Published in final edited form as:

J Virol Methods. 2008 April ; 149(1): 180–183. doi:10.1016/j.jviromet.2008.01.001.

Detection and Isolation of H5N1 Influenza virus from Large Volumes of Natural Water

Alexey Khalenkov¹, W. Graeme Laver², and Robert G. Webster^{1,*}

¹Department of Infectious Diseases, Division of Virology, St. Jude Children's Research Hospital Memphis, TN, 38105, USA

²23047 Barton Highway, Big pond, Murrumbateman, NSW 2582, Australia

Abstract

Various species of aquatic or wetlands birds can be the natural reservoir of avian influenza A viruses of all hemagglutinin (HA) subtypes. Shedding of the virus into water leads to transmission between waterfowl and is a major threat for epidemics in poultry and pandemics in humans. Concentrations of the influenza virus in natural water reservoirs are often too low to be detected by most methods. The procedure was designed to detect low concentrations of the influenza virus in large volumes of water without the need for costly installations and reagents. The virus was adsorbed onto formalin-fixed erythrocytes and subsequently isolated in chicken embryos. Sensitivity of the method was determined using a reverse-genetic H5N1 virus. A concentration as low as 0.03 of the 50% egg infection dose per milliliter (EID₅₀/ml) of the initial volume of water was effectively detected. The probability of detection was ~13%, which is comparable to that of detecting the influenza virus M-gene by PCR amplification. The method can be used by field workers, ecologists, ornithologists, and researchers who need a simple method to isolate H5N1 influenza virus from natural reservoirs. The detection and isolation of virus in embryonated chicken eggs may help epidemiologic, genetic, and vaccine studies.

Keywords

Influenza virus; Virus isolation; Hemagglutination assay

Various species of aquatic or wetlands birds (particularly ducks, geese, swans, gulls, and shorebirds) are considered to be the natural reservoir of avian influenza A viruses of all 16 hemagglutinin and 9 neuraminidase subtypes (Munster et al., 2007). In birds, low-pathogenic influenza viruses primarily infect cells of the intestinal tract and are excreted in high concentrations in their feces. Shedding of the virus into the water leads to transmission among waterfowl via the fecal—oral route (Webster et al., 1992). The recently emerged highly pathogenic H5N1 viruses reproduce mainly in the respiratory tract of infected birds, but also end up in water either because of dabbling or direct transmission from dead birds in the waterways. Influenza viruses from zoonotic wild bird reservoirs can also be transmitted to other species, including humans, pigs, horses, and domestic poultry.

*Corresponding author. Address: 332 N. Lauderdale St., MS 330, Memphis, TN 38103. Phone: +1-901-495-4179. Fax: +1-901-523-2622. E-mail: E-mail: Robert.Webster@stjude.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Influenza viruses of H5 and H7 subtypes can cause a disease outbreak in domestic poultry, with high mortality when transmitted from wild birds. During the last decade, there have been several cases of direct transmission of viruses of the H5, H7, and H9 subtypes from poultry to humans (Fouchier et al., 2004). H5N1 viruses can cause severe respiratory disease and death in humans, posing a major threat of human pandemics. Recent avian influenza viruses of the H6 and H9 subtypes can be not only pathogenic in poultry but also serve as a source of internal genes for highly pathogenic H5N1 viruses, including those isolated from humans (Chin et al., 2002).

Previous studies show that the low-pathogenic influenza viruses remain infectious in lake water for more than 30 days at 0°C and for up to 4 days at 22°C (Webster et al., 1978). Recent studies revealed, that H5 and H7 avian influenza viruses, including highly-pathogenic strains, have the ability to persist in water with wide variety of temperature and salinity for extended periods of time (Brown et al., 2007). Several methods have been employed to determine the viral concentration in water from the site of interest. Early techniques of isolating the influenza virus from lake water *in vitro* used unconcentrated water samples along with isolation in allantoic or amniotic cavities of embryonated chicken eggs or in tissue culture, such as Madin–Darby canine kidney (MDCK) cells, with following hemagglutination inhibition or virus neutralization assays to confirm virus presence. Later, immunofluorescence methods and PCR-based assays were applied. At present, virus detection methods that use different PCR-based techniques, such as PCR amplification of different segments of influenza virus genes by using specific primers with simultaneous subtyping (Stone et al., 2004), are most popular. Multiple development of the PCR-based assay, such as subsequent dot blot and hybridization analysis (Fouchier et al., 2000) or loop-mediated isothermal amplification (RT-LAMP; Ito et al., 2006), are up to a 100 times more sensitive than direct isolation in culture. However, they require costly reagents, such as primers and enzymes, programmed thermostats, and special installations, not necessarily available in all laboratories. Finally, commercially available rapid influenza diagnostic kits are reliable, but require higher concentrations of viral particles in a given volume than other methods do and are mostly oriented for medical applications. In addition, in many applications such as epidemiologic studies, genetics, and vaccine preparation, not only detection but also relatively high volumes of concentrated virus are needed. Thus, even after the virus is detected by a highly sensitive method, its direct isolation is required.

In this study a procedure for detecting and isolating the influenza virus from large volumes of natural water is presented. The method is based on well-known hemagglutination properties of influenza viruses (Hirst, 1941). Concentrations of the influenza virus in large volumes of water can be quite low and frequently undetectable by direct isolation in embryonated chicken eggs or tissue culture. In contrast, the method described below detects very low concentrations of the virus due to “concentration” process which is based on the biologic property of the influenza virus to agglutinate red blood cells (RBCs) by binding to sialyl receptors on the cell surface of the erythrocyte. The influenza virus isolated by this method in embryonated chicken eggs can also be immediately used for subsequent studies or grown for large stock. The sensitivity of method was compared with that of RT-PCR amplification of the M-gene of influenza virus.

RBCs were separated from the chicken whole blood by the following procedure. Chicken blood was divided in 15-ml Falcon™ tubes (BD Biosciences, San Jose, CA) and centrifuged at 5000 rpm for 3 minutes. Sera and white blood cells (WBCs) were pipetted from the top. Sterile phosphate-buffered saline (PBS; pH 7.2) was added and the blood suspension mixed thoroughly by inverting the tubes several times. Centrifugation and washing were repeated 3 times until all sera and WBCs were removed. Packed erythrocytes were used immediately or stored for no longer than 3 days at 4°C.

For long-term storage and avoidance of hemolysis of chicken erythrocytes in water, chicken RBCs (CRBCs) were treated with formaldehyde. One volume of fresh chicken erythrocytes was added to nine volumes of sterile PBS. An appropriate volume of 37% formaldehyde solution in water was transferred into a dialysis sack to achieve a final concentration of 1.5% formalin in the solution or it was added drop-wise with constant stirring. The erythrocyte solution was mixed at 4°C for 18–20 h with constant stirring to prevent erythrocyte clumping. The erythrocyte mixture turned dark brown to indicate reaction with formalin. After fixing, the erythrocyte solution was washed five times with PBS to remove formalin (by a procedure similar to that described for preparing fresh CRBCs). Fixed erythrocytes could be stored indefinitely at 4°C in PBS at 10% (v/v) suspension. To prevent bacterial contamination, 0.01% (by suspension volume) of ethyl-mercuri-thio-salicylic acid or merthiolate was added.

Because the procedure required propagation of water samples in 10-day-old embryonated chicken eggs, a highly concentrated antibacterial medium was prepared to avoid bacterial contamination of the embryos. The antibacterial medium contained a 1:1 PBS/glycerol sterile solution with several antibiotics added. The powdered antibiotics penicillin, nystatin, and polymyxin B were first mixed in PBS and then glycerol was added. Table 1 presents the amounts of antibiotics used to prepare 1 liter of the antibacterial medium. Because the antibacterial medium was too viscous for resuspending the CRBC pellet and bacterial contamination of eggs was still possible, additional antibiotics dissolved in sterile water were added to the medium in a 1:1 proportion (Table 1). This allowed easy resuspension of the pellet and ensured that the antibiotics were present at concentrations not toxic to embryos, thereby improving antibacterial properties of the mixture. The prepared antibiotics mixture was aliquoted and stored at –20°C until used.

The method discussed has been standardized to detect influenza virus in a 1 liter water sample from a natural reservoir (Mississippi river). For lower or higher volumes or for more than one sample, the amounts of reagents and number of steps should be changed accordingly. The water sample was chilled to 4°C and used immediately or stored at 4°C. It was not filtered as the virus at very low concentrations could be lost on the filters; however, natural sedimentation of large sediments is recommended.

To 1 liter of the water sample, 5 ml of the 10% suspension of formaldehyde-fixed erythrocytes in PBS was added. The 1 liter bottle was set on ice and shaken thoroughly manually at 10-min intervals for 1 hour. Water was aliquoted into centrifuge bottles (four 250-ml bottles used in the RC-5B Plus Beckman centrifuge). Samples were centrifuged for 5 min at 5000 rpm in a 4°C prechilled rotor chamber. The supernatant was discarded but 5 ml of liquid was left in each bottle. The pellet was resuspended with the residual supernatant with the bottle on ice. The resuspended RBC mixture was taken into a 50 ml plastic centrifuge tube, centrifuged for 10 min at 5000 rpm, and the supernatant was discarded. The pellet was resuspended in a mixture of 0.3 ml antibacterial media and 0.3 ml PBS with antibiotics. The mixture was pipetted into a microcentrifuge tube and incubated for 1 hour at 37°C. Tubes were mixed by inverting three or four times during the incubation period. The entire volume (including RBCs) was used to inoculate three 10-day-old embryonated chicken eggs with equal volumes of the mixture. Eggs were incubated for 72 hours at 37°C and chilled overnight at 4°C. The allantoic fluid from each egg was collected separately and a standard hemagglutination test was performed to confirm the presence of the virus. Hemagglutination test was performed in 96-wells microtiter plates with 0.5% chicken red blood cells and serial 2-fold dilutions of allantoic fluid. If no virus was observed, the allantoic fluid from all eggs was pooled and used for the second passage in embryonated chicken eggs.

In the present study a nonpathogenic reverse-genetic virus carrying the hemagglutinin and neuraminidase genes of the A/Vietnam/1203/2004 (H5N1) virus in the background of A/

Puerto Rico/8/34 was used (Hoffmann et al., 2002). Virus yield was $8.5 \log_{10}$ 50% egg infection doses per milliliter ($\text{EID}_{50}/\text{ml}$) or $7.9 \log_{10}$ 50% tissue culture infection doses in MDCK cells per milliliter ($\text{TCID}_{50}/\text{ml}$). The end-point sensitivity of the method was determined by using 10-fold virus dilutions (Table 2): 1 ml of each serial 10-fold virus dilution was added to 1 liter of water from natural reservoir to get final concentrations of 3000, 300, 30, 3, and 0.3 $\text{EID}_{50}/100$ ml of water sample. Each water sample was tested by this method and the highest detectable dilution was found. Each isolation experiment was considered positive if the virus was confirmed by a hemagglutination assay in at least one embryo. To compare the sensitivity of method with direct detection of the virus by RT-PCR techniques, 0.2 ml of the test sample was collected before and after concentration. RNA extraction (QIAGEN RNeasy Mini Kit 75182) and one-step RT-PCR amplification (RT-PCR Mini Kit 210212) by using universal primers for the M gene of influenza virus (M-For 5'-TATTCGTCTCAGGGAGCAAAAGCAGGTAG-3') and M-Rev 5'-ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT-3') were performed (Hoffman et al., 2001).

Before the concentration process, neither direct isolation of the virus from the water sample in embryonated chicken eggs nor PCR amplification of the M-gene of the influenza virus could detect the virus in concentrations lower than 3 $\text{EID}_{50}/\text{ml}$ of sample. For sensitivity testing the most straightforward and time-saving technique of PCR amplification was used and therefore it was impossible to obtain the maximum sensitivity equivalent to detection of 0.1 $\text{EID}_{50}/\text{ml}$ sample that could be achieved by rigorous and enhanced PCR-based methods such as RT-LAMP (Ito et al., 2006) or by using specific primers (Fouchier et al., 2000). The concentration process allowed detection of viral concentrations as low as 3 $\text{EID}_{50}/100$ ml of starting volume with a probability $\sim 13\%$, which is slightly less than the limit of detection of influenza virus M-gene by PCR amplification. Table 2 compares results of both methods along with the number of independent tests done. The probability of virus detection was calculated as the ratio of the number of experiments that confirmed virus isolation by the hemagglutination assay and the cumulative number of experiments performed for a given virus concentration.

Conclusion

The worldwide increase in the number of outbreaks of highly pathogenic influenza viruses is a major concern. Recent studies have revealed that many bird species can be reservoirs and intermediate hosts for the highly pathogenic H5N1 influenza viruses, causing major threats to both domestic poultry and humans, at least in Southeast Asia (Chen et al, 2005). Thus, monitoring the aggregation and breeding sites of such waterfowl, chiefly natural lakes or ponds, is very important for early detection of highly pathogenic variants of the influenza virus. Although extensive surveillance along with gathering and analyzing individual samples is essential and can play a key role in the early recognition of and preparation for the outbreak (Laver et al., 2000, Munster et al., 2006), time is of the essence, as control measures could become ineffective when the virus is introduced into the poultry population (Chen et al., 2005).

The method described can detect the influenza virus in large volumes of water without requiring costly installations or reagents. It is sensitive enough to detect viral presence at concentrations as low as 3 $\text{EID}_{50}/100$ ml of virus in water volumes equal to or more than 1 liter. This method can be used by field workers, ecologists, ornithologists, and researchers who need a simple method to detect the influenza virus in sea water, lakes, and rivers. Simultaneous isolation of the virus in embryonated chicken eggs may aid epidemiologic, genetic, vaccine, and water supply contamination studies.

Acknowledgments

Authors thank the scientific editing department for editorial assistance. This project has been funded in part with Federal funds from the National Institute Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract # HHSN266200700005C and by the American Lebanese Syrian Associated Charities (ALSAC).

References

- Brown JD, Swayne DE, Cooper RJ, Burns RE, Stallknecht DE. Persistence of H5 and H7 avian influenza viruses in water. *Avian Dis* 2007;50:285–289. [PubMed: 17494568]
- Chen H, Smoth G, Zhang S, Qin K, Wang J, Li K, Webster R, Peiris J, Guan Y. H5N1 virus outbreak in migratory waterfowl. *Nature* 2005;436:191–192. [PubMed: 16007072]
- Chin PS, Hoffmann E, Webby R, Webster RG, Guan Y, Peiris M, Shortridge KF. Molecular evolution of H6 influenza viruses from poultry in Southeastern China: prevalence of H6N1 influenza viruses possessing seven A/Hong Kong/156/97 (H5N1)-like genes in poultry. *J. Virol* 2002;76:507–516. [PubMed: 11752141]
- Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, Kuiken T, Rimmelzwaan GF, Schutten M, Doornum GJ, Koch G, Bosman A, Koopmans M, Osterhaus AD. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc. Natl. Acad. Sci* 2004;101(5):1356–1361. [PubMed: 14745020]
- Fouchier RA, Besterbroer T, Herfst S, Van Der Kemp L, Rimmelzwaan G, Osterhaus A. Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene. *J. Clin. Microbiol* 2000;38(11):4096–4101. [PubMed: 11060074]
- Hirst GK. The agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. *Science* 1941;94:22. [PubMed: 17777315]
- Hoffmann E, Krauss S, Perez D, Webby R, Webster RG. Eight-plasmid system for rapid generation of influenza virus vaccines. *Vaccines* 2002;20(2526):3165–3170.
- Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. *Arch. Virol* 2001;46(12):2275–2289. [PubMed: 11811679]
- Ito M, Watanabe M, Nakagawa N, Ihara T, Okuno Y. Rapid detection and typing of influenza A and B by loop-mediated isothermal amplification: Comparison with immunochromatography and virus isolation. *J. Virol. Methods* 2006;135:272–275. [PubMed: 16616961]
- Laver W, Bischofberger N, Webster R. The origin and control of pandemic influenza. *Perspect. Biol. Med* 2000;43(2):173–190. [PubMed: 10804584]
- Munster VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, Rimmelzwaan G, Beyer W, Schutten M, Olsen B, Osterhaus A, Fouchier R. Spatial, temporal and species variation in prevalence of influenza A viruses in wild migratory birds. *PLoS Pathogens* 2007;3(5):0630–0638.
- Munster VJ, Veen J, Olsen B, Vogel R, Osterhaus AD, Fouchier RA. Towards improved influenza A virus surveillance in migrating birds. *Vaccine* 2006;24(4446):6729–6733. [PubMed: 16806601]
- Stone B, Burrows J, Schepetiuk S, Higgins G, Hampson A, Shaw R, Kok T. Rapid detection and simultaneous subtype differentiation of influenza A viruses by real time PCR. *J. Virol. Methods* 2004;117:103–112. [PubMed: 15041206]
- Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol. Rev* 1992;56:152–179. [PubMed: 1579108]
- Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG. Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology* 1978;84:268–278. [PubMed: 23604]

Table 1

Preparation of antibacterial media and antibiotic mixture

Antibiotics	Amount added per liter of	
	Antibacterial media	Specific egg antibiotic mixture
Streptomycin sulfate	200 mg	100 g
Penicillin G	2×10^6 units	6.7×10^8 units
Polymyxin B	2×10^6 units	10^8 units
Nystatin	5×10^5 units	—
Ofloxacin	60 mg	—
Gentamycin	250 mg	40 g
Sulfamethoxazole	0.2 g	—

Table 2
Detection of influenza H5N1 virus in samples of natural water

Method	Probability of detection (%) vs. final virus concentration (EID ₅₀ /100 ml) ^a					
	0	0.3	3	30	300	3000
RT-PCR of unconcentrated fluid ^b	n/a [*]	n/a	n/a	0 ^c (0/3) ^d	100 (3/3)	100 (3/3)
Isolation in eggs of unconcentrated fluid	n/a	n/a	n/a	0 (0/3)	50 (2/4)	100 (3/3)
RT-PCR of concentrated fluid ^e	n/a	n/a	0 (0/2)	66 (2/3)	100 (3/3)	100 (3/3)
Isolation in eggs of concentrated fluid	0 (0/2)	6 (1/16)	13 (1/8)	35 (7/20)	85 (11/13)	100 (12/12)

* Experiment was not performed

^a Final concentration in 1 liter of water sample.

^b Water (0.2 ml) was taken for RNA extraction and the M-gene subsequently amplified by RT-PCR.

^c Probability of virus isolation expressed as the ratio of the number of independent experiments that resulted in isolation to the total number of experiments.

^d Ratio of the number of experiments with successful detection of virus either by PCR amplification or isolation in embryonated chicken eggs to the total number of independent experiments using particular method.

^e Concentrated fluid (0.2 ml) was taken for RNA extraction and the M gene subsequently amplified by RT-PCR.