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A virus precipitation method for concentration & detection of avian influenza viruses from environmental water resources & its possible application in outbreak investigations

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***Background & objectives*: Avian influenza (AI) viruses have been a major cause of public health concern. Wild migratory birds and contaminated environmental sources such as waterbodies soiled with bird droppings play a significant role in the transmission of AI viruses. The objective of the present study was to develop a sensitive and user-friendly method for the concentration and detection of AI viruses from environmental water sources.**

***Methods*: Municipal potable water, surface water from reservoirs and sea were spiked with low pathogenic AI viruses. To concentrate the viruses by precipitation, a combination of potassium aluminium sulphate with milk powder was used. Real-time reverse transcription-polymerase chain reaction was performed for virus detection, and the results were compared with a virus concentration method using erythrocytes. Drinking water specimens from poultry markets were also tested for the presence of AI viruses.**

***Results*: A minimum of $10^{1.0}$ EID_{50} (50% egg infectious dose)/ml spiked H5N1 and $10^{1.7}$ EID_{50}/ml spiked H9N2 viruses were detected from spiked potable water; $10^{1.0}$ and $10^{2.0}$ EID_{50}/ml spiked H5N1 virus was detected from surface water and seawater samples, respectively. The present method was more sensitive than the erythrocyte-binding method as approximately 10-fold higher infectious virus titres were obtained. AI H9N2 viruses were detected and isolated from water from local poultry markets, using this method.**

***Interpretation & conclusions*: Viability and recovery of the spiked viruses were not affected by precipitation. The present method may be suitable for the detection of AI viruses from different environmental water sources and can also be applied during outbreak investigations.**

Key words Avian influenza virus - detection - environmental water - outbreak - surveillance - virus precipitation

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Influenza A viruses belong to the *Orthomyxoviridae* family of single-stranded, negative-sense enveloped RNA viruses. They are divided into 18 haemagglutinin and 11 neuraminidase (NA) subtypes[1,2]. Outbreaks of highly pathogenic avian influenza (HPAI) viruses have been reported from 68 countries, as on May 2019[3]. India reported outbreaks of the HPAI H5N1 viruses for the first time in 2006[4]. Since then, more than 137 outbreaks of H5N1 viruses have been reported[3]. H9N2 virus is a low pathogenic avian influenza (LPAI) virus with widespread distribution in poultry in Asia[5]. In Asia, AI H9N2 viruses have been regularly isolated from ducks[6]. It has been reported that AI H9N2 viruses have acquired receptor-binding characteristics typical of human strains, increasing the potential for reassortment in both human and pig respiratory tracts[7]. During AI surveillance, the presence of AI H9N2 virus in poultry from India has been reported[8]. The seroprevalence of antibodies against AI H9N2 among poultry workers in India has also been reported[9].

Wild aquatic birds such as geese, shorebirds and ducks are the natural reservoirs of influenza A viruses[10]. Transmission of avian influenza (AI) viruses occurs by contact between infected and susceptible hosts. Water-borne transmission of AI viruses has been suggested as an important transmission mechanism in domestic ducks and wild birds[6]. AI viruses have been isolated from water sources where wild birds congregate and it has been demonstrated that in these environments, influenza A viruses retain their infectivity for several weeks and the viable virus can be isolated[11,12]. AI viruses have been isolated at a higher frequency from the drinking water containers kept for poultry at live poultry markets (henceforth referred to as poultry drinking water) compared to faecal, tracheal and cloacal swab specimens[13]. Experimentally, both HPAI and LPAI viruses have been shown to persist in water[14]. It has also been shown that the exposure of H5N1 virus to ultraviolet light for 90 min did not inactivate the virus in either dry or wet poultry faeces[15]. The transmission of influenza A viruses among wild birds occurs mainly by the faecal-oral route, which enables the rapid spread of the disease[12]. These points underscore the importance of screening of environmental water specimens for the detection and characterization of AI viruses.

Only a few methods have been published describing the detection of AI viruses from water sources. Based on principles used for enteroviruses, the use of adsorption/elution on electropositive or mixed cellulose filters was reported for the detection of influenza viruses in large volumes of experimentally spiked tap water[16,17]. Deboosere *et al*[18] used a filtration-elution method with polyethylene glycol concentration. In another technique, influenza virus was concentrated by binding on to erythrocytes[11,12]. Some of these methods have limitations of sample volume or are cumbersome to use. The use of potassium aluminium sulphate (potash alum) is a well-known method for clarification of water; its presence in water causes the negatively charged colloidal particles to clump and settle at the bottom[19]. The use of skimmed milk powder has been demonstrated for concentration of adenoviruses from seawater samples[20]. We describe here the combination of potash alum and milk powder for precipitation of AI viruses. The addition of milk powder increases the density and enables the settling of the virus contained in the water due to the effect of gravity. The objective was to develop a sensitive method of virus precipitation as a convenient, low-cost alternative to concentrate AI viruses from a large volume of multiple sources of water.

Material & Methods

An H9N2 virus A/chicken/Pune/099321/2009 (hereafter referred to as AI H9N2 virus) isolated from Maharashtra in 2009 was used in this study. A reverse genetically modified LPAI H5N1 virus A/India/NIV/2006-PR8-IBCDC-RG7 (hereafter referred to as H5N1-RG) was also used[21]. The 50 per cent egg infectious dose (EID_{50}) virus titres were determined using the Reed and Muench method[22]. The haemagglutination (HA) assay and EID_{50} titres were 1024 HAU and $10^{7.33}$/0.2 ml, respectively, for AI H9N2 virus whereas that for H5N1-RG were 512 and $10^{6.5}$/0.2 ml, respectively.

Collection of environmental specimens: The water specimens included potable water supplied by the Pune Municipal Corporation (running tap water): surface water specimens collected from the Veer and Bhor reservoirs (Pune and Satara districts, respectively) which are known overwintering sites for migratory birds and seawater collected from the Arabian Sea at Nagaon (Raigad district), Maharashtra, in the month of January 2015. The water specimens were collected in clean plastic containers of 20 l capacity, transported immediately to the laboratory, ICMR-National Institute of Virology, Pune, India, and stored at room temperature (20-28°C). Thirteen specimens of poultry drinking water in shops in the month of March 2015 and

two specimens from lakes near Pune city in the month of November 2016 were also collected in sterile bottles (100 ml). They were transported to the laboratory, and *p*H and turbidity of the water were recorded.

The study protocol was approved by the ICMR-National Institute of Virology, Pune, India, where the study was conducted.

Preparation of water precipitation kit: Potassium aluminium sulphate (ER, Qualigens, Maharashtra), milk powder (Nestle, New Delhi) and 1 N sodium hydroxide (ER, Qualigens, Maharashtra) were used for precipitation of virus. The reagents were prepared as follows: Reagent A: potassium aluminium sulphate (2 g) dissolved in 10 ml of distilled water to attain a final concentration of 0.02 per cent in the water specimen; Reagent B: milk powder (1 g) dissolved in 10 ml of distilled water to attain a final concentration of 0.01 per cent in the water specimen, and Reagent C: sodium hydroxide (1 N) to adjust the *p*H of water to 6.5-7.0.

Precipitation of avian influenza viruses: The viruses were concentrated using the precipitation method as follows; 10 l water was spiked with AI virus to obtain a final concentration of $10^{1.0}$, $10^{1.7}$ and $10^{2.0}$ EID_{50} virus/ml (which corresponded to 10, 50 and 100 infectious particles (IP)/ml, respectively). These desired infectious virus titres were achieved by diluting the virus suspension as per their EID_{50} values (Table I). After a thorough dispersion of the virus in water by stirring, 1 ml sample of this suspension was withdrawn for testing. Reagent A (10 ml) and reagent B (10 ml) were added to the 10 l water and mixed thoroughly, and the *p*H of this suspension was measured and re-adjusted if necessary to the range 6.5 to 7.0 with reagent C. The water was kept overnight at ambient temperature (20-28°C) for precipitation. After precipitation, supernatants (1 ml) were collected, processed for RNA extraction and tested for residual virus by real-time reverse transcription-polymerase chain reaction (RT-PCR). The precipitate settled at the bottom of the funnel was collected in a tube and centrifuged at 894 × *g* for 2 min at 4°C. The supernatant after centrifugation was discarded and the pellet was resuspended in 10-15 ml of sterile distilled water (*p*H 6.5) and homogenized using a vortex mixer. Resuspended precipitates were diluted five- and ten-folds (1:5 and 1:10) in sterile distilled water; viral RNA was extracted from each dilution for the detection of the virus. Phosphate-buffered saline (PBS) control was used in each experiment instead of virus for spiking, and the precipitates were processed in the same manner as described above. When potable tap water was used, it was equilibrated overnight at ambient temperature in an open plastic container to remove residual chlorine, if any.

Extraction of viral RNA and RT-PCR: Viral RNA was extracted using the QIAamp viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions using a modification in the first step where Trizol LS reagent (Invitrogen, USA) was used instead

Table I. Detection of avian influenza viruses spiked in water using real-time reverse transcription-polymerase chain reaction

Viruses	Types of specimens (dilution)	Ct value average[a]±SD		
		$10^{1.0}$ EID_{50}/ml (10 IP/ml)	$10^{1.7}$ EID_{50}/ml (50 IP/ml)	$10^{2.0}$ EID_{50}/ml (100 IP/ml)
A/India/NIV/2006/PR8-IBCDC-RG-7 (H5N1-RG)	Pre specimen[b] (undiluted)	35±3.5	37±1.2	Undetermined
	Supernatant[c] (undiluted)	Undetermined[e]	Undetermined	Undetermined
	Precipitate[d] (1:5)	32±0.6	25±1.7	27±0
	Precipitate (1:10)	31±1.0	27±2.1	26±0.7
A/chicken/Pune/099321/2009 (H9N2)	Pre-specimen (undiluted)	Undetermined	Undetermined	Undetermined
	Supernatant (undiluted)	Undetermined	Undetermined	Undetermined
	Precipitate (1:5)	Undetermined	34±1.0	33±0.6
	Precipitate (1:10)	Undetermined	32±0.6	33±0.6

Three different concentrations (10, 50 and 100 IP/ml) of the two viruses were spiked and the prespecimen supernatant and precipitate samples were tested by real-time RT-PCR. [a]Average Ct values obtained for the real-time RT-PCR of three replicates each. Cut-off ≤35. [b]Pre-specimen. The water sample collected after spiking of virus. [c]Supernatant. The supernatant after the settling of the precipitate in the potash alum method. [d]Precipitate obtained after virus concentration using the potash alum method. The precipitate was diluted five- (1:5) and 10-fold (1:10) in distilled water. [e]Undetermined Ct values. IP, infectious particles; EID_{50}, 50% egg infectious dose; SD, standard deviation; RT-PCR, reverse transcription-polymerase chain reaction; Ct, threshold cycle

of QIAamp viral lysis buffer keeping rest of the steps same. One-step real-time RT-PCR was performed for the detection of influenza type A viruses using the M-gene-specific primers[23]. Following was the composition of each real-time RT-PCR reaction: 5 µl of nuclease-free water, 12.5 µl of 2x RT-PCR buffer, 0.5 µl each of forward primer, reverse primer and probe (Applied Biosystems, USA) and 1 µl of 25x RT-PCR enzyme mix. Real-time RT-PCR assays were performed using the ABI 7300 real-time PCR platform (Applied Biosystems, USA) using the following conditions: reverse transcription 30 min at 50°C and Taq inhibitor inactivation 5 min at 95°C, followed by 45 cycles of 95°C for 15 sec and 55°C for 30 sec. Appropriate, negative and positive controls were used in the assays.

Viability of avian influenza viruses in water: To study viability, 10 and 100 IP/ml of A/India/NIV/2006/PR8-IBCDC-RG-7-H5N1 virus were spiked in water from reservoirs and the Arabian Sea, respectively. After precipitation, precipitates were diluted two-fold (1:2) in viral transport medium (VTM, Hanks balanced salt solution) supplemented with gentamicin (250 mg/l), penicillin (2×10^6 U/l) and streptomycin (200 mg/l) (supplemented VTM), maintained at room temperature (20-28°C) for 1 h and centrifuged at 313 × *g* for 2 min at 4°C. This mixture was filtered using the 0.22 micron syringe filter (Merck Millipore, USA), and 200 µl of the filtrate was inoculated in 10 day old embryonated chicken eggs (allantoic route). Eggs were incubated at 37°C for 72 h. Allantoic fluids were harvested and tested using the HA[24] assays.

Estimation of virus recovery: In a separate experiment, AI H9N2 and H5N1-RG viruses with EID_{50} titres of $10^{3.48}$ EID_{50}/0.2 ml and $10^{3.4}$ EID_{50}/0.2 ml, respectively, were spiked in 2 l water to estimate the virus recovery after precipitation. The concentrated virus in the precipitates was inoculated in embryonated chicken eggs as described above. The allantoic fluids from the inoculated eggs were tested by HA. The infectious virus (EID_{50}) titres were calculated using the Reed and Muench method[22]. Each experiment was performed three times.

Virus isolation from poultry drinking water: A total of 15 water specimens from poultry markets were tested for the presence of AI viruses using the potash alum precipitation method as described earlier. To circumvent potential issues caused by the initial turbidity of the poultry drinking water, 100 ml of the source water was diluted to a final volume of 500 ml using sterile distilled water. After precipitation, pellets were diluted (1:2) in supplemented VTM and processed for virus isolation.

Haemagglutination (*HA*) *and HA inhibition assays*: Allantoic fluids from inoculated eggs were tested by HA to detect the presence of virus using 0.5 per cent turkey erythrocytes. Briefly, virus titration was performed in 96 well V bottom microplates (Tarsons, West Bengal). Two-fold serial dilutions of the specimens were prepared in PBS containing sodium chloride, potassium chloride, sodium hydrogen phosphate and monopotassium phosphate (PBS, prepared in-house), *p*H 7.2, and incubated for 30 min with 50 µl 0.5 per cent Turkey erythrocytes. HA titre was expressed as reciprocal of the highest dilution showing complete haemagglutination[24].

The HAI assay was conducted for identification of virus from poultry drinking water samples using influenza A anti-serum raised in fowl (*Gallus gallus domesticus*) against AI H5N1, H9N2, H11N1 and H4N6 viruses. Anti-serum samples were treated with the receptor-destroying enzyme (Denka Seiken, Japan) at 37°C for 16 h, inactivated at 56°C for 30 min and then used in the assay. Appropriate cell control and serum controls were used[24].

Comparison of the present method with virus concentration using erythrocytes: The potash alum precipitation method was compared with virus concentration using chicken erythrocytes[13]. Briefly, chicken erythrocytes were collected in Alsever's solution (prepared in-house) and washed with PBS (*p*H 7.2). Three aliquots of municipal water (50 ml each) were spiked with $10^{1.0}$, $10^{1.7}$ and $10^{2.0}$ EID_{50}/ml AI H9N2 virus, respectively. To each aliquot PBS (10x, 5 ml) and pre-washed erythrocytes (100 µl) were added and were maintained on ice for 1 h for binding of the virus to erythrocytes. Suspensions were centrifuged at 500 × *g* for 5 min at 4°C. Pellets were resuspended in 1 ml of supplemented VTM and maintained at 37°C for 30 min for virus elution, followed by centrifugation. The eluate was collected in a separate tube, and the pelleted erythrocytes were resuspended in 1 ml distilled water. Appropriate negative controls were used. The eluates were inoculated in 10 day old embryonated chicken eggs and EID_{50} titres were determined. The pre- (spiked water before treatment), eluate and pellet samples were also tested for the presence of viral RNA using real-time RT-PCR. The EID_{50} titres were also determined for the

Table II. Comparison of the present method with virus concentration using erythrocytes;

AI H9N2 virus spiked/ml	Virus precipitation method		Virus concentration using erythrocytes	
	Types of specimens (dilution)	Ct value average±SD	Types of specimens	Ct value average±SD
$10^{1.0}$ EID_{50} (10 IP/ml)	Pre-specimen[a]	Indeterminate[f]	Pre-specimen	Indeterminate
	Supernatant[b]	Indeterminate	Eluate[d]	Indeterminate
	Precipitate[c] (1:5)	Indeterminate	Erythrocyte pellet[e]	Indeterminate
	Precipitate (1:10)	34±0.7		
$10^{1.7}$ EID_{50} (50 IP/ml)	Pre-specimen	Indeterminate	Pre-specimen	Indeterminate
	Supernatant	Indeterminate	Eluate	Indeterminate
	Precipitate (1:5)	33±2.7	Erythrocyte pellet	34±1.7
	Precipitate (1:10)	32±0.2		
$10^{2.0}$ EID_{50} (100 IP/ml)	Pre-specimen	36±0.5	Pre-specimen	35±0.8
	Supernatant	Indeterminate	Eluate	34±0.7
	Precipitate (1:5)	30±0.1	Erythrocyte pellet	31±2.9
	Precipitate (1:10)	31±0.4		

Ct value cut-off ≤35. Average values of three replicates have been represented, [a]Pre-specimen. The water sample collected after spiking of virus, [b]Supernatant. The supernatant after the settling of the precipitate in the potash alum method, [c]Precipitate obtained after virus concentration using the potash alum method. The precipitate was diluted five-fold and ten-fold in distilled water. [d]Eluate. After virus release from the erythrocytes in erythrocyte-binding method, [e]Erythrocyte pellet. The erythrocytes settled after removal of eluate, [f]Indeterminate Ct value

precipitates obtained after spiking with $10^{1.0}$, $10^{1.7}$ and $10^{2.0}$ EID_{50}/ml AI H9N2 virus. The virus concentrated by the precipitation method was also tested using real-time RT-PCR.

Results

Properties of water specimens: Water samples from municipal supply were clear in appearance, while surface water and seawater were visibly turbid and exhibited neutral *p*H (*p*H 7.0). Specimens of poultry drinking water were highly turbid and acidic (*p*H 6.0-6.5).

Extraction of viral RNA and RT-PCR: Real-time RT-PCR was performed for the detection of concentrated virus from the precipitates. Ct values ≤35 were considered positive[25]. A minimum of 10 IP/ml spiked H5N1 virus and 50 IP/ml spiked H9N2 virus were detected from the water (Table I).

Viability of avian influenza viruses in water: To study viability, H5N1-RG virus was spiked in reservoir and seawater and precipitated. The precipitates were inoculated in embryonated chicken eggs for virus isolation. After virus isolation, 10 IP/ml spiked virus was detected from water specimens from reservoirs, whereas 100 IP/ml spiked virus was detected from the seawater after precipitation. The allantoic fluids from the inoculated eggs showed a titre of 128 HAU. This demonstrated that the process of precipitation did not affect the viability of viruses.

Estimation of virus recovery: AI H9N2 ($10^{3.48}$ EID_{50}/0.2 ml) and H5N1-RG ($10^{3.4}$ EID_{50}/0.2 ml) viruses were spiked separately in two separating funnels containing 2 l water in each. For both the viruses, no significant drop in the mean log EID_{50} titres of virus from precipitates was observed indicating comparable virus recovery.

Virus isolation from poultry drinking water: Six of the 15 water specimens were positive by real-time RT-PCR for the presence of AI viral RNA. Of these six positive specimens, two were positive for virus isolation. These viruses were identified as AI H9 virus by the HAI assay.

Comparison of the present method with virus concentration using erythrocytes: $10^{1.0}$, $10^{1.7}$, and $10^{2.0}$ EID_{50}/ml H9N2 virus was spiked and the infectious virus titres obtained by precipitation were $10^{2.60}$, $10^{2.63}$ and $10^{2.63}$ EID_{50}/ml, respectively, which were about 10-fold higher than the erythrocyte-binding method.

For the water spiked with $10^{1.0}$ and $10^{1.7}$ EID_{50}/ml virus, the precipitates were positive for the presence of AI virus by real-time RT-PCR, whereas the eluate obtained after concentration using erythrocytes was negative (Table II). The pelleted erythrocytes for the water spiked with $10^{1.7}$and $10^{2.0}$ EID_{50}/ml virus were positive for the presence of viral RNA, indicating a possibility of incomplete elution of the spiked virus from the erythrocytes (Table II).

Discussion

The persistence of AI viruses in the environment, particularly in the aquatic habitats of wild birds, plays an important role in its transmission. In the present study, AI H9 virus was detected in six of the 15 poultry drinking water samples, of which two were positive for virus isolation, demonstrating the utility of this method for AI surveillance and outbreak investigations. More number of positive samples by real-time RT-PCR as compared to virus isolation may be due to the fact that the real-time RT-PCR assay is highly sensitive and detects viral RNA from live as well as inactivated virus. In addition, the possibility of inactivation of viruses present in poultry drinking water due to adverse environmental conditions cannot be ruled out. Although certain methods involving the use of membrane SMWP filters have been found to be practical for recovery of AIVs in water[17], the advantage of the present method is its adaptability. It is possible to concentrate virus from the volumes of water ranging from 500 ml to 2 l. This could find its application during outbreak investigations and AI surveillance activities since the probability of AI virus detection could be higher at sites such as ponds and ditches in the vicinity of larger waterbodies where a large number of birds congregate.

The present method was used for the concentration of virus from 10 l of water, eventually scaling it down to 2 l, since transport and handling of such large volumes of water from the field is a logistical challenge. The present method can also be downscaled to lesser volumes up to 500 ml. The optimum *p*H of water for this method was found to be 6 to 6.5 as precipitation did not occur beyond this *p*H. Different concentrations of milk powder and potash alum were also tested for standardization (data not shown).

The combination of potash alum and milk powder along with the optimization of *p*H has not been used earlier for the concentration of AI viruses. However, only potash alum has been used for the concentration of chikungunya virus from grown virus stocks[26]. Erythrocyte methods for the concentration of AI viruses have been used previously. Thus, for comparison purposes, the erythrocyte-based sedimentation method was used. In the erythrocyte-binding method described by Okuya *et al*[12], the concentrated virus is eluted from the erythrocytes in the eluate and processed for virus isolation. In the present study, the viral RNA was detected by real-time RT-PCR in the erythrocyte pellet as well indicating the possibility of incomplete elution. The total volume of the precipitate in the present method was 10-15 ml whereas that of the resuspended erythrocyte pellet in the erythrocyte-binding method was only 1 ml. This further indicated the sensitivity of the precipitation method due to the fact that it was able to detect virus from larger volumes of water.

When real-time RT-PCR was used for virus detection, it was found that virus suspension before precipitation and supernatant after precipitation were either negative or borderline positive; conversely, precipitates were positive for the presence of the virus. This demonstrated that diluted virus could not be detected from water without precipitation, highlighting the utility of this method. Adjustment of the *p*H of the water to 6.5-7.0 was found crucial for virus precipitation since precipitation does not occur beyond this range. The Ct values of 1:5 and 1:10 dilutions of the precipitates were compared. The ten-fold diluted precipitates showed lower Ct values indicating that dilution of the precipitate improved the sensitivity of virus detection which could be due to the fact that the RNA associated with the precipitate becomes available or accessible for detection. It was observed that when lysis buffer was used for extraction of viral RNA, the silica membrane of the spin column was blocked by the precipitate. Since Trizol LS dissolved the precipitate and only aqueous phase was used for extraction, blocking of silica membrane of the column was prevented. Therefore, Trizol LS was found appropriate for extraction of viral RNA.

The viability of AI viruses in the environment depends on the cumulative effects of chemical and physical factors, such as humidity, temperature, *p*H, salinity and organic compounds, as well as differences in the virus itself[27,28]. It has been shown that the viral infectivity is reduced at *p*H 6.0-4.6 depending on the strain, and acidic *p*H affects the conformation of the virus[29]. The precipitation of virus in water from municipal, surface and seawater sources did not affect

the viability of the virus since the virus could grow in embryonated chicken eggs. This underscores the utility of the present method for isolation of AI viruses from different environmental water sources.

The limitation of the present study was that only two AI subtypes were used. Further studies with other subtypes, as well as other water-borne viruses, are needed. In summary, the present method was novel, sensitive and user-friendly which offered the potential for the detection of AI viruses from water during surveillance and outbreak investigation studies.

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***Conflicts of Interest*:** None.

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