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VIRMET 12420 1–5
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Journal of Virological Methods xxx (2014) xxx–xxx

Contents lists available at ScienceDirect
Journal of Virological Methods
journal homepage: www.elsevier.com/locate/jviromet

1 # A simple and efficient method for detecting avian influenza virus in
2 water samples

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11 *Article history:*
12 Received 26 December 2012
13 Received in revised form 15 January 2014
14 Accepted 21 January 2014
Available online xxx

15
16 *Keywords:*
17 Influenza virus
18 Virus concentration method
19 Positively charged membrane
20 Water
21 Reverse transcription PCR

A B S T R A C T

Waterborne transmission plays an essential role in the transmission and spread of avian influenza viruses. The abundance of influenza viruses in environmental water is usually extremely low and viruses or viral genomes can hardly be detected by conventional reverse transcription (RT-) PCR without concentration. In the present study, an electropositive filter membrane was used to concentrate influenza viruses from water sample, in addition, a glass fiber filter has been used prior to positive charged membrane for the prefiltration. Unlike the traditional adsorption–elution method, Trizol-LS reagent was used to lyse the viruses attached directly to the electropositive filter membrane and the influenza virus genomic RNA was extracted, followed by RT-PCR analysis. The method established in this study could improve the efficiency of the conventional RT-PCR technique used to detect the M, NP, and HA genes of influenza virus in natural water samples. This method could also reduce the time taken for the traditional adsorption–elution concentration procedure.

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22 ## 1. Introduction

23 Migratory birds that carry avian influenza viruses might shed
24 viruses into the environment along their migration routes. After
25 birds leave an area, the environmental persistence of viruses may
26 play an important ecological role in virus transmission (Lang et al.,
27 2008; Brown et al., 2009). The shedding of viruses into water could
28 infect any other waterfowl that visit the same area by direct or
29 indirect fecal-oral routes (Webster et al., 1992). It is accepted that
30 water is an important component of the transmission cycle of avian
31 influenza viruses (Ito et al., 1995; Khalenkov et al., 2008; Zhang
32 et al., 2011a,b). Recently, the presence of avian influenza viruses
33 in water samples from aquatic bird habitats was described. How-
34 ever, there have been relatively few studies on the concentration
35 of avian influenza viruses from natural water samples (Khalenkov
36 et al., 2008; Dovas et al., 2010; Deboosere et al., 2011). The titer
37 of influenza viruses in water samples associated with infected
38 birds is generally too low to be detected by most standard reverse
39 transcription (RT-) PCR methods. Therefore, virus concentration
40 procedures are required to detect viruses present at low titer in
41 water samples prior to RT-PCR, and several concentration methods
42 have been described previously for viruses other than influenza
43 virus (Gilgen et al., 1997; Katayama et al., 2002; Moce-Llivina et al.,
44 2002; Kittigul et al., 2005; Haramoto et al., 2007). In the conven-
45 tional methods used for the concentration of viruses from water
46 samples, the first step involves filtration, which is followed by
47 further concentration steps, including washing, elution, ultracen-
48 trifugation, or precipitation. In the present study, to avoid recovery
49 losses during the secondary concentration step, which affect the
50 ultimate sensitivity, elution and lysis steps had been combined
51 together prior to RNA extraction. Water samples from Dongting
52 Lake were inoculated with known amounts of influenza viruses
53 and filtered through positively charged membranes, before the
54 viruses were lysed directly on the membranes without any addi-
55 tional steps. The extracted RNA was used for reverse transcription
56 and the HA, NP, and M gene fragments of the influenza viruses were
57 detected by PCR using specific primers. This method may increase
58 the efficiency of detecting influenza viruses in water samples and
59 no complex equipment is involved. This method also requires less
60 time than other methods.

61 ## 2. Materials and methods

62 ### 2.1. Virus strains

63 The viruses A/duck/Huan/9/2009(H4N2) (Zhang et al., 2012),
64 A/environment/Hunan/1-8/2007 (H5N1), A/chicken/Hunan/

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0166-0934/$ – see front matter © 2014 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jviromet.2014.01.013

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Please cite this article in press as: Zhang, H., et al., A simple and efficient method for detecting avian influenza virus in water samples. J. Virol. Methods (2014), http://dx.doi.org/10.1016/j.jviromet.2014.01.013

2/2008 (H9N2) (Zhang et al., 2011b), and A/environment/Dongting Lake/Hunan/3-9/2007 (H10N8) (Zhang et al., 2011a) were inoculated into the allantoic cavities of 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (Merial Vital, Beijing, China) and incubated at 37 °C for 24–72 h. After incubation, the allantoic fluid was harvested and centrifuged at 12,000 rpm for 10 min at 4 °C. The confirmed influenza virus stock was divided into aliquots and stored at −80 °C until use. Fifty percent egg infection dose (EID_{50}) titers of each virus were determined in SPF eggs by 10-fold serial dilution of viruses, and endpoints were calculated by Reed and Munch formula (Reed and Muench, 1938). The titer of the virus stocks is: H4N2: $10^{8.5}$ EID_{50}/ml; H5N1: $10^{9.3}$ EID_{50}/ml; H9N2: $10^{10.3}$ EID_{50}/ml; H10N8: $10^{9.3}$ EID_{50}/ml.

2.2. Concentration and enrichment of influenza viruses from water

To develop the method for concentrating influenza viruses from water samples, water samples were taken from Dongting Lake, China. The Dongting Lake wetland is an important habitat and an over-wintering area for East Asian migratory birds, which is located at 28°30′–30°20′ N and 111°40′–113°40′ E in the Northeastern part of Hunan Province, China. The pH value of the water samples is 6.95. The influenza virus stock was used to produce tenfold serial dilutions (10^4–10^8 EID50) and 0.1 ml of each dilution was inoculated into 1 L Dongting Lake water samples. Dongting Lake water contained large amounts of microorganisms and other impurities, so direct filtration through the electropositive membrane would have blocked the filter membrane. Thus, sedimentation of the water samples had been done after the addition of influenza virus. A 47-mm diameter glass fiber filter with a low adsorption rate was then placed on the surface of the electropositive membrane before filtration. Finally, the water was filtered through a glass fiber filter (pore size = 1.0 μm, APFB04700, Millipore, Billerica, USA) and the electropositive membrane (pore size = 0.45 μm, INYC00010, Millipore, Billerica, USA) at a flow rate of 80–100 ml/min (Fig. 1). More information about the glass fiber filter could be retrieved from this linkage: http://www.millipore.com/catalogue/item/apfb04700. More information about the electropositive membrane could be retrieved from this linkage: http://www.millipore.com/catalogue/item/inyc00010.

2.3. Extraction of viral RNA genomes

After the filtration, the glass fiber filter membrane contained much micro-organism and other foreign substance, which made the RNA extract become difficult and the quality of the extracted RNA was not good enough for the sequent experiment. So the glass fiber filter membrane was abandoned after the filtration.

The viral RNA was directly extracted from the positively charged membrane as described previously with some modification (Gentry-Shields and Stewart, 2013). The positively charged membrane adsorbed influenza viruses from the water sample. It was taken from the filter and placed in a 60-mm diameter dish and covered with 2 ml Trizol LS reagent (Gaithersburg, USA) for 10 min. The lysate was divided equally and transferred to two 1.5 ml RNAse-free Eppendorf tubes (Axygen, New York, USA), then mixed completely with 400 μl chloroform. After incubation for 2–3 min, the viral genomic RNA was extracted according to the following protocol: centrifugation at 12,000 rpm for 10 min and collection of the supernatant; addition of an equal volume of isopropanol and incubation for 30 min at −20 °C; centrifugation at 12,000 rpm for 10 min at 4 °C, before discarding the supernatant, followed by the addition of 1 ml 70% ethanol; final centrifugation at 12,000 rpm for 10 min at 4 °C and discarding the supernatant. After 10-min air-drying completely, 13 μl RNAase-free water was used to dissolve the RNA precipitates in the two 1.5 ml Eppendorf tubes, before reverse transcription was conducted.

2.4. Reverse-transcription PCR

Reverse transcription was carried out in a reaction mixture (25 μl) that contained 5 μl of 5× reaction buffer (Promega, Madison, USA), 4 μl dNTP mixture (2.5 mM each of four dNTPs, Promega), 1 μl M-MLV reverse transcriptase (2000 U/μl, Promega), 1 μl RNase inhibitor (40 U/μl, Promega), 1 μl Uni12 primer, and 13 μl of RNA extract (about 100 ng). The 13 μl of RNA was the maximum template volume according to the manufacturer's protocol.

In this study, the primers M-229f and M-299r were used to detect the M genes of four influenza virus strains. The primers NP-1200f and NP-1529r were used to detect NP genes. The primers H4-8f and H4-777r, H5-155f and H5-699r, H9-151f and H9-638r, H10-521f and H10-932r were used to detect the HA genes of H4N2, H5N1, H9N2, and H10N8 influenza viruses, respectively. All of the primer sequences have been reported previously (Lee et al., 2001), and all of the primers used in this study were synthesized by Sangon Biotech (Shanghai, China) and diluted to the 20 μM for use.

The HA genes of H5N1, H9N2, and H10N8 were detected by PCR using the following conditions: an initial denaturation step of 3 min at 95 °C, followed by 35 cycles at 95 °C (denaturation) for 30 s, 50 °C (annealing) for 40 s, 72 °C (extension) for 40 s, and a final extension step at 72 °C for 10 min. The annealing temperature used to detect the HA gene of the H4N2 virus was 55 °C but the other conditions were the same. The annealing temperature used to detect the NP and M genes from each virus strain was 52 °C but all other conditions were the same (Lee et al., 2001). In addition, the PCR reaction volume is 25 μl containing 5 μl cDNA, 2 μl dNTP mixture (2.5 mM each), 2.5 μl 10× PCR Buffer (Mg^{2+} Plus), 0.2 μl TaKaRa Taq DNA polymerase 5 U/μl (Dalian, China), 0.5 μl the upper and lower primers each (20 μM each) and 14.3 μl ddH_2O. One tube was tested for each PCR.

2.5. Real-time PCR

The M gene fragment of the H10N8 virus was inserted into the pMD18-T vector (TaKaRa, Dalian, China) and this plasmid was used to construct the standard curve. A set of primers (5′-AAGACCAATCCTGTCACCTCTGA-3′ and 5′-CAAAGCGTCTACGCTGCAGTCC-3′) designed by Karlsson et al. (2007) was used in the real-time PCR analysis. An SYBR® Premix Ex Taq™ kit (TaKaRa, Dalian, China) was used for real-time PCR, according to the manufacturer's instructions. The real-time PCR reaction system is 20 μl containing 10 μl SYBR® Premix Ex Taq (Tli RNaseH Plus) (2×), 0.4 μl PCR forward primer (10 μM), 0.4 μl PCR reverse primer (10 μM), 0.4 μl ROX reference dye II (50×), 2 μl cDNA and 6.8 μl ddH_2O. The real-time PCR was performed using a 7900HT Real-Time PCR System (Applied Biosystems, Foster City, USA) according to the following protocol: an initial denaturation step at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, and 60 °C for 30 s, followed by dissociation stage at 60 °C for 1 min, and 95 °C for 15 s. All of the samples were analyzed in triplicate for each reaction. The data were analyzed using 7900HT System SDS Version 2.4 (Applied Biosystems, Foster City, USA).

3. Results

3.1. Virus concentration and RT-PCR

The H10N8 virus was tenfold serially diluted (10^4–10^8 EID_{50}) and added to 1 L Dongting Lake water. The water samples were then filtered through the glass fiber filter and positively charged membrane at a flow rate of 80–100 ml/min. After filtration, the virus

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