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Research Note—

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SUMMARY. The utility of using Nobuto paper strips for the detection of avian influenza antibodies was examined in mallards (*Anas platyrhynchos*) experimentally infected with low pathogenic avian influenza viruses. Blood was collected 2 wk after infection and was preserved either as serum or whole blood absorbed onto Nobuto strips. Analysis of samples using a commercially available blocking enzyme-linked immunosorbent assay revealed comparable results (≥96% sensitivity for all methods) between sera stored at −30 C and the Nobuto strip preservation method even when the Nobuto strips were stored up to 3 mo at room temperature (RT). Significant differences were detected in the ratio of sample absorbance to negative control absorbance for Nobuto strips stored at RT compared with sera stored at −30 C, although these differences did not affect the ability of the test to reliably detect positive and negative samples. Nobuto strips are a convenient and sensitive alternative to the collection of serum samples when maintaining appropriate storage temperatures is difficult.

RESUMEN. *Nota de Investigación*—Evaluación de las tiras de papel filtro Nobuto para la detección de anticuerpos virus de influenza aviar en aves acuáticas.

Se evaluó la utilidad de las tiras de papel Nobuto para la detección de anticuerpos contra la influenza aviar en patos reales (*Anas platyrhynchos*) infectados experimentalmente con un virus de baja patogenicidad de la influenza aviar. Se recolectaron muestras de sangre dos semanas después de la infección y se conservaron como muestras de suero o de sangre entera absorbida en tiras de papel Nobuto. Mediante el análisis de las muestras con un estuche de inmunoensayo con enzimas ligadas competitivo disponible comercialmente se obtuvieron resultados comparables (sensibilidad mayor o igual al 96% para todos los métodos) entre los sueros almacenados a −30 °C y el método de preservación en las tiras de Nobuto, incluso cuando las tiras de Nobuto se almacenaron hasta 3 meses a temperatura ambiente. Se detectaron diferencias significativas en las proporciones entre la absorbancia de las muestras y de los controles negativos de las muestras en tiras de papel conservadas a temperatura ambiente en comparación con los sueros almacenados a −30 °C, aunque estas diferencias no afectaron la capacidad de la prueba para detectar de manera confiable muestras positivas y negativas. Las tiras de papel Nobuto son una alternativa conveniente y sensible para la colección de muestras de suero cuando resulta difícil mantener temperaturas adecuadas de almacenamiento.

Key words: antibody, avian influenza, blocking enzyme linked immunosorbent assay, method, Nobuto

Abbreviations: AI = avian influenza; AIV = avian influenza virus; bELISA = blocking enzyme-linked immunosorbent assay; dpc = days postcollection; dpi = days postinoculation; ELISA = enzyme-linked immunosorbent assay; HPAIV = highly pathogenic avian influenza virus; LPAIV = low pathogenic avian influenza virus; RT = room temperature; RT-PCR = reverse transcription-polymerase chain reaction; S/N ratio = ratio of sample absorbance to negative control absorbance

Due to the emergence of highly pathogenic avian influenza virus (HPAIV) H5N1 in Asia in the late 1990s and early 2000s and its subsequent spread to Europe and Africa, there is increased interest in both HPAIV and low pathogenic avian influenza virus (LPAIV), especially in wild birds. Advancements in the ability to detect both avian influenza viruses (AIVs) by reverse transcription-polymerase chain reaction (RT-PCR) and specific avian influenza (AI) antibody by enzyme-linked immunosorbent assay (ELISA) have made the study of these pathogens more efficient as they allow testing of large numbers of samples (5,12). However, these techniques need to be validated as their uses are applied in new ways.

Paper strip blood sampling techniques for the detection of antibodies to various pathogens, including plague (*Yersinia pestis*) and Newcastle disease have been available for >50 yr (2,3,10). Nobuto developed a standard method using a filter paper strip (commonly known as the "Nobuto strip") for the diagnosis of toxoplasmosis (11). The Nobuto strip method simplifies postcollection handling and processing of blood samples for subsequent serologic analysis and has been used for the diagnoses of antibodies to a variety of diseases in many different species (3,13).

Field studies of wildlife disease present many logistical problems. Surveillance for HPAIV H5N1 in Alaska (and other remote locations) illustrates many of these issues, such as the need to take limited field equipment and supplies, because often these remote locations are only accessible by plane or helicopter (8). Using Nobuto strips for the collection of blood samples would greatly simplify field studies if analyses produced accurate results. With the advent of new ELISA techniques, most notably a commercially available blocking ELISA (bELISA) for the detection of avian influenza antibodies (4,5), the utility of using Nobuto strips to simplify sample collection in the field needs to be evaluated. In this study, we compared the ability of the bELISA to detect specific AI antibodies in blood samples collected onto Nobuto filter strips that were stored for various lengths of time and conditions versus sera from experimentally infected mallards (*Anas platyrhynchos*).

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Table 1. Results (data shown as S/N ratio[A]) of serologic testing by bELISA of sera and Nobuto paper strips collected from mallards experimentally infected with LPAIVs and stored at –30 C and RT and tested on specific dpc.

	Sera −30 C	Nobuto strip −30 C			Nobuto strip RT	
Bird ID	10 dpc	9 dpc	38 dpc	99 dpc	38 dpc	99 dpc
1[B]	**0.81**	**1.18**	**0.97**	**1.07**	**0.78**	**0.88**
26[B]	**0.79**	**1.04**	**0.81**	**0.81**	**0.73**	**0.83**
2[C]	0.23	0.19	0.21	0.19	0.19	0.29
3[C]	0.27	0.31	0.23	0.26	0.23	0.28
4[D]	0.13	0.18	0.18	0.15	0.13	0.15
5[D]	0.17	0.24	0.25	0.23	0.16	0.21
6[D]	0.22	0.2	0.18	0.15	0.25	0.36
7[D]	0.24	0.26	0.22	0.2	0.22	0.27
8[D]	0.28	0.29	0.27	0.26	0.32	0.38
9[D]	0.34	0.34	0.28	0.32	0.37	0.45
10[D]	0.16	0.16	0.13	0.15	0.13	0.15
27[C]	0.3	0.45	0.32	0.31	0.23	0.4
28[C]	0.37	0.35	0.29	0.29	0.32	0.38
29[D]	0.31	0.43	0.31	0.25	0.28	0.39
30[D]	0.15	0.18	0.15	0.14	0.14	0.16
31[D]	0.25	0.25	0.2	0.18	0.17	0.22
32[D]	0.21	0.15	0.16	0.15	0.15	0.19
33[D]	0.4	0.3	0.24	0.27	0.3	0.37
34[D]	0.29	0.29	0.25	0.23	0.29	0.44
35[D]	0.26	0.21	0.19	0.19	0.2	0.23
51[D]	0.3	0.31	0.29	0.27	0.3	**0.55**
52[D]	0.33	**0.59**	**0.56**	**0.53**	0.26	0.31
53[D]	0.23	0.21	0.16	0.17	0.22	0.32
54[D]	0.11	0.19	0.16	0.16	0.11	0.15
55[D]	0.27	0.28	0.24	0.24	0.21	0.24
56[D]	0.24	0.26	0.19	0.2	0.22	0.32

[A]S/N ratio represents the unknown sample absorbance divided by the average absorbance of negative serum controls provided by the manufacturer. Results >0.50 are considered negative (shown in bold).
[B]Uninoculated controls.
[C]Received inoculation of a single virus.
[D]Received inoculation of two viruses.

MATERIALS AND METHODS

Twenty-four 30-day-old mallard ducklings were inoculated intranasally and intrachoannally with LPAIV, subtypes H5N2 (A/northern pintail/California/44242-758/2006) and/or H7N3 (A/northern pintail/Nevada/44252-242/2007), with cohorts receiving one or both viruses (Table 1). Two additional uninoculated birds served as negative controls. All birds were randomly assigned to cohorts and had approximately 1 ml of pre-exposure blood samples collected before inoculation of AI viruses (0 days postinoculation [dpi]). Pre-exposure blood samples were placed directly into serum separator tubes and held on wet ice until centrifugation. Serum was then decanted and placed into individually labeled cryovials and stored at −30 C until testing. These birds were part of a broader study and were handled and housed according to Institutional Animal Care and Use Committee-approved protocols. All inoculated birds became infected with AIVs, and negative control birds tested negative for AIVs, based on RT-PCR analyses of oral and cloacal swabs (data not shown). At 15 dpi, blood samples were collected by jugular venipuncture from all experimental and control birds (0 days postcollection [dpc]). Blood samples were treated in two ways. First, approximately 1 ml of whole blood was collected from each bird and placed directly into serum separator centrifuge tubes and handled as described previously for preinoculation samples. Second, whole blood was absorbed onto Nobuto blood filter paper (Advantec® Type I Blood Sampling Paper, Toyo Roshi Taishu, Ltd., Tokyo, Japan; Nobuto strips) following the manufacturer's instructions. In brief, the Nobuto strips (minimum of 5 strips per bird) were placed in collection tubes containing whole blood, and the blood was allowed to wick evenly onto the blood-absorbing portion of the strip. The blood absorption area of the strips absorbs approximately 40 µl of blood according to the manufacturer's instructions. Nobuto strips were removed and allowed to air dry. For each mallard, Nobuto strips were randomly divided into two groups, and then the strips were placed into manila envelopes and stored at either room temperature (RT) or at −30 C.

Following the manufacturer's instructions, Nobuto strips were eluted by cutting the blood-absorbing portion of the Nobuto strip into three or four pieces with sterile scissors and then transferring the pieces to a microcentrifuge tube containing 400 µl of phosphate-buffered saline solution (pH 7.2), resulting in an approximate 1:10 dilution of the original blood sample. Tubes were briefly mixed and incubated at room temperature for 60 min, and the eluate was transferred to a sterile tube.

Samples were tested for the presence of influenza antibodies using the bELISA Avian Influenza MultiS-Screen Antibody Test Kit (IDEXX Laboratories, Westbrook, ME), following the manufacturer's instructions. Eluates were loaded directly into the test wells as 1:10 dilutions as the instructions directed. Absorbance readings were obtained on a model ELx808 plate reader (BioTek Instruments, Inc., Winooski, VT). Samples with a ratio of sample absorbance to negative control absorbance (S/N ratio) of <0.5 were defined as positive for AI antibodies as defined by the manufacturer.

Sera collected on 0 dpi was tested on 25 dpc, and sera collected on 15 dpi were tested on 10 dpc. Nobuto strips stored at −30 C were tested on days 9, 38, and 99 dpc, and Nobuto strips stored at RT were tested on 38 and 99 dpc. Nobuto strips were tested within 24 hr of the Nobuto strip being eluted.

Data were analyzed using a paired *t*-test comparing the S/N ratio of each Nobuto strip treatment group against the S/N ratio of the sera (14). Sensitivity was calculated by dividing the number of true positives by the number of true positives plus the number of false negatives. True positives were identified based on the RT-PCR results of the oral and cloacal swab samples tested previously.

RESULTS

Sera collected from all 24 birds that received doses of virus and tested on 10 dpc were positive by bELISA for AI antibodies (Table 1). For Nobuto strips stored at −30 C and tested at 9, 38, and 99 dpc, 23 of 24 infected birds were positive. The negative sample found for each group tested was from the same bird. For Nobuto strip samples held at RT and tested on 38 dpc, all 24 infected birds were positive and when tested on 99 dpc, 23 of 24 infected birds were positive. The negative sample in the RT group was from a different bird than the samples that were negative in the Nobuto strips stored at −30 C. The 2 uninoculated control birds tested negative at each time point for all treatment groups.

The sensitivity was 100% for both sera tested at 10 dpc and for Nobuto strips stored at RT and tested at 38 dpc. In all other treatment groups the sensitivity was 96% (95% confidence interval, 87.6%–100%). In every case, the control samples collected from preinoculation birds were correctly identified as being serologically negative. No significant differences in S/N ratios were detected between sera stored at −30 C, and Nobuto strips stored at −30 C and tested at 9 ($P = 0.1387$), 38 ($P = 0.2671$), and 99 ($P = 0.0805$) dpc. Significant differences in S/N ratios were detected between sera stored at −30 C and Nobuto strips store at RT and tested at 38 ($P = 0.0012$) and 99 ($P = 0.0030$) dpc.

DISCUSSION

Nobuto strips have been in use for >50 yr as a diagnostic tool for the detection of various diseases (10), although the technique has not been used previously for detection of specific AI antibody. The reasons that this technique has not been used in AI studies are not known, but it may be that traditional serological methods used by researchers, such as hemagglutination inhibition, do not work with Nobuto strip eluates. In this study, we found that using Nobuto strips in an experimental setting for blood collection and testing them with the recently developed bELISA provided comparable results to testing of sera for the detection of AI antibodies. These results were consistent even when Nobuto strip samples were stored up to 3 mo at RT. Although we did detect significant differences in the S/N ratio of Nobuto strips stored at RT compared with sera these difference did not result in a reduced ability to detect positive or negative samples reliably. We found using Nobuto strips to be a sensitive alternative to the use of serum for detection of AI antibodies under varied storage conditions; however, a future study in wild birds should further evaluate this technique as has been done for studies of other disease agents (6).

Having additional techniques to study AIV will aid in understanding this disease in wild birds. Although collection of serum samples for detection of AI antibodies is likely to always be the preferred technique, using Nobuto strips to collect samples may, at times, provide a better alternative. Nobuto strips may be a more suitable technique when collecting samples from dead birds (e.g., hunter-killed animals) and when working in remote locations or with untrained handlers and samplers (6). Due to the ease of use of Nobuto strips, hunters could collect samples from their own harvests to broaden the geographic range of sampling or when working with species that are hunted broadly but where only a few individuals are shot at a time (6).

Due to the diversity of AIV, the interpretation of seroprevalence data without hemagglutinin and neuraminidase subtypes can be problematic. However, seroprevalence studies may be useful in understanding the occurrence of AIV in those species such as shorebirds where low detection rates of AIV are common (1,7,9). This may be because birds are being sampled during periods of time or at locations where AI transmission is low and previous infections have been cleared. Testing for antibodies in these species by bELISA is a relatively simple, cost-effective method to screen large numbers of birds for exposure to AIV, and the use of Nobuto strips presents another tool for researchers to use. The data generated from this study can lead to more effective sampling and surveillance strategies in regard to targeting the species of birds and the locations that are most relevant.

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