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Effect of Preservative on Recoverable RT-PCR Amplicon Length from Influenza A Virus in Bird Feces

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Abstract

SUMMARY—Surveillance for avian influenza A viruses in wild bird populations is often limited by requirements for a cold chain from time of specimen collection, by availability of ultra-low temperature specimen storage within a few hours or days, and by laborious classical virologic procedures. Successful storage of specimens in preservatives at ambient temperature and subsequent detection of RNA by reverse transcriptase–polymerase chain reaction (RT-PCR) would assist in helping influenza surveillance efforts become more widespread in remote areas, as well as more timely and inexpensive. Here, we describe bird feces spiked with influenza A virus preserved with guanidine and commercial buffers or alcohols at ambient temperature and analyzed by RT-PCR protocols. Virus-specific RT-PCR products of, at most, 206 bp were recovered for samples preserved with alcohols and up to 521 bp for samples preserved with guanidine or commercial buffers. These results suggest that this approach is feasible in the field and that preserved specimens might be better assayed molecularly when preserved in guanidine or commercial buffers.

Keywords

feces; influenza; RNA extraction; RT-PCR

Influenza A virus causes respiratory illnesses in humans, producing seasonal outbreaks, epidemics, and occasional pandemics and causes outbreaks and epizootics in lower mammals and birds (16). This virus is a major public health concern because it continually circulates in mammals and birds, and strains are subject to rapid genetic changes that can increase transmissibility and virulence in livestock, poultry, and humans. Because migratory waterfowl are considered to be the ancestral reservoir for influenza A viruses (10), they are a primary focus of surveillance. The recent emergences of high-pathogenicity H5 and H7 avian influenza viruses in poultry in South America, Australia, Europe, Asia, and North America in the last 10 years and human infections and even deaths have increased surveillance efforts in wild birds and prompted the development of more rapid detection methods (6,7).

The current system of influenza A virus surveillance is to grow live viral cultures from field specimens. The need to chill the specimens immediately and to cryo-freeze specimens within 3 days, and preferably much sooner, in virus transport medium limits the geographic scope of the surveillance network. Because field viruses are grown in embryonated hen's eggs or cell culture and viruspositive samples are frequently identified by classical cytopathic effect or

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hemagglutination assays (13), live cultures are time consuming and expensive to process. We became interested in preserved samples as an alternative surveillance method because they are expected to be cheaper, faster, less labor intensive, and more practical in remote areas, which would provide the capability of a world-wide influenza A virus surveillance network (3). However, the data that can be obtained from live virus cultures are currently more robust than those from fixed samples. Preserved samples are not renewable, and their genomic analysis is limited by the quality of the RNA recovered followed by molecular methods such as reverse transcriptase–polymerase chain reaction (RT-PCR) (3). With short segments of recovered RNA, hemagglutinin (HA) and neuraminidase (NA) classifications become more difficult and inefficient.

Because all currently identified high-pathogenicity avian influenza virus isolates are subtype H5 or H7, surveillance efforts are concentrated in identifying these subtypes of influenza and to determine indicators of pathogenicity, such as the genomic or amino acid sequence surrounding the HA cleavage site (6,7). Subtyping is determined by reference antibodies, cross reactivity between the live or inactivated virus isolate in question, and the reference viral strains that define subtypes of HA and NA viral surface proteins (15). A variety of real-time and RT-PCR subtyping assays have been described (5,11,12, and references therein), but most are specific to HA subtypes, and a simple system to screen and unequivocally determine all subtypes by RT-PCR is not yet available.

Our experience with fixed specimens in virus surveillance previously employed ethanol as a preservative (3,9). Although ethanol has been useful in the identification of influenza A–positive samples, it was insufficient to efficiently identify subtypes by RT-PCR (data not shown). Published primers intended to subtype influenza isolates by sequence homology typically rely on full-length gene segments (1) or amplicon sizes of 640 (8) or 231–770 bp (4). Because the design of primers and probes for real-time PCR identification of samples fixed in ethanol is optimal for short (<100 bp) sequences (3), we reasoned that ethanol preservation might result in RNA degradation. It seemed that other preservatives might result in successful PCR amplification of larger size fragments. For example, it was reported that a 4 M guanidine reducing buffer offered superior preservation of rotavirus RNA in gorilla stool compared with ethanol or other preservatives (14). Others reported degradation of RNA in nasal aspirates and serum samples stored at –70 C and described frustrations with sample preservation (2). This study was initiated to examine the effects of different preservatives on RT-PCR amplicon size from influenza A virus RNA in bird feces.

MATERIALS AND METHODS

Sample collection, fixation, and extraction

Ten to 30 min after defecation, droppings from resident wild mallards (*Anas platyrhynchos*) and wild Canada geese (*Branta canadensis*) were placed into 50-ml polypropylene tubes and stored at –80 C for less than 2 mo. An equal mixture of thawed duck and goose feces (3 g) was mixed with 3 ml of influenza A/Puerto Rico/8/34 (H1N1) at a titer of approximately 10^5 50% tissue culture infectious dose (TCID₅₀)/ml. We mixed the two kinds of feces in an effort to represent the range of fecal material from waterfowl in the environment and so as not to be specific to one avian diet or digestive system. Bird feces were not tested for the presence of avian influenza RNA before the addition of A/Puerto Rico/8/34 virus. From this slurry, 0.2 ml was transferred to each microfuge vial, and 1.2 ml of preservative was added to each vial. Ethanol (100%) was purchased from Warner-Graham (Cockeysville, MD). Methanol and isopropanol were purchased from MG Scientific (Pleasant Prairie, WI). The commercial preservative was RNeasyTM (Ambion; Austin, TX). Guanidine buffer (4 M guanidine isothiocyanate, 50 mM Tris-HCl [pH 7.5], 25 mM ethylenediaminetetraacetic acid [EDTA]) was purchased from Invitrogen (Carlsbad, CA). After a 2-wk incubation period at room

temperature, the samples preserved with alcohols were dried to approximately 0.2 ml on a Speed-Vac and adjusted to 250 μ l with diethyl pyrocarbonate (DEPC)–treated distilled deionized water. The RNA from all samples except those preserved with the commercial buffer was extracted with Trizol LS (Invitrogen) according to the manufacturer's instructions. We found that the commercial preservative was not miscible with Trizol LS, and we purified this RNA with a QIAamp viral RNA mini kit (Qiagen; Valencia, CA) as described (13). None of the RNA samples were tested for spectroscopic purity or average length. Positive control samples consisted of the extracellular media from Madin-Darby canine kidney cells infected with A/Puerto Rico/8/34 (H1N1). The samples were titrated at 10^5 TCID₅₀/ml, and RNA was isolated according to the procedure described above.

RT-PCR

RNA was resuspended in 50 μ l of water and cDNA was prepared according to the manufacturer's instructions with Moloney murine leukemia virus reverse transcriptase (Invitrogen) primed with random hexamers. Accounting for all volume changes, the amount of input virus that was processed for alcohols, commercial preservative, and guanidine buffer was approximately 5:1:1, respectively, for each preservative. cDNA samples were used as template for RT-PCR with the primers listed in Table 1. Two and one half units of AmpliTaq Gold (Invitrogen) in the manufacturer's buffer with 2.5 mM MgCl₂, 200 μ M deoxynucleoside triphosphate, and 1 μ M primers were used to amplify 1.5 μ l of cDNA in a total volume of 50 μ l. For PCR products of 640 bp, we employed the conditions described previously (8). For PCR products from 521, or 432 bp, conditions were 95 C for 9 min followed by 40 cycles of 94 C for 30 sec, 56 C for 30 sec, and 72 C for 3 min. For PCR products of 322 to 137 bp, conditions were 95 C for 9 min followed by 40 cycles of 94 C for 30 sec, 53 C for 30 sec, and 72 C for 1.5 min. PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and photographed. RT-PCR reactions were repeated at least once for each of the five independent replicative experiments performed.

RESULTS AND DISCUSSION

We found that commercial and guanidine buffer preservatives allowed for the amplification of PCR products ranging from 137 to 432 bp (Fig. 1). In contrast, none of the alcohols tested were reproducibly capable of preserving samples to allow for specific PCR amplification of greater than 206 bp. The PCR signal intensities of the samples preserved by alcohols at 136 and 206 bp were generally weaker than those for the commercial or guanidine buffers. Although isopropanol and methanol allowed for stronger 136- and 206-bp signals than did ethanol (Fig. 1), on the basis of presence or absence of signal, differences among alcohols were not reproducible. Because none of the preservatives were reproducibly capable of amplifying the 640-bp amplicon (Fig. 1), we added a 521-bp amplicon (Table 1) to our analyses. The results of two experiments indicated that none of the alcohols, but both commercial and guanidine buffers, were capable of producing RT-PCR products of the appropriate size, although with signals of less than 20% of those from positive controls (data not shown).

There are some caveats to these data. Although five times the input RNA was employed for analyses with alcohols, it is possible that unidentified RT-PCR inhibitor concentrations were also increased by the same amount. Also, because different PCR conditions were employed on the basis of product size, it is possible that some of the differences in consistent amplicon length amplification were due to PCR reaction and primer efficiencies.

The amount of influenza A virus in our virus-spiked feces was compared to that from cloacal swabs of influenza-positive birds. This was measured by real-time PCR with a conserved 93-bp region of the matrix gene, as described (3). Although the data were not quantitative, we found that samples preserved with either commercial or guanidine buffers had approximately

10- to 100-fold less amplifiable viral RNA than observed for cloacal swabs on the basis of C_T values (data not shown), whereas alcohols had approximately 1000- to 10,000-fold less (data not shown). This suggests that our assay conditions were unlikely to overestimate the amount of influenza RNA likely to be present in field samples. It also suggests that amplifiable viral RNA concentrations for feces stored in alcohol were 100 to 1000-fold less than those in commercial or guanidine buffers. This could be because of increased sample degradation during alcohol storage. However, although the overall efficiencies of sample processing were measured, RNA preservation was not determined directly. Taken together, the data suggest that commercial or guanidine buffer preservatives allow for the amplification of influenza RNA in mixed bird feces of up to 521 bp and are superior preservatives to common laboratory alcohols.

We provide evidence that environmental samples or cloacal swabs preserved in a concentrated guanidine or commercial buffer and held at ambient temperatures might be useful for the identification of waterfowl infected by influenza A viruses with increased confidence and that additional molecular assays can be performed successfully. We anticipate that this work will assist influenza A virus surveillance efforts become more widespread, rapid, and inexpensive.

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Abbreviations

DEPC, diethyl pyrocarbonate; EDTA, ethylenediaminetetraacetic acid; HA, hemagglutinin; M, matrix; NA, neuraminidase; RT-PCR, reverse transcriptase–polymerase chain reaction; TCID₅₀, 50% tissue culture infectious dose.

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Fig. 1.

Effect of preservative on RT-PCR amplification of various influenza viral amplicons in bird feces. Agarose gels stained with ethidium bromide show the RT-PCR amplification efficiencies for different amplicon sizes of influenza A/Puerto Rico/8/34 (H1N1) stored with various preservatives. The data shown are consistent with those results that were reproducible in five independent experiments. - = negative control (water template); + = positive control (viral RNA template); I = isopropanol preservative; E = ethanol preservative; M = methanol preservative; C = commercial preservative; G = guanidine buffer preservative. The left column gives amplicon size (bp). For the 137 bp product, upper band is specific, whereas the products underneath are probably primer dimers.

Table 1

Deoxynucleotide primers used in this study. Sequences are oriented 5' to 3' and positions are given relative to influenza A/Puerto Rico/8/34 (H1N1) gene segments.

Gene segment ^A	Amplicon size (bp)	Forward primer (position in bp)	Reverse primer (position in bp)
HA	640	GGAATGATHGAYGGNTGGTATGG ^B (1110–1132)	ATATCGTCTCGTATTAGTAGAAACAAGGGTGT ^B (1778–1759)
M	521	GAACGGGGATCCAAATAACA (283–303)	TGCGGCAATAGCGAGAG (803–787)
M	432	AGCCGAGATCGCACAGAGACTT (88–109)	ATTTCCTATGAGACCGATGCT (519–498)
M	322	TGGGGCCAAAGAAATCTCACTCAG (355–378)	TCTCATCGCTGCACCATTGTCT (676–653)
M	206	AGCCGAGATCGCACAGAGACTT (88–109)	GATCCCCGTTCCCATTA (294–277)
M	137	AGCCGAGATCGCACAGAGACTT (88–109)	TGAGCGTGAACACAAATCCTAAAA (224–200)

^A M = matrix.

^B As reported by Phipps *et al.* (8).