024, 1:256, and 1:1,024 for coyotes A to E, respectively (10). In the F1-LPA, sera (1:4) from coyotes at 0 dpi yielded an average MFI of 1,695, with background signal extinction (average MFI of $<$250) occurring at a dilution of 1:512. F1-LPA analysis of sera (1:4 to 1:512) collected at either 140 or 156 dpi produced an average signal MFI of $>$25,000, which was the

TABLE 1 Reproducibility of the F1-LPA for canids

	Fluorescence signal[a]					
	Intra-assay			Interassay		
Standard[b]	Mean (MFI)	SD	% CV	Mean (MFI)	SD	% CV
S3	25,047.6	109.8	0.4	24,648.4	201.9	0.8
S2	9,876.6	263.9	2.7	10,328.1	885.7	8.6
S1	82.5	4.7	5.6	83.9	19.4	23.1

[a]Five replicates were used for both intra-assay and interassay groups. SD, standard deviations; CV, coefficient of variation (SD assay results/mean assay results).
[b]S1, S2, and S3 were designed to produce a negative, moderately positive, and strongly positive response, respectively, in the F1-LPA.

determined saturation point of the assay. Thus, compared to the gold standard method, PHA, the diagnostic sensitivity of the F1-LPA was 100%. The F1-LPA allowed for detection (>10× background) of positive samples at dilutions of 1:262,144, 1:524,288, 1:65,536, 1:16,384, and 1:65:536 for coyotes A to E, respectively. These results suggest that the limit of detection of the F1-LPA was 32× to 64× that of PHA for F1-specific IgG.

In order to establish F1-LPA intra-assay and interassay reproducibility, repetitive testing ($n = 5$) of coyote Nobuto serum samples consisting of a negative standard (S1), a moderately positive standard (S2), and a strongly positive standard (S3) was performed (Table 1). Intra-assay variance ranged from 0.4% to 5.6%. Interassay variance of S3 and S2 was 0.8% and 8.6%, respectively. Interassay variance of S1 was 23.0%, but differences in MFI only ranged from 66 to 110, which are both below the established background MFI threshold of 250.

Analysis of the F1-LPA with a canid Nobuto serum test panel. A panel of canid serum samples from areas where plague is endemic was eluted from Nobuto strips and tested by PHA and the F1-LPA for comparative analyses (Fig. 2, Table 2; see also

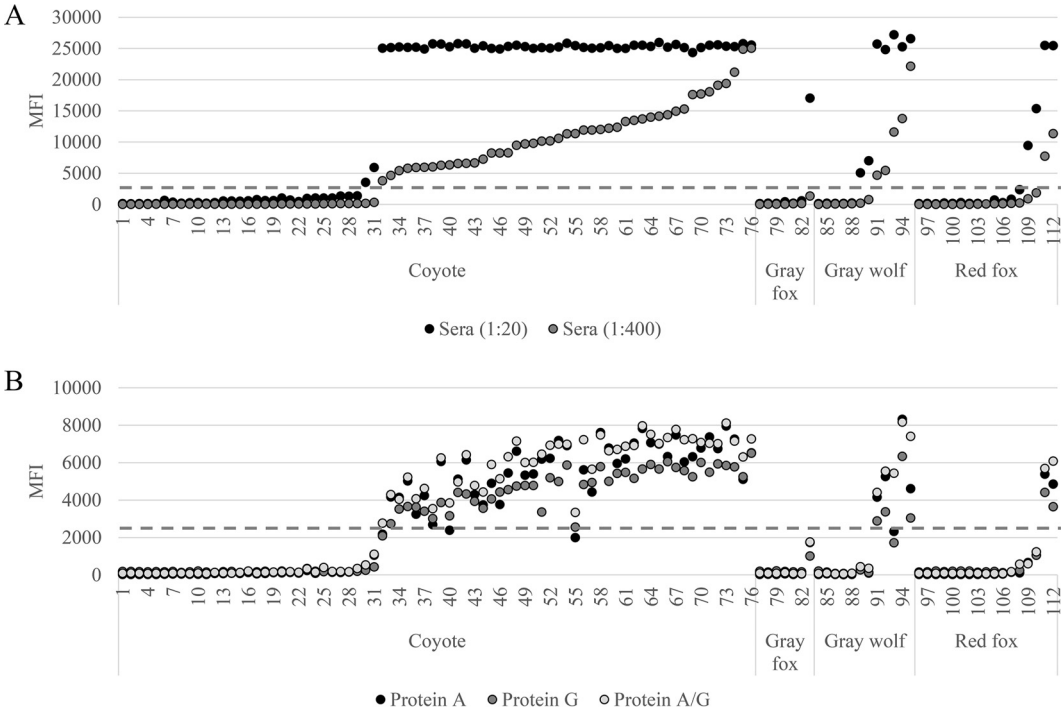

FIG 2 Signal response of Nobuto serum samples from 112 canids profiled with the F1-LPA. (A) Detection of F1 immunoreactivity was accomplished using a genus-specific secondary anti-IgG antibody. (B) Detection of F1 immunoreactivity was accomplished using biotinylated conjugates of staphylococcal protein A, streptococcal protein G, and staphylococcal protein A and streptococcal protein G (A/G), with sera diluted 1:20. The dashed line indicates the threshold for positive and negative samples using an S/N ratio of 10.

TABLE 2 Canid Nobuto serum sample test panel and comparative analysis of F1-LPA and PHA results

Species (no. of samples)	No. of Nobuto serum samples			Locations (no. of samples)	Diagnostic sensitivity[a] (%) of F1-LPA
	Female	Male	Sex unknown		
Coyote (76)	26	49	1	AZ (2), CO (9), ID (3), MT (11), NE (4), NM (21), NV (4), OK (1), TX (1), UT (14), WY (6)	85.5
Gray fox (7)	4	3	0	AZ (3), TX (3), NM (1)	71.4
Gray wolf (12)	5	7	0	ID (2), MT (9), WY (1)	75.0
Red fox (17)	10	5	2	Afghanistan (1), CO (1), ID (1), MT (1), ND (6), NE (1), UT (2), WY (4)	88.2

[a]Percentage of agreement between F1-LPA and PHA in the determination of samples as positive or negative.

Table S1 in the supplemental material). This panel encompassed both male and female animals from 12 U.S. states, one sample from Afghanistan, 76 samples from coyotes, seven samples from gray foxes (*Urocyon cinereoargenteus*), 12 samples from gray wolves (*Canis lupus*), and 17 samples from red foxes (*Vulpes vulpes*).

Comparing F1-LPA to the gold standard of PHA, the diagnostic sensitivity of the F1-LPA was 80.4% (90 of 112 samples) for detection of F1-specific IgG in canid Nobuto serum samples (1:20). A total of 16 of the 112 samples were differentially classified as positive or negative by PHA and F1-LPA. Six samples were classified as indeterminate by F1-LPA.

Given that F1-LPA targets IgG and that PHA is likely biased to IgM detection, anti-F1 IgG immunoblots were performed to clarify the immunological status of samples with discrepant results. Of the 16 samples differentially classified as positive or negative by PHA and F1-LPA, immunoblot results were in agreement with the F1-LPA for 15 samples (Table S1). Insufficient volume prevented immunoblot analysis of the remaining sample. These results strongly suggest that samples were correctly classified as positive or negative by the F1-LPA. Of the six samples classified as indeterminate by the F1-LPA, four of these samples were negative by both PHA and IgG immunoblotting. This result indicates that the analytical sensitivity of the F1-LPA exceeds that of PHA and immunoblotting or the potential for F1 cross-reactivity. Two samples classified as indeterminate by the F1-LPA were positive by PHA but not by IgG immunoblotting, suggesting that these samples were acquired soon after *Y. pestis* exposure before a robust anti-F1 IgG antibody response developed.

Wildlife occupy diverse habitats where they are exposed to largely uncharacterized microbiomes. Thus, to evaluate the potential for cross-reactivity in the F1-LPA, 302 randomly selected canid Nobuto serum samples (1:20) collected from 10 U.S. states where plague is absent from the landscape were analyzed (Table S2). Only one of these samples was classified as positive by the F1-LPA, indicating an assay specificity of 99.7%. Sixteen of these samples were classified as indeterminate by the F1-LPA; however, the average MFI of these samples was only 32% of that required to produce a positive result in the F1-LPA. Additionally, 11 coyote Nobuto serum samples with known exposure to *Francisella tularensis* were tested, and these sera were all classified as negative by the F1-LPA (Table S2). In total, these results are in agreement with previous studies that indicate that F1 is the most specific serodiagnostic antigen for plague (12–16). Nevertheless, any signal above background indicates antigen cross-reactivity, and limited evidence for F1 cross-reactivity has been described previously when a crude native F1 antigen preparation was used for plague serodiagnosis (26).

Testing was performed on the canid Nobuto serum samples diluted 1:400 to further evaluate sample background relative antibody concentration and to ensure continuity of the F1-LPA across presumptive working concentrations of antibodies (Fig. 2). In this testing, the average MFI of negative canid samples was 484 and 58 for Nobuto serum samples diluted 1:20 and 1:400, respectively. The signal responses between samples were as expected, where MFI of samples diluted 1:20 was always greater than that of samples diluted 1:400. When Nobuto serum samples were assayed directly (1:20), the majority of positive canid samples were detected at the saturation limit of the F1-LPA,

whereas samples diluted 1:400 yielded a spectrum of signal intensities. This suggests that a sample should be assayed directly (1:20) for maximum analytical sensitivity, and that semiquantitative determinations of serum titer using F1-LPA are possible with appropriate dilution of samples.

A limitation for serological monitoring of wildlife is the lack of readily available immunological reagents for some genera/species. Therefore, as a proof of concept, we evaluated biotinylated conjugates of staphylococcal protein A, streptococcal protein G, and a combination of protein A and protein G (protein A/G) to serve in place of genus-specific secondary antibodies in the F1-LPA (Fig. 2 and Table S1), since these proteins are recognized for their affinities to antibodies from a broad range of vertebrates. Canid antibodies (IgG, IgM, and IgA) are recognized to strongly interact with protein A, and canid IgG weakly associates with protein G (27, 28). In serum samples (1:20) that were considered to be negative by the F1-LPA (using the anti-dog secondary antibody for detection), the average background MFI was 70, 171, and 131 when protein A, protein G, and protein A/G were used, respectively. The MFI of positive samples averaged 5,461 for protein A, 4,496 for protein G, and 6,048 for protein A/G. Considering a signal-to-noise (S/N) ratio of $\geq$10 (using an MFI of 250 as baseline background noise), detection using protein A, protein G, or protein A/G was in agreement with F1-LPA results (using the anti-canid secondary antibody) for 90.6%, 94.3%, and 98.1% of samples, respectively. For samples established as indeterminate or negative using the anti-canid secondary antibody, the S/N ratios of these samples detected with protein A and G were $<$5. Thus, trends in MFI from samples analyzed using protein A/G were consistent with genus-specific secondary antibodies, albeit with reduced signal intensity for positive samples (Fig. 2).

F1-LPA performance in noncanids. Wildlife plague monitoring programs routinely collect samples from noncanids, highlighting the need for a multispecies plague assay. Thus, all available Nobuto serum samples from bobcats (*Lynx rufus*), mountain lions (*Puma concolor*), and raccoons (*Procyon lotor*) were evaluated with genus-specific secondary antibodies as well as protein A and protein G using the F1-LPA (Fig. 3, Table 3, and Table S3). Both anti-raccoon-biotin IgG and anti-dog-biotin IgG were tested for raccoon samples. Enzyme conjugates of anti-dog IgG have been successfully used for the detection of raccoon antibodies in multiple immunoassay formats (29, 30). Background of the raccoon antibodies was high (average MFI of 1,803 for negative raccoon samples) compared to that of the anti-dog antibodies (average MFI of 74 for negative raccoon samples), and the anti-dog antibody had a higher maximum signal intensity for a positive raccoon sample. Therefore, anti-dog antibodies were used for subsequent analyses of raccoon samples.

Compared to PHA, the diagnostic sensitivity of F1-LPA was 85.5% for bobcat samples (n = 13), 94.1% for mountain lion samples (n = 17), and 75.0% for raccoon samples (n = 8). The average background MFI was $<$250 for negative felid and raccoon samples when tested at dilutions of both 1:20 and 1:400. The average MFI of positive samples was 23,026 and 16,369 for samples diluted 1:20 and 1:400, respectively. IgG immunoblots were used to clarify the anti-F1 status of the four samples that were differentially classified by PHA and F1-LPA, and these analyses indicated that F1-specific IgG immunoreactivity was correctly classified as determined by F1-LPA (Table S3). One bobcat sample was classified as indeterminate by the F1-LPA and was negative by PHA and immunoblotting (Table S3).

Felid IgGs are reported to strongly react with protein A and weakly with protein G (31). Of felid samples (1:20) determined to be positive with the F1-LPA using a genus-specific secondary antibody (n = 8), the average MFI was 5,981, 252, and 6,772 when protein A, protein G, and protein A/G was used, respectively (Fig. 3). For the negative felid samples (n = 21), the average MFI was 48 when protein A, 109 when protein G, and 82 when protein A/G was used for detection. As with canid samples, trends in MFI with protein A/G were consistent with genus-specific secondary antibodies but had a lower overall signal intensity (Fig. 3). The interpretations of samples as

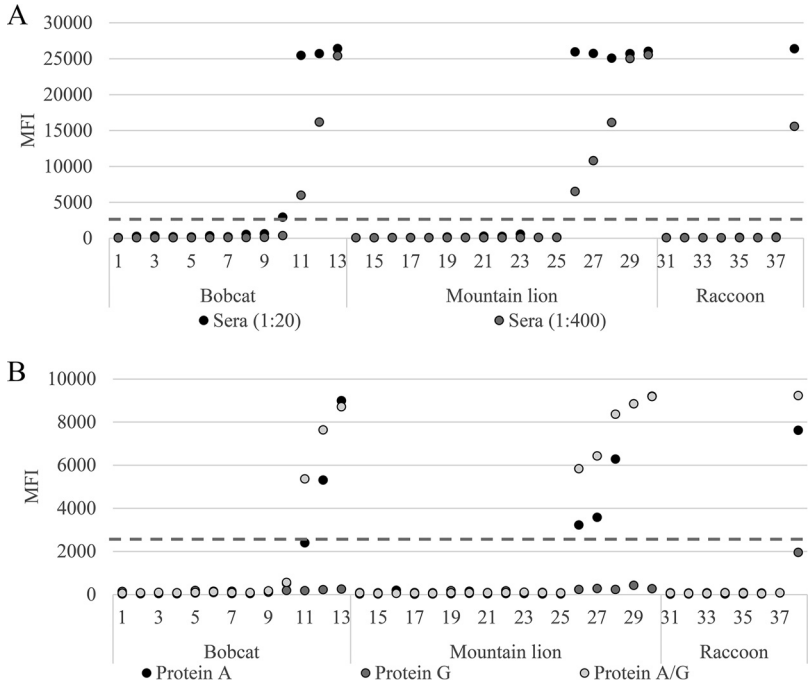

FIG 3 Signal response of Nobuto serum samples from 30 felids and eight raccoons profiled with the F1-LPA. (A) Detection of F1 immunoreactivity was accomplished using anti-cat IgG and anti-canid IgG for felids and raccoons, respectively. (B) Detection of F1 immunoreactivity was accomplished using biotinylated conjugates of staphylococcal protein A, streptococcal protein G, and staphylococcal protein A and streptococcal protein G (A/G), with sera diluted 1:20. The dashed line indicates the threshold for positive and negative samples using an S/N ratio of 10.

positive or negative using protein A/G was identical to those obtained using the genus-specific secondary antibody, with an average S/N ratio of 30.2. Negative samples had an average S/N ratio of <0.5 by both protein A and A/G.

Raccoon antibodies are reported to react with protein A (32). Only one of eight serum samples (1:20) was positive when anti-dog secondary antibodies were used in the F1-LPA, and this sample displayed an MFI of 7,623, 1,950, and 9,228 when protein A, protein G, and protein A/G was used for detection, respectively (Fig. 3). The average MFI of raccoon samples determined to be negative was <80, with S/N ratios of <0.5 for protein A, protein G, and protein A/G.

DISCUSSION

The F1-LPA developed here was demonstrated to be an effective tool for serodiagnosis of *Y. pestis* exposure in canids and felids, which are associated with a number of plague cases in the United States (33), and raccoons, which can be exposed to plague on the landscape. Intra- and interassay variance was low, F1-conjugated beads were stable for long periods of time, data interpretation was unambiguous, and coupling of this system with semiautomated bead handling minimized technician effort and allowed for high reproducibility. Comparative analysis suggested that the F1-LPA assay

TABLE 3 Felid and raccoon Nobuto serum sample test panel and comparative analysis of F1-LPA and PHA results

Species (no. of samples)	No. of Nobuto serum samples				Diagnostic sensitivity[a] (%) of F1-LPA
	Female	Male	Sex unknown	Locations (no. of samples)	
Bobcat (13)	5	8	0	AZ (4), CO (2), KS (2), NM (3), TX (1), UT (1)	84.6
Mountain lion (17)	10	6	1	AZ (6), MT (1), NV (5), UT (5)	94.1
Raccoon (8)	3	4	1	CO (4), NV (1), UT (3)	75.0

[a]Percentage of agreement between F1-LPA and PHA in the determination of samples as positive or negative.

had an analytical sensitivity that was 32× to 64× greater than that of PHA for F1-specific IgG detection and that there was low potential for F1 cross-reactivity. Therefore, the F1-LPA can be used for reliable screening of wildlife samples for plague exposure across time and space. The high analytical sensitivity of this assay could shed new light on the dynamics of a pathogen whose epidemic cycles are still poorly understood in the United States. In addition, application of this method to wildlife and domestic animal samples associated with case investigations of human plague infections may further help to clarify human risk.

The F1 antigen used in the F1-LPA is recognized as the preeminent plague serodiagnostic antigen for its immunoreactivity, specificity, and presence in virulent *Y. pestis* strains (12–16). However, use of F1 as the sole serodiagnostic antigen for plague has been debated due to the identification of F1-negative *Y. pestis* strains. In our experience characterizing *Y. pestis* strains collected from around the world, alluded to previously, F1-negative strains are likely a result of laboratory passage (34). Without the selective pressure encountered in the natural life cycle of *Y. pestis*, the plasmid carrying *caf1* (the gene encoding the F1 antigen) is lost on agar passage or incorporated into the chromosome. There is an absence of data in the literature indicating detection of F1-deficient *Y. pestis* strains in clinical or animal samples (independent of culture). As modeled by Sebbane et al., rarity of F1-deficient strains in nature is at least in part linked to the vector ecology of *Y. pestis*, which would drive these strains to extinction (35). Thus, although the F1-LPA would not be capable of detecting exposure to F1-deficient *Y. pestis* strains, this limitation is not thought to be significant for plague monitoring of wildlife.

The majority of plague-positive canid Nobuto serum samples (1:20) produced a saturating signal in the F1-LPA (approximately 26,000 MFI), whereas the average MFI of a negative sample was 708.3. This large difference in signal to background allowed for straightforward classification of most samples as positive or negative. Additionally, these results suggest that the small intra- and interassay variance of the F1-LPA would not be significant in the classification of most samples as positive or negative. Only 22 of 425 (5.1%) of the canid Nobuto samples tested here were classified as indeterminate by the F1-LPA (see Tables S1 and S2 in the supplemental material). This result was echoed in plague monitoring subsequently performed by the NWDP using the F1-LPA in which 300 of 3,993 (7.5%) canid Nobuto serum samples were classified as indeterminate (data not shown). Such a result was not unexpected due to differential exposures of wildlife to *Y. pestis*, waxing/waning of the humoral immune response, and that the potential for antigen cross-reactivity can never be accounted for with complete certainty within a serodiagnostic assay. Nevertheless, sporadic detection of F1 immunoreactivity by immunoblotting was possible for several of these indeterminate samples, particularly those with relatively high F1-LPA signal intensities (Table 4 and Table S4), suggesting these samples are useful in plague monitoring. Additional studies are under way in our laboratories to further resolve the status of F1 immunoreactivity in these samples.

Analytical sensitivity of the F1-LPA was potentially influenced by the composition of the sample matrix. As reviewed elsewhere, a number of naturally occurring compounds that interfere with serodiagnostics are endogenous to whole-blood samples (36, 37). Additionally, some antibodies have been found to directly associate with the solid phase of bead-based flow cytometric assays, although this phenomenon can be assessed using unlabeled paramagnetic polystyrene beads and is generally controllable using appropriate antigen conjugation and blocking procedures (38). In the F1-LPA, relatively high background levels (average background MFI of approximately 1,700 with a 1:4 dilution of sera) were observed from 0 dpi serum samples obtained from experimentally infected coyotes. However, interfering background was largely abrogated when analyzing serum samples from Nobuto strips. Direct analysis of Nobuto serum samples classified as negative by the F1-LPA typically had an MFI of less than 400. The relatively low background of Nobuto-preserved samples is partially attributable to the dilution effect resulting from antibody elution, but it was also apparent that

TABLE 4 F1-LPA normalized signal response of canid Nobuto serum samples compared to immunoblot-based interpretations of F1 reactivity

F1-LPA normalized signal intensity[a]	No. of samples tested[b]	Immunoblot interpretation (no.)	
		Negative	Positive
1.1×–1.2×	4	0	4
1.21×–1.3×	7	5	2
1.31×–1.4×	7	1	6
1.41×–1.5×	7	1	6
1.51×–1.6×	4	0	4
1.61×–2.0×	13	0	13

[a]The MFI for each canid Nobuto serum sample obtained using the F1-LPA was normalized to standards S1 and S2 using the following equation: (sample MFI − S1 MFI)/(S2 MFI − S1 MFI). Samples with normalized signal intensities ranging between 1.1× and 2.0× were compared with their respective immunoblotting-based interpretation of F1 reactivity to establish a robust cutoff for a positive sample in F1-LPA.

[b]Following the screening of 3,993 Nobuto samples using the F1-LPA (data not shown), only these 42 (1.05%) samples had normalized signal intensities ranging between 1.1× and 2.0× (Table S4). The average normalized signal intensities for negative and positive samples in the F1-LPA was 0.12× and 2.6×, respectively (Tables S1 and S2).

not all blood components were desorbed, as reflected by retained pigmentation of the Nobuto strip postelution. Thus, it is possible that the Nobuto strips adsorbed serological components which interfere with immunological assays. Early evaluations identified that plague serum titers were overestimated when samples were eluted from Nobuto strips compared to direct testing using PHA, suggesting that inhibitory compounds can be retained by the Nobuto strip (39). The application of Nobuto strips in sample cleanup is particularly intriguing, given their versatility, low cost, and ease of use. Further, Nobuto strips in combination with refrigerated storage are recognized for their ability to preserve whole-blood samples for up to 2 years for serological analysis of plague (11), and here we successfully detected F1-specific antibodies preserved on a Nobuto strip at −20°C for over 12 years.

The F1-LPA was demonstrated to be amenable to the detection of serological responses to *Y. pestis* in noncanids, including felids and raccoons, although relatively few samples were available for analysis. The F1 antigen is used for plague serodiagnostics for multiple species, including humans (10, 16, 40). Therefore, it is likely that the F1-LPA is capable of serodiagnosis of *Y. pestis* exposure in a much greater array of hosts than that tested in this study. Adapting the F1-LPA to additional host species can be accomplished through the use of biotinylated-conjugated genus/species-specific secondary antibodies, cross-reactive secondary antibodies, or ligands such as protein A and/or protein G. The utility of each of these strategies was demonstrated here. Given that genus/species-specific secondary antibodies are not always available and antibody cross-reactivity data are not known for many wildlife species, ligands such as protein A and/or G with broad affinities to host immunoglobulins may be particularly useful in the F1-LPA. Both protein A and protein G conjugates have been used in other platforms for the serodiagnosis of *Y. pestis* exposure. For example, a plague assay based upon immunochromatography and protein A was used to detect antibodies directed against *Y. pestis* capsule in *Rattus rattus*, *Rattus norvegicus*, and humans (40). Protein G was used within a lateral flow device used to screen for antibodies directed against F1 or the LcrV antigen in coyotes, prairie dogs (*Cynomys ludovicianus*), lynx (*Lynx canadensis*), and black-footed ferrets (*Mustela nigripes*) (41). In the current study, the signal response was lower when protein A and/or G was used for detection compared to anti-IgG secondary antibodies. However, using an S/N ratio of ≥10 as the threshold for positive samples demonstrated that qualified immunoreactivity to protein A/G was nearly identical to that of target-specific secondary antibodies.

In conclusion, we present a bead-based flow cytometric assay for serodiagnosis of *Y. pestis* exposure in wildlife, the F1-LPA. This assay is currently under evaluation as a replacement for PHA as the primary tool for plague monitoring by the NWDP. Additionally, retrospective studies of archived Nobuto serum samples are planned using the

F1-LPA to determine if this method can offer increased resolution of plague exposure in wildlife populations.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00273-18.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Sam Lehman, Kerri Pedersen, Rachel Maison, Hailey McLean, and Kylie Schumacher for assisting with Nobuto serum sample processing for the NWDP plague monitoring program. In addition, we thank USDA-APHIS-WS wildlife disease biologists and other Wildlife Services personnel, as well as biologists with state agencies, whose time and effort for sample collection made this research possible. The following reagent was obtained through BEI Resources, NIAID, NIH: fraction 1 capsular antigen (F1) from *Yersinia pestis*, expressed in *Escherichia coli*, NR-44223.

The views and opinions expressed here are those of the authors alone and do not represent the official position of the Centers for Disease Control and Prevention (CDC).

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