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Research Article

The effect of sediment composition and polyethylene glycol precipitation on the detection limit of H6N2 influenza virus in sediment samples

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ABSTRACT

Influenza A viruses (IAVs) are naturally carried by wild aquatic birds and generally cause asymptomatic gastroenteric disease in their natural reservoir hosts. Because the viruses follow oral-fecal route in the avian host, they could be shed into water bodies through feces. Furthermore, IAVs that are secreted to abiotic sources might be preserved in the environment for a period, facilitating the transmission of viruses between individuals or species. Viral stability could be affected by several factors such as pH, salinity, and temperature of water. Therefore, this study aims to investigate the lowest amount of infectious IAVs that could be detected in sediment samples via molecular and virus isolation methods, and to compare the sediment composition with the efficiency of detection/isolation of IAVs and viral persistence. For this purpose, an H6N2 virus (A/Aquatic bird/Gediz Delta/1/2018) of avian origin was used for artificially seeding the sediment samples that were collected from Gediz Delta, Izmir, Turkey. Molecular methods showed that lower amount of H6N2 virus could be detected in sediment sample collected from freshwater area (FS) in comparison with the sediment samples that were collected from salty water area (SS). Furthermore, virus precipitation method using polyethylene glycol increased the efficiency of virus isolation by 10-fold in FS, but not in SS. On the other hand, although the detection limit for IAVs was higher in SS than in FS, viral fitness was better maintained in SS. Moreover, high number of cations in the composition of SS along with larger surface area facilitated virus adsorption on SS complicating the virus to detach from sediment particles. Thus, the result of this study remarks that the environmental origin of abiotic sources could affect the viral stability and fitness; therefore, it could affect the transmission dynamics of the virus in different environments.

Introduction

Influenza A viruses (IAVs) are one of the greatest public concerns affecting millions of people during pandemics and seasonal influenza infections. They belong to the Orthomyxoviridae family and diverge into subtypes based on their surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). Wild aquatic birds (Anseriformes and Charadriiformes) are the natural reservoir for IAVs and cause asymptomatic or mild gastroenteric disease in its natural host (Webster et al., 1992). So far, 16 HA and 9 NA subtypes have been identified in wild aquatic birds. The RNA genome of the virus is composed of eight unique gene segments that encode 10-12 proteins. Lack of proofreading mechanism of RNA dependent RNA-polymerase (RdRp) and the segmented nature of the viral genome facilitate point mutations and reassortment events, respectively, which may cause the virus to gain interspecies transmission ability.

Abiotic sources such as water and sediment may play a significant role as an indirect route for the virus transmission between individuals or species. Wild aquatic birds excrete IAVs into water bodies through their feces which may contain high titers of IAVs for at least six days (Webster et al., 1978). Viruses in fecal material may further precipitate onto sediment and recirculate in water bodies via the effects of physical factors such as animal activities or water movements. Several studies showed that IAVs were detected in water and sediment via molecular methods. For instance, 4.8% of the water and ice

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samples collected from migratory stopover sites in Michigan were detected as IAV-positive based on the presence of matrix (M) gene (Lickfett et al., 2018). Another study showed that 56% of the sediment samples that were collected from ponds with high abundance of aquatic birds were IAV-positive (Lang et al., 2008). Furthermore, IAVs may remain infectious in water for a period of time, even after the birds' departure from the aquatic ecosystems. For instance, IAVs were successfully isolated from water samples collected from several Alaskan lakes in autumn after ducks migrated away (Ito et al., 1995). However, it is not known whether IAVs remain infectious after they precipitate in sediment.

The persistence and stability of IAVs in aquatic environments depend on several environmental factors such as pH, salinity, and temperature which are defined as neutral pH, low temperatures, and fresh to brackish salinities (Stallknecht et al., 1990a; Brown et al., 2009). There are several studies in the literature that focused on how environmental factors may affect the infectivity of IAVs. For instance, IAVs could perpetuate their infectivity up to 207 days at 17°C according to the experiments using artificially seeded samples (Stallknecht et al., 1990b). Moreover, viral infectivity seemed to be maintained longer times at lower temperatures (4°C) when the virus was kept in distilled water (Stallknecht et al., 1990a). In another study, the infectivity of human H1N1 virus was maintained for more than one day, although the temperature and salinity of water were high (35°C and 270 ppt, respectively) (Dublineau et al., 2011). Considering the idea that IAVs which maintain the infectivity in water bodies may be adsorbed onto the colloidal particles suspended in water, they might precipitate together into the sediment. This could be possible through the electrostatic charge differences of IAVs and sediment particles which are negatively and positively charged, respectively (Bitton, 1975). It was shown that IAVs could be adsorbed onto the surface of several minerals such as hematite (iron oxide) or clay containing Na, Cl and Al elements (Warren et al., 1966; Bitton, 1975). Consequently, this might also increase the preservation of viruses on sediment particles as preventing the negative effects of UV-light or heat.

Several methods can be used to separate and precipitate viruses from complex environments. For instance, viruses can be suspended in solutions such as beef extract, glycine buffer, sodium pyrophosphate, or potassium citrate for the virus separation step (Williamson et al., 2003) followed by precipitation and concentration step. Polyethylene glycol (PEG) together with NaCl can be used for its high efficiency in virus precipitation (Guan et al., 2008).

Altogether, our study aims to determine the detection limits of IAVs in freshwater and salty water sediment samples via molecular and culture methods. We also aimed to investigate the effects of environmental factors such as inorganic composition and surface area of sediment samples on the viral fitness and virus adsorption processes. To achieve these aims, we artificially inoculated freshwater and salty water sediment samples using an H6N2 virus of avian origin with mammalian adaptation markers and detected the lowest amount of IAVs in those sediment samples via molecular and egg culture experiments. Furthermore, elemental/mineral compositions and specific surface areas of sediment samples were detected via XRF/XRD and BET analyses, respectively. Considering the fact that IAVs with H6 subtype are able to transmit to mammals without prior adaptation and have the ability to recognize human-type receptors (Gillim-Ross et al., 2008; Wang et al., 2014), the findings of this study remark the ecology of H6 viruses outside the host and highlights the role of abiotic sources in viral transmission of IAVs which pose a risk for human and veterinary health.

Materials and Methods

Virus

The virus used in this study was an H6N2 (A/Aquatic bird/Gediz Delta/1/2018) virus which was previously isolated from the fecal sample of an aquatic bird (Mercan et al., 2021). The initial titer of the virus was calculated as 6.5 $\log_{10}$ 50% egg infective dose per ml (EID_{50}/ml). All experimental work was conducted at a BSL-2 laboratory under a Class II A2 biosafety cabinet.

Sediment samples

To determine the limit of detection for IAVs in sediment samples via molecular and culture methods, IAV-negative freshwater sediment (FS) and salty water sediment (SS) samples were used. These sediment samples were collected from Izmir Bird Paradise, Gediz Delta in January 2018 by the scientific research permission (No: 72784983-488.04-232438) that was granted by the General Directorate of Nature Conservation and National Parks, Republic of Turkey Ministry of Agriculture and Forestry. The sediment samples were collected in transport media which contained PBS, glycerol, antibiotics (20000 U/ml Penicillin, 400 µg/ml Streptomycin, 300 µg/ml Gentamicin Sulfate, 10 U/ml Polymyxin B) and an antifungal (5 U/ml Nystatin).

Confirmation of the absence of IAVs in sediment samples

To ensure that the sediment samples were IAV-negative prior to seeding with a known virus, they were subjected to molecular analysis via RT-PCR and virus culture in embryonated chicken eggs (ECEs). First, the sediment samples that were collected from freshwater and salty water sources in transport media were combined separately to obtain approximately 9 gr from each sediment type (excluding the tube weight). This was necessary to obtain enough sediment samples to perform further experiments. Then, the sediment samples were centrifuged at 4400 rpm for 5 min and the supernatant was used to perform RNA isolation using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The absence of IAVs in sediment samples were first tested using molecular methods. Conventional RT-PCR reactions were performed using One-step RT-PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. IAV-specific primers targeting 234 bp region of the M1 portion of M gene (M30F 5'-ATGAGYCTTYTAACCGAGGTCGAAACG-3', M264R

5'- TGGACAAANCGTCTACGCTGCAG-3') (WHO, 2021) (partial M gene) and 634 bp region of the HA-2 portion of the HA gene (HA1144F 5'-GGAATGATAGATGGNTGGTAYGG-3', HA1778R 5'-ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT-3') (WHO, 2002) (partial HA gene) were used in separate reactions. The final concentration of each reaction consisted of 2 µl of extracted RNA, 4 µl of nuclease-free water, 1x RT-PCR Buffer, 400 µM of each dNTP, 0.4 µl of Enzyme Mix and 0.6 µM of each primer. The thermal cycler conditions were 50°C for 60 min, then 95°C for 15 min, followed by 40 cycles of 95°C for 1 min, 56°C (partial HA gene) or 58°C (partial M gene) for 1 min and 72°C for 2 min, and a final extension for 10 min at 72°C. The sediment samples were also inoculated into the allantoic cavities of ECEs for five consecutive passages (Eisfeld et al., 2014) to ensure no virus propagation was observed. Briefly, each sample was centrifuged at 4400 rpm for 5 min, then 0.1 ml supernatant/egg mixed with concentrated antibiotic mixture (2000 U/ml Penicillin, 400 U/ml Streptomycin, 200 U/ml Polymyxin B, 0.05 mg Gentamicin) at 1:1 ratio was inoculated into the allantoic cavities of the 10-days-old ECEs in triplicate. Then, eggs were incubated at 35°C for three days. The viability of the embryos was checked daily. The eggs were then chilled at 4°C overnight. The allantoic fluids were harvested into sterile tubes and tested for the absence/presence of IAVs via hemagglutination (HA) assay using chicken red blood cells (CRBCs) or turkey red blood cells (TRBCs) depending on the ability of IAVs to attach red blood cells. Because there are many mammals (jackal, fox, wild rabbit, weasel, badger, wild cat, jungle cat, seal, wild boar, and horse) residing in the sampling area, TRBCs were used to detect mammalian-like IAVs, if any. Briefly, 0.5% red blood cells (in PBS) were mixed with serial two-fold dilutions of allantoic fluids and incubated at room temperature for 30 minutes. A drop of allantoic fluids were inoculated on blood agar plates (5% sheep blood) to check bacterial or fungal contamination and incubated at 37°C for up to 2 days. In case of bacterial/fungal contamination, the allantoic fluids were sterilized through 0.44 and 0.22 µm filters, checked once again as mentioned above. After contamination was cleared, the HA assay was repeated. Finally, the allantoic fluids were also tested via molecular methods to detect viral RNA as described above to ensure no virus propagation was observed.

Determination of the limit of detection via molecular and culture methods

To determine the limit of detection, first, the freshwater and salty water sediments were aliquoted and the dilutions (1:10, 1:100, 1:1000, 1:10000, 1:100000) of an H6N2 virus (A/Aquatic bird/Gediz Delta/1/2018) with an initial titer of 6.5 $\log_{10}$ EID_{50}/ml were artificially seeded into the sediment aliquots. To determine the molecular detection limits immediately after virus seeding, viral RNA isolation and RT-PCR reactions were carried out for each virus dilutions as described above. To determine the detection limit via egg culture, all virus dilutions prepared in freshwater and salty water sediments were centrifuged at 4400 rpm for 5 min, then the supernatant was inoculated into ECEs and passaged for five times. The presence of IAVs was tested via HA assay using CRBCs after each egg passage. The presence of IAVs in allantoic fluids was also confirmed via molecular methods using partial M and partial HA gene primers as described above.

Determination of viral titers

Viral titers in allantoic fluids from each passage were measured in terms of HA unit and EID_{50}. To measure HA titer of each sample, 2-fold serial dilutions of the sample were prepared in PBS. Then, 0.5% CRBC solution was added to each well and the end-point dilution was noted as the HA titer of the virus. To measure the titer of the virus in terms of EID_{50}, 10-fold serial dilutions of the sample were inoculated into 10-days old ECEs in triplicate. Then, eggs were incubated at 35°C for 3 days. Presence of the virus was tested via HA assay using 0.5% CRBC solution. EID_{50} value was calculated for each sample according to the Reed and Muench method (Reed and Muench, 1938).

Virus purification and precipitation

To purify and precipitate IAVs from sediment samples, PEG precipitation method supplemented with beef extract solution was applied on the sediment dilutions (Guan et al., 2008). First, five volumes of 3% beef extract (Sigma, Darmstadt, Germany) solution (pH = 6.72) were mixed with all sediment samples that were artificially seeded with the dilutions of an H6N2 virus. Then, the mixture was agitated at 300 rpm for 50 minutes at 4°C to facilitate the separation of viruses from sediment particles. The mixture was centrifuged at 5000 × g for 60 minutes at 4°C and the supernatant was collected. To precipitate the viruses in the beef extract solution, equal volume of 16% PEG-6000 (Sigma, Darmstadt, Germany) solution in 0.01 M PBS (pH = 7.48) was mixed with the supernatant (final PEG concentration: 8%). The mixture was agitated at 200 rpm for 30 minutes at 4°C and incubated overnight at 4°C. Finally, the mixture was centrifuged at 10000 × g for 90 minutes at 4°C and the pellet was resuspended in 2 ml sterile PBS. RNA extraction was performed from the PEG-precipitated samples and RT-PCR reactions targeting specific regions of M and HA genes were carried out as described above. The PEG precipitated sediment samples were also inoculated into ECEs for five consecutive egg passages and the presence of IAVs was tested via HA assay using CRBCs. Viral RNAs were also extracted from the allantoic fluids after each egg passage and subjected to RT-PCR reactions targeting partial HA and M genes using gene-specific primers as described above. The viral titers were also determined for PEG-precipitated samples after each passage in eggs in terms of HA unit and EID_{50} as described previously.

Sediment composition and specific surface area analyses

The elemental/mineral composition, organic matter density and specific surface area of sediment samples were investigated in non-seeded FS and SS samples to understand the possible effect of sediment composition on virus adsorption process, thus the efficiency of IAV detection. First, the impurities were removed from FS and SS via two different washing methods. The first method was used to eliminate organic impurities

from the samples which were used as a template for inorganic component analyses. This method consisted of several washing steps with isopropanol alcohol (≤ 99.5%) followed by washing with dH_2O. The second method was to make possible to get rid of the impurities without damaging organic components of the samples. For this, sediment samples were washed several times with 10% NaCl followed by cleaning with dH_2O. Then, all wet sediment samples were dried at 50°C for 3 days. The elemental compositions, mineralogical structures and the specific surface areas of sediment samples were determined via XRF (AMATEK-Spectro IQ II), XRD (X'Pert ProX-ray, Phillips, The Netherlands) and multi-point BET method (Quantochrome NovaWin 2) analyses, respectively. These analyses were carried out at the Center for Materials Research, Izmir Institute of Technology. Prior to BET analyses, the samples were degassed at 300°C overnight (Guven and Akinci, 2013). Organic matter contents of sediment samples were determined gravimetrically by ignition of the dried sediment samples at 550°C for 4 hours at the Solid Waste and Soil Pollution Laboratory at the Department of Environmental Engineering, Faculty of Engineering, Dokuz Eylul University.

Results

Confirmation of the absence of IAVs in sediment samples prior to artificial seeding

Before we artificially seeded the sediment samples with a known H6N2 virus, both FS and SS samples were tested via molecular and egg culture methods to ensure that the sediment samples were IAV-negative. To test the samples via molecular methods, we first performed viral RNA extraction from FS and SS, followed by RT-PCR reactions with the primers targeting specific regions of M and HA genes of IAVs. Both sediment samples were confirmed IAV-negative based on the amplification of partial M and HA genes (Figure 1).

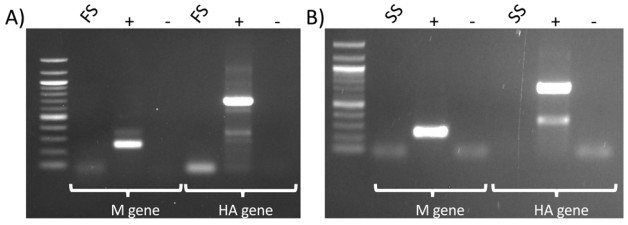

Figure 1. Confirmation of the absence of IAVs in sediment samples via molecular methods before artificially seeding with a known virus. The absence of IAVs were tested for FS (A) and SS (B) via RT-PCR targeting partial M gene (left panel) and partial HA gene (right panel). The viral RNA of H6N2 virus and nuclease-free water were used as a positive and negative control, respectively.

Then, the samples were inoculated into ECEs and passaged for five times. Allantoic fluid from each egg passage was tested via HA assay for the presence of IAVs. Since the sampling sites are shared by birds and mammals such as horses, pigs etc., we used CRBCs for avian-like IAVs and TRBCs for mammalian-like IAVs to detect both avian and mammalian origin IAVs. Our results showed that no virus propagation was detected up to five consecutive passages in ECEs via HA assay confirming that the sediment samples were IAV-negative (Figure 2). These results were also confirmed by performing RNA isolation and RT-PCR reactions from the allantoic fluids of each passage targeting specific regions of M and HA genes of IAVs, as no amplicon was observed for both genes (Figure 3).

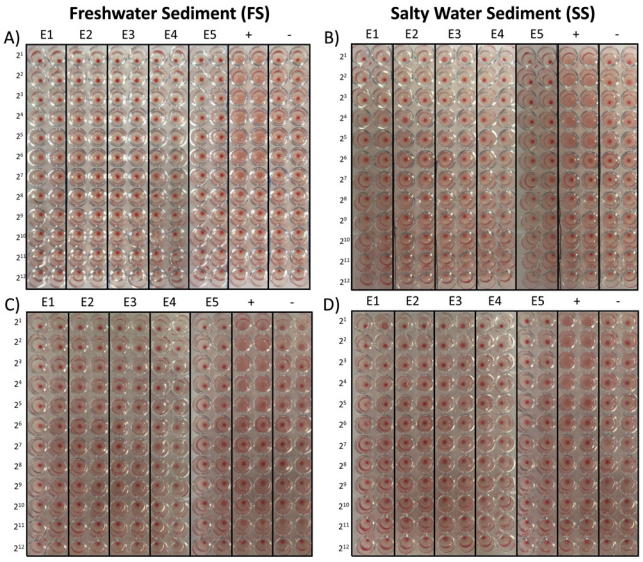

Figure 2. Confirmation of the absence of IAVs in sediment samples via HA assay before artificially seeding with a known virus. Presence of the HA activity was tested for FS (A, C) and SS (B, D). Serial dilutions of allantoic fluids obtained from five consecutive egg passages were treated with CRBCs (A, B) for avian-like IAVs or TRBCs (C, D) for mammalian-like IAVs. The absence of the HA activity at any dilution in any of the egg passage proved that both samples were IAV-negative. The consecutive egg passages were depicted as E1, E2, E3, E4 and E5, respectively. The viral RNA of H6N2 virus and nuclease-free water were used as a positive and negative control, respectively.

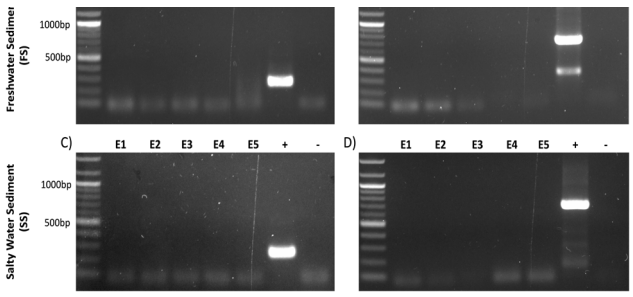

Figure 3. Confirmation of the absence of IAVs in five consecutive egg passages via molecular methods. The presence/absence of IAVs was tested for FS (A, B) and SS (C, D) via RT-PCR targeting specific regions of M (A, C) and HA (B, D) genes. Virus propagation could not be detected in any egg passage based on M and HA gene amplification. The consecutive egg passages were depicted as E1, E2, E3, E4 and E5, respectively. The viral RNA of H6N2 virus and nuclease-free water were used as a positive and negative control, respectively.

Determination of the molecular detection limit after artificial seeding with a known IAV

To investigate the detection limit via molecular methods, FS and SS aliquots were separately seeded with five different dilutions (1:10, 1:100, 1:1000, 1:10000, 1:100000) of an H6N2 virus (A/Aquatic bird/Gediz Delta/1/2018) that was previously isolated (Mercan et al., 2021). Then, viral RNA isolation and RT-PCR reactions targeting the specific regions of M and HA genes were conducted immediately after adding the virus. As a result, we detected amplicons for both the partial M and partial HA gene in FS at as low as 1:10000 dilution (2.5 $\log_{10}$ EID_{50}/ml) (Figure 4A and 4B). On the other hand, the detection limits varied for the amplification of partial M gene (at 1:1000 dilution; 3.5 $\log_{10}$ EID_{50}/ml) and HA gene (at 1:10 dilution; 5.5 $\log_{10}$ EID_{50}/ml) in SS (Figure 4C and 4D).

We also investigated the effect of virus separation from sediment samples using beef extract followed by PEG precipitation on the virus detection limit via molecular methods. Amplicons for neither gene were not obtained upon virus precipitation indicating that this approach might result in inhibition in RT-PCR reactions (data not shown).

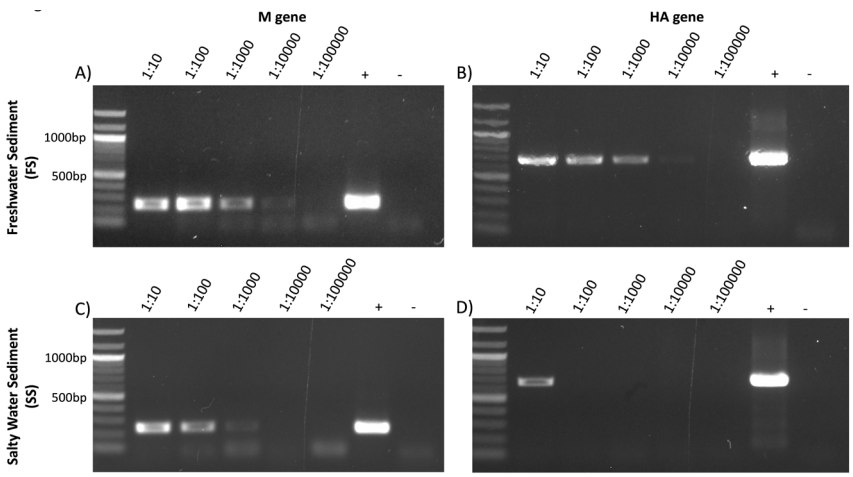

Figure 4. Determination of detection limit for an H6N2 virus in sediment samples via molecular methods. H6N2 virus (initial virus titer: 6.5 $\log_{10}$ EID_{50}/ml) was serially diluted in 10-fold in FS (A, B) and SS (C, D), then partial M (A, C) and HA gene (B, D) were amplified. Both partial M (A) and HA gene (B) amplicons were observed at 1:10000 dilution (2.5 $\log_{10}$ EID_{50}/ml) in FS. On the other hand, M gene amplicon (C) was observed at 1:1000 dilution (3.5 $\log_{10}$ EID_{50}/ml), while HA gene amplicon was observed only at 1:10 dilution (5.5 $\log_{10}$ EID_{50}/ml) in SS. The viral RNA of H6N2 virus and nuclease-free water were used as a positive and negative control, respectively.

Determination of the detection limit via culture method after artificial seeding with a known IAV

In addition to detecting viral genes based on RT-PCR amplifications, we also investigated the lowest dilution at which virus propagation could be detected in the artificially seeded sediment samples. For that purpose, same dilutions of an H6N2 virus in sediment samples were inoculated into ECEs and passaged for five times consecutively. Virus propagation could be detected in each egg passage of 1:100 dilution (4.5 $\log_{10}$ EID_{50}/ml) of FS and SS via HA assay using CRBCs. However, no virus propagation was observed in lower dilutions.

Then, virus separation with beef extract solution followed by PEG precipitation method was performed to investigate whether PEG precipitation increases the yield of the virus isolation from lower dilutions. PEG-precipitated sediment samples were also inoculated into ECEs for five consecutive passages and the presence of the H6N2 virus was tested via HA assay using CRBCs. Our results showed that PEG precipitation procedure increases the sensitivity of the detection limit via culture method by 10-fold for FS (3.5 $\log_{10}$ EID_{50}/ml, 1:1000 dilution), while it remained same for SS (4.5 $\log_{10}$ EID_{50}/ml).

Viral titers were also determined in terms of HA units for each egg passage of each H6N2 dilution in FS and SS. Our results showed that viral titers in the dilutions of H6N2 viruses in FS and SS varied between 32-512 and 4-256 HA units, respectively (Table S1). Although the HA titers of H6N2 viruses for PEG-precipitated sediment samples were similar to those for non-PEG-precipitated sediment samples (32-512 and 16-128 HA units for FS and SS, respectively) (Table S1), the overall HA titers of H6N2 viruses were slightly lower in SS. This might indicate that the ability of the virus to attach to the sialic acid receptors on the CRBCs might decrease for the viruses that are adsorbed onto sediment particles in salty environment.

Because the molecular methods can be more sensitive for the detection of lower amount of viruses as compared to HA assay, we also investigated the presence of IAVs in the allantoic fluids from each egg passage based on the amplification of partial M and HA genes. Overall, the results of gene amplifications were consistent with the HA assay results indicating that the viral RNA could not be detected in the allantoic fluids of passages that did not show HA activity (Figure 5, Figure S1). The amplicons for both genes that were obtained from the first egg passage are shown in Figure 5. For instance, HA activities were recorded at 1:100 and 1:1000 dilutions in FS before and after PEG precipitation, respectively (Table S1). Similarly, the amplicons for partial M and partial HA genes were also detected at 1:100 and 1:1000 dilutions in FS before (Figure 5A and 5B) and after PEG precipitation (Figure 5E and 5F), respectively. On the other hand, HA activities (Table S1) and gene amplifications were detected at as low as 1:100 dilutions in SS before (Figure 5C and 5D) and after PEG precipitation (Figure 5G and 5H).

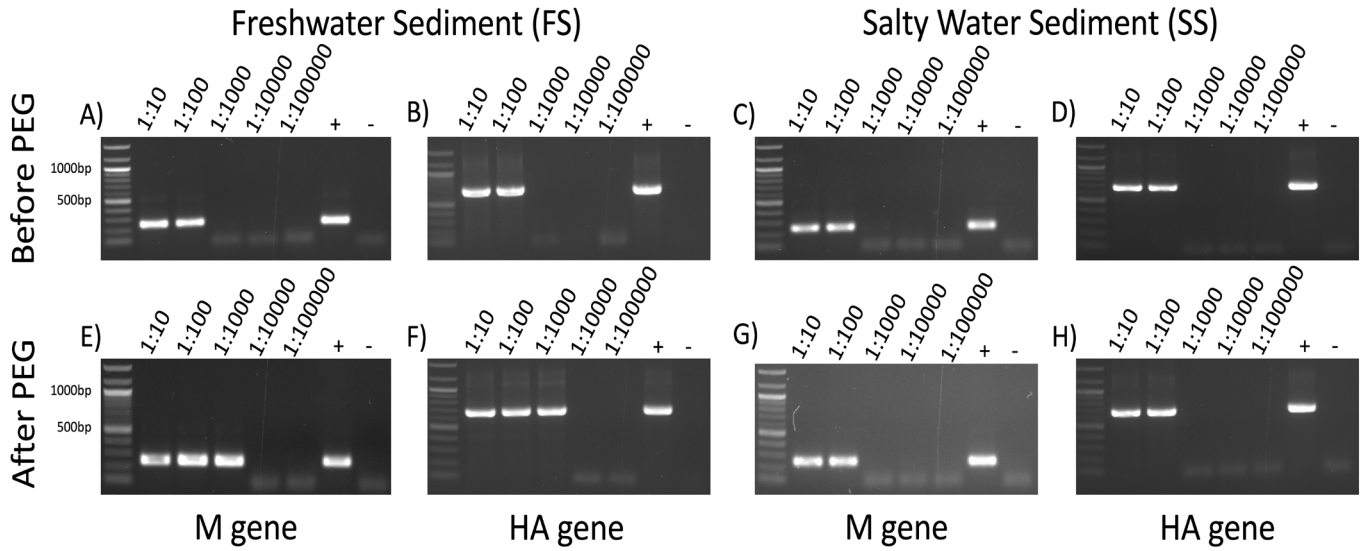

Figure 5. Confirmation of HA assay results obtained from the first passages of each dilution via molecular methods. The presence of IAV was tested for FS (A, B, E, F) and SS (C, D, G, H) before (A, B, C, D) and after PEG precipitation (E, F, G, H) via RT-PCR targeting partial M (A, C, E, G) and partial HA (B, D, F, H) genes. The expected size amplicons were detected up to 1:100 dilution (4.5 $\log_{10}$ EID_{50}/ml) for FS (A, B) and SS (C, D) before PEG precipitation. On the other hand, expected size amplicons were observed up to 1:1000 dilution (3.5 $\log_{10}$ EID_{50}/ml) for FS (E, F) and up to 1:100 dilution (4.5 $\log_{10}$ EID_{50}/ml) for SS (G, H) after PEG precipitation. The viral RNA of H6N2 virus and nuclease-free water were used as a positive and negative control, respectively.

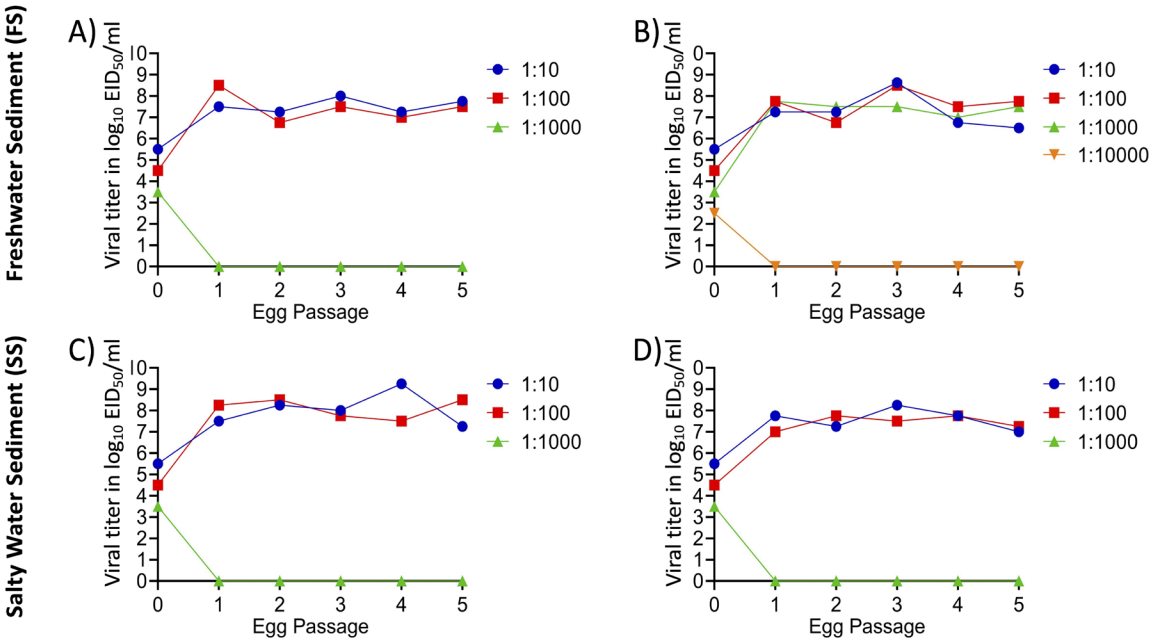

Figure 6. Infectious viral titers of H6N2 viruses isolated from consecutive egg passages of virus dilutions. Viral titers of each egg passages were measured for FS (A, B) and SS (C, D) before (A, C) and after (B, D) PEG precipitation in terms of EID_{50}. Statistical significance was not calculated because the viral titers were calculated as a single data point.

Effects of initial virus titer, PEG precipitation, and sample type on the viral infectivity

To investigate the effects of initial virus titer, PEG precipitation, and sample type on the infectivity of H6N2 viruses, viral titers were measured from the viruses that were obtained from each egg passage of each dilution in terms of EID_{50}.

Inoculation of the virus with an initial titer of 5.5 log_{10} EID_{50}/ml (1:10 dilution) caused 2 log increase in the viral titers during the first passage for both FS (Figure 6A) and SS (Figure 6C) before PEG precipitation. Then the viral titers were quite similar in consecutive egg passages except for the sudden increase at the fourth egg passage in SS (viral titers in E4 were detected as 7.25 and 9.25 log_{10} EID_{50}/ml in FS and SS, respectively). On the other hand, the viral titers increased 4 log in the first passages for both FS and SS before PEG precipitation when the initial titer of the virus was 4.5 log_{10} EID_{50}/ml (1:100 dilution) (Figure 6A and 6C). However, the viral titer decreased 2 log in the second passage of FS, while it stayed same for SS. Then, the viral titers were quite similar in further passages for both sample type. Thus, our results showed that 4.5 log_{10} EID_{50}/ml was the ideal initial viral load for maintaining high viral titers in the first passage for both sample type. However, viral fitness was better maintained in SS at the initial titer of 5.5 log_{10} EID_{50}/ml as the viral titer gradually increased up to 9.25 log_{10} EID_{50}/ml during first four passages, while the titer did not significantly change for FS in corresponding passages.

The viral titers were also determined for the five consecutive passages of the same virus dilutions in FS and SS after PEG precipitation. Because the efficiency of the virus isolation increased by 10-fold after PEG precipitation in FS, the viral titers of the egg passages with the initial titer of 3.5 log_{10} EID_{50}/ml (1:1000 dilution) could be determined for FS. Overall, the viral titers were comparable for all passages of FS and SS when the initial virus titer was 5.5 log_{10} EID_{50}/ml (1:10 dilutions) (Figure 6B and 6D). Like non-PEG-precipitated samples, the viral titers increased ~2 log at the first passage and stayed steady after four consecutive passages. However, our results showed that the sudden increase at the fourth egg passage in SS could not be observed after PEG precipitation. Moreover, 3 log increase in viral titers were observed at the first passage of FS and SS at the initial titer of 4.5 log_{10} EID_{50}/ml after PEG precipitation. This indicated that the infectivity and viral fitness were negatively affected by PEG precipitation procedure regardless of the sample type. On the other hand, the efficiency of virus isolation increased by 10-fold after PEG precipitation for FS, thus the titers in each passage could be measured at the initial titer of 3.5 log_{10} EID_{50}/ml. Our results showed that the initial titer increased by 4 log at the first passage, then stayed steady at the following four passages in FS after PEG precipitation (Figure 6B). Therefore, our data suggested that, regardless of the PEG precipitation status of the sample, the viral infectivity peaks for FS when the initial viral load is the lowest dilution where the infectivity is observed.

Effects of sediment properties on the detection and isolation of H6N2 virus

To investigate the effects of the sediment type and composition on the virus detection and isolation processes, inorganic (elemental/mineral) structures and specific surface areas of FS and SS were analyzed via XRF, XRD and BET methods. According to XRF analysis, concentrations of 22 elements were out of the detection range, thus they were excluded from the study (Table S2).

The concentrations of the remaining 18 elements in the samples were compared according to the fold changes (FCs) which were calculated in log2 base by dividing the given elemental concentration in FS to the corresponding element in SS. In case element concentration was higher in SS, the FC was pointed out as a negative value. The FC values of seven elements (Mg, Na, P, Mn, Sr, Nb, and Ba) were below 1.5-fold, thus they were considered as non-significant. The remaining 11 elements were divided into three groups as their FCs were between 1.5-2-fold, 2-4-fold and greater than 4-fold (Table 1). Our results indicated that the concentrations of eight elements (Al, S, Fe, Si, K, Ti, Y, and Cl) were high in SS at a varied degree; FCs of three elements were between 1,5-2-fold (Al, S and Fe), four elements were between 2-4-fold (Si, K, Ti and Y) while one was over 4-fold (Cl). Seven of these elements were identified as the cations, thus availability of more cations in SS might result in increased adsorptive capacity for IAVs in SS as the viral particles are negatively charged. Thus, high number of cations in SS might negatively affect the efficiency of virus separation and precipitation.

Table 1. Comparison of the important elemental concentrations for freshwater and salty water sediment samples

Symbol	Element	FS (Conc. %)	SS (Conc. %)	Fold Change[a]
Cl	Chlorine	0.01222	0.05079	-2,06***
Ca	Calcium	36.83	7.297	2,34***
Si	Silicon	12.85	26.04	-1,02**
K	Potassium	1.105	2.646	-1,26**
Ti	Titanium	0.31	0.6344	-1,03**
Y	Yttrium	0.00221	0.00625	-1,50**
Al	Aluminum	5.065	9.301	-0,88*
S	Sulfur	0.02156	0.03796	-0,82*
Fe	Iron	2.6	4.222	-0,70*
Cu	Copper	0.1184	0.0668	0,83*
Se	Selenium	0.00342	0.00223	0,62*

[a]Fold changes (FCs) were given in log2 base. * = greater than 1.5-fold increase and less than 2fold increase, ** = between 2-fold and 4-fold increase, *** = greater than 4-fold increase. Negative FCs indicate that the concentrations were higher in SS.

On the other hand, the concentration of three elements (Ca, Cu and Se) were detected at higher degrees in FS than SS; two elements (Cu and Se) were detected as trace elements (Cu: 0.1184% and Se: 0.00342%) and their FC values were between 1.5-2-fold. The FC value of Ca, which is also a cation that might interact with IAVs, was almost 5-fold high in FS. Although the content of sediment particles in terms of the number of cations

did not affect the efficiency of virus isolation before PEG precipitation, low number of cations in FS might be the result of an increased efficiency for virus isolation after PEG precipitation as the detachment of virus from sediment particles could be easier with less adsorptive capacity of the sediment.

We also checked the mineral compositions of the samples to further investigate the adsorption processes between IAVs and sediment particles. According to XRD analyses, quartz and calcite minerals were found as the main minerals in both sediment samples. The main differences between two sediment samples were detected in trace minerals. Albite was found as a common mineral in both sample type. On the other hand, muscovite and aluminum phosphate minerals were only observed in SS, while calcium aluminum silicate, moganite and anorthoclase minerals were detected in FS.

Apart from the composition of sediments, specific surface area might have a contribution to virus adsorption processes. Thus, we investigated the surface area (m^2/g) of FS and SS via BET analyses. Our results showed that the specific surface area of SS (16.1025 m^2/g) was higher than FS (6.8842 m^2/g). This clearly indicated that IAVs could have more surface area for adsorption in SS. Since the organic contaminants might interfere with the molecular methods, we also investigated the organic matter contents of the FS and SS. No significant difference was observed between organic matter contents of FS (5.77%) and SS (6.04%).

Discussion

In this study, we aimed to investigate the effects of initial virus load and PEG precipitation on the detection of IAVs from sediment as well as the type of abiotic source (freshwater versus salty water) in terms of sediment composition. For that purpose, we artificially seeded the sediment samples with an H6N2 virus (A/Aquatic bird/Gediz Delta/1/2018) to obtain five 10-fold dilutions (1:10 to 1:100000) after confirming that the environmental samples were IAV-negative. Based on the amplifications of specific regions of M and HA gene, the viral RNA was detected at as low as 2.5 $\log_{10}$ EID_{50}/ml initial virus titer in FS (Figure 4A and 4B). However, the viral RNA was detected at higher initial virus titers based on M gene (3.5 $\log_{10}$ EID_{50}/ml) and HA gene (5.5 $\log_{10}$ EID_{50}/ml) in SS (Figure 4C and 4D). Since the concentrations of inorganic/organic contaminants that might affect the molecular methods could be higher in SS (Schrader et al., 2012), amplification of viral genes might be inhibited during RT-PCR reactions. On the other hand, because the shorter amplicons could be amplified with ease (Debode et al., 2017), specific regions of M gene (234 bp) and HA gene (634 bp) could be detected at different dilutions in SS.

The supernatants of same dilutions were also inoculated into ECEs to investigate the effects of initial titer on virus propagation. Based on HA assay followed by molecular confirmation, the presence of viruses was detected at 4.5 $\log_{10}$ EID_{50}/ml (1:100 dilution) for FS and SS at the first passage (Figure 5). Because additional passages would increase the amount of viruses (Zowalaty et al., 2011), allantoic fluids of first passages were inoculated into ECEs up to five consecutive passages. This process did not change the results and viruses were detected at the initial titer of 4.5 $\log_{10}$ EID_{50}/ml in all passages for FS and SS (Figure S1). Further experiments were performed to separate viruses from sediment particles using beef extract solution and concentrate by PEG precipitation protocol. Because the beef extract and PEG solution could inhibit the molecular methods (Abbaszadegan et al., 1993), we could not amplify any genes from any dilution (data not shown). On the other hand, this process did not negatively affect the viral propagation in egg culture; in fact, PEG precipitation caused 10-fold increase on the efficiency of virus isolation for FS (3.5 $\log_{10}$ EID_{50}/ml) (Figure 5E and 5F). However, the detection limit for virus isolation remained same for SS (4.5 $\log_{10}$ EID_{50}/ml) after PEG precipitation (Figure 5G and 5H). Thus, it was suggested that the composition of SS could affect the efficiency of PEG precipitation in terms of having more adsorptive capacity.

We also investigated the viral titers in the allantoic fluids of each egg passage of each virus dilution in terms of HA unit and EID_{50}. Our results showed that overall HA titers of viruses in FS were relatively higher than those in SS (Table S1). This emphasized that the binding affinity of HA protein to sialic acid receptors on CRBCs decreased when the virus was isolated from SS. The interaction between HA and sialic acid receptor of red blood cells could be inhibited by the effects of natural contaminants such as sera, urine, and edible bird nests, thus binding of HA protein to sialic acid receptor could be partially abolished (Matrosovich and Klenk, 2003). Thus, there could be differences in terms of the amount of natural contaminants in FS and SS, affecting the slight variation in HA titers. Our results also demonstrated that the initial titer and PEG precipitation have an important role on the infectivity of viruses. The most ideal initial viral titer was observed at 1:100 dilution (4.5 $\log_{10}$ EID_{50}/ml) for FS and SS before PEG precipitation as the titer increased by ~4 log at the first passage for both sample type (Figure 6A and C). On the other hand, viral fitness was better maintained in SS when the initial titer was 5.5 $\log_{10}$ EID_{50}/ml. This suggested that although the binding ability of HA slightly decreased, the viral fitness was better maintained for SS as the viral titer gradually increased up to fourth passage. Further experiments are needed to investigate the interactions between the binding capacity of HA and the mutations on HA gene that arise in different passages. Additionally, PEG precipitation procedure increased the sensitivity of the virus isolation by 10-fold FS, thus the titers of viruses in each passage could also be measured at the initial titer of 3.5 $\log_{10}$ EID_{50}/ml (Figure 6B). Our results demonstrated that the lowest amount of virus that could be propagated in eggs was the ideal viral load for maintaining viral fitness in FS before and after PEG precipitation (Fig 6A and B). On the other hand, although the PEG precipitation method increased the sensitivity of virus isolation for FS, it has negative effects on the viral fitness regardless of the sample type.

Altogether, these results led us to investigate the organic and inorganic compositions of the sediment samples to better

understand the effect of sediment composition on the detection of viruses and viral fitness. For this, element and mineral compositions as well as specific surface areas of FS and SS were investigated via XRF, XRD and BET analyses. Comparison of elemental concentrations indicated that Al, S, Fe, Si, K, Ti, Y and Cl were higher in SS, whereas Ca, Cu and Se were higher in FS (Table 1). Among them, Ca element was ~5-fold higher in FS and it is one of the cations. Because PEG precipitation method increased the sensitivity of virus detection in FS, it is possible that adsorptive impact of Ca^{+2} might be reversible. Therefore, PEG precipitation procedure might be enough for the termination of the potential interactions between IAVs and Ca element. On the other hand, high number of cations (Al, S, Fe, Si, K, Ti, and Y) were detected in higher concentrations in SS. Previous studies showed that ferric compounds such as iron oxide and porous silicon surfaces are used in the adsorption process of IAVs (Warren et al., 1966; Gonchar et al., 2020). Considering that the cations could increase the adsorptive capacity of the compound (Bitton, 1975), it was speculated that SS might have more adsorptive capacity than FS. This might be the reason for why virus separation using beef extract and PEG precipitation did not increase the efficiency of virus isolation and detection in SS. This assumption could be intensified by the mineral composition of SS that contains aluminum phosphate as a trace mineral. The information about the adsorptive capacities of the trace minerals that showed differences between FS and SS is quite limited in the literature. However, it was reported that aluminum phosphate mineral is used as an adsorbent in the IAV purification (Miller and Schlesinger, 1955). Together with the fact that PEG precipitation method is less efficient in SS, aluminum phosphate mineral could play a role in increasing the adsorptive capacity of the sediment for virus. Additionally, providing more surface area could play a role on the virus adsorption. Regarding to this, sediment particles of SS provided specific surface area that was 2.5-fold higher than FS, which indicated that SS had more surface area for virus adsorption. Larger surface area and high number of cations might provide an advantage for the virus as adsorption of the virus onto the sediment particles might serve as a shelter from damaging environmental factors maintaining virus viability. This could provide additional fitness for the viruses in the environmental sources, especially in salty water habitats, as the virus could maintain its viability. Additionally, organic matter densities of FS and SS were similar, thus it might not have effects on the detection of viruses or viral fitness.

Conclusion

In conclusion, this study clearly indicates that the amount of viruses that are shed into environment and elemental/mineral compositions of sediment particles have significant contribution on the infectivity, viability and detection range of the IAVs. Using additional protocols for virus separation and concentration might also increase the efficiency of detection, however this might be correlated with the composition and adsorptive capacity of the sediment. Understanding the interactions between viruses and the inorganic composition of the environmental sources could help us develop novel adsorptive agent(s) to improve the virus detection in different settings.

In this study, we used an H6N2 virus of avian origin to investigate the detection efficiency. Further investigations with other subtypes of IAVs would help us better evaluate the dynamics between viruses and their environment as we expand our knowledge about the role of environmental sources in the ecology and transmission of IAVs.

Supplementary Material

Data underlying the reported findings have been provided as part of the submitted article and are available at: Azbazdar et al. (2022) Supplementary Materials.

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COMPLIANCE WITH ETHICAL STANDARDS

Authors' Contributions

MEA and ZAK conceived and designed the experiments. MEA and GA performed the experiments. MEA, GA, EG, and ZAK analyzed the data. ZAK acquired financing. MEA and ZAK wrote the original paper. MEA, GA, EG, and ZAK reviewed and edited the paper.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

This study is not conducted by any human material thus no ethical committee approval was needed. Sediment samples used in this study were collected by the scientific research permission (No: 72784983-488.04-232438) that was granted by the General Directorate of Nature Conservation and National Parks, Republic of Turkey Ministry of Agriculture and Forestry.

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