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USE OF WHOLE BLOOD SAMPLES PRESERVED IN DNA LYSIS BUFFER FOR SEROLOGICAL DETECTION OF AVIAN MALARIA IN HAWAIIAN FOREST BIRDS

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ABSTRACT

Two buffers that are commonly used to preserve whole blood for polymerase chain reaction (PCR) diagnostics, tris-ethylenediaminetetraacetic acid (TEN) and tris-sodium dodecyl sulfate-ethylenediaminetetraacetic acid (SDS-EDTA), were evaluated to determine whether they can also be used to preserve blood for serological studies to detect antibodies to avian malaria. TEN buffer had no effect on antibody binding as measured by enzyme-linked immunosorbent assay (ELISA) or Western blotting. By contrast, the SDS-EDTA buffer completely abolished all antibody binding. Efforts to restore binding by dialysis and concentration of the samples were not successful. Addition of sodium dodecyl sulfate (SDS) and Proteinase K to samples preserved in TEN buffer was also evaluated, because this treatment is sometimes used to render samples non-infectious prior to shipping. This treatment abolished all antibody binding by both ELISA and Western blotting. TEN buffer appears to be good for preserving whole blood samples for both PCR and serological studies, making it possible to simultaneously preserve blood samples for both PCR and serological diagnostic tests in a single tube.

INTRODUCTION

Accurate diagnosis of malarial infections in vertebrate hosts is challenging because (1) asexual stages of the parasite may alternate between fixed tissues of the host and circulating blood cells, (2) the dynamics of infection in circulating erythrocytes may vary by orders of magnitude as parasite growth rates are limited by the host immune response, and (3) detection of parasites that remain dormant and sequestered within cells of the reticuloendothelial system may be difficult at times of the year when arthropod vectors are not active (Valkiūnas 2005). Methods for diagnosing infections have relied on microscopic detection of parasites within circulating erythrocytes through preparation of fixed and stained blood smears (Garnham 1966) and more recently through use of polymerase chain reaction (PCR) amplification of parasite DNA in blood samples using primers designed to different regions of the parasite genome (Feldman *et al.* 1995, Fallon *et al.* 2003, Hellgren *et al.* 2004). Both methods rely on detection of erythrocytic parasites in the peripheral circulation and are prone to false negative results when parasitemia drops below test sensitivity or parasites disappear from the circulation and become sequestered within non-erythroid tissues of the avian host. A third approach based on detection of circulating antibodies to the parasites by either ELISA (enzyme-linked immunosorbent assay) or Western blotting is effective for diagnosing infections with low parasitemia or sequestered asexual parasites and can provide independent verification of infection status for diagnostic results based on microscopy and PCR (Graczyk *et al.* 1993, Atkinson *et al.* 2001).

Recent detections of avian malarial parasites in native and non-native forest birds at Hakalau Forest National Wildlife Refuge in Hawai'i and reports of epidemic transmission of the disease in high elevation habitats (Freed *et al.* 2005) as well as controversy over accuracy of the PCR diagnostic test that was being used (Jarvi *et al.* 2002) led to a request by U.S. Fish and Wildlife Service to see if existing blood samples that were preserved in a DNA lysis buffer could be used for independent confirmation of the findings with antibody based serological methods (Atkinson *et al.* 2001). The primary objective of this study was to test whether some DNA buffers used for preservation of blood samples (Feldman *et al.* 1995, Jarvi *et al.* 2002) cause denaturation and loss of antigenicity of antibody molecules. If the buffer does not destroy antigenicity of these

molecules, then the samples can be used in serological assays to provide an independent assessment of the accuracy of the PCR test.

METHODS

Work was conducted at the U.S. Geological Survey, Kīlauea Field Station, Hawai‘i Volcanoes National Park between 2005 and 2006. Blood samples were collected by jugular venipuncture from eight captive Hawai‘i ‘amakihi (*Chlorodrepanis virens*) with chronic experimental infections with the avian blood parasite *Plasmodium relictum* plus one uninfected control bird. The birds were survivors from prior experimental studies, and in the case of infected birds, still had detectable numbers of circulating parasites by microscopy of Giemsa-stained blood smears. The birds were held under University of Hawai‘i IACUC Protocol 00-035-5 for experimental studies of avian malaria and pox virus. Up to 100 µl of heparinized whole blood was drawn from each of the birds and divided into three equal portions. One part was mixed 1:10 with phosphate buffered saline (PBS), pH 7.0, as an untreated positive control for serological analysis. The second portion was mixed 1:10 with a DNA buffer used by Feldman *et al.* (1995), tris-ethylenediaminetetraacetic acid (TEN; 10 mM Tris, 10 mM NaCl, 2 mM EDTA, pH 8.0), and frozen. The third portion was mixed 1:10 with a DNA lysis buffer used by Jarvi *et al.* (2002), tris-sodium dodecyl sulfate-ethylenediaminetetraacetic acid (SDS-EDTA; 0.1 M Tris-HCl, pH 8.0, 0.1 M EDTA, 2% SDS), and frozen.

The DNA samples were subsequently thawed several weeks after being frozen. Samples that were preserved in SDS-EDTA buffer were dialyzed overnight against ice cold PBS in dialysis tubing (Sigma D-0405, molecular weight cutoff = 12,400) to attempt to remove SDS (sodium dodecyl sulfate) and EDTA (Dong *et al.* 1997). After removal from the tubing the following morning, the liquid was centrifuged 15 min at 28,200 g to pellet any cellular debris, transferred to Centricon YM-50 centrifugal filter devices with a nominal molecular weight limit of 50,000 daltons (Millipore Corporation, Billerica, Massachusetts), and centrifuged 15 min at 13,000 g to concentrate antibodies. If samples preserved in SDS-EDTA buffer were too gelatinous for use in the Centricon filters, they were briefly sonicated on wet ice to liquefy the pellet with a Microson XL200 ultrasonic cell disruptor (Medsonic, Inc., Farmingdale, New York). The concentrate was removed from the upper wells of the filters, transferred to vials, and either refrigerated or frozen prior to evaluation by ELISA or Western blotting (Atkinson *et al.* 2001). Samples preserved in TEN buffer and PBS were used without additional processing.

For the ELISA analysis, samples were serially diluted with PBS at 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, and 1/1280 and then tested by a modification of the ELISA procedure described by Graczyk *et al.* (1993). Briefly, a crude *P. relictum* antigen extract was prepared from infected Pekin duckling erythrocytes by lysing the cells with saponin to rupture erythrocyte membranes, pelleting the lysed cells, and washing them with sodium carbonate buffer, pH 9.0, to remove hemoglobin. The cells were pelleted and sonicated to disrupt cellular membranes and centrifuged at 20,000 g to pellet cellular debris. The supernatant was used to coat individual wells of ELISA plates (Immulon 4HBX flat bottom plates, Thermo Fisher Scientific, Waltham, Massachusetts) by overnight incubation at 4°C. The ELISA procedure followed that described by Graczyk *et al.* (1993), using a rabbit anti-chicken IgG alkaline phosphatase conjugate (Sigma-Aldrich Corp., St. Louis, Missouri, catalog number A9171) that cross-reacts with native forest bird IgG as a secondary antibody. Absorbance values were expressed as a percent ELISA value (% EV) of positive and negative Pekin duckling plasma controls that were run on each plate.

The % EV was calculated as (mean absorbance of triplicate samples – the mean absorbance of triplicate negative controls)/(mean absorbance of triplicate positive controls – mean absorbance of triplicate negative controls)*100. Samples tested by Western blotting followed the procedures described by Atkinson *et al.* (2001).

To evaluate whether antibody binding is affected by supplemental addition of SDS and Proteinase K, a blood sample from an infected Hawai'i 'amakihi that had been preserved in PBS and TEN buffers was thawed. One 250 µl aliquot of the sample preserved in PBS was transferred to a new tube, and two 250 µl aliquots of the sample preserved in TEN were transferred to each of two new tubes. Fifteen microliters (15 µl) of distilled water were added to the tubes containing blood samples that were preserved with PBS or TEN buffer. Thirteen microliters (13 µl) of a 20% solution of SDS and 2 µl of a 25 mg/ml solution of Proteinase K (Roche Diagnostics, Indianapolis, Indiana) were added to the third tube containing the blood sample that was preserved in TEN buffer. All tubes were mixed by vortexing and centrifuged at 20,000 g for 15 min at 5°C. The sample containing SDS and Proteinase K was gelatinous and briefly sonicated on wet ice to liquefy the pellet. All samples were concentrated with Centricon YM-50 filters, resuspended with 200 µl of PBS, and re-concentrated with an additional centrifugation through the YM-50 filters. The remaining concentrate was resuspended to a final volume of 20 µl with PBS and used for ELISA and Western blotting as described earlier.

Differences between mean % EV for various treatments were evaluated by a repeated measures ANOVA for comparisons of serially diluted samples (Systat Software 2004). When significant differences were detected, pairwise comparisons of means were made with a Bonferroni adjustment (Systat Software 2004). Differences were considered statistically significant when $P < 0.05$. Raw data from the project with accompanying metadata are archived at ScienceBase (Atkinson 2020).

RESULTS

There were significant treatment differences in antibody binding as measured by % EV among samples preserved in PBS (controls), TEN buffer, and SDS-EDTA buffer (Figure 1; $F = 4.871$, $df = 2$, $P = 0.021$). Mean % EV were highest for samples diluted 1/10 and then declined with each dilution, with mean % EV approaching 0 at dilutions of 1/320 and higher. Samples diluted in SDS-EDTA buffer had much higher variability and significantly higher % EV values than samples diluted in PBS buffer at dilutions 1/10 ($P = 0.026$), 1/20 ($P = 0.031$), and 1/40 ($P = 0.040$; Figure 1). Other comparisons among treatments at each dilution were not statistically significant ($P > 0.05$; Figure 1). Significant within-subjects effects were evident for serial dilutions for each treatment, with expected higher % EV for less diluted samples ($F = 43.322$, $df = 7$, $P < 0.0001$; Figure 1).

When negative control plasma was evaluated, there was no difference in % EV when the sample was diluted in PBS or TEN. However, the % EV for the negative control sample preserved in SDS buffer was substantially higher than those preserved in PBS or TEN buffer, indicating that denaturation of the sample by SDS led to non-specific antibody binding (Figure 2).

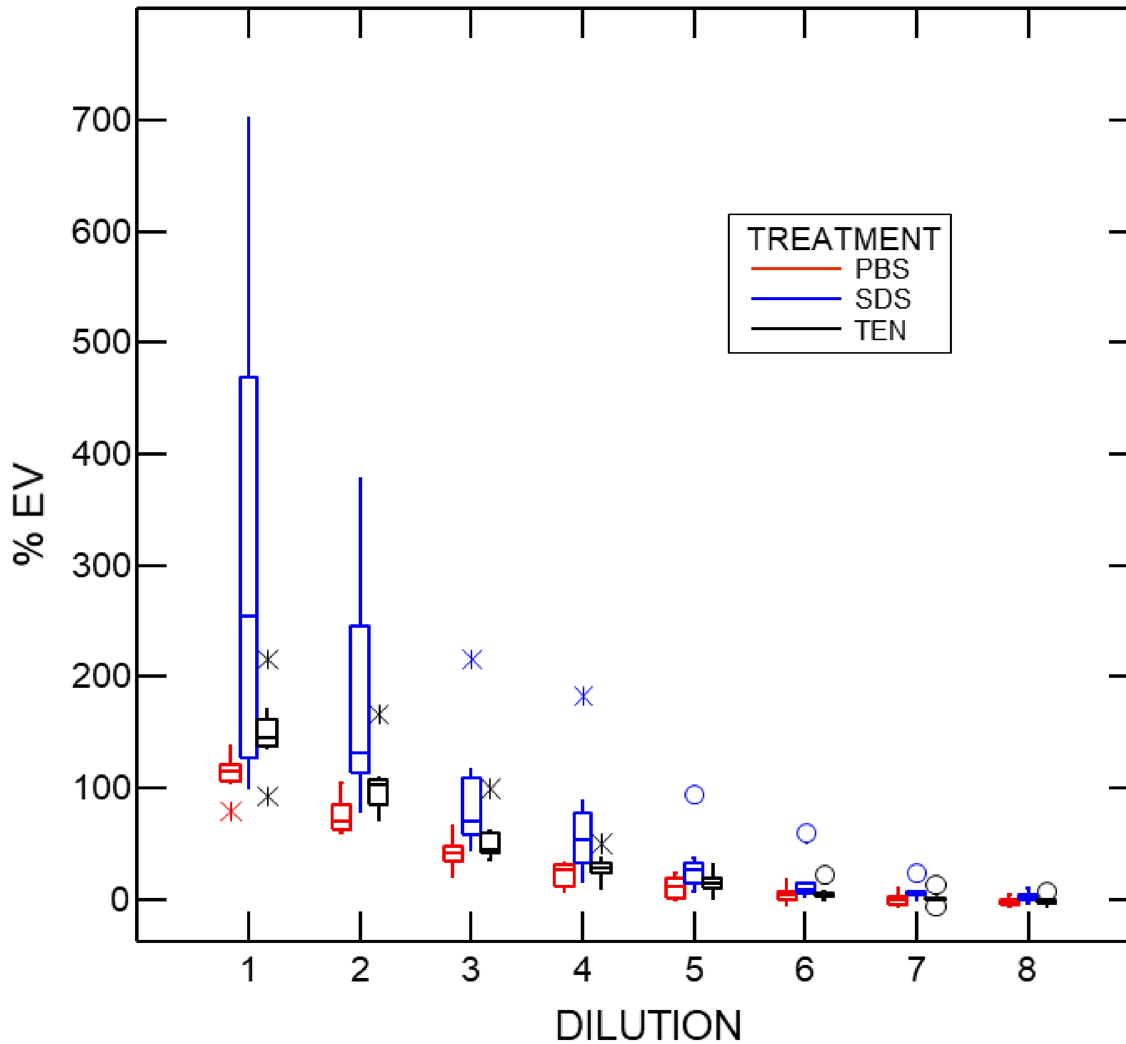

Figure 1. Box plots of mean % ELISA values (% EV) of serially diluted blood samples from 7 experimentally infected Hawai'i 'amakihi (*Chlorodrepanis virens*; Bands 130, 137, 139, 142, 147, 148, 77735) that were preserved in PBS, SDS-EDTA, and TEN buffers. Samples were dialyzed and concentrated with Centricon YM-50 centrifugal filters (as described in Methods). Serial twofold dilutions of samples were done from 1/10 (dilution 1) to 1/1280 (dilution 8). The central horizontal line in each box plot marks the median of each sample, the length of each box shows the range within which the central 50% of the values fall (between 25th and 75th percentiles), and the vertical blue lines and markers designate the 10th and 90th percentiles. Values between the inner and outer fences are plotted with asterisks. Values beyond the outer fences are plotted with empty circles.

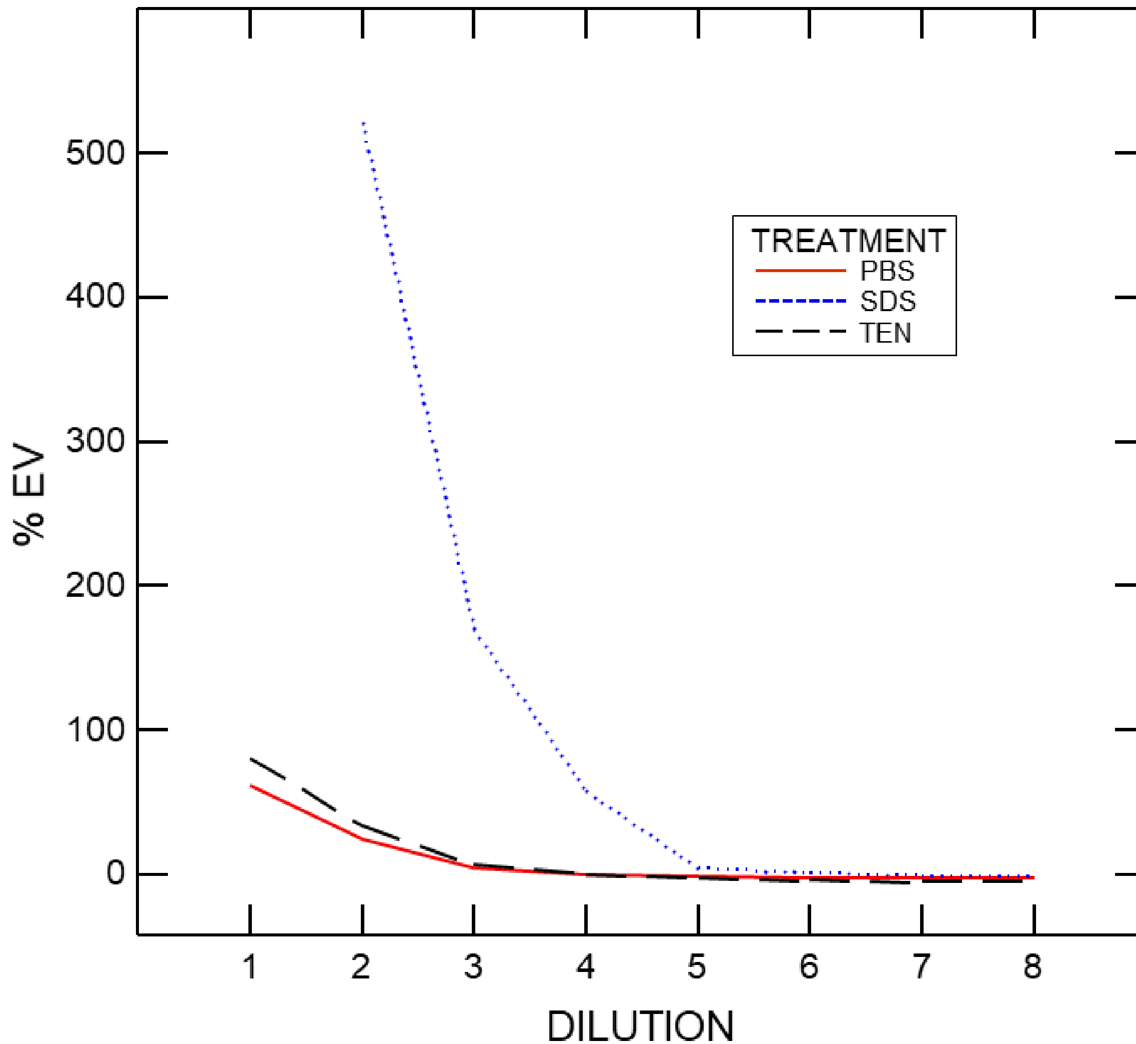

Figure 2. Percent ELISA values (% EV) of a serially diluted blood sample from an uninfected Hawai'i 'amakihi (*Chlorodrepanis virens*; Band = 6) that was preserved in PBS, SDS-EDTA, or TEN buffer. Samples were dialyzed and concentrated with Centricon YM-50 centrifugal filters (as described in Methods). Serial twofold dilutions of samples were done from 1/10 (dilution 1) to 1/1280 (dilution 8).

To test whether the higher ELISA values among samples preserved in SDS buffer were a result of antibody binding to the ELISA plates or non-specific interactions of blood proteins, birds were also screened by Western blotting to look for characteristic bands that are present when birds have chronic malarial infections (Atkinson *et al.* 2001). There were 180, 66, and 55 kilodaltons (kDa) bands among samples that were preserved in PBS (control) and TEN buffers and diluted at 1/200 with PBS in the blotting protocol, but no antibody binding among samples that were preserved in SDS-EDTA buffer that were serially diluted from 1/200 to 1/1600 with PBS (Figure 3).

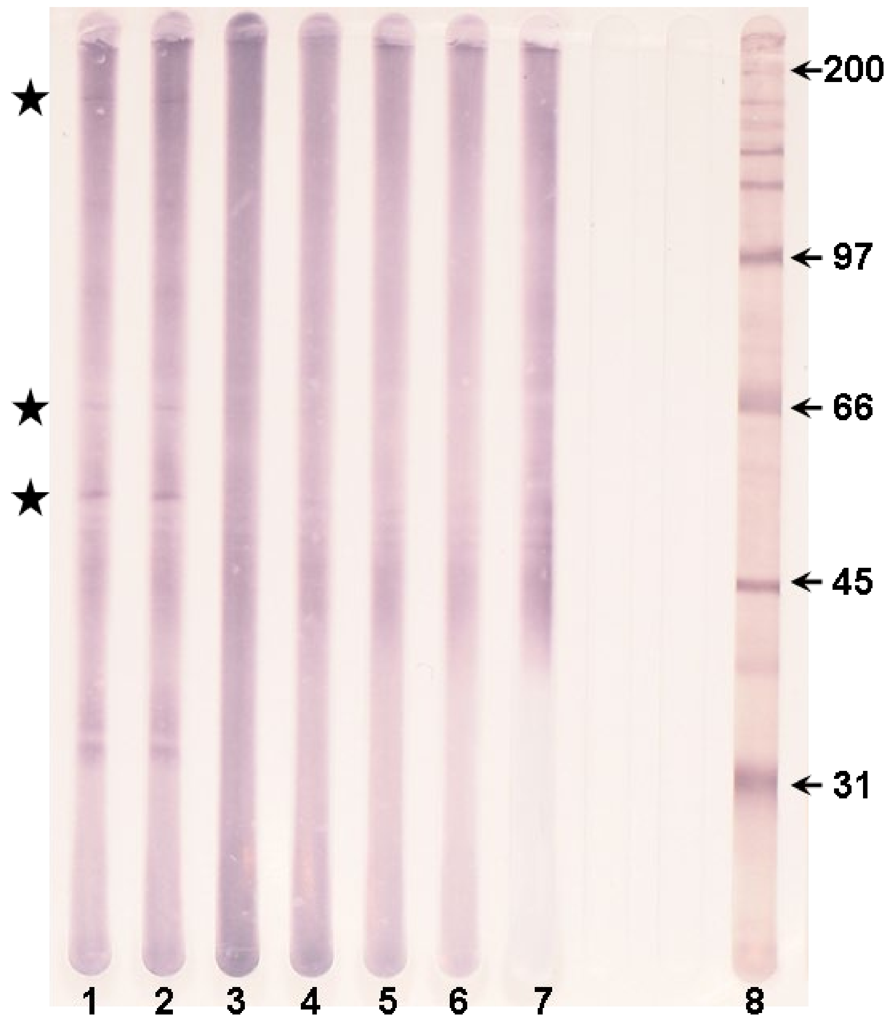

Figure 3. Western blot of a blood sample from experimentally infected Hawai'i 'amakihi (*Chlorodrepanis virens*; HAAM 142) that was divided into three parts and preserved in either PBS (Control, lane 1), TEN (lane 2), or SDS-EDTA buffer (lanes 3–6). Characteristic bands (stars) were evident for samples preserved in PBS and TEN buffers, but no antibody binding was evident for the sample preserved in SDS-EDTA buffer. Samples in lanes 1 and 2 were diluted with PBS at 1/200. Dilutions with PBS in lanes 3–6 were 1/200 (lane 3), 1/400 (lane 4), 1/800 (lane 5), and 1/1600 (lane 6). Lane 7 is Rabbit anti-forest bird antibody control (incubations with secondary antibodies and enzyme to illustrate levels of non-specific background). Lane 8 is molecular weight markers in kilodaltons.

Finally, the effect of adding SDS and Proteinase K to preserved samples was tested because this treatment is sometimes done to render the samples non-infectious prior to shipping. When SDS and Proteinase K were added at low concentrations to samples that were preserved in TEN buffer, all antibody binding was abolished (Figure 4).

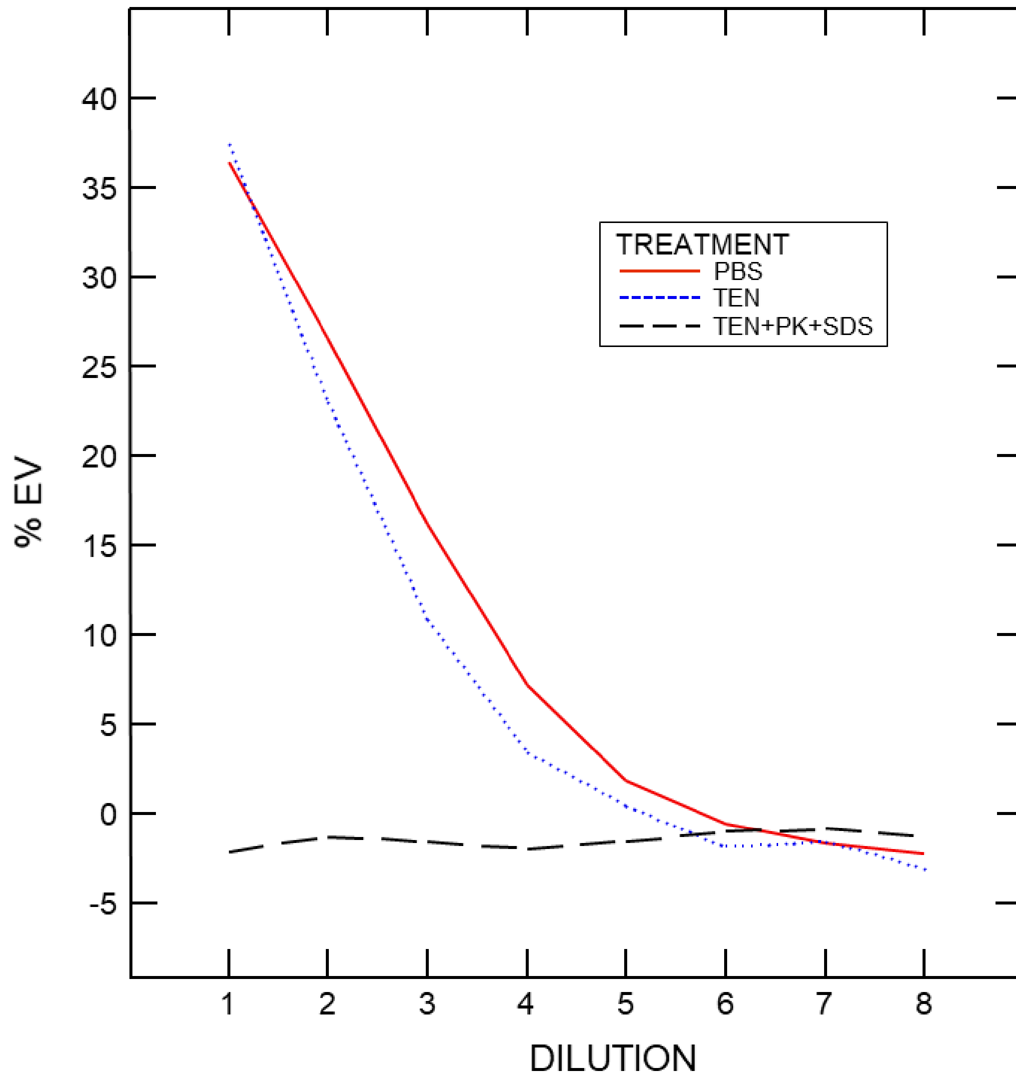

Figure 4. Percent ELISA values (% EV) from a blood sample from experimentally infected Hawai'i 'amakihi (*Chlorodrepanis virens*; HAAM 142) that was preserved in PBS, TEN, or TEN with SDS and Proteinase K added later. Serial twofold dilutions of samples were done from 1/10 (dilution 1) to 1/1280 (dilution 8).

To verify that treatment with SDS and Proteinase K completely abolished antibody binding, the same samples were tested by Western blotting. Antibody binding was evident for samples in PBS and TEN that were diluted 1/200 for the blotting procedure. No antibody binding was evident for the sample in TEN with added SDS and Proteinase K (Figure 5).

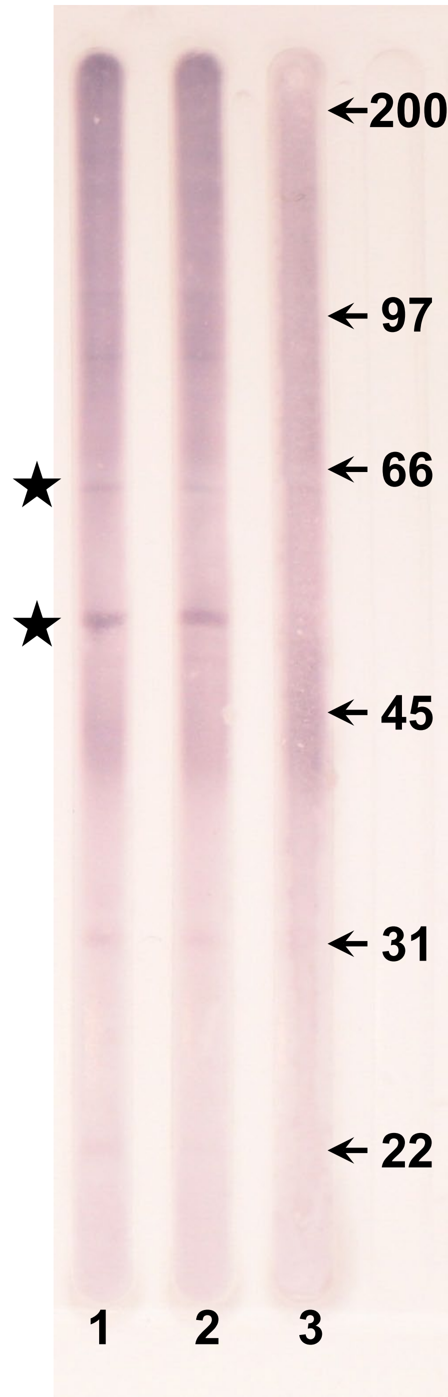

Figure 5. Western blot illustrating the effects of treatment with SDS and Proteinase K on samples preserved with TEN buffer. These additives completely abolished antibody binding (lane 3), as indicated by absence of 55 kDa and 66 kDa bands (stars). Lane 1 = sample preserved in PBS (positive control). Lane 2 = sample preserved in TEN. Lane 3 = sample preserved in TEN with SDS and Proteinase K added after thawing to duplicate shipping conditions. Arrows indicate molecular weight standards in kDa. All samples were diluted 1/200 with PBS prior to use in the blotting protocol.

Discussion

No significant loss of malarial antibody binding by either ELISA or Western blotting was detected for samples preserved in TEN DNA buffer, indicating that this treatment can provide excellent preservation of antibodies when samples are frozen. By contrast, DNA buffers containing SDS caused high background readings at low dilutions in the ELISA protocol (Figures 1 and 2) and appeared to completely abolish antibody activity when samples were tested by Western blotting (Figure 3). Attempts to restore activity by overnight dialysis against cold PBS and concentration with Centricon YM-50 centrifugal filters were not successful.

Addition of both SDS and Proteinase K to samples preserved in TEN buffer completely abolished antibody binding. This was probably through the combined denaturing effects of SDS and the enzymatic activity of Proteinase K which degraded proteins in the samples (Ebeling *et al.* 1974, Hilz *et al.* 1975). Thus, treatment of samples preserved in TEN buffer to render them non-infectious prior to shipping is not recommended.

The project demonstrated that TEN buffer is an effective preservative for whole avian blood for both PCR and serological studies. Use of TEN buffer makes it possible to simultaneously preserve blood samples for both PCR and serological diagnostic tests in a single tube, without the need for a field centrifuge to separate plasma from erythrocytes. This allows confirmatory, parallel diagnostics when results from either serology or PCR are questionable and opens the possibility that retrospective serological studies for a wide variety of avian diseases can be performed on frozen blood samples that are preserved in TEN buffer.

Acknowledgements

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Literature Cited

Atkinson, C. T. 2020. Hawaii Island forest bird avian malaria detection using whole blood preserved in lysis buffer, 2005–2006. U.S. Geological Survey data release, https://doi.org/10.5066/P98CVJJG.

Atkinson, C. T., R. J. Dusek, and J. K. Lease. 2001. Serological responses and immunity to superinfection with avian malaria in experimentally-infected Hawaii amakihi. Journal of Wildlife Diseases 37:20–27.

Dong, M., L. G. Baggetto, P. Falson, M. Le Maire, and F. Penin. 1997. Complete removal and exchange of sodium dodecyl sulfate bound to soluble membrane proteins and restoration of their activities, using ceramic hydroxyapatite chromatography. Analytical Biochemistry 247:333–341.

Ebeling, W., N. Hennrich, M. Klockow, H. Metz, H. D. Orth, and H. Lang. 1974. Proteinase K from *Tritirachium album* Limber. European Journal of Biochemistry 47:91–97.

Fallon, S. M., R. E. Ricklefs, B. L. Swanson, and E. Bermingham. 2003. Detecting avian malaria: an improved polymerase chain reaction diagnostic. Journal of Parasitology 89:1044–1047.

Feldman, R. A., L. A. Freed, and R. L. Cann. 1995. A PCR test for avian malaria in Hawaiian birds. Molecular Ecology 4:663–673.

Freed, L. A., R. L. Cann, M. L. Goff, W. A. Kuntz, and G. R. Bodner. 2005. Increase in avian malaria at upper elevation in Hawai'i. The Condor 107:753–764.

Garnham, P. C. C. 1966. Malarial parasites and other haemosporidia. Blackwell Scientific Publications, 1132 pp.

Graczyk, T. K., M. R. Cranfield, and C. J. Shiff. 1993. ELISA method for detecting anti-*Plasmodium relictum* and anti-*Plasmodium elongatum* antibody in infected duckling sera using *Plasmodium falciparum* antigens. Journal of Parasitology 79:879–885.

Hellgren, O., J. Waldenström, and S. Bensch. 2004. A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. Journal of Parasitology 90:797–802.

Hilz, H., U. Wiegers, and P. Adamietz. 1975. Stimulation of Proteinase K action by denaturing agents: application to the isolation of nucleic acids and the degradation of 'masked' proteins. European Journal of Biochemistry 56:103–108.

Jarvi, S. I., J. J. Schultz, and C. T. Atkinson. 2002. PCR diagnostics underestimate the prevalence of avian malaria (*Plasmodium relictum*) in experimentally-infected passerines. Journal of Parasitology 88:153–158.

Systat Software. 2004. SYSTAT 11. Systat Software, Inc., Richmond, CA.

Valkiūnas, G. 2005. Avian malaria parasites and other haemosporidia, CRC Press, Boca Raton, FL. 946 pp.