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EFFECT OF STORAGE TIME AND STORAGE CONDITIONS ON ANTIBODY DETECTION IN BLOOD SAMPLES COLLECTED ON FILTER PAPER

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ABSTRACT: Using filter paper to collect blood from wildlife for antibody analysis can be a powerful technique to simplify the collection, transport, and storage of blood samples. Despite these advantages, there are limited data that detail how long these samples can be stored and how storage conditions affect antibody longevity. We used blood samples collected on filter paper from coyotes experimentally infected with *Yersinia pestis* to determine optimum sample storage conditions over time. Blood samples collected on filter paper were stored for 454 d or more in four groups: 1) at ambient temperature and at ambient relative humidity, 2) at ambient temperature with desiccant, 3) at 4 C with desiccant, and 4) at –20 C with desiccant. Samples stored at 4 C or –20 C with desiccant had detectable antibody for a longer period of time than the samples stored at room temperature.

Key words: Antibody, blood, coyote, filter paper, Nobuto, plague, *Yersinia pestis*.

INTRODUCTION

Collecting blood samples from wildlife is often time and labor intensive. It can also be logistically difficult to maintain sample viability when samples are transported from remote field locations where centrifuging, refrigeration, or freezing are not feasible. Transporting heavy coolers or ice packs is also not always an option when supplies need to be carried a long distance. Methodologies that simplify blood collection in the field can increase the chance of viable sample collection. In addition, sample collection methods that are simple and efficient can increase sample numbers by providing collection strategies that require limited training for field personnel.

Nobuto filter paper strips (hereafter FP strips, Advantec, Inc., Newark, California, USA) and other filter paper products are often used in place of blood collection tubes to collect samples in a variety of settings (Burke et al. 1985; Prado et al. 2005; Dusek et al. 2011). Diagnostics that use filter paper–collected blood samples are widely used for neonatal screening, but some of the earliest uses were for veterinary diagnostics (Nobuto 1966). Once a blood-saturated FP strip is dried, the sample is stable and can be easily transported to a laboratory. It can then be stored until elution and the eluate can be used for a variety of diagnostic tests. The sensitivity and specificity of antibody assays using FP strip blood samples have been determined for a variety of pathogens including canine heartworm (Sacks et al. 2002), *Neospora caninum* and West Nile virus (Curry et al. 2014a), dengue virus (Prado et al. 2005), Japanese encephalitis virus (Burke et al. 1985), and others (Dusek et al. 2011; Abbott et al. 2014).

Although FP strips can be successfully used for blood collection and antibody detection for a suite of diseases, less is known about how long these samples can be stored and still provide viable data. In addition, there are limited data available on how FP strip blood samples should be stored and if storage conditions can limit or extend the amount of time for antibody detection (Dusek et al. 2011; Curry et al. 2014b). We used samples collected during an experimental infection study (Baeten et al. 2013) in coyotes (*Canis latrans*) to examine antibody reactivity in FP

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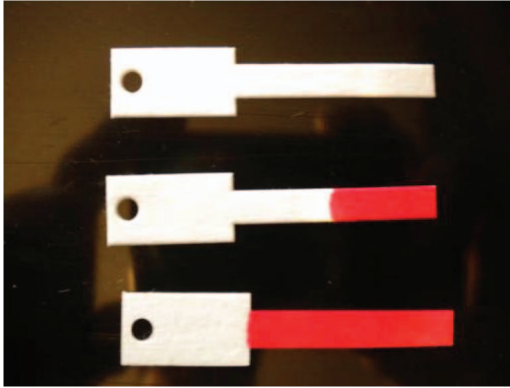

FIGURE 1. Images of Nobuto filter paper strips depicting an unused strip (top), an incorrectly saturated strip (middle), and a correctly saturated strip (bottom). The correct blood saturation level equals 100 μL of whole blood.

strips subjected to different storage conditions over time.

MATERIALS AND METHODS

Venous blood samples were collected from six manually restrained or chemically anesthetized coyotes that were experimentally infected with *Yersinia pestis* (Baeten et al. 2013), the causative agent of plague (Wolff and Hudson 1974). Blood samples were collected in sodium-heparin tubes or ethylenediaminetetraacetic acid (EDTA) vacuum tubes (Vacutainer, Becton Dickinson, Franklin Lakes, New Jersey, USA). Filter paper strips were then saturated to the recommended level on both sides (Fig. 1) by transferring whole blood from blood collection tubes using disposable blood bank pipettes (Fisherbrand, Fisher Scientific, Pittsburgh, Pennsylvania, USA). Serum was also collected from each animal and tested at day 0 to establish concordance between FP strip results and serum results.

Two replicate FP strips were collected from each animal. Blood collected on FP strips was separated into four storage treatment groups: 1) at ambient temperature and relative humidity, 2) at ambient temperature with a desiccant pack (1 g silica gel color indicating packs, www.desiccantpackets.com, Concord, New Hampshire, USA), 3) in a refrigerator at 4 C with a desiccant pack, and 4) in a freezer at –20 C with a desiccant pack. The four different treatment groups, combined with replicate sampling of each animal, resulted in eight FP strip blood samples that were collected and tested from each animal at each time point. A minimum of 112 FP samples were collected and tested from each of the six animals, with additional samples collected if sufficient blood volume was available.

Mean temperatures for each treatment were continuously monitored using HOBO® data loggers (Onset, Bourne, Massachusetts, USA). Desiccants in freezers can improve the viability of dried blood spot samples (McDade et al. 2007) and were used in all treatment groups except for the treatment that simulated FP strips being stored in ambient laboratory conditions. Relative humidity was also monitored for the treatment group where FP strips were left at ambient temperature and without a desiccant pack (ambient humidity). Humidity levels were assumed to be lower for all other treatment groups because they employed desiccant packs.

Samples were screened for antibody titers to the *Y. pestis* fraction 1 capsular protein using a previously described passive hemagglutination assay (Wolff and Hudson 1974; Chu 2000). Briefly, FP strips were cut in half and placed at the bottom of the vial. Then 0.4 mL of borate elution buffer (Chu 2000) was added to the vial, which was covered with Parafilm M® (Bemis, Neenah, Wisconsin, USA) and incubated overnight at 4 C. After the incubation, eluted vials were partially submerged in a 56 C water bath for 30 min for heat inactivation. A single drop of packed sheep red blood cells (SRBC) was added to each vial, which was mixed by inversion and allowed to sit at room temperature for another 30 min as previously described (Chu 2000). The vials were spun to pellet the SRBC and the resulting supernatant was pipetted for testing.

Samples were screened at day 0 to determine the baseline titer, and thereafter on d 2, 6, 8, 12, 14, 26, and thereafter during wk 6, 10, 14, 18, 22, 26, 52, and 65. The amount of blood collected differed among coyotes and samples from some animals had been exhausted by wk 65. Therefore, limited testing was also carried out on any remaining FP strips on wk 104, 130, and 155.

Patterns of antibody viability over time and by storage condition were analyzed using a generalized linear mixed model with a multinomial distribution and a cumulative logit link function for ordinal data. Categorical independent variables were time (d) and storage conditions (treatment). Titer was the response variable and it was adjusted to the percent of the original titer at time point 0. This was done to control for variation in day 0 titers across animals. Replicate within experimental animal was a random effect. A Wilcoxon signed-rank test also compared the titer of paired EDTA and serum-separating tube samples to determine if blood-collection tubes influenced titers over time. All analyses were run in SAS v.9.2 (SAS Institute, Cary, North Carolina, USA).

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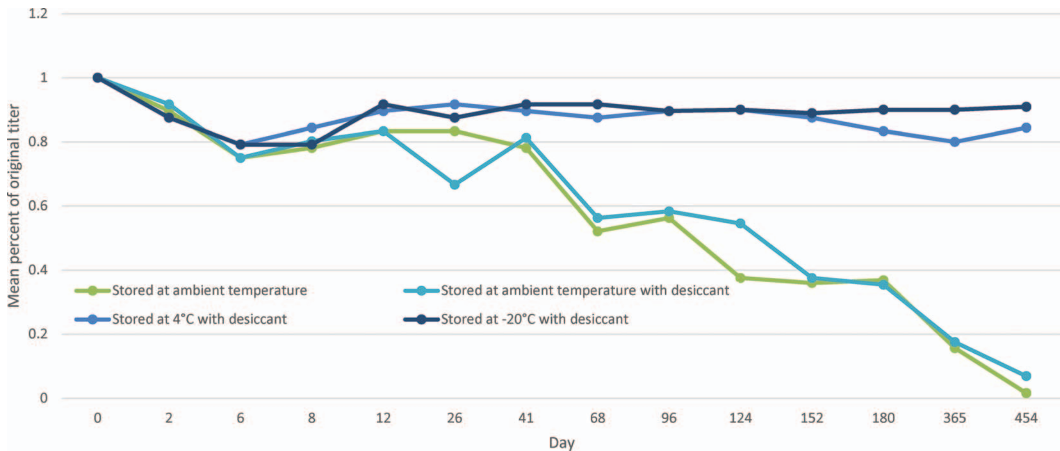

FIGURE 2. Mean titers of blood samples collected on Nobuto strips, adjusted by percent of the original titer at time point 0, for four storage conditions over time.

RESULTS

Temperatures of the four storage treatments were recorded throughout the study, with the two ambient temperature treatment groups having an average temperature of 22.2 C (SD=0.50). The average temperatures for the 4 C and –20 C treatment groups were 5.5 C (SD=0.4) and –24.6 C (SD=5.02), respectively. The relative humidity of the storage treatment group at ambient temperature and ambient humidity was 37.1% (SD=5.5). We collected 849 samples on FP strips by saturating them with blood from EDTA or serum-separating tubes prior to centrifuging. All serum samples and filter paper samples were positive for *Y. pestis* antibody (sensitivity=100%). Additional posteuthanasia samples that were collected on filter paper from incision sites associated with organ and tissue collection indicated that collecting directly from the incision site does not significantly affect antibody titer compared to samples collected from EDTA or serum separating tubes (L.B. unpubl. data).

Titers were significantly affected by both the storage condition treatment group (F=9.2, P<0.001) and by the length of time the samples were stored (F=87.6, P<0.001). Titers decreased over time for all storage conditions; however, the titers of samples stored at –20 C or at 4 C with desiccant packs were higher at the final time point than were samples that stored at ambient temperature with a desiccant pack or samples stored at ambient temperature and relative humidity (Figs. 2, 3). Odds ratios (OR) and 95% confidence intervals (CI) on the estimates that compared each treatment to samples stored at ambient temperature and ambient humidity demonstrated that storing samples with desiccant packs at –20 C (OR=0.023, 95% CI=0.003–0.162) or at 4 C (OR=0.028, 95% CI=0.004–0.196) significantly increased the detection of titers over time. There was no significant difference in titer between samples stored with or without desiccant packs at ambient temperature and humidity.

Predictably, the titer of detected antibody decreased as storage time increased (Figs. 2, 3). A limited amount of blood resulted in FP strips not being available for all animals after 65 wk of storage because samples from those animals were used up during earlier time points. Those that were available were tested at wk 104 (≈24 mo), wk 130 (≈30 mo), and wk 155 (≈36 mo). For FP strips stored at ambient temperature and ambient humidity, *Y. pestis* antibody was only detected in 11% (1/9) of the samples at wk 104 and no samples were reactive during the subsequent testing periods. For samples stored at ambient temperature and with a desiccant pack, antibody was detectable in 33% (3/9) of FP strips at wk 104, but the antibody signals had

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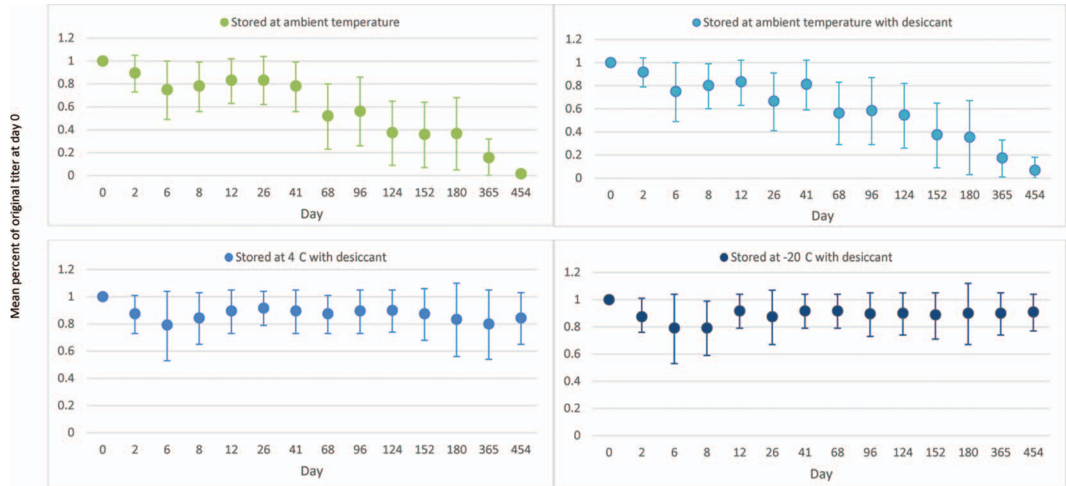

FIGURE 3. Mean titers of blood samples collected on Nobuto strips, adjusted by percent of the original titer at time point 0, for filter paper strips kept under four storage conditions. Error bars represent 95% confidence intervals.

degraded below detectable limits at subsequent time points. At wk 104, 130, and 155, antibody was still detected in 88% (8/9), 57% (4/7), and 66% (4/6), respectively, of FP strips stored at 4 C with a desiccant pack. For FP strips stored at –20 C with a desiccant pack, 100% (22/22) of samples still had detectable antibody when tested on wk 104, 130, and 155.

DISCUSSION

Nobuto filter paper strips can be used to collect blood samples for convenience or when sample collection is logistically difficult. Results reported here indicate that storing saturated FP strips at –20 C maintains sample viability for >1 yr. In addition, most samples stored with desiccant packs at 4 C and at –20 C still produced a titer within one or two dilutions below the titer at time point 0 >2 yr after the original collection date. Conversely, antibodies detected from FP strips stored for >10 wk at ambient conditions inside a laboratory (22 C and 37% relative humidity) might degrade to the point where they were no longer detectable. Data from this specific study suggest that antibody titers of FP strips stored in ambient conditions begin to degrade as early as 6 wk after collection, although consistent statistical differences in antibody longevity between samples stored in ambient conditions and samples stored at colder temperatures did not emerge until day 124 (4 mo of storage).

These results are in line with previously published research that has used FP strips to detect antibodies or antigen in wild animals. Dusek et al. (2011) found that samples collected from birds on FP strips gave reliable positive and negative results for avian influenza, even after being stored at room temperature for 3 mo. However, they noted a difference in the quantitative antibody value when compared to sera, similar to what we report. Additional research on a suite of pathogens in caribou (*Rangifer tarandus* ssp.) and reindeer (*Rangifer tarandus tarandus*) found that in some cases, FP strip samples stored for 1 yr at room temperature could have similar sensitivity and specificity to sera stored at –20 C (Curry et al. 2014b); but again, the authors saw successively greater decreases in sensitivity of FP sample testing as storage time increased, and this may reflect degradation of antibodies in the filter paper matrix over time.

Our results reflect the pathogen (*Y. pestis*) and assay (passive hemagglutination) used in this study; however, others have shown that

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blood samples collected on filter paper provide reliable results for multiple types of pathogens and assays (Machado-Coelho et al. 1995; Phetsouvanh et al. 2009; Desvars et al. 2011). These findings suggest that filter paper samples collected for use in antibody analysis could benefit by being stored in a freezer, regardless of the assay being performed. Despite the benefits of cold temperatures on long-term storage, our data and data from previous studies demonstrate that blood collected on filter paper can still be stored in ambient conditions if they are being tested relatively quickly (i.e., within 10–12 wk) after collection (Dusek et al. 2011; Curry et al. 2014b). The length of time that FP blood samples can be stored may differ, however, based on the target (nucleic acid, antibody, etc.) and the assay being utilized. The viability of long-term storage for filter paper samples should therefore be evaluated based on the specifics of the research. In addition, filter paper blood samples may not provide high-enough sensitivity and specificity for some pathogens or assays (Figueiredo et al. 2010). It is important that research using blood samples collected on FP strips have high-quality samples. Nobuto filter paper strips that are not fully saturated (see Fig. 1) will not give reliable results. Results will also be unreliable if samples are collected from animals that have been dead for a long period of time, although the exact length of time after death when viable samples can still be collected depends upon the assay being used (Tryland et al. 2006).

The storage conditions in this study also used silica desiccant to lower the humidity in the storage treatments. While antibodies were detectable for the greatest length of time in samples stored at cold temperatures with desiccant packs, the study did not compare samples stored at cold temperatures with and without desiccant. Desiccants were used because damp conditions can affect the integrity of filter paper samples (Smit et al. 2014), but these desiccants also are most efficient at room temperatures, so it was difficult to determine if they significantly contributed to the preservation of detectable antibodies in FP strips.

Our findings shed light on how storage conditions affect antibody detection in blood samples collected using FP strips. Using this technique for antibody analysis in wildlife is a simple and cost-effective way to test a large number of samples. Recent calls to increase the use of filter paper samples to develop low-cost diagnostic assays (Solomon et al. 2012) highlight the need to better understand the conditions that best maintain sample viability and to validate the use of this sample type in more assays.

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