



Field detection of avian influenza virus in wild birds: Evaluation of a portable rRT-PCR system and freeze-dried reagents

John Y. Takekawa^{a,*}, Samuel A. Iverson^a, Annie K. Schultz^a, Nichola J. Hill^a, Carol J. Cardona^b, Walter M. Boyce^c, Joseph P. Dudley^d

^a U.S. Geological Survey, Western Ecological Research Center, San Francisco Bay Estuary Field Station, 505 Azuar Drive, Vallejo, CA 94592, USA

^b Department of Population Health and Reproduction, School of Veterinary Medicine, 1 Shields Avenue, University of California, Davis, CA 95616, USA

^c Wildlife Health Center, School of Veterinary Medicine, 1 Shields Avenue, University of California, Davis, CA 95616, USA

^d Science Applications International Corporation, 12530 Parklawn Drive, Suite 350, Rockville, MD 20852, USA

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Wild birds have been implicated in the spread of highly pathogenic avian influenza (HPAIV) of the H5N1 subtype, prompting surveillance along migratory flyways. Sampling of wild birds is often conducted in remote regions, but results are often delayed because of limited local analytical capabilities, difficulties with sample transportation and permitting, or problems keeping samples cold in the field. In response to these challenges, the performance of a portable real-time, reverse transcriptase-polymerase chain reaction (rRT-PCR) unit (RAPID[®], Idaho Technologies, Salt Lake City, UT) that employed lyophilized reagents (Influenza A Target 1 Taqman; ASAY-ASY-0109, Idaho Technologies) was compared to virus isolation combined with real-time RT-PCR conducted in a laboratory. This study included both field- and experimental-based sampling. Field samples were collected from migratory shorebirds captured in northern California, while experimental samples were prepared by spiking fecal material with an H6N2 AIV isolate. Results indicated that the portable rRT-PCR unit had equivalent specificity to virus isolation with no false positives, but sensitivity was compromised at low viral titers. Use of portable rRT-PCR with lyophilized reagents may expedite surveillance results, paving the way to a better understanding of wild bird involvement in HPAIV H5N1 transmission.

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1. Introduction

The spread of avian influenza viruses (AIVs) in nature is intrinsically linked with the migratory movements of wild birds (Webster et al., 2007). Wild birds are the reservoirs for low pathogenic avian influenza viruses (LPAIVs) (Webster et al., 1992) and their migration facilitates the circulation of LPAIVs between breeding grounds at high latitude and wintering areas at lower latitudes (Hinshaw et al., 1980, 1985). There is growing evidence that migratory birds may also be directly involved in the spread of highly pathogenic avian influenza (HPAIV), particularly in view of the ability of some waterfowl species to carry the virus without clinical symptoms (Gaidet et al., 2008; Keawcharoen et al., 2008). The transnational movement of HPAIV, in particular, the lethal H5N1 subtype, which has a fatality rate of 60% in infected humans and up to 100% in infected poultry (W.H.O., 2009), presents a major challenge for public health and agricultural biosecurity worldwide (Dudley, 2008). Consequently, surveillance of wild birds along migratory flyways

has been adopted by many countries as an early-warning system for the detection of AIV.

Surveillance of live and dead migratory birds is often carried out in remote regions, consequently, laboratory results are often delayed or compromised because of limited local analytical capabilities, difficulties with transportation of samples and regulatory permitting, or problems maintaining cold chain in the field. On-site diagnosis of host status would prove useful not only for containing outbreaks, but also for identifying and studying infected individuals in wild populations. The opportunity to collect information on the immunological and physiological response of infected wild birds and their flight performance will provide insights into the extent to which they can act as vectors for LPAIV and HPAIV over long distances. While virus isolation remains the gold standard test for AIV detection, the need for a readily available supply of embryonating chicken or duck eggs and the length of time required to culture the virus (up to several weeks), coupled with the need for a high biosecurity laboratory for safely conducting analyses, precludes the field use of this diagnostic technique (Suarez et al., 2007).

Advances in molecular testing over the last decade have allowed for diagnosis of AIV with both sensitivity and specificity equivalent to virus isolation (Fouchier et al., 2000; Slomka et al., 2007;

* Corresponding author. Tel.: +1 707 562 2000; fax: +1 707 562 3001.

E-mail address: john.takekawa@usgs.gov (J.Y. Takekawa).

Soares et al., 2005; Spackman et al., 2003). However as with virus isolation, molecular diagnostic tools such as real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) require sophisticated laboratory settings with trained personnel that are not available or affordable in all countries. A drawback preventing the use of portable rRT-PCR in a field setting has been the need to maintain wet reagents at a constant cold temperature and follow multi-step specimen preparations in a sanitary, controlled environment. The development of lyophilized reagents that do not require refrigeration with sensitivity at or above the level of wet reagents (Das et al., 2006) has brought on-site remote testing to a practical goal. These reagents eliminate errors caused by improper storage and handling of wet reagents and contain all of the necessary components in a single tube: primers, probes, enzymes, buffers and internal positive controls. However, as yet there has been no systematic validation of molecular tools for detecting AIV in wild bird samples in the field—a crucial step for ensuring accurate diagnosis during wild bird outbreaks.

To evaluate the utility of rRT-PCR equipment and reagents that are available commercially for field diagnosis of AIV in wild birds, the performance of a portable rRT-PCR unit (Ruggedized Advanced Pathogen Identification Device or RAPID® 7200, Idaho Technologies, Salt Lake City, UT) that employed lyophilized reagent technology (Idaho Technologies Influenza A Target 1 Taqman; ASAY-ASY-0109) was compared to virus isolation conducted in a traditional laboratory setting. The experimental design, including analysis of a combination of field and experimental samples, followed the validation testing outlined by Suarez et al. (2007). Field samples were collected from five species of migratory shorebirds captured in northern California, while experimental samples were prepared by spiking fecal material with high and low level titer H6N2 AIV isolated from chickens. The ultimate objective was to assess the feasibility of molecular screening for AIV in a field setting where surveillance of wild birds is conducted.

2. Materials and methods

2.1. Field sample collection

Migratory shorebirds were captured with mist nets in the San Francisco Bay and Delta region of California (37°25'19.1"N, 122°05'06"W). Capture took place between 8 January and 9 May 2007, coinciding with spring migration when birds depart from wintering grounds. Full details of shorebird capture are presented in Iverson et al. (2008) and animal handling followed protocols specified by the Animal Care and Use Committee of the U.S. Geological Survey Western Ecological Research Center. Three species were identified as priority candidates for HPAIV H5N1 surveillance in this study (U.S. Avian Influenza Interagency Working Group, 2006), on the basis of range overlap with Asian-origin birds, habitat affiliations and population size: the Long-billed Dowitcher (*Limnodromus scolopaceus*), Western Sandpiper (*Calidris mauri*) and Dunlin (*Calidris alpina*).

Samples for AIV screening were collected by gently inserting a rayon-tipped swab (MicroPur™, PurFybr Inc., Munster, IN) into the cloaca of the bird. The tip of the swab was removed and preserved in cryovial tubes (Remel Inc., Lenexa, KS) containing 1.5 mL viral transport media. Samples were kept on ice for up to 12 h before storage in liquid nitrogen dry shippers (−70 °C). Two cloacal samples were taken from each bird. The first sample (A) was shipped for confirmation testing at the University of California at Davis (UCD). The second sample (B) was stored in a freezer (−10 °C) for analysis with the portable rRT-PCR unit in a field setting. Our prior study indicated no decrease in viral load detectable by RT-PCR, between cloacal samples collected sequentially (Iverson et al., 2008).

2.2. Experimental sample preparation

An isolate of H6N2 AIV obtained from chickens (A/Chicken/California/4077/04(H6N2)) was used to prepare the experimental samples. Viruses of the H6 subtype are among the most prevalent of the AIVs circulating in waterbirds (Hill et al., 2010; Sharp et al., 1993; Slemons et al., 2003) and have also caused outbreaks in poultry in North America (Webby et al., 2003; Woolcock et al., 2003). Three viral titers of H6N2 AIV were prepared; high, low and blank. The 'high' titer group (21 vials) had a total concentration of 10^{8.2} EID₅₀ (50% Egg Infectious Dose, the titer of virus at which half the eggs become infected), the 'low' titer group (20 vials) had a total concentration of 10^{3.3} EID₅₀, and the blank group (20 vials) contained no virus. The concentration used for the low titer samples approximated values observed in weakly positive cloacal samples from wild birds. The virus was added to 350 µl of previously tested negative cloacal swab media, and all samples were run as blind tests.

2.3. AIV testing under laboratory conditions

In the laboratory, field samples (A) and experimental samples were screened for AIV by virus isolation in embryonating chicken eggs followed by RNA extraction and rRT-PCR on the harvested allantoic fluids using published methods (Spackman et al., 2003; Swayne et al., 1998). In brief, each sample was inoculated into the allantoic cavity of 9- to 11-day old embryonating chicken eggs and incubated at 37.5 °C for 6 days or until embryo death, as detected by daily candling. Viral allantoic fluid (VAF) from live embryos was tested for hemagglutinating activity with chicken erythrocytes following standard methods (Swayne et al., 1998). RNA was extracted from VAF harvested from all dead embryos, and the hemagglutinating VAF from live embryos using the MagMAX-96 Viral Isolation Kit (Ambion Inc. Austin, TX). RNA was screened for AIV with RT-PCR targeting the matrix gene (Spackman et al., 2003). Assays were run on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA).

2.4. AIV testing under field conditions

Testing of field samples (B) and experimental samples was conducted in a mobile trailer at the San Francisco Estuary Field Station in Vallejo, California. The trailer provided a non-laboratory setting and emulated conditions similar to those under which samples would be processed in remote locations. To conduct analyses, we used the RAPID® 7200 system that included a PCR unit, laptop computer, software, micro-centrifuge, backpack, ruggedized case and user's manuals, in addition to the Vortex Genie™ (Scientific Industries, Bohemia, NY), RNeasy® Mini kit (Qiagen, Valencia, CA) and pipettes (Fig. 1).

RNA extraction was performed with the RNeasy® Mini kit with minor modifications to the manufacturer's instructions following Spackman et al. (2002). A volume of 350 µl of swab medium was purified in a spin column and eluted with 50 µl of nuclease-free water. To avoid contamination, RNA extraction was performed in a dedicated area of the trailer away from the PCR unit. Lyophilized reagents were prepared for use in the PCR assay according to instructions from the Idaho Technologies Freeze-Dried Reagent Detection Kit for TaqMan Probes Influenza A Target 1 (ASAY-ASY-0109). Positive and negative controls were prepared in duplicate and sample tubes were prepared by adding 20 µl of extracted RNA to the 'unknown' reagent tubes. All reactions were mixed in the Vortex Genie™ followed by brief centrifugation to rehydrate reagents before transfer to LightCycler® capillary tubes. Each reagent vial yielded duplicate 20 µl reactions.



Fig. 1. Sampling and testing techniques used during this study; (A) capture of a Western Sandpiper with a mist net; (B) collection of cloacal samples; (C) performing RNA extraction; and (D) set-up of the RAPID® 7200 portable rRT-PCR unit in a mobile trailer.

Assays were run in batches of 10 ‘unknown’ tubes with one negative and two positive controls on the portable RAPID® 7200 following pre-programmed RT-PCR conditions. The RT step involved incubation at 40 °C for 30 min and denaturation at 94 °C for 120 s. A two-step PCR cycling protocol was used to amplify the Influenza A virus matrix gene involving 45 cycles of 94 °C for 0 s and 60 °C for 20 s. Fluorograms were displayed with the RAPID® LightCycler Data Analysis graphing software on a laptop computer and interpreted on the basis of the cycle threshold (C_T) and final baseline fluorescence values (Fig. 2). A value $0 < C_T \leq 35.0$ was regarded as positive, while a C_T value = 0 was regarded as negative (Spackman and Suarez, 2008b).

3. Results

3.1. Field samples from shorebirds

A total of 336 shorebirds were captured from five migratory species: Western Sandpiper, Long-billed Dowitcher, Dunlin, Least Sandpiper (*Calidris minutilla*), and Semipalmated Plover (*Charadrius semipalmatus*). All 336 samples were determined to be negative by the confirmation laboratory. For field testing, a sub-set of 100 cloacal samples were tested with the RAPID® 7200. Samples were divided between the three primary target species (Table 1). Consistent with the laboratory results, the RAPID® 7200 also determined

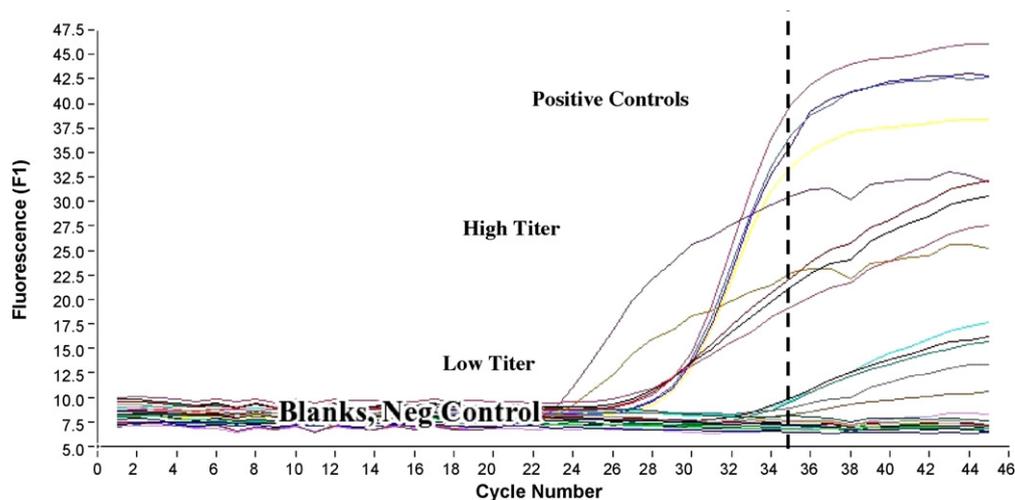


Fig. 2. Fluorogram generated by the RAPID® 7200 portable rRT-PCR unit for the H6N2-spiked samples (high titer, low titer and blanks) and positive and negative controls using the LCDA software (Idaho Technologies Inc.) The 35 C_T cut-off is indicated by the dashed line.

Table 1

Comparison of AIV field test results for shorebird cloacal samples obtained in laboratory (A) and field (B) testing by rRT-PCR.

Species	No. of samples	Laboratory testing (A)			Field testing (B)		
		No. of tested	No. of negatives	Specificity ^a (C.L.) ^b	No. of tested	No. of negatives	Specificity ^a (C.L.) ^b
Western Sandpiper	192	192	192	100.00 (98.10–100)	50	50	100.00 (92.89–100)
Long-billed Dowitcher	80	80	80	100.00 (95.49–100)	25	25	100.00 (86.28–100)
Dunlin	36	36	36	100.00 (90.26–100)	25	25	100.00 (86.28–100)
Other shorebirds ^c	28	28	28	100.00 (87.66–100)	–	–	–
Total	336	0	0		100	0	

^a Specificity was calculated as the number of negatives accurately diagnosed divided by the total number of samples tested, expressed as a percentage.^b Confidence limits.^c Species included the Least Sandpiper and Semipalmated Plover.

the shorebird samples to be negative for Influenza A with no C_T values registered. All positive controls yielded a C_T value (28.67 ± 0.26 SD) indicating that no false negatives were diagnosed, and none of the negative controls generated a C_T value, indicating an absence of false positives.

3.2. Spiked experimental samples

The H6N2-spiked fecal trial indicated comparable detection rates between field and confirmation laboratory. One discrepancy occurred for the high viral titer samples, as the portable PCR diagnosed 20 of 21 samples as positives ($C_T = 27.70 \pm 3.89$ SD). Hence, the portable unit had a sensitivity of 95% (C.L. 77.16–99.88) at high viral loads compared to 100% (C.L. 83.89–100) for virus isolation (Table 2). All of the sample was consumed in the first reaction and could not be retested to assess whether operator error was involved in the single failure to detect a positive sample.

For the low titer H6N2-spiked samples ($n = 20$), all were positive ($C_T = 32.66 \pm 1.27$ SD). Hence the portable unit and confirmation laboratory testing both achieved 100% (C.L. 83.16–100) sensitivity at low viral loads (Table 2). However, for two of the low viral titer samples, one of the two replicates produced C_T values marginally higher than the cut-off for positive results ($C_T \leq 35.0$, sample 462a; 33.73, 37.11 and 37ac; 34.86, 35.83). Of the 20 blank samples, all were correctly identified, suggesting that the procedure can be run in a field setting without false positives due to contamination. Overall, sensitivity of the portable rRT-PCR unit was determined to be 98%.

4. Discussion

This study provides the first systematic evaluation of the performance of a portable thermocycler unit with freeze-dried reagents for the detection of AIV in wild bird samples, a necessary step before deploying any testing equipment for use during AIV outbreaks in wild birds. Although based on a small sample size, the use of both field and experimentally derived samples provided a rigorous test of the performance of the RAPID[®] 7200 and lyophilized reagents. Analysis indicated that the portable rRT-PCR unit had identical specificity (100%) and comparable sensitivity (98%; low titer 100%

and high titer 95%) to virus isolation performed in a laboratory setting. The portable rRT-PCR unit did not achieve 100% sensitivity because of diagnosis of a single high viral titer sample as a false negative. Previous validation studies have demonstrated that false negatives are the most common error with rRT-PCR because of the large potential for improper sample preparation, presence of PCR inhibitors in fecal material or degradation of the lyophilized reagent bead (Das et al., 2006; Spackman et al., 2002; Spackman and Suarez, 2008a)

Minor limitations in the sensitivity of portable rRT-PCR became apparent during analysis of low viral titer samples. One replicate from two low viral titer samples generated values above the 35 C_T cut-off for positive samples. This did not compromise the sensitivity of the technique at low viral titer (100%), but emphasized the importance of running assays in duplicate and even re-screening samples that generate a C_T value marginally higher than the cut-off. We anticipate that the limited sensitivity of lyophilized reagents at low viral titer would not interfere with diagnosis during a wild bird outbreak, because of the high viral titers typically shed by wild birds with infections of HPAIV H5N1 (Brown et al., 2008; Chen et al., 2006; Pantin-Jackwood et al., 2007). To ensure optimal sensitivity of lyophilized reagents in view of rapid HPAIV evolution, primers and probes issued for use with portable rRT-PCR require constant re-validation. For example, reagents specific for American and Eurasian lineages of the H5 and H7 subtypes are now recommended due to the divergence of the virus according to biogeographic region (Pasick, 2008). As with all other field testing, suspected HPAIV positives should be transported to an approved facility for final confirmation testing with virus isolation for highest specificity and sensitivity (O.I.E., 2004).

Shortcomings associated with reagent sensitivity were outweighed by features of the system that facilitated use in remote field situations, such as ease of sample storage and preparation. Unlike virus isolation, molecular methods do not rely on live viral particles; hence, storage of RNA at subzero temperatures is not essential. Therefore wild bird samples may be kept at 4 °C for 3 weeks without degradation of RNA integrity (Munster et al., 2009). The much less stringent specimen storage requirements of rRT-PCR are suitable for remote situations where maintenance of a cold chain may be impractical if liquid nitrogen shippers or dry ice is not

Table 2

Comparison of AIV experimental results for spiked samples obtained with laboratory (A) and field (B) testing by rRT-PCR.

Sample type	No. of samples	Laboratory testing (A)		Field testing (B)		
		No. of positives	Sensitivity ^a (C.L.) ^b	No. of positives	Sensitivity ^a (C.L.) ^b	Mean C_T value (\pm SD)
High titer – H6N2	21	21	100.00 (83.89–100)	20	95.24 (77.16–99.88)	27.70 (\pm 3.89)
Low titer – H6N2	20	20	100.00 (83.16–100)	20	100.00 (83.16–100)	32.66 (\pm 1.27)
Blanks	20	0	–	0	–	0
Positive controls	32	32	100.00 (89.11–100)	32	100.00 (89.11–100)	26.67 (\pm 0.26)
Negative controls	16	0	–	0	–	0

^a Sensitivity was calculated as the number of test positive samples divided by the total number of true positive samples, expressed as a percentage.^b Confidence limits.

available. In addition, sample analysis with freeze-dried reagents proved straightforward enough to be performed by field biologists with minimal knowledge of laboratory-based molecular analysis. The use of lyophilized reagents simplifies sample preparation by providing all of the necessary components for testing at appropriate concentrations, including primers, probes, enzymes, buffers and internal positive controls. As a consequence, the technique was time-efficient and also cost-effective. With one operator, it was feasible to run three batches, resulting in 42 screenings for AIV each day in a field setting. All materials needed to run this system could be brought to any location and the methodology could be taught to an operator over the course of a day. The limiting factor in remote field situations is the power supply required to run the portable rRT-PCR unit and attached computer, however, this may be mitigated through the use of a voltage-regulated portable electrical power generator.

In conclusion, this study demonstrated that portable rRT-PCR is suitable for the purpose of screening cloacal samples from wild birds for AIV in non-laboratory settings. The primary advantage of this technique is to expedite diagnosis of wild birds, increasing the chances of containing an HPAIV outbreak in a remote location. Portable rRT-PCR also represents a major breakthrough for researchers that seek to unravel the role of wild birds in the spread of HPAIV. The ability to diagnose host status at the time of sampling makes it possible to collect biological information to address key questions about immunological and physiological responses to infection, or genetic characteristics associated with natural resistance in wild birds. Moreover, deploying satellite transmitters to track the movement of the host (see Gaidet et al., 2008) would be invaluable for identifying which species act as long-distance carriers of HPAIV H5N1 as well as assessing their migratory performance, habitat preferences and levels of interaction with poultry. Evidence for the survival of HPAIV-infected wild birds suggests their role in the long-distance spread of the virus may change (Kim et al., 2009; Webster et al., 2007), highlighting the importance of surveillance of wild bird populations along their flyways. Future operational testing of the instrument in areas of international concern for AIV, such as China, South-east Asia, India and Africa, would conclusively determine how rapid rRT-PCR units perform under outbreak scenarios when diagnosis of a large number of positive samples is time-critical.

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