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FILTER-PAPER BLOOD SAMPLES FOR ELISA DETECTION OF *BRUCELLA* ANTIBODIES IN CARIBOU

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ABSTRACT: We evaluated blood collected on Nobuto filter-paper (FP) strips for use in detecting *Brucella* spp. antibodies in caribou. Whole blood (for serum) and blood-saturated FP strips were obtained from 185 killed arctic caribou (*Rangifer tarandus groenlandicus*). Sample pairs (serum and FP eluates) were simultaneously tested in duplicate using competitive enzyme-linked immunosorbent assay (c-ELISA) and indirect ELISA (i-ELISA) for *Brucella* spp. Prior work based on isolation of *Brucella* spp. revealed sensitivity (SE) and specificity (SP) of 100% and 99%, respectively, for both these serum assays in caribou. Infection status of the animals in the current study was unknown but recent sampling had revealed clinical brucellosis and >40% *Brucella* antibody prevalence in the herd. To assess the performance of FP relative to serum in these assays, serum was used as the putative gold standard. On both assays, the findings for duplicate runs (A and B) were similar. For c-ELISA run A, the FP *Brucella* prevalence (47%) was lower than serum prevalence (52%), with SE 89% (95% confidence interval [CI]: 82–95%) and SP 99% (97–100%). For i-ELISA run A, serum and FP *Brucella* prevalence rates were identical (43%), and the SE and SP of FP testing were 100% and 99% (97–100%), respectively. The findings suggest better FP test performance with i-ELISA than with c-ELISA; however, i-ELISA does not distinguish cross-reacting antibodies induced by *Brucella* vaccination or exposure to certain other Gram-negative pathogens. Results for duplicate FP eluates (prepared using separate FP strips from each animal) were strongly correlated for both protocols (r=0.996 and 0.999 for c-ELISA and i-ELISA, respectively), indicating minimal variability among FPs from any individual caribou. Dried caribou FP blood samples stored for 2 mo at room temperature are comparable with serum for use in *Brucella* spp. c-ELISA and i-ELISA. Hunter-based FP sampling can facilitate detection of disease exposure in remote regions and under adverse conditions, and can expand wildlife disease surveillance across temporospatial scales.

Key words: Arctic, *Brucella*, caribou, disease surveillance, filter paper, Nobuto, *Rangifer tarandus*, serology.

INTRODUCTION

Wildlife disease researchers face a variety of difficulties collecting and deriving useful information from biologic specimens (Kuiken et al., 2005). The sampler needs practical, reliable tools that can be transported easily, perform well under field conditions, and provide multifaceted information about health status. Collecting blood on filter paper (FP) may be a simple way to address some of these problems. This method can be performed by laypeople and circumvents many of the logistic and cost issues associated with obtaining, processing, and shipping conventionally sampled blood (Mei et al., 2001; McDade et al., 2007). Advantages include the elimination of tube breakage, reduced processing time and labor in the field, and no requirement for special equipment, such as centrifuges and freezers, that can be difficult to transport, operate, and maintain in the field.

Filter-paper blood sampling is not new; this method has been widely used in various forms in human medicine since the 1960s and its applications continue to expand (Mei et al., 2001). The current and prospective human-related uses of FP for clinical chemistry and in remote situations are diverse. Recent field-related human

FP publications focus on nucleic acid- and antibody-based detection of human immunodeficiency virus, and the agents of malaria, dengue fever, and other infectious diseases (Lederman et al., 2007; Balmaseda et al., 2008; Castro et al., 2008; Corran et al., 2008). This mode of blood collection has parallel benefits for assessing animal health (e.g., Beard and Brugh, 1977; Hopkins, et al. 1998; Thangavelu et al., 2000; Dubay et al., 2006; Yu et al., 2007) and its advantages as a field tool suggest a range of applications for wildlife health analysis. However, validation for detection of biochemical analytes and pathogen exposure in free-living species is lacking. This research addresses this for an important infectious disease of wild ungulates, brucellosis.

Brucellosis is a zoonosis caused by bacteria of the genus *Brucella.* This disease has a multisystemic pathogenesis, numerous clinical signs, and tends to affect the reproductive system in particular (Romich, 2008). Severe losses through infertility/abortion and reduced productivity make brucellosis one of the most serious diseases of livestock (Romich, 2008). *Brucella suis* biotype 4 is the causative agent in caribou and reindeer (both *Rangifer tarandus* subspecies) and the clinical signs and lesions are similar to those seen in cattle and other domestic species, but are often more severe (Forbes, 1991). Rangiferine brucellosis occurs across northern Canada and in Alaska and Russia, and poses a potential human health risk if proper precautions are not taken with carcass handling and cooking (Forbes, 1991; Bradley et al., 2005). Certain favored caribou "country foods" are eaten raw or undercooked. Natural *Brucella* infections have been documented in muskoxen, moose, captive Rocky Mountain bighorn sheep, and predator species, and researchers have demonstrated experimental transmission from naturally infected reindeer to cattle (Gates et al., 1984; Forbes and Tessaro, 1993; Honour and Hickling, 1993; Kreeger et al., 2004). There remain major gaps in scientific knowledge about the epidemiology of *Brucella* in *Rangifer*, including transmission patterns and effects at individual and population levels (Forbes, 1991; Forbes and Tessaro; 2003). Extensive surveillance for brucellosis in these animals has been limited by the high cost and logistic difficulties of sampling in the North. A broader sampling strategy is needed to help provide better understanding of the patterns and ecology of this disease in caribou. Our aim was to assess the efficacy of blood collected on FP relative to serum derived from clotted blood for *Brucella* serologic testing in caribou. In contrast to conventional blood sampling of caribou, FP sampling could facilitate widespread hunter-based surveillance of *Brucella* exposure in circumpolar herds.

MATERIALS AND METHODS

Samples and processing

In March 2008, paired serum and FP blood samples were obtained from each of 185 barren-ground caribou killed for a territorial government scientific study near the community of Coral Harbour (64°11′24″N, 83°21′36″W) on Southampton Island, Nunavut, Canada (Nunavut Wildlife Research Permit WL 000892). All sampling was done outside in the extreme cold (temperature range −39.2 C to −18.3 C). Each sample pair (blood tubes and blood-soaked FP strips) was collected as soon after death as possible and kept inside a shelter and above freezing until end-of-day processing 2–12 hr later. Serum was obtained by collecting jugular or femoral venous blood into a glass Vacutainer® (Becton-Dickinson, Mississauga, Ontario, Canada) tube without coagulant. Filter paper samples were collected from the same source by saturating the full length of all FP strips with blood and shaking off any excess (Fig. 1). For each animal, 15 Nobuto blood filter strips (Toyo Roshi Kaisha, Ltd., Tokyo, Japan; distributor Advantec MFS Inc., Dublin, California, USA) mounted in sets of five strips on a handmade lightweight cardboard "handle" (Fig. 2a) were collected. According to manufacturer specifications, the blood-absorbing section of each strip holds 100 μl of whole blood (approximately 40 μl of serum depending on hemat-

FIGURE 1. Collecting filter-paper blood samples from the jugular vein of a hunter-killed caribou.

ocrit). Each animal's FP sets were kept in an antimicrobial-lined #10 envelope (Quality Park, St. Paul, Minnesota, USA) inside a reclosable (zipper-lock) plastic bag. Care was taken to avoid touching FP strips during preparation of FP sets for sampling and throughout collection, processing, and storage. For processing, blood tubes were centrifuged (15 min at 3,500 revolutions per minute) and aliquots of serum were stored at −20 C until analysis. Collected FP sets were air-dried in racks at room temperature overnight (Fig. 2b) and returned to a dry antimicrobial envelope. Multiple envelopes (up to 25) were placed together in a large reclosable plastic bag with eight to 10 desiccant packs (Humidity Sponge™, VWR International LLC, Mississauga, Ontario, Canada) and stored at room temperature. Desiccant was checked regularly and replaced as required on the basis of the manufacturer's color indicator insert.

Tissue specimens were not obtained for culture and *Brucella* infection status was unknown for the animals in this study. Recent annual sampling of the herd had revealed clinical evidence of disease and >40% antibody prevalence (M. Campbell, unpublished data). As the aim was to evaluate FP results relative to serum findings, serum was used as the gold standard indicator of *Brucella* exposure.

Filter-paper elution

After 2 mo of storage, two eluates (A and B) were prepared from each animal's FPs. A stock solution was made consisting of Dulbecco's phosphate-buffered saline with CaCl and MgCl (D-PBS 1×, Gibco® Invitrogen™, Burlington, Ontario, Canada) and an antibiotic mixture (penicillin–streptomycin liquid, Invitrogen). The final penicillin and streptomycin

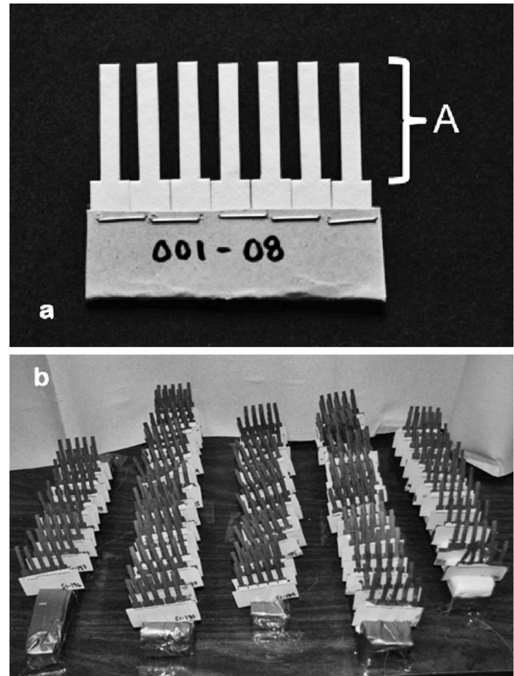

FIGURE 2. (a) A set of seven Nobuto filter-paper (FP) strips mounted on light cardboard and with the 3-cm absorbent portion (A) of each strip identified; (b) saturated FP sets in simple drying racks made from hard foam material and duct tape.

concentrations in the stock solution were 100 U/ml and 100 μg/ml, respectively. For each eluate, clean (flamed and cooled) small scissors were used to cut the absorbent portions (Fig. 2a) of two FP strips into five or six pieces directly into a 1.5-ml microcentrifuge tube (MCT-200-C tubes, Axygen Scientific, Union City, California, USA). Eight-hundred microliters of stock solution were added (as per the Nobuto FP manufacturer's instructions of 400 μl per strip) and the tube was finger-flicked to ensure all fragments were in full contact with the fluid. Tubes were stored at 4 C for 16 hr, and all (dark red) fluid was pipetted from each into a new, labeled 1.5-ml microcentrifuge tube. Eluates were spun very briefly (15 sec) to draw all fluid to the tube bottom, and then stored at −20 C until testing. Each resultant two-strip eluate was 400–440 μl and estimated to be 1:10 serum concentration, according to the FP manufacturer's specifications.

Immunoassays

All samples were tested at the Brucellosis Centre of Expertise (BCE) in Ottawa, Canada

(Canadian Food Inspection Agency, Government of Canada). The BCE uses enzyme-linked immunosorbent assay (ELISA) protocols and a fluorescence polarization assay (FPA) that were developed for diagnosing brucellosis in Cervidae. Gall et al. (2001) evaluated these tests in *R. tarandus* ssp. (reindeer and woodland caribou) and other cervids, and some of the reindeer sera tested were from animals that were culture-positive for *B. suis* biovar 4. The data from that study support the use of competitive ELISA (c-ELISA) and FPA for diagnosing brucellosis in caribou, and the authors identified FPA as the diagnostic test of choice for this purpose. However, multiple attempts to run FPA with our caribou FP eluates failed. Thus, the two assays we used for our study were the c-ELISA and indirect ELISA (i-ELISA) assessed by Gall et al. (2001); protocols were detailed by Nielsen et al. (1994,1996). Both assays are based on antigen of *Brucella abortus*, which is the main cause of brucellosis in cattle and known to infect bison and elk. Gall et al. (2001) tested caribou serum samples using the *Brucella* c-ELISA with a cutoff value of 16% inhibition (I) and observed sensitivity (SE) 100% (n=102) and specificity (SP) 99% (n=308). The corresponding n values, SE, and SP for i-ELISA (cutoff 11% positivity [P]) were identical to those observed with c-ELISA. Currently, the BCE uses higher cutoffs for both these tests (30% I for c-ELISA and 20% P for i-ELISA). Raising these thresholds had minimal effect on SE and SP (K. Nielsen, unpublished data) and the BCE has adopted these higher cutoffs in an effort to set universal values for brucellosis testing across animal species. Note that the *Brucella* i-ELISA does not distinguish antibodies induced by *Brucella* spp. exposure from antibodies elicited by *Brucella* vaccination or certain infectious agents, such as *Yersinia enterolitica* (Nielsen, 1990).

Nobuto FP eluates are estimated to be 1:10 serum concentration; therefore, initial dilution steps in the ELISA protocols were adjusted such that the assays would yield results for eluates and 1:10 serum (one serum/FP pair per animal tested simultaneously in each of two duplicate runs). The 185 serum/FP Southampton Island caribou sample pairs were tested with c-ELISA in July 2008, and sera/eluates were refrozen and kept at −20 C until i-ELISA was done in September 2008. Both immunoassays were carried out in duplicate (i.e., run A: 1:10 serum vs. eluate A; run B: 1:10 serum vs. eluate B). Sera for runs A and B in both assays were drawn from the same serum aliquot per animal. As noted, eluates A and B were prepared from different FPs from the same animal.

Statistical analysis

To evaluate the performance of FP testing relative to serum (the putative gold standard), data from each test run (run A and run B) were categorized and analyzed using Win Episcope 2.0 (de Blas et al., 2005) to generate prevalence and SE and SP values. To evaluate variability between FP tests and between serum tests (i.e., multiple testing of samples collected from each animal), the correlation between duplicate results for each sample type (serum A vs. serum B, eluate A vs. eluate B) was determined for c-ELISA and for i-ELISA (Microsoft® Office Excel 2003, Microsoft Corp., Redmond Washington, USA).

RESULTS

With c-ELISA, the measures of FP test efficacy for the duplicate runs (A and B) were similar (Table 1). For run A, analysis of results using the laboratory's established cutoff value (30% I) revealed a 52% prevalence of *Brucella* antibody on the basis of serum, compared with 47% prevalence on the basis of FP (SE 89% and SP 99%) (Table 1, Fig. 3). With i-ELISA, the measures of FP test efficacy for the duplicate runs (A and B) were also similar (Table 1). For run A, the serum- and FP-based *Brucella* antibody prevalence rates were identical (43%), and FP SE and SP were 100% and 99%, respectively (Table 1). There was only one FP serum results mismatch throughout the entire two runs of 185 i-ELISA sample pairings (370 tests). The i-ELISA *Brucella* antibody prevalence values were lower than observed with c-ELISA, and SE and SP were both higher than observed with c-ELISA.

The c-ELISA FP eluate A and eluate B results were strongly correlated, as were the c-ELISA serum results from runs A and B (r=0.996 and 0.994, respectively; Fig. 4). The i-ELISA eluate A and eluate B results were also strongly correlated, whereas the serum results for run A vs. run B were slightly more variable (r=0.999 and 0.974, respectively; Fig. 5).

TABLE 1. Findings for filter-paper (FP) blood testing relative to serum for *Brucella* spp. competitive and indirect immunosorbent assays (c-ELISA and i-ELISA) in wild caribou (n=185). Results for duplicate test runs (eluates A and B) are shown. The cutoff for c-ELISA is % inhibition (sample's optical density [OD] relative to that of the buffer well [uninhibited control]). The cutoff for i-ELISA is % positivity (sample's OD expressed as a percentage of the OD of a positive control).

Variable	c-ELISA Cutoff: 30% serum and FP		i-ELISA Cutoff: 20% serum and FP	
	Eluate A	Eluate B	Eluate A	Eluate B
Sensitivity (%)	88.5	89.4	100	100
CI[a] (%)	82.2–94.9	83.1–95.6		
Specificity (%)	98.9	98.9	99.1	100
CI (%)	96.7–100	96.8–100	97.2–100	
Serum prevalence (%)	51.9	50.8	42.7	42.7
CI (%)	44.7–59.1	43.6–58.0	35.6–49.8	35.6–49.8
FP prevalence (%)	46.5	45.9	43.2	42.7
CI (%)	39.3–53.7	38.7–53.1	36.1–50.4	35.6–49.8
FP predictive value (+) test (%)	98.8	98.8	98.8	100
CI (%)	96.6–100	96.5–100	96.3–100	
FP predictive value (−) test (%)	88.9	90.0	100	100
CI (%)	82.7–95.1	84.1–95.9		

[a] CI = confidence interval.

DISCUSSION

Previous investigators have assessed FP blood samples in ELISAs for brucellosis diagnosis in humans and for *B. abortus* antibody detection in cattle (McLean and Hilbink, 1989; Takkouche et al., 1995). We examined FP blood testing as a means of identifying *Brucella* spp. exposure in caribou (*Rangifer* spp. in general). Bacteriologic isolation remains the only absolute method for establishing *Brucella* infection status (Gall and Nielsen, 2004). This information was not available for the 185 caribou we investigated; thus, the true brucellosis status of these animals was unknown. As mentioned, the accuracy of caribou serum testing for brucellosis has been investigated previously (Gall et al., 2001). Our intent was to assess the efficacy

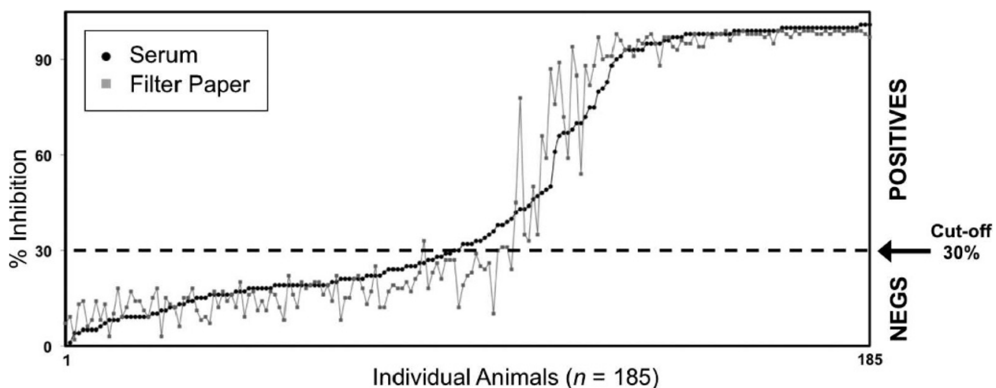

FIGURE 3. *Brucella* competitive enzyme-linked immunosorbent assay (c-ELISA) run A results: The paired serum and filter-paper (FP) values for each animal (plotted according to ascending serum values) in test run A (n=185).

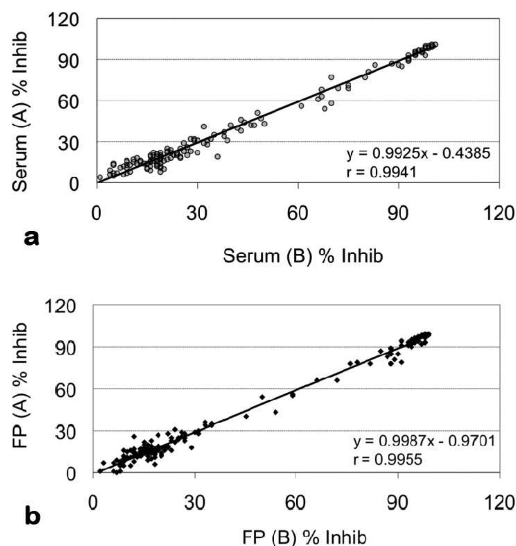

FIGURE 4. Correlation of *Brucella* competitive enzyme-linked immunosorbent assay (c-ELISA) results (% inhibition) for the duplicate runs of caribou (a) serum and (b) filter-paper (FP) eluates (run A vs. run B pairs; $n=185$).

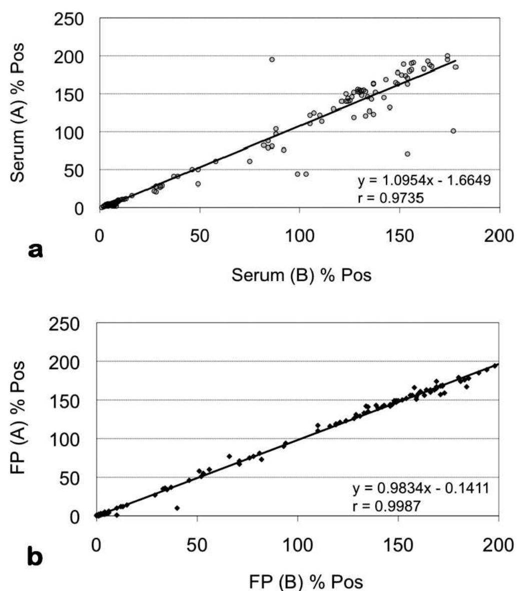

FIGURE 5. Correlation of *Brucella* indirect enzyme-linked immunosorbent assay (i-ELISA) results (% positivity) for the duplicate runs of caribou (a) serum and (b) filter-paper (FP) eluates (run A vs. run B pairs; $n=185$).

of FP relative to serum for detecting anti-*Brucella* antibodies in *Rangifer*.

***Brucella* antibodies: Filter paper vs. serum**

For c-ELISA, the FP and serum results were in close agreement and we conclude that FP c-ELISA is effective (comparable with serum c-ELISA) for detecting *Brucella* antibodies (i.e., exposure) in caribou. Our data indicate that FP i-ELISA is also comparable with serum i-ELISA for detecting *Brucella* exposure in caribou; however, there were some interesting findings. Considering that *Brucella* i-ELISA is known to detect cross-reacting antibodies, and on the basis of the literature (Nielsen, 1990; Gall et al,. 2001; Gall and Nielsen, 2004; Nielsen et al., 2004) one would anticipate higher prevalence with i-ELISA than with c-ELISA; yet we found the opposite. The BCE laboratory i-ELISA yielded serum *Brucella* antibody prevalence 43%, 8% lower than the prevalence detected with c-ELISA. Running the two separate ELISAs involved a freeze/thaw cycle and there was an 8-wk interval between c-ELISA and i-ELISA testing. However, it is unlikely that these factors would cause enough antibody degradation to explain this extent of divergence. *Brucella* c-ELISA and i-ELISA are different test methods and standard controls were run on each antigen-coated plate. The discrepancy between these results may reflect interference with the monoclonal antibody used in the c-ELISA, which would yield apparently higher levels of inhibition. This effect is occasionally observed with sera from other animals, including cattle and pigs, and the cause is unknown (K. Nielsen, unpubl. data). Our results suggest that, if this effect occurs with *Rangifer* specimens, it occurs to similar degrees in serum and FP samples.

Variability among filter papers from individuals

Analysis of variation between duplicate FP tests and between duplicate serum tests done on the same animal revealed very strong correlations for both c-ELISA

and i-ELISA (Figs. 4,5). This encompassed data generated by four FP strips from each individual (each eluate was prepared from two FP strips because of the volume required for testing).

Test choice

Although Gall et al. (2001) support the use of c-ELISA and FPA for serodiagnosis of brucellosis in caribou, our attempts to use FPA with FP elutions failed. Excessive background fluorescence was the suspected problem, and possible reasons for this include 1) protein aggregation after drying on FP and subsequent elution; 2) release of cellulose from the FP causing interference with light transmission; 3) emission of an autofluorescing chemical from the paper matrix. Our experience with FPA underlines the importance of evaluating individual assays before use in new applications.

We found that caribou FP samples worked very well in both *Brucella* i-ELISA and c-ELISA, and the FP i-ELISA showed slightly better agreement with serum values. Although this appears to promote i-ELISA as the better FP screening test for *Brucella* in caribou, this assay does not distinguish antibodies induced by *Brucella* exposure from antibodies elicited by vaccination or *Y. enterolitica* (Nielsen, 1990). Considering this, FP c-ELISA is likely the more informative *Brucella* assay for *Rangifer* population surveillance, where disease and pathogen exposure levels and, in some cases, vaccination status in a population may be unknown or only estimated.

Benefits of filter paper

Filter papers are inexpensive sample media that are well suited for harsh environmental conditions as they facilitate simple, rapid blood collection in the field and can be dried, transported, and stored relatively economically. Filter-paper blood testing has been used and studied in various veterinary and wildlife applications (Beard and Brugh, 1977; Stallknecht and Davidson, 1992; Yamamoto et al., 1998; Sacks et al., 2002; Chomel et al., 2004; Jordan et al., 2005; Dubay et al. 2006; Trudeau et al., 2007; Yu et al., 2007; Duscher et al., 2009); however, the full potential of FP for wildlife disease testing and monitoring has yet to be realized. One important feature of this tool is that it requires no special training and can thus be used by hunters, biologists, and others. For many Northerners, caribou are a local affordable food source and part of culture/tradition. Filter-paper blood collection during hunting would increase sample size and expand temporal and spatial scales of data collection. Such samples would otherwise be lost from animals killed for subsistence. It is apt that those who rely on caribou should be key agents in monitoring of populations. A team strategy with collection by hunters and analysis by scientists engages communities in caribou health assessment. Additionally, harvesters' observational skills and traditional knowledge can inform scientific perspectives on wildlife disease investigation (Brook et al., 2009). To our knowledge, validated FP blood testing has not been applied in a community-based wildlife or domestic animal health-monitoring context anywhere. This mode of disease surveillance is the ultimate goal of our FP efficacy research.

Two common challenges in assessing diagnostic tests for wildlife are sample size and availability of assays designed for wildlife species. We analyzed FP samples from 185 barren-ground caribou using two serologic assays that were developed for cervids and evaluated using caribou serum samples (Gall et al., 2001). Our FP-serum comparisons and FP-FP (eluate) correlations present strong evidence that FP is an effective tool (comparable with serum) for *Brucella* ELISA screening in *Rangifer* species. Confidence in use of the FP method for detecting antibody to *Brucella* and other infectious agents in *Rangifer* will help pave the way for researchers to acquire baseline data on circumpolar caribou and better understanding of disease in these populations. Future work will explore the efficacy of caribou FP testing for *Brucella* after 1 and 2 yr of storage. The FP method

will also be investigated in several other infectious-disease contexts for *Rangifer*, and with samples subjected to different collection-temperature and storage-time regimes that mimic field conditions.

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