



Major article

Viral contamination of aerosol and surfaces through toilet use in health care and other settings



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Background: The airborne spreading of enteric viruses can occur through the aerosol and droplets produced by toilet flushing. These can contaminate the surrounding environment, but few data exist to estimate the risk of exposure and infection. For this reason environmental monitoring of air and selected surfaces was carried out in 2 toilets of an office building and in 3 toilets of a hospital before and after cleaning operations.

Methods: To reveal the presence of norovirus, enterovirus, rhinovirus, human rotavirus, and Torque teno virus and to quantify human adenovirus and bacteria counts, molecular and cultural methods were used.

Results: On the whole, viruses were detected on 78% of surfaces and in 81% of aerosol. Among the researched viruses, only human adenovirus and Torque teno virus were found in both surface and air samples. In several cases the same adenovirus strain was concurrently found in all matrices. Bacterial counts were unrelated to viral presence and cleaning did not seem to substantially reduce contamination.

Conclusions: The data collected in our study confirm that toilets are an important source of viral contamination, mainly in health care settings, where disinfection can have a crucial role in preventing virus spread.

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Surfaces such as door handles, banisters, flush handles on toilets, toys, telephones, drinking cups, and fabrics have all been implicated in the transmission of enteric viruses,¹ namely norovirus, rotavirus, and rhinovirus,² in localized cases and outbreaks. These surfaces can be contaminated directly by contact with infected material or indirectly by dirty hands or settling of large aerosol droplets. Evidence for the role of air and surfaces for viral transmission can be drawn from studies regarding air and fomite contamination, studies of the transfer and survival of viruses, from experimental trials in human volunteers, from epidemiologic data, and from disinfection studies.³

Hygiene and disinfection intervention studies have demonstrated that proper cleaning of hands and efficient disinfection of fomites decreases surface contamination and may interrupt the spread of disease caused by norovirus, rotavirus, and coronavirus.⁴

The role of airborne viral spreading is not well defined because of the difficulties in identifying this transmission route for single cases or outbreaks. Nevertheless, person-to-person transmission could

also be due to environmental contamination by suspended or settled aerosol droplets. For example, during the severe acute respiratory syndrome epidemic transmission was believed to occur primarily by direct physical contact with ill persons or by large-droplet spread; however, several clusters of infection were difficult to explain by these routes and many health care workers were infected despite compliance with World Health Organization infection control guidelines.^{5,6} The role of aerosol and surface contamination in the transmission of viral infection in hospitals is generally recognized,^{7,8} but its sources and dimensions are not deeply studied because of the difficulties in measuring environmental virus contamination and its relation to specific clinical cases.^{9,10}

In addition to respiratory droplets, toilets should be considered as a possible source of indoor air and surface viral contamination. In fact, consistent microbial contamination of the indoor environment typically occurs after a toilet flush, and this can be an important source of diffusion, not only for enteric viruses, but also for respiratory ones, which are also often eliminated by the fecal route. A toilet flush generates a large number of droplets of different dimensions: the largest droplets settle rapidly on the surrounding surfaces, whereas the smallest can be inhaled or remain airborne for a long time.^{11,12} Despite the long-standing evidence of toilet flush as an important source of infective aerosol, a systematic study of toilet

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flush contamination as it affects risk assessment has not yet been completed, especially regarding viral contamination. Nevertheless, from the perspective of risk management, the quantitative assessment of this putative method of exposure would be of utmost importance to select control measures and define the points where they can be successfully applied. To this aim, environmental monitoring was carried out in toilets of a hospital unit (nephrology) and of an office building. Aerosol and surface samples were collected and analyzed for total bacterial count (TVBC), assessing hygienic conditions and the effectiveness of cleaning procedures, and the presence of human viruses. These were chosen taking into account the results of our previous study⁹ and to represent different mechanisms of diffusion: norovirus, genogroups I and II (NoV GI and GII) and human rotavirus (HRV) for the fecal–oral route, rhinovirus (RV) and TTV for the respiratory route, and human adenovirus (HAdV) and enterovirus (EV) for both.

MATERIALS AND METHODS

Study setting and sampling

The study was carried out from December 2009 to April 2010, examining 5 toilets of the nephrology ward of Leghorn Hospital and 2 toilets in an office building, in which roughly 30 persons were usually present during working hours. Among the hospital toilets, 1 was dedicated to health care personnel and the other 4 were adjacent to patient rooms (3 from 2-bed rooms and 1 from a 4-bed room). For each toilet, at least 5 replicate sampling campaigns were conducted. In each campaign, 2 sets of samples were collected: 1 before and 1 after the application of a cleaning procedure. In each toilet and for each set of samples, 1 aerosol and 4 surface samples were collected. The surfaces were chosen to reflect the potential for hand contamination: the toilet seat and its cover, the flushing handle/button, and the internal door handle. In addition to air and surfaces, the water inside each toilet was also sampled as the possible source of the environment contamination. In total, 172 surfaces (108 and 64 for hospital and offices, respectively), 43 air (27 and 16 for hospital and offices, respectively), and 19 water (4 and 15 for hospital and offices, respectively) samples were collected.

Both surface and air samples were analyzed for HAdV, NoV GI, NoV GII, EV, RV, HRV, TTV, and TVBC. Water samples were analyzed only for HAdV.

Sampling procedures

Surfaces

Three adjacent 36 cm² squares were sampled.⁹ The first, for the detection of the RNA viruses (NoV GI, NoV GII, RV, HRV, and EV), was swabbed with cotton swabs soaked in 1 mL 3% beef extract at pH 9. The eluate was then neutralized with 1 M hydrogen chloride and 140 µL of it was used for viral nucleic acids extraction using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany).¹³ The second square area was sampled for the detection of DNA viruses (TTV and HAdV), using a commercial kit (DNAIQ System, Promega, Fitchburg, Wis) designed for forensic use and modified for the detection of virus on surfaces.¹⁴ For bacteriologic analyses, the third square surface was eluted with cotton swabs soaked in a 0.9% w/v sodium chloride solution. Swabs were then incubated in 2 mL nutrient broth for 20 minutes at 37°C. The whole solution was then seeded onto plates containing plate count agar and incubated for 48 hours at 37°C.

Air

Air samples were collected with an impactor sampler (Microflow, Aquaria, Italy).⁹ For virus detection, 1,000 L air was sampled

on replicate organism detection and counting (Rodac) plates containing tryptone soy agar. The agar was then eluted in 3% beef extract at pH 9, and viral RNA and DNA were isolated using a QIAamp RNA Mini Kit and a QIAamp DNA mini Kit (Qiagen, Hilden, Germany), respectively.¹⁵

For bacterial counts, 180 L were sampled using an impactor sampler (Microflow), with Rodac plates containing plate count agar. The plates were then incubated for 48 hours at 37°C.

Water

Water was withdrawn directly from the toilet in a 50-mL plastic tube. Water samples were directly analyzed by isolating DNA with QIAamp DNA mini Kit from 200 µL.

Virus detection

For NoV GI and NoV GII, EV, RV, HRV, and TTV, the isolated nucleic acids were analyzed using nested reverse transcription polymerase chain reaction (RT-PCR) according to previous protocols.⁹⁻¹⁶ For each virus, the PCR products were detected under ultraviolet light after horizontal electrophoresis in 2% agarose gel.

HAdV was detected and its genomic concentration was quantified using real time quantitative PCR according to published protocols.¹⁷ The samples tested were analyzed in 96-well optical plates and read with an ABI 7300 sequence detector system (Applied Biosystems by Life Technologies Corporation, Monza, Italy). The genome copy numbers of HAdV in tested nucleic acid extracts were extrapolated from the equation of the standard curve that was generated from the dilution series (range, 10²–10⁷) of known amounts of nucleic acids. The standard curves were constructed by cloning the entire hexon region of Ad41 into pBR322.¹⁸

For each series of samples both for RT-PCR than for quantitative PCR, neat and a 10-fold dilution of the RNA or DNA suspensions were run in duplicate; for quantitative PCR each dilution of standard DNA suspensions was run in triplicate. Standard precautions were applied in all assays, including separate areas for the different steps of the protocol and addition of nontemplate control and nonamplification control to each run. The presence of enzymatic inhibitors was evaluated by adding target DNA or RNA as an external control to a separate tube that was assayed with the same protocol condition of extracted nucleic acids.

Virus identification

Positive PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and confirmed by sequencing with an ABI PRISM 373 DNA Sequencer (Applied Biosystems by Life Technologies Corporation, Monza, Italy). The results were analyzed using the Basic Local Alignment Search Tool (BLAST; www.blast.ncbi.nlm.nih.gov) and the sequence analyses were carried out using the National Center for Biotechnology Information GeneBank.

HAdV infectivity test

Positive HAdV samples were assessed to reveal infectivity using cell cultures. The samples, after decontamination with chloroform, were cultivated on the A549 cell line (European Collection of Cell Cultures, Public Health England, Porton Down, Salisbury, UK) in Dulbecco's Modified Eagle's Medium (Euroclone, Milan, Italy) with 2% of fetal bovine serum (Euroclone, Milan, Italy). The culture was incubated at 37°C and observed daily by optical microscope for 2 weeks until typical cytopathic effects were detected. The first culture was followed by 2 subsequent confirmation steps.¹⁸

Table 1
Frequency of virus positivity and total bacterial counts over limits and differences between hospital and office toilets

| Test result | Office | | Hospital | | P value* | |
|--|----------|---------|----------|---------|----------|-------|
| | Surfaces | Air | Surfaces | Air | Surfaces | Air |
| Total samples | 64 | 16 | 108 | 27 | — | — |
| Total positive | 46 (71) | 12 (75) | 89 (82) | 23 (85) | .09 | .11 |
| Human adenovirus positive | 43 (67) | 10 (62) | 78 (72) | 21 (77) | .53 | .03 |
| Toque teno virus positive | 6 (9) | 3 (18) | 48 (44) | 4 (15) | <.001 | .70 |
| Norovirus positive | 0 (0) | 0 (0) | 1 (1) | 0 (0) | — | — |
| Human adenovirus + Toque teno virus positive | 3 (4) | 1 (6) | 38 (35) | 2 (7) | <.001 | 1 |
| Total bacterial count > limits [†] | 2 (3) | 13 (81) | 4 (3) | 11 (41) | .67 | <.001 |

NOTE. Values are presented as n (%).

* χ^2 test.

[†]Samples over the limit for low contamination in surfaces and aerosol samples²⁰; surfaces: total bacterial count >10 CFU/cm²; aerosol: total bacterial count >100 CFU/m³.

Data analysis

Frequency of positive data were analyzed using a χ^2 test. A paired Student *t* test was used to evaluate the differences before and after cleaning; a Pearson test and calculation of the *R*² value were used for the evaluation of linear correlation between HAdV titers and TVBC values. The statistic program MedCalc Software (version 11.5; Ostend, Belgium) was used for statistical analyses.

All bacterial and viral counts were normalized as follows: air samples to a volume of 1 m³; surface samples to 1 cm²; water samples to a volume of 1 mL. For *t* test and regression analyses, a viral load corresponding to half of the detection limit (10 GC/mL) was attributed to samples that were negative for viral presence by quantitative PCR for HAdV.¹⁹

RESULTS

Viral occurrence on surfaces and in air and water samples

Viruses were detected on 135 surfaces (78% of the total tested), in 35 aerosol samples (81%), and in 17 water samples (89%). Among the researched viruses only HAdV, TTV, and NoV GII were revealed. In particular HAdV was found on 121 surfaces (70%) and in 31 air samples (72%); TTV was detected on 54 surfaces (31%) and in 7 air samples (16%). Both viruses were detected on 24% of the surface samples and in 7% the of air samples. NoV GII was detected on only 1 hospital surface sample. Table 1 shows the frequencies of virus-positive samples for hospital and office surfaces and air samples. The surface total positivity was 82% in the hospital and 71% in offices; aerosol sample viral presence was 85% and 75% in hospital and office samples, respectively, without significant differences between samples for each matrix. Regarding aerosol contamination, 21 samples (77%) from the hospital setting were positive for HAdV only, 4 samples (15%) were positive for TTV only, and 2 samples (7%) were positive for both virus types. For the office samples the frequencies were: 10 samples (62%) positive for HAdV only, 3 samples (18%) positive for TTV only, and 1 (6%) sample tested positive for both. The only significant difference between hospital and offices settings was the total percentage of samples exhibiting HAdV contamination, which was higher in hospital samples (Table 1). A significant difference was found also between the frequencies of air samples positive for TVBC over the limits allowed for low contamination areas according French normative recommendations.²⁰ No differences were found between HAdV positivity in water samples from hospital and office settings.

The most contaminated surfaces were door handles (66%) followed by flushing buttons (62%), toilet seats (59%), and toilet covers (52%). Nevertheless, no significant differences were found among these areas for HAdV and TTV positivity. The highest frequency of TTV contamination in hospital samples was recorded for each surface.

Thirty-two office samples and 48 hospital samples were obtained both before and after cleaning. The frequency of TTV positivity on surfaces decreased after cleaning, both in offices and the hospital, although the difference was not always statistically significant. We found neither setting had samples over the limits set for detection after disinfection. For TVBC at both sites, no samples over limits were detected after disinfection. Surprisingly, the frequency of HAdV positivity increased on surfaces after cleaning, although not significantly.

HAdV titers and relations with TVBC

The geometric means and standard deviations of HAdV titers in offices were $3.9 \times 10^4 \pm 1,921$ genome copies (GC)/m³ in air, 91 ± 31 GC/cm² on surfaces, and $1.2 \times 10^7 \pm 5$ GC/mL in water. In the hospital, the corresponding titers were $5.8 \times 10^6 \pm 5,712$ GC/m³, 483 ± 53 GC/cm², and $6.6 \times 10^7 \pm 668$ GC/mL, all significantly (*P* < .01) higher than in offices. Such values were not related to the corresponding TVBC values in offices (147 ± 251 CFU/m³ in air and 1.44 ± 5 CFU/cm² on surfaces) or in the hospital (293 ± 209 CFU/m³ in air and 1 ± 5 CFU/cm² on surfaces).

The geometric means of viral titers on specific surfaces were not significantly different. Viral titers were 270 ± 40 GC/cm² for the door, 144 ± 60 GC/cm² for the flushing button, 222 ± 44 GC/cm² for the toilet seat, and 312 ± 63 GC/cm² for the toilet cover.

No significant differences were found between HAdV titers before and after cleaning (Table 2); however, in accordance with the frequency of positive data, the values seemed to increase following disinfection in hospital. On the contrary, the TVBC values were significantly reduced after cleaning.

Viral identification and HAdV infectivity

Viral presence was confirmed by sequencing the amplified nucleic acids, which also allowed identification of detected viruses. In offices where only HAdVs were detected, 18 different strains were found, belonging to 12 genotypes. Genotype 5 was found most often (detected in 17 samples), followed by the genotype 1 (detected in 7 samples). Eight strains were simultaneously detected in separate samples collected from the same toilet on the same sampling date; the frequency of this correspondence (number of sampling days with correspondence/total number of sampling days) between air and surfaces was 25% and between water and surfaces was 50%. In the hospital, 20 different strains of HAdV, belonging to 12 genotypes and 14 strains of TTV, were found. Sixteen strains of HAdV and 1 strain of TTV were simultaneously detected in separate samples collected from the same toilet on the same sampling date. The frequency of correspondence (as previously defined) was 77% between water and surfaces, 44% between water and air, and 22% among the 3 matrices.

The HAdV strains detected in offices and the hospital were different except the 1 strain (Accession no.: EF564601). Among these positive samples, only HAdV can be isolated in cell cultures, but none of them resulted infective in A549 cells.

DISCUSSION

Our work sought to investigate viral contamination of air and surfaces of toilets in different settings, to assess the role of this

source in the spread of virus, and to determine the best control measures and points where they can be successfully applied.

From our observations we can conclude that toilets are doubtless a very important source of this contamination; this is confirmed by the higher positivity and titers found here as opposed to the data from our previous study¹⁸ on other hospital rooms. A very important source of contamination from toilets seems to be droplets coming from toilet flush and settling on surfaces, as demonstrated by the high correspondence between the HAdV strains found in water and on surfaces. Nevertheless, another important mechanism of diffusion is hands, which cause the high amount of contamination observed for door handles and flushing buttons.

Moreover, our results confirm the data already found in our previous study⁹ revealing the wide dissemination of HAdV and, to a lesser extent, TTV in health care settings, but also indicate the presence of these viruses in indoor working environments (namely offices). HAdV showed the highest dissemination, thus confirming its possible role as an indicator of viral contamination of air and surfaces, as already proposed for water.²¹ On the contrary, for TTV, a marked difference was observed between the hospital and offices; it is possible that in health care settings this virus is more widely spread due to the greater presence of people with impaired immune response.²²

On the other hand, as we previously observed, but in contrast with other studies,⁸ the other viruses we investigated were almost absent: only 1 sample was found to be positive for NoV GII and no NoV GI, EV, RV, or HRV were detected. A possible explanation of this observation can be the different epidemiologic diffusion of these agents. In fact, both HAdV and TTV are normally eliminated by feces even in the absence of clinical disease and are very common in the population, as demonstrated by sewage monitoring,²³ whereas the circulation of the other viruses we searched is more limited, more often linked to clinical cases, and dependent on alternative mechanisms of transmission based on the oral–fecal route. During virologic monitoring, an epidemiologic surveillance of symptomatic infections, based on the observation of fever and diarrhea, did not show evident cases of simultaneous presence of target viruses in environment and patients samples (data not shown).

Similar to previously published works,⁸⁻²⁴ virologic monitoring was carried out with molecular methods, which are widely recognized as very sensitive and specific, although they are unable to indicate viability.²⁵ In some studies¹⁰⁻²⁶ the PCR positivity of surface and air samples has been confirmed by cell culture but, here, none of the HAdV PCR-positive samples were confirmed as infective. This poses a problem particularly when virologic monitoring is used to verify the efficacy of disinfection procedures. Studies on HAdV survival in comparison with DNA persistence following chemical disinfection⁹ showed a difference of 1 log due to PCR detection of DNA originating from inactive virus. Moreover, when comparing the results of the virologic analysis of surfaces before and after disinfection, the HAdV positivity and titers seem to increase even if not significantly, whereas the values of TVBC decrease, mainly in the hospital. The reason for this contradiction seems to be the interference of dirtiness on PCR enzymes, as demonstrated by our experiments on artificially contaminated surfaces, as well as previous studies.²⁷ Thus, before using molecular assays to verify the efficiency of disinfection measures, the sensitivity of analytical tests should be carefully evaluated in the presence of different concentrations of organic substances. From these data, we can conclude that molecular data on surfaces before disinfection are affected by underestimation due to interference by organic substances on PCR and data obtained after disinfection are affected by overestimation due to the detection of noninfective DNA. Thus, the validity of TVBC abatement for predicting virus removal should be assessed through further investigation.

Table 2

Geometric means (\pm standard deviation) of human adenovirus (HAdV) titers and total bacterial counts (TVBC) on surfaces before and after disinfection in hospital and office toilets

| | Mean concentration \pm standard deviation | | | |
|---------------------|---|----------|-----------------------------|----------|
| | HAdV (GC/cm ²) | P value* | TVBC (CFU/cm ²) | P value* |
| Offices | | | | |
| Before disinfection | 124 \pm 42 | .12 | 1.2 \pm 2 | .16 |
| After disinfection | 67 \pm 23 | | 0 | |
| Hospital | | | | |
| Before disinfection | 349 \pm 51 | .08 | 1.57 \pm 3 | .015 |
| After disinfection | 1,371 \pm 49 | | 1.15 \pm 1 | |

*Student *t* test for paired samples.

Although further studies are necessary for more precise estimation, the data we collected in this study indicate that the risk of exposure to virus from toilet use results mainly from contact with infected surfaces, confirming the importance of handwashing and disinfection for infection control. This approach will be useful to develop a quantitative microbial risk assessment, to better define risk management strategies in health care settings.

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