

Survival of Influenza Virus on Banknotes[∇]

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Successful control of a viral disease requires knowledge of the different vectors that could promote its transmission among hosts. We assessed the survival of human influenza viruses on banknotes given that billions of these notes are exchanged daily worldwide. Banknotes were experimentally contaminated with representative influenza virus subtypes at various concentrations, and survival was tested after different time periods. Influenza A viruses tested by cell culture survived up to 3 days when they were inoculated at high concentrations. The same inoculum in the presence of respiratory mucus showed a striking increase in survival time (up to 17 days). Similarly, B/Hong Kong/335/2001 virus was still infectious after 1 day when it was mixed with respiratory mucus. When nasopharyngeal secretions of naturally infected children were used, influenza virus survived for at least 48 h in one-third of the cases. The unexpected stability of influenza virus in this nonbiological environment suggests that unusual environmental contamination should be considered in the setting of pandemic preparedness.

To control influenza outbreaks or a pandemic, it is of utmost importance to identify and characterize the different vectors that could promote influenza virus transmission between humans. The respiratory tract of influenza virus-infected individuals is the main reservoir for the chain of transmission in a community. Based on experiments with animal models and observational field studies, large respiratory droplets are considered to be the most frequent vectors sustaining this transmission (4). However, experimental studies with animals with no direct contact have demonstrated that aerosols also play a significant role. In humans, the hypothesis that there is an aerosol route of transmission is supported by indirect evidence in special circumstances, such as confinement for a prolonged period of time in an airplane in the presence of a patient infected with influenza virus (1, 6, 13, 16, 24). In addition, it has been documented that human influenza A viruses can survive for a prolonged period on different types of surfaces once they are present in the environment. Although controversial (2, 6, 24), the possibility that contaminated surfaces and fomites could act as vectors of transmission needs to be considered in the context of overall influenza pandemic preparedness.

For any environmental contamination to be relevant, the virus should not only remain infectious but also persist at a sufficient concentration to enable it to reach the respiratory tract via finger contamination. Rhinovirus is the most common respiratory virus known to be easily transmitted by this route (12). Whether influenza virus is also commonly transmitted by this route remains a subject of debate (4, 24). However, given

that the biological properties of a potential influenza virus pandemic strain have not been established, this route of transmission has to be considered. The severe acute respiratory syndrome coronavirus highlighted the ability of respiratory viruses to act in unconventional ways since environmental contamination by stools played a significant role in some population clusters (3, 15, 18). All these questions should be considered not only from a scientific standpoint. We must also take into account and provide answers to the many possible questions raised by various communities and public health authorities.

We hypothesized that banknotes may be one of various possible influenza vectors and may offer opportunities for infection. In Switzerland, a small country with a population of approximately 7 million, it is estimated that 20 to 100 million banknotes are exchanged each day, and billions of individual notes are exchanged daily worldwide. Here, we report the results of an analysis of the stability of human influenza A and B viruses on banknotes.

MATERIALS AND METHODS

We conducted a series of experiments designed to assess the survival and duration of infectiousness of human influenza viruses on banknotes. The term “survival” is defined in this study as the persistence of cultivable virus on cells.

The main experiments were performed with four isolates, influenza A/Moscow/10/99 (H3N2), A/Wisconsin/67/2005 (H3N2), A/New Caledonia/20/99 (H1N1), and B/Hong Kong/335/2001, which were used in the first experiments at stock concentrations of 1.8×10^8 , 10^6 , 10^6 , and 3.2×10^3 50% tissue culture infective doses (TCID₅₀)/ml, respectively. These strains, which were initially isolated with eggs (kindly provided by Alan Hay, Mill Hill, London, United Kingdom), were passed on Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) several times in our laboratory. A diluted viral suspension was obtained using the same medium as the medium used for cell culture after viral inoculation. Eagle minimal essential medium containing 25 mM HEPES supplemented with Earle's salts (catalog no. 21430020; Invitrogen, Paisley, United Kingdom), 0.22% NaHCO₃, 1% penicillin, 1% streptomycin, 1% gentamicin, 1% amphotericin B (Fungizone), and 1% glutamine (Gibco Europe). Rhinovirus prototype strains HRV2 and HRV37 purchased from ATCC (VR-482 and VR-

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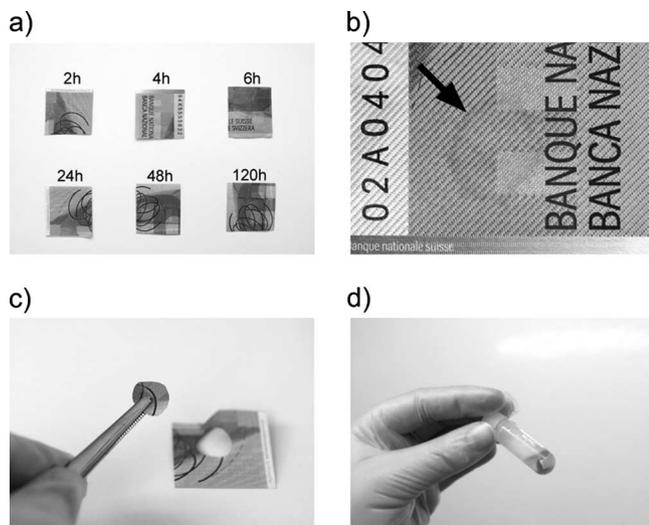


FIG. 1. Methods used for banknote inoculation and subsequent viral isolation. (a) Portions (50 μ l) of a viral suspension are deposited on a predefined area of banknotes and allowed to dry for different periods of time. (b) After 1 h of incubation at room temperature, the liquid has evaporated, but a trace remains quite visible (arrow). (c) A standardized piece of banknote containing viral particles is cut from a banknote. (d) Viral particles are collected from the piece of banknote by immersion in a tube containing cell culture medium, and after 10 min the eluate is inoculated onto a cell culture.

510; LGC Promochem, Molsheim, France) were used at a concentration of 10^6 TCID₅₀/ml. Human rhinoviruses were obtained after passages on HeLa Ohio cells (kindly provided by F. G. Hayden, University of Virginia, Charlottesville).

Determination of infectiousness. Infectiousness was determined using the following protocols. A 50- μ l portion of a viral suspension was deposited on a 1-cm² area of a banknote and then kept under laminar airflow at room temperature and exposed to light (Fig. 1a and b). The banknotes were standard 50-Swiss franc notes that had been widely used and withdrawn from circulation for destruction. During the experiments, the average recorded temperature was 22°C, the minimum temperature was 21°C, and the maximum temperature was 28°C; the relative humidity ranged from 30 to 50%. At different time points, a 1-cm² piece of banknote containing the inoculated area was immersed in 500 μ l of Eagle minimal essential medium containing 25 mM HEPES (Fig. 1c and d) for 15 min. A 0.4-ml portion of the eluate was then used for cell inoculation. Each experiment was performed in triplicate. Propagation of influenza viruses and rhinoviruses was performed using MDCK and HeLa Ohio cells, respectively. Cells were incubated at 37°C for 10 days and harvested for immunofluorescence testing using influenza A virus-specific monoclonal antibody (Chemicon, Temecula, CA) or Pan-Enterovirus Blend antibody for picornavirus detection (Chemicon, Temecula, CA).

RNA stability. Aliquots (10 μ l) of influenza A/Moscow/10/99 (H3N2) and influenza A/New Caledonia/20/99 (H1N1) viral suspensions containing 10^6 TCID₅₀/ml were deposited onto standardized 1-cm² pieces of banknotes and air dried at room temperature. At different time points, the pieces were directly immersed in lysis buffer AVL of a QIAamp viral RNA mini kit (Qiagen, Hombrechtikon, Switzerland) supplemented with carrier RNA to increase the efficiency of extraction of RNA molecules at low concentrations. After addition of 140 μ l of 1 \times phosphate-buffered saline, RNA extraction was performed according to the manufacturer's recommendations, and RNA was stored at -80°C. Influenza A virus RNA was quantified in triplicate in a single experimental run by performing a one-step TaqMan real-time reverse transcriptase (RT) PCR using a Quantitect probe RT-PCR kit (Qiagen, Hombrechtikon, Switzerland). The primers and probe targeting the influenza A virus matrix gene were derived from those developed by Spackman et al. (22), with modifications adapted to the variability of circulating strains and to recently published sequences. A standard 25- μ l RT-PCR mixture contained 5 μ l template DNA, 900 nM forward primer InfA-2-For (5'-AGA TGA GYC TTC TAA CMG AGG TC-3'), 900 nM reverse primer InfA-2-Rev (5'-GCA AAI ACA TCY TCA AGT YTC TG-3'), and 200 nM dual-labeled fluorogenic probe InfA-2 (6-carboxyfluorescein-5'-TCA GGC

CCC CTC AAA GCC GA-3'-carboxytetramethylrhodamine). RT-PCR was performed with an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). The threshold cycle for each RT-PCR amplification plot was compared to a standard curve for RNA extracts of quantified influenza A viruses.

Nasopharyngeal secretions. The potential beneficial effect of respiratory secretions on viral survival was tested first by using an experimental design using nasopharyngeal secretions collected from children with respiratory symptoms. Twenty specimens, each of which was negative for respiratory viruses as determined by RT-PCR (9), were pooled and then used to dilute our viral stocks. For each experiment testing the effect of respiratory mucus, 500 μ l of quantified influenza virus supernatant was mixed with 2 ml of nasopharyngeal secretions. In the second step, 14 natural nasopharyngeal secretions were collected from children under 11 years old between January and March 2007. The cases were selected based on positive results of a rapid enzyme immunoassay (BinaxNow; Emergo Europe, The Hague, The Netherlands) and then confirmed by culture or RT-PCR. Fifty-microliter portions of homogenized secretions were deposited on banknotes that were tested for infectiousness after 24 and 48 h as described above.

RESULTS

The duration of infectiousness was initially tested using native viral supernatants deposited on pieces of banknotes at room temperature and under daylight conditions. Testing of the infectiousness of influenza A (H1N1) and influenza B viruses was limited to durations of 1 and 2 h, respectively (Fig. 2). The survival of the influenza A (H3N2) viruses influenza A/Wisconsin/67/05 and A/Moscow/10/99 was significantly longer (up to 1 and 3 days, respectively) (Fig. 2).

In the second set of experiments, we tested the potential effects of both inoculum concentration and respiratory secretions on the duration of infectiousness. Influenza A/Moscow/10/99 (H3N2) and influenza B/Hong Kong/335/2001 viral supernatants at defined concentrations were used directly or mixed with secretions in parallel experiments (Fig. 3).

The first observation was that the recovery rate was directly related to the inoculum size, with rapid abolition of the infectiousness when the inoculum was diluted (Fig. 3A to D). The duration of infectiousness without mucus of influenza A/Moscow/10/99 (H3N2) virus at the highest concentration tested (8.9×10^5 TCID₅₀/ml) was 2 days, while at the lowest concentration tested (1.1×10^5 TCID₅₀/ml) the duration was only 1 h.

The second observation was that respiratory secretions increased the duration of influenza virus infectiousness. In the presence of mucus, influenza A/Moscow/10/99 (H3N2) virus remained infectious for 8 days after inoculation, whereas without mucus the same inoculum remained infectious for less than 2 h (Fig. 3D). The duration of infectiousness observed with the higher concentration of native influenza A/Moscow/10/99 virus similarly increased in the presence of mucus from 2 to 17 days (Fig. 3A to C). The results obtained with the influenza A/Moscow/10/99 strain confirmed the effects of both the inoculum size and the presence of mucus on the duration of infectiousness. Similar observations were made with the influenza B/Hong Kong/335/2001 virus. Influenza B virus at a concentration of 3.2×10^5 TCID₅₀/ml on banknotes in the presence of mucus was still infectious after 1 day, whereas in the absence of mucus it remained infectious on banknotes for no more than 2 h (Fig. 3E). The residual viral load was quantified in one experiment using influenza A/Moscow/10/99 (H3N2) virus at an inoculum size of 8.9×10^5 TCID₅₀/ml. After 2 h and 7 and 14 days, the viral loads decreased rapidly to 5.6×10^3 , 18, and 5 TCID₅₀/ml, respectively.

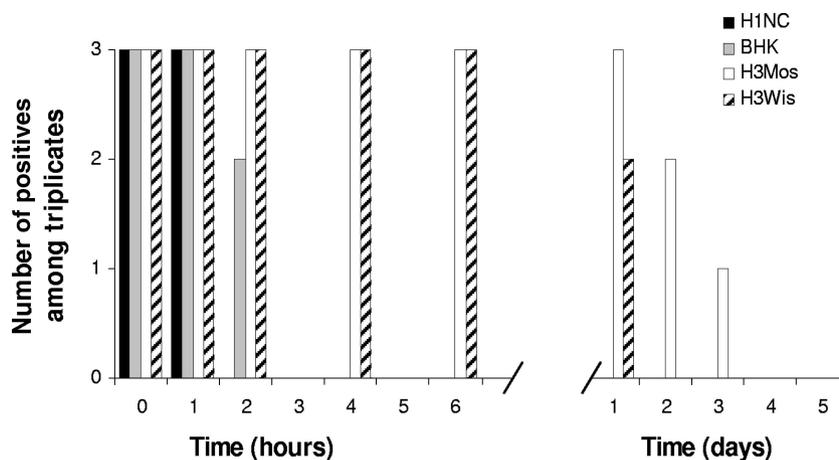


FIG. 2. Duration of infectiousness of four different influenza virus subtypes after inoculation onto banknotes at room temperature. The following viral suspensions were used in triplicate for each experiment: influenza A/New Caledonia/20/99 (H1N1) virus (H1NC) at a concentration of 2.8×10^5 TCID₅₀/ml; influenza B/Hong Kong/335/2001 virus (BHK) at a concentration of 1.6×10^4 TCID₅₀/ml; influenza A/Moscow/10/99 (H3N2) virus (H3Mos) at a concentration of 8.9×10^6 TCID₅₀/ml; and influenza A/Wisconsin/67/2005 (H3N2) virus (H3Wis) at a concentration of 5×10^4 TCID₅₀/ml.

To assess whether our observations under *in vitro* conditions could be reproduced under natural conditions, we used nasopharyngeal secretions from children with an influenzalike illness for whom an influenza test was required. Native nasopharyngeal secretions from 14 influenza-positive cases, identified by a rapid enzyme immunochromatographic assay and confirmed by cell culture, were inoculated onto banknotes and tested for influenza recovery. Influenza virus survived for at least 24 h in 7/14 (50%) cases and for at least 48 h in 5/14 (36%) cases (Fig. 4). In one case, influenza virus retained its capacity to infect cells for 12 days after the secretion was deposited on a banknote.

RNA stability was tested in similar infectiousness duration experiments in which influenza A (H1N1) and influenza A (H3N2) virus genomes were detected by using real-time RT-PCR. The targeted stretch of genome of the different viral subtypes was detected for more than 10 days, and there was progressive but slow quantitative loss (Fig. 5).

Preliminary experiments were also carried out to investigate whether our findings for influenza A virus could be extended to other common respiratory viruses. In tests using the human rhinoviruses HRV2 and HRV37, both viruses survived for several days on banknotes without respiratory mucus. HRV2 survived for 48 h, and HRV37 survived for more than 120 h (data not shown).

DISCUSSION

Our main observation was that human influenza viruses can survive and maintain their infectiousness for several days when they are deposited on banknotes. The duration of viral infectiousness was related to both the concentration of the inoculum and the presence of a beneficial microenvironment. When high-concentration inocula were mixed with respiratory mucus, the infectiousness of influenza A (H3N2) and influenza B viruses increased in an unexpected way by up to 17 days. The concentrations of virus needed to achieve this prolonged survival were around 8.9×10^5 TCID₅₀/ml. This concentration is

within the range of concentrations found in clinical specimens since at the peak of symptoms during naturally acquired influenza A, the median virus titers in nasopharyngeal secretions can reach 6.3×10^4 to 10^7 TCID₅₀/ml (6, 11, 14). This protective role of respiratory secretions for the survival of a virus is in agreement with previous studies performed in the 1940s in which Parker et al. showed that there was increased viral stability in the presence of human mucus (17). However, despite the fact that the virus could be cultivated over a long sampling period, the concentration of infectious virus diluted in mucus and deposited on banknotes dropped quite rapidly (approximately 10^2 -fold after 2 h and 10^5 -fold after 2 weeks) (data not shown).

The clinical relevance of these observations was confirmed by using the nasopharyngeal secretions collected from children with influenzalike illnesses which had been screened in our routine laboratory during the ongoing season. Respiratory secretions from these influenza A (H3N2) virus-positive cases were directly deposited on banknotes. The virus survived for at least 24 h in 50% of the cases and for 48 h or more in more than one-third of the cases. These findings are similar to our experimental findings and confirm that the viral load in naturally infected individuals is high enough to significantly contaminate the environment. Unfortunately, we are unable to provide quantitative data for this part of the study. It should also be kept in mind that children harbor a higher viral load, which suggests that environmental contamination might be more frequent in this population.

A study of influenza virus stability in the environment needs to take many different factors into consideration, which must be identified and evaluated. A striking example in our study was the protective effect of respiratory mucus on influenza viruses. Other factors that have been recognized and whose effects on virus infectivity have been evaluated include the type of surface (nonporous versus porous) (2, 7, 19, 20), the type of virus used, viral concentration, temperature, humidity (19, 20), and the light and UV conditions (7), as well as the pH. These

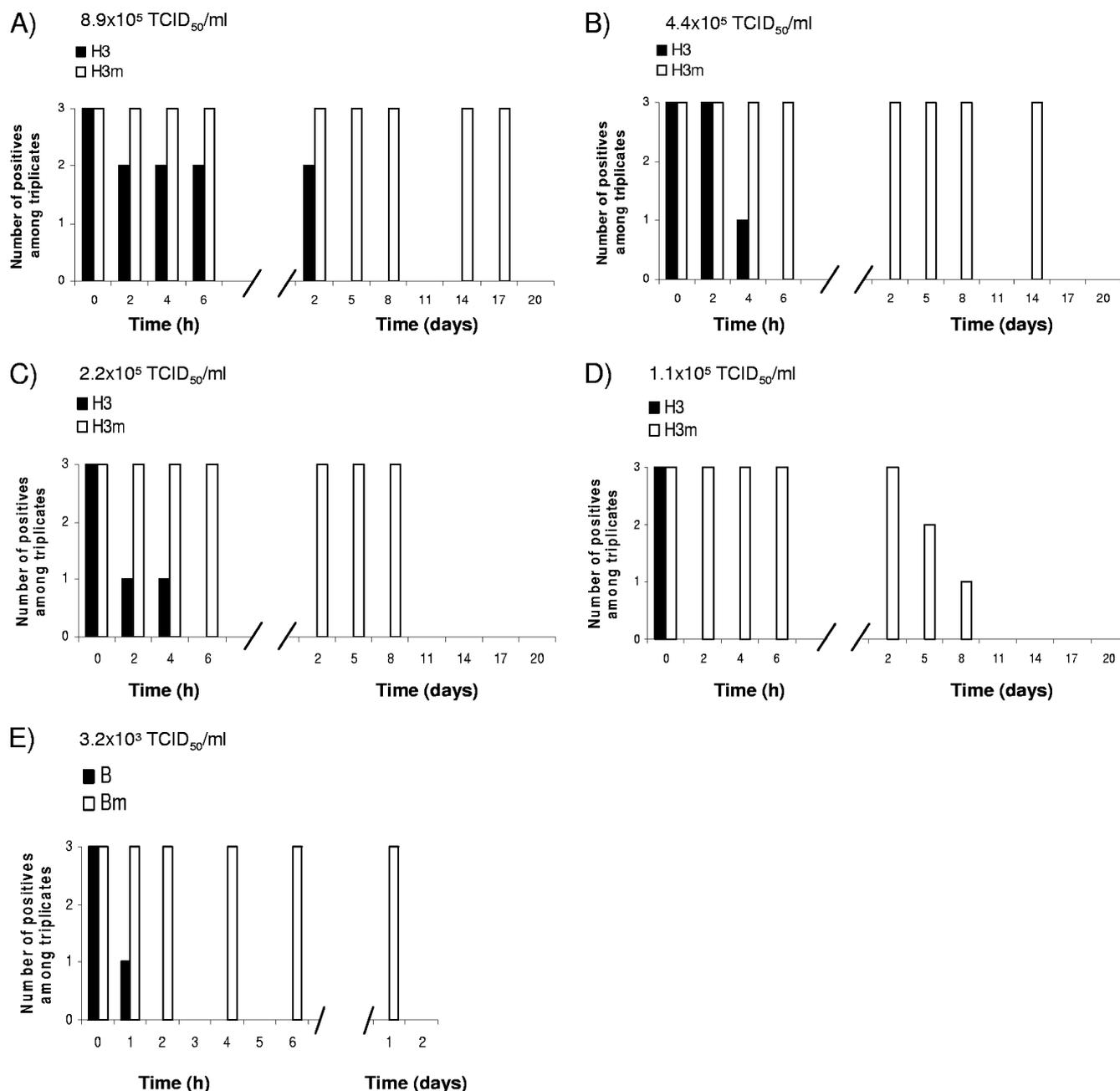


FIG. 3. Duration of infectiousness according to the size of the initial inoculum and the presence or absence of mucus. Influenza A/Moscow/10/99 (H3N2) virus was deposited in triplicate on banknotes at the following concentrations, each in the presence (H3m) or absence (H3) of respiratory mucus: (A) 8.9×10^5 TCID₅₀/ml; (B) 4.4×10^5 TCID₅₀/ml; (C) 2.2×10^5 TCID₅₀/ml; (D) 1.1×10^5 TCID₅₀/ml. (E) Similarly, influenza B/Hong Kong/335/2001 virus was deposited at a concentration of 3.2×10^3 TCID₅₀/ml in the presence (Bm) or absence (B) of respiratory mucus.

factors were not considered in the present experiments. Furthermore, given that many environmental studies were performed several decades ago, differences in methodology, such as the detection methods (egg culture, cell culture, in vivo inoculation) should also be borne in mind. Thus, in agreement with previous results (2, 7, 17), we found that influenza A viruses can survive on inert and nonporous surfaces for days or even weeks. On porous surfaces, such as paper or tissue, the survival rate appeared to be shorter and limited to 12 and 8 h for influenza A and B viruses, respectively. Although bank-

notes could be considered to be an inhospitable surface for any biological agent, we learned that the main raw material for the fabrication of Swiss banknotes is cotton which is covered by a resin (kinogram). This resin represents a nonporous surface, which we found to exhibit no significant pH variation (data not shown). Whether similar results would be obtained with banknotes from other countries and with different characteristics needs to be studied.

Survival in the environment is, however, not sufficient to sustain transmission and represents only the first requirement

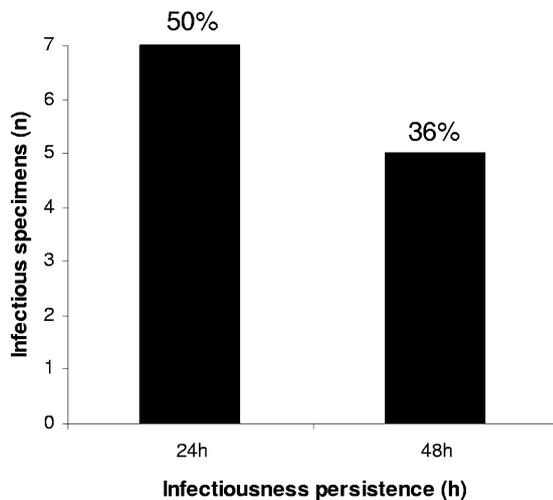


FIG. 4. Persistence of infectiousness over time of influenza virus-positive respiratory secretions. Fourteen influenza A (H3N2) virus culture-positive human secretions detected during the 2006–2007 winter season were directly deposited on banknotes and tested for persistence of influenza virus infectiousness after 24 and 48 h.

and the first step before possible self-inoculation via fingers. The nasal route for establishing influenza virus infection is known to be effective in both humans and animals (8, 13, 21). Some observations suggest that this fact needs to be considered, and a recent review even concluded that contaminated fomites play a predominant role in influenza virus transmission between humans (4). Moreover, contamination of hands after contact with an influenza virus-contaminated surface has been demonstrated. Indeed, infectious influenza virus was isolated from hands after contact with a porous surface contaminated for 15 min, as well as after contact with a nonporous surface contaminated for 24 h (2). Subsequent hand-to-hand transmission was also demonstrated, and competent influenza virus was recoverable for at least 5 min from fingers after brief contact with the previously experimentally contaminated hand of a patient infected with influenza virus.

Similarly, nasal inoculation with other respiratory viruses via contaminated fingers is also known to be effective, particularly for rhinovirus (10, 12). Our observations demonstrate that even in the absence of mucus, HRV2 and HRV37 survived for 2 and 5 days, respectively, on banknotes. Rhinovirus is known to survive on fomites (10, 12), and previous studies showed that HRV14 and HRV37 could survive for 14 and 24 h on nonporous surfaces (12, 20). This suggests that our findings might also be relevant for other respiratory viruses.

From the perspective of a possible influenza A (H5N1) pandemic, specific biological properties of avian strains have to be considered. In humans, the virus is excreted at high concentrations (1.8×10^4 to 9.8×10^4 copies per ml) in both respiratory secretions and feces (5). Avian influenza A viruses that are excreted at very high levels in bird feces were able to survive for more than 60 days in distilled water at 28°C and for 91 days at 4°C (23). All these data suggest that environmental contamination, not only from respiratory secretions but also from feces, might be more frequent than expected in the event of an H5N1 pandemic. The severe acute respiratory syndrome

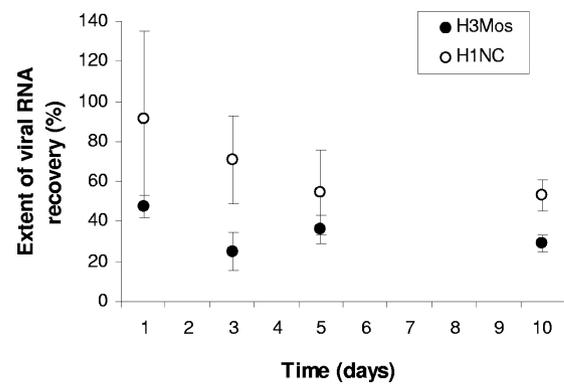


FIG. 5. Influenza A virus genome persistence on banknotes. Influenza A/Moscow/20/99 (H3N2) (H3Mos) and influenza A/New Caledonia/20/99 (H1N1) (H1NC) virus RNA were quantified (see Materials and Methods) and expressed as a proportion of the initial amount of RNA present in the inoculum. The bars indicate the standard deviations calculated for triplicates.

experience has also shown that emerging enveloped RNA viruses can develop versatile biological properties that enable them to be transmissible in an efficient manner via environmental contamination (15).

An interesting observation in our study was that a constant level of viral RNA could be detected for more than 10 days. It should be noted that a real-time PCR assay that targets small stretches of nucleic acid was used and was still positive, while the larger genomic RNA could be fragmented. These results suggest that real-time PCR can be considered an appropriate detection tool for environmental screening.

We showed that infectious virus can survive for several days on banknotes. This requires a relatively large inoculum and the presence of a protective matrix, such as respiratory mucus. Pandemic events depend on the presence of sufficient quantities of virus with pandemic properties, as well as suitable vehicles for its transmission, including environmental vectors, such as banknotes. The results of our study show that influenza virus stability is not the sole determining factor in a pandemic. As hundreds of billions of banknotes are probably exchanged every day worldwide, infection from hands contaminated with virus picked up from virus-contaminated banknotes cannot be totally ignored. Given the unexpected stability of influenza virus in this nonbiological environment, our current understanding of the conditions favoring influenza virus survival needs to be revised, particularly in the context of pandemic preparedness.

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