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Survival of H9N2 Avian Influenza Virus in Natural Water Bodies

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Abstract
The present study was conducted to determine the survival period of H9N2 avian influenza virus in natural water bodies at different temperature and time of exposure and to observe the effects of various physiochemical factors on the survival and transmission of this virus via natural water bodies. The study was conducted between October 2015 to January 2016. Influenza virus H9N2 strain was isolated from Bangladesh Livestock Research Institute through Poultry Research and Training Center, Chittagong, Bangladesh. Five ponds water samples were collected from five sub-districts of Chittagong, Bangladesh. The slide haemagglutination test was performed to determine highest possible survival time or infectivity of the H9N2 virus. The findings of this study indicate that water is likely a vital route for the epidemic transmission of avian influenza viruses, especially during the winter season.H9N2 virus can survive up to 4 hours at (22-30˚C) temperature in slightly alkaline pH (7.44 - 7.56) of water, but at 37˚C it can survive hardly for 4 hours. Survival capacity of the H9N2 virus is decreasing with the increasing of environmental temperature and exposure time. Water with high pH value and bicarbonate concentration can reduce survival period of the H9N2 virus. The water samples of this study were soft in nature (<60 PPM). Soft waters may permit the viral persistence, however, the effects of pH and bi-carbonates together concealed the observation of actual effects of the hardness on H9N2 viral survival.
Keywords: Avian influenza virus, H9N2, water samples, temperature, slide haemagglutination test.

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Introduction

The Poultry industries of Bangladesh play a major role in income generation and employment opportunity for a significant section of people. It is also the predominant source for supplying animal protein at the lowest price to the people [1]. However, since 2007 its growth has been seriously inhibited by the emergence of low pathogenic avian influenza (LPAI) strain H9N2 which is a haemagglutinating virus. The natural water bodies are important routes for spreading and transmission of avian influenza virus [2]. The contaminated water for poultry farming, especially in backyard farming is being used in most of our poultry farmers [3,4]. Sources of drinking water that may be contaminated with the avian influenza virus include natural water bodies (e.g. ponds, lakes, canals, rivers etc.), groundwater aquifers and rainwater collection systems [5]. Of these sources, open water bodies are often infected by migratory waterfowl which are the most likely potential route of entry of the virus into the drinking-water supply [6].

The factor of determination for an avian influenza virus to transmit through diffusion in water is the term of the viability of the virus in the water body.

To better understand the survival period of avian influenza virus in natural water bodies and thus the risk of avian influenza virus spreading and transmission via such bodies a systematic study was conducted to determine the inactivation dynamics of H9N2 strain at different temperatures in water bodies of critical migratory bird's habitats in Chittagong district which also represent the entire country. The impacts of pH, hardness, and bicarbonates of water bodies, on the survival of H9N2 avian influenza virus, were also observed by this study. HPAI viruses cause a high mortality; in contrast, mortality attributable to LPAIV is negligible but may be great if concomitantly infected with other pathogens [7]. Such a widely reported strain is H9N2. Both HPAIV H5N1 and LPAIV H9N2 have been reported from commercial poultry in Bangladesh [8,9].Strict enforcement of biosecurity procedures and possible vaccination programs of all poultry flocks with continuous monitoring of poultry stations that may ensure minimization of avian influenza A prevalence and

avoid the emergence of more pathogenic strains [10,11].

The findings of this study indicate that water is very likely an important route for spreading of avian influenza virus epidemically, especially at low temperatures during the winter season. The survival time of avian influenza H9N2 virus at different temperatures in different water samples was determined in this study, which can reflect environmental conditions in which viruses can grow. Use of the contaminated water sources in poultry farming may be minimized by this study for those periods in which avian influenza H9N2 virus is remaining infective in those water bodies.

Materials and Methods

Five Upazilas (sub-districts) of Chittagong district of Bangladesh were selected based on the previous history of the abundance of migratory birds in natural water reservoirs (ponds or tanks), especially during winter season.

Selection of water sources and collection of water samples

Five large sized ponds (Dighi) from five Upazilas in Chittagong district were selected where migratory birds frequently visit in winter. The local name of the selected ponds is Nithyananda school dighi, Shuklambor dighi, Ashram dighi, Padma dighi, Varsity dighi located in Anowara, Chandanaish, Banshkhali, Rangunia and Hathazari Upazila, respectively. One liter of water sample per pond per Upazila was obtained and transferred immediately to Microbiology Laboratory at CVASU, Bangladesh. Water samples were used as a media for exposure to the H9N2 avian influenza virus.

Storage of water samples

The water samples were stored at room temperature before starting the study in the Microbiology laboratory of CVASU, Bangladesh.

Grouping of collected water samples

At first, every water sample was divided into two aliquots in an equal volume of 500 ml by using a conical flask. One of the aliquots was boiled at 100˚C for 15 minutes on electric heater followed by filtration with the help of syringe filter (pore size 0.2 µm) to remove microbes and other extraneous particles. Another aliquot was remaining in raw condition (unfiltered and non-boiled).

Determination of pH and temperature of water samples

The pH value and temperature of water samples were determined by using AD1030 pH/mV and Temperature Meter.

Estimation of bicarbonates concentration in water samples

The bicarbonate ion is an alkaline anion with the empirical formula HCO_3^-. Bicarbonates are the major constituents to maintain the alkalinity of water. Buffer solution, methyl orange indicator, and 0.02N H_2SO_4 solution were used to estimate the bicarbonates of water samples. At first, 100 ml of each collected water sample was taken into a conical flask. Then 2 ml of buffer solution was added into the sample. Ten drops of methyl orange were poured as an indicator which turned the sample into yellow. The solution was then titrated with 0.02N H_2SO_4 until the color become orange. Then titer value was documented. Then calculations were done by the following formula and results were determined.

Total bi-carbonates concentration (PPM) = titer value × 10

Detection of bicarbonate concentration in water samples

Water hardness or temporary hardness is a type of water hardness that occurs due to the presence of calcium and magnesium cations (Ca_2^+ and Mg_2^+) and carbonate and bicarbonate anions (CO_3^{2-} and HCO_3^-). It can be minimized either by boiling the water or by the addition of lime (calcium hydroxide). Hardness is most commonly expressed as mg's of calcium carbonate equivalent per liter.

Water sample, 0.01M EDTA solution, Buffer solution, Erichrome Black-T (EBT) indicator was used to determine the hardness concentration of collected water samples.

100 ml water sample was taken in a conical flask. Two ml of buffer solution was added into the sample. Then 4 drops of Erichrome Black-T indicator was added and the mixed solution was turned into blue color according to the color of the indicator. If the blue color is unchanged, then it indicates the absence of hardness and then further titration is not necessary. If the color is changed, then the solution was titrated with 0.01M EDTA until the color was disappeared. The protocol was followed for each collected water sample. Then calculations were done by the following formula and results were recorded.

Total hardness concentration (PPM) = titer value × 10

Antibiotic treatment to the water samples

Both raw and treated parts of each water samples were mixed with 10% solution of Gentamycin (Inj.Genta-10) @ 0.2 ml/100 ml of water. 0.2 ml antibiotic solution contains 20 mg of Gentamycin.

Collection of embryonated eggs

Six numbers of 9 days old embryonated chicken eggs were bought from Regional Poultry Farm, Pahartoli, Chittagong. Then the eggs were shifted into Microbiology laboratory. Eggs were candled properly to mark the circular air sac area and also marked a blood vessel free drilling point on the circular mark by the help of a Candler and pencil.

Avian influenza H9N2 virus collection and propagation

Avian influenza H9N2 field virus was collected from BLRI through PRTC, which was previously isolated from infected poultry farms of the country and stored at -80˚C temperature. Then, the drilling points were properly swabbed with alcohol by cotton to destroy extraneous microbes on the egg shell. After that, the virus suspension was kept at room temperature (26˚C) for few minutes for melting. By that time, the marked points of eggs were drilled properly.

Avian influenza H9N2 field virus was propagated in those chicken eggs via allantoic route for further use. At first, melted viral suspensions were inoculated @ 0.1 ml to the allantoic route of those embryonated chicken eggs. After completion of inoculation in all the eggs, those were transferred into an incubator for 48 hours at 37˚C. Allantoic fluids were collected via 10 ml sterile syringe and stored at - 80˚C temperature in 15 ml tubes for further use.

Collection of chicken blood and preparation of 1% chicken RBC

At first, a broiler chicken was bought from the market. Then 1 ml of blood was drawn from wing vein by using a 1ml insulin syringe that contains few granules of anticoagulant EDTA. After gentle mixture with EDTA, the blood sample was transferred slowly to a large, conical centrifuge tube for washing. An equal amount of 1×phosphate-buffered saline (PBS) was added and the suspension was centrifuged at 1000 rpm for 10 minutes. The supernatant was poured off, and 20 to 30 volumes of 1×PBS were added to the packed cells. The cells were re-suspended gently, and the centrifugation step was repeated two more times. The cells can then be used to prepare a 1% cell suspension in isotonic 1×PBS. 1 ml of chicken RBC's suspension was mixed with 99 ml of 1×PBS in a conical flask to prepare 1% chicken RBC. This suspension was stored at 4°C for 48 hours.

Preparation of 4 ml of 8 HA unit virus

At first, 50 µl of 1× PBS was dispensed into each well of a plastic V-bottomed microtiter plate. 50 µl of virus suspension was placed in the first well and mixed properly. Two-fold dilution of 50 µl volumes of the virus suspension was made across the plate. 50 µl of 1× PBS was dispensed to each well. Then, 50 µl of 1% (v/v) chicken RBC was dispensed to each well and mixed by tapping the plate gently. Then, RBC's were allowed to settle for about 40 minutes at room temperature. After that, the results were read and recorded. For each run, a positive and a negative control were included.

A positive result was indicated by a thin film of red blood cells (presence of haemagglutinin) and a negative result was indicated by a sharp button of red blood cells (absence of haemagglutinin).

The last well that showed complete haemagglutination is said to contain one hemagglutinating (HA) unit. From that, the titer of the original undiluted sample had been calculated. 1 HA unit virus was found in number 5 well, that means 1 HAU = 25 or 1: 32. So, 8 HA unit virus was contained in number 2 well and the titer was 22 or 1: 4. That means, 4 ml of 8 HA unit virus was made by mixing 1 ml of virus suspension to the 3 ml of 1× PBS. After preparation that virus suspension was stored at -80 ℃ for further use.

Viral culture in embryonated chicken eggs

Stored viral suspensions were kept at room temperature for melting and then 4 ml of 8 HA unit virus suspension was mixed with 4 ml of each water sample in a 10ml syringe. Then 4 ml of H9N2 virus suspension was mixed with 4ml both of the filtered and unfiltered water samples and incubated for at 1,2,3 and 4 hours in the temperature of 22˚C, 30˚C, and 37˚C. Then, 2 ml from each mixture or suspension was removed after being incubated for each hour at 22˚C, 30˚C, and 37˚C. That incubation procedure was maintained for both raw and treated (filtered and boiled) categories of all the water samples. After exposing the virus to different times and temperatures, 0.1 ml of viral suspension was inoculated into the allantoic route of 9-11 days old embryonated chicken eggs for each temperature, time, categories and water samples. Then they were

incubated at 37˚C for 48 hours. After 48 hours of incubation at 37˚C, the allantoic fluids were harvested by 10 ml syringe. 1ml of allantoic fluid was harvested from every inoculated embryonated egg and the haemagglutinating activity of H9N2 viruses was observed with the help of Slide Haemagglutination test for each temperature (22˚C, 30˚C, and 37˚C), time (1,2,3 and 4 hours), categories (raw and treated) and water samples sources (five ponds) of the study.

Slide haemagglutination test

It is very easy and rapid technique to detect the persistence of haemagglutinating viruses. In the case of slide haemagglutination test, 5% chicken RBC (5 ml of RBC's was mixed with 95 ml of PBS) was prepared for clear detection of haemagglutination instead of 1% chicken RBC.

Otherwise, the same procedure was followed as like as preparation of 1% chicken RBC. Then an equal volume of 5% chicken RBC and virus containing allantoic fluid on a glass slide were poured. Then the gentle mixture was done. In the case of positive results, agglutinated RBC's on the glass slides were observed with naked eyes within 5-10 minutes. On the other hand, blood was as usual with negative results.

Statistical analysis

The estimated data were entered into spreadsheet program Microsoft Office Excel- 2007 and T- test was carried out to observe the differential significance of two groups of data (raw water and treated water) for several chemical parameters. Differences were considered significant when those had P value < 0.05.

Results

The pH values of raw and treated water samples were shown in the Table-1. The average pH values of raw and treated water samples are 7.72 and 7.60 respectively. No significant difference in pH was observed between two groups of water where P value is 0.30 (P < 0.05) (**Table 1**).

Regardless of chemical types concentration reduced after treatment, however, there was no significant difference in bicarbonate concentration between two groups of water where P value was 0.37 (P < 0.05), but the significant difference was observed in hardness concentration where P value is 0.02 (P < 0.05) (**Table 2**).

Table 1: Estimated pH values of raw (unfiltered and non-boiled) and treated (filtered and boiled) water samples

Water Samples	pH values		Differences	P value
	Raw water	Treated water		
1	7.85	7.72	0.13	
2	7.97	7.88	0.09	
3	7.60	7.48	0.12	
4	7.56	7.44	0.12	**0.35**
5	7.62	7.51	0.11	
Average	**7.72**	**7.60**	**0.11**	

In Table (3 to 8), (+) indicates haemagglutination positive; (-) indicates haemagglutination negative. The yellow and blue highlighted cells in every table indicate major variations in haemagglutination activity of virus between raw and treated water samples in same time and temperature of exposure.

Table 2: Determination of bi-carbonate and hardness (Ca++ and Mg++) concentrations from the collected water samples

Chemical parameters	Sample No.	Raw water	Treated water	Differences	P values
Bi-carbonates (PPM)	1	24	23	1	0.37
	2	26	25	1	
	3	22	20	2	
	4	19	17	2	
	5	21	18	3	
	Average	22.4	20.6	1.8	
Hardness (Ca++ and Mg++) (PPM)	1	47	46	1	0.02
	2	46	43	3	
	3	45	37	8	
	4	46	44	2	
	5	49	38	11	
	Average	46.6	41.6	5	

Water sample-1 was collected from Anowara Upazila of Chittagong District (**Table3**). Avian influenza H9N2 virus was exposed at 22, 30 and 37 ˚C for 1, 2, 3 and 4 hours in both types (raw and treated) of this water sample. The maximum viral survival times were found in raw water sample-1 up to 3 hours of exposure and up to 4 hours of exposure in treated

water-1 at 22˚C temperature. The virus can survive up to 3 hours of exposure at a 30˚C temperature in both cases of raw and treated water sample-1. However, in the case of raw water sample-1, the virus can survive only up to 2 hours of exposure at 37˚C temperature. In the case of treated water sample-1, the virus can survive at 37˚C for maximum 3 hours of exposure.

Table 3: Slide Haemagglutination test showing survival of avian influenza H9N2 virus in collected water sample-1 (Anowara)

Natural water Types	Temperature of Exposure	Times of Exposure (Hour/s)	Results of Slide Haemagglutination test
Raw	22 ˚C	1	+
		2	+
		3	+
		4	-
	30˚C	1	+
		2	+
		3	+
		4	-
	37˚C	1	+
		2	+
		3	-
		4	-
Treated	22˚C	1	+
		2	+
		3	+
		4	+
	30˚C	1	+
		2	+
		3	+
		4	-
	37˚C	1	+
		2	+
		3	+
		4	-

Water sample-2 was collected from Chandanaish Upazila of Chittagong District (**Table 4**). Avian influenza H9N2 virus was exposed at 22, 30 and 37 ˚C for 1, 2, 3 and 4 hours in both types (raw and treated) of this water sample. The maximum viral survival times were found in raw water sample-2 up to 3 hours of exposure and up to 4 hours of exposure in treated water-2 at 22˚C temperature. The virus can survive up to 2 hours of exposure at a 30˚C temperature in the case of raw water and up to 3 hours of exposure in treated water sample-2. However, in the case of raw water sample-2, the virus cannot survive even up to 1 hour of exposure at 37˚C temperature. In the case of treated water sample-1, the virus can survive at 37˚C for maximum 1 hour of exposure.

Water sample-3 was collected from Banshkhali Upazila of Chittagong District (**Table5**). Avian influenza H9N2 virus was exposed at 22, 30 and 37 ˚C for 1, 2, 3 and 4 hours in both types (raw and treated) of this water sample. The maximum viral survival times were found in both raw and treated water sample-3 up to 4 hours of exposure at 22˚C temperature. The virus can survive up to 2 hours of exposure at a 30˚C temperature in the case of raw water and up to 3 hours in treated water sample-3. However, in the case of raw water sample-3, the virus can survive only up to 2 hours of exposure at 37˚C temperature. In the case of treated water sample-3, the virus can survive at 37˚C for maximum 3 hours of exposure.

Table 4: Slide Haemagglutination test showing survival of avian influenza H9N2 virus in collected water sample-2 (Chandanaish)

Natural water Types	Temperature of Exposure	Times of Exposure (Hour/s)	Results of Slide Haemagglutination test
Raw	22 ˚C	1	+
		2	+
		3	+
		4	-
	30˚C	1	+
		2	+
		3	-
		4	-
	37˚C	1	-
		2	-
		3	-
		4	-
Treated	22˚C	1	+
		2	+
		3	+
		4	+
	30˚C	1	+
		2	+
		3	+
		4	-
	37˚C	1	+
		2	-
		3	-
		4	-

Water sample-4 was collected from Rangunia Upazila of Chittagong District (**Table 6**). Avian influenza H9N2 virus was exposed at 22, 30 and 37 ˚C for 1, 2, 3 and 4 hours in both types (raw and treated) of this water sample. The maximum viral survival times were found in both raw water and treated water sample-4 up to 4 hours of exposure at 22˚C temperature. The virus can survive up to 3 hours of exposure at a 30˚C temperature in the case of raw water sample-4 and 4 hours of exposure in treated water sample-4. However, in the case of raw water sample-4, the virus can survive up to 3 hours of exposure at 37˚C temperature. In the case of treated water sample-4, the virus can survive at 37˚C for maximum 4 hours of exposure.

Table 5: Slide Haemagglutination test showing survival of Avian influenza H9N2 virus in collected water sample-3 (Banshkhali)

Natural water Types	Temperature of Exposure	Times of Exposure (Hour/s)	Results of Slide Haemagglutination test
Raw	22 ˚C	1	+
		2	+
		3	+
		4	+
	30˚C	1	+
		2	+
		3	-
		4	-
	37˚C	1	+
		2	+
		3	-
		4	-
Treated	22˚C	1	+
		2	+
		3	+
		4	+
	30˚C	1	+
		2	+
		3	+
		4	-
	37˚C	1	+
		2	+
		3	+
		4	-

Table 6: Slide Haemagglutination test showing survival of avian influenza H9N2 virus in collected water sample-4 (Rangunia)

Natural waterTypes	Temperature of Exposure	Times of Exposure (Hour/s)	Results of Slide Haemagglutination test
Raw	22 ˚C	1	+
		2	+
		3	+
		4	+
	30˚C	1	+
		2	+
		3	+
		4	-
	37˚C	1	+
		2	+
		3	+
		4	-
Treated	22˚C	1	+
		2	+
		3	+
		4	+
	30˚C	1	+
		2	+
		3	+
		4	+
	37˚C	1	+
		2	+
		3	+
		4	+

Water sample-5 was collected from Hathazari Upazila of Chittagong District (**Table 7**). Avian influenza H9N2 virus was exposed at 22, 30 and 37˚C for 1, 2, 3 and 4 hours in both types (raw and treated) of this water sample. The maximum viral survival times were found in both raw water sample-5 and treated water sample-5 up to 4 hours of exposure at 22˚C temperature. The virus can survive up to 2 hours of exposure at a 30˚C temperature in the case of raw water sample-5 and up to 3 hours of exposure in treated water sample-5. However, in the case of raw water sample-5, the virus can survive only up to 1 hours of exposure at 37˚C temperature but in the case of treated water sample-5, the virus can survive at 37˚C for maximum 3 hours of exposure.

Table 7: Slide Haemagglutination test showing survival of avian influenza H9N2 virus in collected water sample-5 (Hathazari)

Natural waterTypes	Temperature of Exposure	Times of Exposure (Hour/s)	Results of Slide Haemagglutination test
Raw	22 ˚C	1	+
		2	+
		3	+
		4	+
	30˚C	1	+
		2	+
		3	-
		4	-
	37˚C	1	+
		2	-
		3	-
		4	-
Treated	22˚C	1	+
		2	+
		3	+
		4	+
	30˚C	1	+
		2	+
		3	+
		4	-
	37˚C	1	+
		2	+
		3	+
		4	-

From Table (3 to 7) it is clear that virus can survive at low temperature for a long time, but increasing temperature reduces the infectivity as well as survival time. The highest survival time of H9N2 avian influenza virus was found in water sample No.-4. In the case of treated water samples, survival times were increased even after prolonged exposure to study temperatures. Viruses can hemagglutinate chicken RBC, s in treated (filtered & boiled) type of water sample No.-4 at the temperature 22˚C and 30˚C up to 4 hours of exposure. That could be due to the effects of pH which might be controlled by the concentration of bicarbonates. This study found that there were low pH values and low bicarbonates concentrations in the

water samples no.-4. That's why in water sample 4 virus survival is higher than the other samples.

At 37˚C infectivity of the H9N2 viruses sustains hardly for 4 hours in the water samples-4. In which only the treated samples supported the persistence of H9N2 viruses. There were no haemagglutination activities in chicken RBC's observed after 4 hours of exposure at 37˚C in raw and processed water sample no.-1, 2, 3 and 5.

Filtration and boiling also reduce the bicarbonates concentration as well as pH. As a result, in treated (filtered and boiled) water virus can survive more even after long time exposure compared with raw (unfiltered & non-boiled) water samples. High pH and bi-carbonates concentrations minimize the haemagglutination capacity of H9N2 avian influenza virus rapidly by comparing with relatively low pH and bi-carbonates concentrations in water.

In this study, maximum survival periods of avian influenza H9N2 virus were found at 22℃ exposure temperature in different water samples (**Table 8**).

Table 8: Table showing maximum survival periods of avian influenza H9N2 virus at **22℃** exposure temperature in different water samples at a glance

Collected water samples	Type of water samples	Times of exposure (Hour/s)	Results of Slide Haemagglutination test
1	Raw	1	+
		2	+
		3	+
		4	-
	Treated	1	+
		2	+
		3	+
		4	+
2	Raw	1	+
		2	+
		3	+
		4	-
	Treated	1	+
		2	+
		3	+
		4	+
3	Raw	1	+
		2	+
		3	+
		4	+
	Treated	1	+
		2	+
		3	+
		4	+
4	Raw	1	+
		2	+
		3	+
		4	+
	Treated	1	+
		2	+
		3	+
		4	+
5	Raw	1	+
		2	+
		3	+
		4	+
	Treated	1	+
		2	+
		3	+
		4	+

Discussion

This study found that pond water is the most important source for persistence and transmission of the avian influenza H9N2 virus, especially in the low temperature. The findings also reveals that, avian influenza H9N2 virus can persist in our seasonal condition up to 4 hours in slightly alkaline pH (7.44-7.56) at (22-30)˚C temperature and when water containing (17 – 19) PPM bicarbonates, and (44 – 46) PPM Ca++ and Mg++ concentrations. At 37˚C the virus can survive hardly up to 4 hours of exposure. As the temperature and pH value increase in water, reduce the infectivity of H9N2 avian influenza virus. This research finding is that H9N2 virus can survive in pond water even up to 4 hours if it was exposed at 30˚C. It was reported that avian influenza H9N2 virus could survive up to 5 hours in lake water at the temperature 28˚C [2].

They reported that the duration of infectivity of the virus could be extended by the decreasing of temperature. From that point of view, the currently estimated result is closely similar to the concluded result [2].

The previous study also showed that avian influenza H9N2 virus [2] could survive 75, 17 & 4 hours at the temperature 4˚C, 16˚C & 28 ˚C respectively in unfiltered water wherein filtered water survival was 96, 23, 5 hours at the temperature 4˚C, 16˚C & 28 ˚C respectively.

This study also found that filtered water supports the extended persistence of avian influenza virus than the unfiltered one [12].

It was reported that avian influenza virus remains infectious for few days at a low temperature in water bodies [13]. Survive even at 22 ˚C for up to 7 hours. The current study found that H9N2 avian influenza virus remains infectious up to 4 hours. That could be due to the variation of chemical compositions, pH value, soil compositions among the water bodies. Strain differences also have effects on the duration of survival. Microbial contents in natural water also have influences upon viral persistence [2].

It was reported that RNA virus HIV was showing the persistence of 8 hours in a treatment at the temperature 37ºC [14]. H9N2 avian influenza virus is also an RNA virus, but in this study, it shows survival hardly for 4 hours at 37ºC. Poliovirus could survive at 26°C in well water samples for 5 to 15 hours [15]. It might be due to the differences between virus type and other associated factors.

The ND virus remains infective on surfaces of the environment over the month. It survives on feathers

for 12 days, in moist soil for 2 days and in pond water for 9 hours at 20°C [16].

The pH values (**Table 1**) of all the collected water samples of both raw and treated were measured by using AD1030 pH/mV & Temperature Meter. There were found that, all the samples containing the pH values higher than the neutral level (pH-7). The pH value ranges in those water samples from 7.44 to 7.97. That means there all the collected pond water samples were slightly alkaline in nature. However, little bit differences in pH value were found between two groups of each sample. The highest value was found in sample No. 2 (Chandanaish) [pH- 7.97 and 7.88] and lowest value in sample No. 4 (Rangunia) [7.56 and 7.44].

It was studied four lake water samples in China and found that the pH of those water ranges from 6.93 to 8.89 [2]. They were found both acidic and alkaline waters. The variation of pH values in lake or pond water samples may depend upon the soil constituents of water reservoirs, utilization of water by local people, the presence of crop lands and industries besides the water reservoirs. Chemical wastage and pesticides might have possibilities to mix up to the natural water via rain or drainage systems. Another finding is that after filtration and boiling of water pH values are reduced in all the cases. Raw water pH value ranges from 7.56 to 7.97 and treated water samples pH values ranges from 7.44 to 7.88. It might be due to entrapment of little quantity of bicarbonates through the syringe filter, evaporation, and deactivation via boiling.

A previous study showed that the survival and subsequent transmission would be greatest in cold water bodies, with pH values ranging from 7.40 to 7.80 [17]. Although, that conclusion was based on results obtained by using distilled water. The research also showed that avian influenza virus is most stable in pH ranging 7.4 to 8.2 if the environment contains low salt concentration and the temperature persists below the 17˚C [18]. The findings of this study also support that conclusion. Because here estimated pH values range is remaining within the reported range but current study temperature is higher than the reported temperature (17˚C). That has likely effects on the stability of virus.

In the present study, the bicarbonate and hardness (Ca++ and Mg++) concentrations of collected water samples were measured by titration method and found that concentration of chemical ingredients was reduced due to filtration and boiling of water samples. Entrapment and evaporation of alkaline bicarbonates might result because of reduction of pH values in treated water samples than those of raw samples. Bi-carbonates concentration in five water samples ranges from 17 to 26 PPM. In the case of raw water, it ranges from 19 to 26 PPM, where it ranges from 17 to 25 PPM in the case of treated water samples. The highest value of bicarbonate concentration was found in sample no. 2 (Chandanaish) (26 and 25) and lowest value in sample no. 4 (Rangunia) [19 and 17]. It was stated that some viruses could survive only a few minutes to hours > 90 PPM concentration of bicarbonates in water and within 15 to 30 PPM most of the enveloped viruses can survive mostly for 5-7 hours at 20˚C depends on virus type [19].

Here, estimated result is within 15 to 30 PPM concentration of bicarbonates in water samples and highest 4 hours survival at 22˚C was found. On the other hand, due to the dominant effects of pH & bicarbonates, it was not possible to observe the actual effect of hardness on the survival of the virus. For studying the effects of hardness on viral persistence, further investigation is required by using the water samples from different types of water reservoirs like Lake, river, sea, rain, distilled water etc. It was established previously that, any water containing hardness (Ca++ and Mg++) concentration < 60 PPM, it is soft water [20]. In this study, only the pond water samples were used as viral survival media which contain hardness (Ca++ and Mg++) level < 50 PPM (**Table 2**).

Conflicts of interest

The authors declare no conflicts of interest.

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Author's Contribution

All authors contributed equally and approved the final manuscript.

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