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Using Whatman FTA® cards to collect DNA for bird-strike identifications

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Abstract: Identification of species of birds is fundamental to implementation of all management and mitigation issues involving bird–aircraft collisions (bird strikes). Methods of identifying bird remains have recently been enhanced to include the use of molecular techniques but the degraded condition of the field sample remains problematic. The Whatman FTA® card is a novel way to instantly fix DNA and inhibit degradation of DNA samples post-collection. During 2008–2009, we analyzed >200 FTA cards containing tissue blots, body fluids, or blood samples from bird strikes to evaluate the performance of these cards in real-time situations. We analyzed the success of extracting DNA from the FTA cards, compared the FTA disc punch extractions to dried tissue extractions from the same birdstrike event, and evaluated the blotting density (heavy versus light) for each FTA card punch. FTA cards alone yielded successful extractions in 63% of the trials whereas dried tissue samples were successful 75% of the time. FTA cards that were scored as being blotted with dense (heavy) samples were more successful (63%) than the light or liquid samples (48%). We encourage the use of the FTA cards or similar products as a valid option for the collection of degraded birdstrike remains because the FTA cards resulted in successful extractions in >50% of the cases. The cards are ideal for shipping and require no special storage; the cards are dry and lightweight; immediate fixation of the sample helps prevent mold and bacterial growth; and FTA cards are listed by the U.S. Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) as an approved method of treatment for foreign shipments of bird remains.

Key words: bird strike, DNA, FTA Whatman cards, human–wildlife conflicts

THE IDENTIFICATION OF SPECIES of birds from minute evidence recovered from bird-strike events continues to assist in management and prevention measures and helps define what birds are most problematic to aviation safety. Scientists are using feather identification techniques in combination with molecular methods to identify species of birds from fragmentary evidence in a variety of fields including bird-strike identification (Dove et al. 2008, 2009; Marra et al. 2009), ecological studies of prey remains (Day 1966, Griffin 1982, Dove et al. 2011), anthropology (Rogers et al. 2002, Dove and Peurach 2002) and human forensics (Deedrick and Mullery 1981, Dove and Koch 2010).

Whatman (Whatman International Ltd., United Kingdom) FTA® cards have been used for many years (Vanek et al. 2001) as a method of collecting, transporting, storing, and purifying DNA safely and securely at room temperature. Although the card was designed primarily for sampling fresh human fluids, it has since been used for a variety of zoological materials (Crabbe 2003, Smith and Burgoyne 2004, Harvey 2005, Borisenko et al. 2008). FTA cards have also been used for plant (Ndunguru et al. 2005, Owor et al. 2007) and fungi sampling (Barlocher et al. 2010). The collecting procedure for the card is simple and involves pressing the biological sample on to the card's filter paper and allowing it to air dry. The filter paper is impregnated with proprietary chemicals that lyse cell membranes on contact, inactivate pathogens, and immobilize nucleic acids immediately fixing the DNA from further degradation. This technology makes the card ideal for forensic types of investigations because it allows for rapid sampling, requires minimal storage facility, helps reduce DNA degradation, and is easy to transport. These cards have recently been shown to provide viable DNA extractions after 8 years of storage (Mullen et al. 2009).

Beginning in 2006, the Smithsonian Institution's Feather Identification Lab encouraged the use of these cards as an option for collecting bird remains from bird-strike events. Prior to this, sampling methods involved wiping bird evidence from aircraft impact points using paper towels, but this method is less than ideal because it does nothing to protect the sample from DNA degradation, mold growth, and rot. Obtaining DNA sequences from field samples has become an important tool for the identification of bird-strike remains (Dove et al. 2008). In addition, robotic workstations with high throughput FTA extraction protocols already have been developed to meet the demand of future increasing extraction needs (Tack et al. 2007, Rockenbauer et al. 2009, Stangegaard et al. 2009). The objective of this study is to evaluate the performance of these sampling cards as an alternative method for collecting DNA samples from birdstrike events.

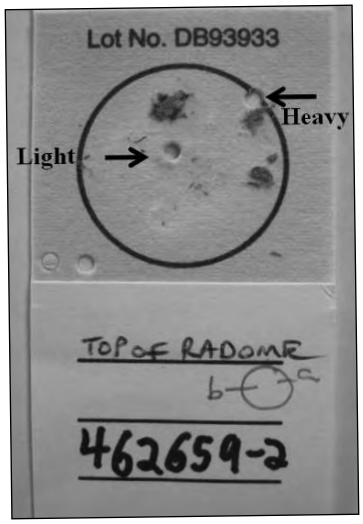

Figure 1. The Whatman FTA® card sampled with a tissue and fluid smear from a bird-strike event. The card is air-dried and submitted for species identification. To remove material for processing in this study, 2 holes (2.0 mm) were punched in sample areas deemed heavy or light.

Methods

Sample collecting

A total of 249 FTA® cards received from various U.S. military and civilian airfields (2008–2009) were examined in this study. In many cases (207), we also collected samples of dried tissue associated with or attached to the same FTA card. This allowed us to compare both tissue and FTA extractions from the exact same sample. We punched 2 small (2.0 mm) holes in most FTA cards with the Harris Uni-Core ™ -2.0 tool (Shunderson Communications Inc., Ontario, Canada, U.S. Pat. No. 7093508). Because the amount of bird remains on individual FTA cards varied, we quantified the denseness of each individual punch by scoring each punch as either heavy or light. Heavy samples contained visible dried tissue mass or clotted blood, while light samples consisted of clear liquid or very faint tissue or fat smears (Figure 1). Most cards were punched twice and ranked accordingly; a single card could receive heavy, light, or both ratings. The DNA extractions were considered successful if species identifications were obtained from a single-pass extraction attempt. Samples received were either on FTA indicating or non-indicating cards. Indicating cards differ by having pink-colored filter paper and were developed to assist in locating the placement of clear liquid samples on the cards; the color turns from pink to white at the location where the clear liquid is placed on the card. Extraction protocols are the same for both card types.

We tested the equality of proportions among the sample groups using a normal deviate test of proportions. We compared z-scores in 2-tailed tests at $P = 0.05$ and rejected the null hypothesis that proportions are equal if $z > 1.75$.

Tissue protocol

From the 249 FTA cards, 207 samples contained both dried tissue and FTA punches. The cytochrome oxidase 1 (CO1) portion of the mitochondrial gene was selected as the marker because it has been shown to identify most species of birds involved in birdstrikes (Dove et al. 2008). DNA was extracted from the tissue using the Qiagen DNeasy® Blood and Tissue Kit, or the Qiagen BioSprint® 96 DNA Blood Kit (Qiagen Inc., Valencia, Calif.). Polymerase chain reaction (PCR) amplification of CO1 gene portion was done on a MJ Research Tetrad Thermal Cycler® (Bio-Rad Laboratories, Hercules, Calif.). The 10.0 µl amplification reaction contained 1.0 µl of genomic DNA from a 200.0 µl extraction volume (containing anywhere from 10-100 ng/µl DNA), 1.0 µl Bioline® dNTP mix at 10 µM, 1.0 µl Bioline 10x NH_4 Reaction buffer, 0.3 µl Bioline® $MgCl_2$ solution at 50

μM, and 0.2 μl BIOLASE™ Taq Polymerase (BioLine USA, Randolf, Mass.), 5.5 μl dH_2O, 0.5 μl CO1 F primer (5′-TTCTCGAACCAGAAA GACATTGGCAC-3′) at 10 μM, and 0.5 μl CO1 R primer (5′-ACTTCTGGGTGGCCAAA GAATCAGAA-3′) at 10 μM. We set the thermal cycler for an initial denaturation at 94°C for 2 minutes followed by 25 cycles of 94°C for 20 seconds, 48°C for 45 seconds, and 72°C for 30 seconds, a final extension at 72°C for 3 minutes, and an indefinite hold at 10°C. Our PCR products were then cleaned with a diluted solution of ExoSAP-IT® (USB Corporation, Cleveland, Oh.). We diluted ExoSAP-IT 10-fold in dH_2O and added 1.0 μl added to each 10.0 μl PCR sample. Our samples were then heated to 37°C for 30 minutes, and at 80°C for 15 minutes. We cycle-sequenced the samples using BigDye® Terminator v.3.1 (Applied Biosystems, Foster City, Calif.) in both forward and reverse directions. Our 10.0 μl reactions contained a 1.0 μl cleaned PCR product, 0.75 μl BigDye, 1.65 μl 5x cycle sequencing buffer, 0.5 μl CO1 F or CO1 R primer, and 6.1 μl dH_2O. We conducted cycle sequencing on the MJ Research Tetrad Thermal Cycler in 25 cycles of 94°C for 30 seconds, 50°C for 30 se-conds, and 60°C for 4 minutes, with an indefin-ite hold at 10°C. Our reactions were purified using Sephadex-G50® (Sigma-Aldrich, St. Louis, Mo.), and loaded on an Applied Biosystem 3130 DNA Analyzer® (Applied Biosystems, Foster City, Calif.) with a 36-cm array. We analyzed and trimmed trace files on a Finch server v2.20.4 software (Geospiza Inc., Seattle, Wash.). Our sequences were entered into the Barcode of Life Database (BoLD; <http://www.barcodinglife.org>) for quality checks and species identifications (Ratnasingham and Hebert 2007).

FTA protocol

FTA extractions on all 249 FTA cards were processed strictly following the manufacturer's protocol. To prevent cross-contamination, the Harris Uni-Core ™ tool was cleaned between each sample by punching on a fresh, unused FTA card. In summary, each of the 2.0 mm punched discs were dropped into a 1.5ml tube and processed through 2 washes for 5 minutes at room temperature using the FTA purification reagent. This was followed by two more washes with TE buffer (10 mM tris, 0.1 mM EDTA) at pH 8 at room temperature. The washed punches were then dried at 55°C for 10 minutes. PCR was done to amplify the CO1 gene *in situ* in strips or individual PCR tubes immediately after drying. The final volume added to the dried punches was 50 μl. The solution contained 32.5 μl dH_20, 5 μl of the 5x reaction buffer (Promega, Madison, Wisc.), 5 μl of dNTP, 1.5 μl of 50 mM $MgCl_2$, 2.5 μl of 10 μM CO1 forward and reverse primers (see above) and 1 μl of Promega GoTaq® DNA polymerace (Madison, Wisc.). Promega and Bioline® Taq were used interchangeably for the PCR reaction. Cycle sequencing and Sephadex cleanup followed the tissue extraction methods described above; sequence identifications were checked on BoLD.

Results

The 249 FTA cards analyzed here yielded 63% successful sequences for avian species identifications on the first-pass attempt. Most of the FTA punches (2 per card) were scored as light (306) as opposed to heavy (139) for sampling density. Punches scored as heavy were significantly different (normal deviate z = 2.93; P = 0.05) from the light samples (Table 1).

Of the 207 cases that were sub-sampled for both tissue and FTA cards (Table 1), the dried tissue samples yielded successful results in 75% of the cases, whereas the FTA cards were successful 60% of time (normal deviate z = 2.86; P = 0.05). The FTA cards were successful in 9 cases when we did not obtain DNA from the tissue sample, but the dried tissue was successful in 40 cases that had FTA failures. Both FTA and tissue samples from the same bird-strike event resulted in successful sequences for identifications in 56% of the trials.

Table 1. Success of DNA identifications from the 207 FTA cases that contained both dried tissue and FTA punches (left side of table). Comparison of all FTA cards analyzed for heavy (dried tissue mass or clotted blood) and light (faint tissue or fat smear) sampling effort (right side of table).

	FTA®	Tissues	FTA® heavy	FTA® light
Success	124	155	87	146
Failure	83	52	52	160
Total	207	207	139	306

Discussion

The material being evaluated in this study was undoubtedly degraded and compromised even before it was collected. Therefore, we could compare only the material that we had available to us for study. Our assumption was that because the FTA cards and the dried tissue samples were from the same event, we could at least begin to compare the success of both collecting methods. The tissue protocol is different from the FTA manufacturer's protocol. The FTA card was designed to store fresh, clean human tissue and blood samples, preferably dropped on the filter paper in small amounts, instead of large chunks that overwhelm the reagents on the card. In our survey, many cases contained clotted blots or large tissue chunks of degraded material often mixed with soot, fuel and debris. These conditions are a major challenge for molecular identifications. Additionally, Smith and Burgoyne (2004) noted that avian blood may actually contain too much DNA for even the smallest punched discs to be used directly in PCR reactions because it contains nucleated red blood cells and can create a challenge for mitochondrial DNA. Mullen et al. (2009) reported the presence of PCR inhibitors in some blood that also may affect PCR results.

In our analysis of the sampling density, the punches ranked as heavy outperformed those scored as light. This may be due to the mixed type of material on the heavy cards (tissue and blood) or to the possibility that the light cards did not actually contain any bird material. Light cards often appeared to have only clear fluid on the filter paper.

Although the dried tissue protocol was successful more often than the FTA card protocol in this study, we are encouraged by the 63% FTA success results from these degraded avian samples. This is excellent progress and an improvement over the former collecting methods of wiping samples with wet paper towels (Dove et al. 2008). Further, these cards have been approved as a treatment method for the prevention of avian diseases by the U.S. Department of Agriculture Animal and Plant Health Inspector Services (APHIS) and allows for easy shipment from overseas airfields. Because of these positive aspects and the fact that the FTA cards are still being tested and modified, we continue to encourage the use of these cards, or similar products for bird-strike sampling.

Management implications

Improving species identification is crucial to proper implementation of bird-strike management plans on airfields, the design of new aircraft and engines, and in creating computer models to predict bird-strike risks. Although the identification of species of birds continues to rely on morphological methods when ample feather material is available, field personnel are now more skilled at finding bird remains that are minute or consist only of tissue and blood. Therefore, collecting and preserving the freshest samples for DNA analysis is paramount to successful DNA identifications. The use of DNA fixing cards is a novel attempt to solve common problems of DNA degradation, storage and transport issues and may help improve bird identifications to the species level for a variety of scientific studies.

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