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Cold oxygen plasma technology efficiency against different airborne respiratory viruses

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ABSTRACT

Background: Respiratory infections caused by viruses are major causes of upper and lower respiratory tract infections. They account for an important mortality and morbidity worldwide. Amongst these viruses, influenza viruses and paramyxoviruses are major pathogens. Their transmission is mainly airborne, by direct transmission through droplets from infected cases.
Objectives: In the context of an influenza pandemic, as well as for the reduction of nosocomial infections, systems that can reduce or control virus transmission will reduce the burden of this disease. It may also be part of the strategy for pandemic mitigation.
Study design: A new system based on physical decontamination of surface and air has been developed. This process generates cold oxygen plasma (COP) by subjecting air to high-energy deep-UV light. To test its efficiency, we have developed an experimental device to assess for the decontamination of nebulized respiratory viruses. High titer suspensions of influenza virus type A, human parainfluenza virus type 3 and RSV have been tested.
Results: Different experimental conditions have been evaluated against these viruses. The use of COP with an internal device allowed the best results against all viruses tested. We recorded a reduction of 6.5, 3.8 and 4 log(10) TCID50/mL of the titre of the hPIV-3, RSV and influenza virus A (H5N2) suspensions.
Conclusions: The COP technology is an efficient and innovative strategy to control airborne virus dissemination. It could successfully control nosocomial diffusion of respiratory viruses in hospital setting, and could be useful for the reduction of influenza transmission in the various consultation settings implemented for the management of cases during a pandemic.

1. Background

Respiratory syncytial virus (RSV) and human parainfluenza virus type 3 (hPIV-3) infections are two leading causes of lower respiratory illness (LRI) in young children and also in elderly.[1,2] These infections are associated to high morbidity. Global annual mortality worldwide for RSV, for example, is estimated to be 160,000 and many efforts are actually done in order to develop vaccines and antiviral drugs against these viruses.[3,4] Influenza virus is one of the most important viruses responsible for upper respiratory tract infection regarding morbidity and mortality. Prevention and treatment of influenza viruses rely on inactivated vaccines and antiviral drugs. The prospect of future influenza pandemics, potentially caused by avian influenza has raised the question of pandemics preparedness.[5,6]

Airborne transmission, either direct or secondary, has been postulated to be involved in the dissemination and spread of several microorganisms.[7] Several reports have shown that fine particle aerosols may play a role in respiratory virus infection. It is now well established for influenza virus, but it may not be the primary way of spreading for RSV and hPIV-3.[7,8] To protect human population, several air disinfection systems have been developed, based on different technologies. Classic approaches consist in air filtration,[9] ionization,[10] and ultraviolet irradiation.[11] Other recent approaches implicate air oxi-

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dation by photocatalytic process,[12,13] ozone [14] or plasma-based disinfection.

Gas plasmas can be considered as the fourth state of matter, following by order of increasing energy, the solid, liquid and gaseous states. Man-made cold gas plasmas are usually produced by subjecting a gas to an electric field. Gas plasmas are composed of ions, electrons, uncharged particles such as atoms, molecules (e.g. O_3) and radicals (OH-, NO-, etc.).[15] These ions and uncharged particles can be in an excited state and can become to a normal state by emitting a photon or through collisions with a surface for example. These events can induce chemical reactions such as oxidations and lipid/protein peroxidations.[15] The possibility to use plasma-sterilizing properties was first introduced in the end of 60s and first works with a plasma made with oxygen were proposed in 1989. Nelson and Berger[16] have shown that O_2 plasma could be a very efficient biocidal tool against bacteria. More recently, Biozone scientific firm has developed a new process for the generation of a cold oxygen plasma (COP) by subjecting air by high-energy deep-UV light with a effective radiation spectrum between 180 nm and 270 nm. This cold gas plasma is composed of several species like negative and positive ions, free radical molecules, electron, UV-photons and ozone. The ozone production is controlled and maintained to a maximum level of 0.04 ppmv (parts per million by volume). This technology is dedicated to be used in human environment for the decontamination of both surface and air.

2. Objectives

To our knowledge, no attempts have been made to evaluate the efficiency of cold oxygen plasma against virus and more precisely airborne respiratory viruses. To address this issue we have set up an experimental device in the purpose of testing the efficiency of Biozone technology COP against nebulizated preparation of three respiratory viruses of significant clinical importance: RSV, hPIV-3 and A (H5N2) influenza viruses.

3. Study design

3.1. Cells and viruses

LLC-MK2 cells (Monkey kidney cells) were obtained from American type culture collection (ATCC reference CCL-7) and were grown in Eagle's minimal essential medium (EMEM) with 5% foetal calf serum. MDCK (Madin-Darby canine kidney cells) were obtained from American type culture collection (ATCC reference CCL-34) and were grown in ultra MDCK medium (Lonza-Biowhittaker). RSV-A Long strain and hPIV-3 C-243 strain were obtained from the ATCC (respectively ATCC VR-26 and ATCC VR-93). Since the influenza A (H5N1) virus is strongly pathogenic, the study was performed with the A (H5N2) strain chosen as the conventional research model for the influenza virus A (H5N1) strain (H5N2 A/Finch/England/2051/2001).

3.2. Viral production and purification

In order to produce large quantities of hPIV-3 and RSV, three 175 cm^2 flasks of LLC-MK2 cells were infected for each virus at a multiplicity of Infection (MOI) of 10^{-3} [17] and supernatants were harvested 3 days post-infection. After a centrifugation at 1200 × *g* at 4 °C for 10 min, supernatants were centrifuged at 25,000 × *g* at 4 °C for 2 h on a 20% saccharose cushion in phosphate buffered saline (PBS; pH 7.4). Viral pellets were resuspended in 50 mL of PBS; pH 7.4 and stocked at 4 °C before nebulization step. Influenza A (H5N2) strain was cultivated on MDCK cells and the viral suspension was prepared in a same way as hPIV-3 and RSV.

3.3. Cold oxygen plasma experimental device

The efficiency of the gas plasma process in air disinfection was studied directly by a pilot reaction core manufactured by Biozone scientific. A schematic drawing of the testing system is depicted in Fig. 1. The reaction core is composed of external and internal cold oxygen plasma device (COP) and an internal classic UV-C lamp (254 nm).

The system consists of a one-pass flow tunnel with a reaction core to be tested situated inline such that air samples can be taken before and after the reaction core. For safety, the entire system was installed in a BSL3 laboratory with the entry and exit of the flow system located inside biological safety hoods within the laboratory. Samplers to determine upstream and downstream outlet airborne levels of infectious virus where also located inside safety hoods. At the entry of the flow tunnel, a viral aerosol suspension was generated using a 6-jet Collision spray nebulizer (Model CN311, BGI, INC). The suspension was aerosolized by applying compressed air to the Collision nebulizer at 1.8 bars of pressure. Under these conditions, the mean diameter of the droplets is 1.9 µm. During these tests the air speed through the system was stabi-

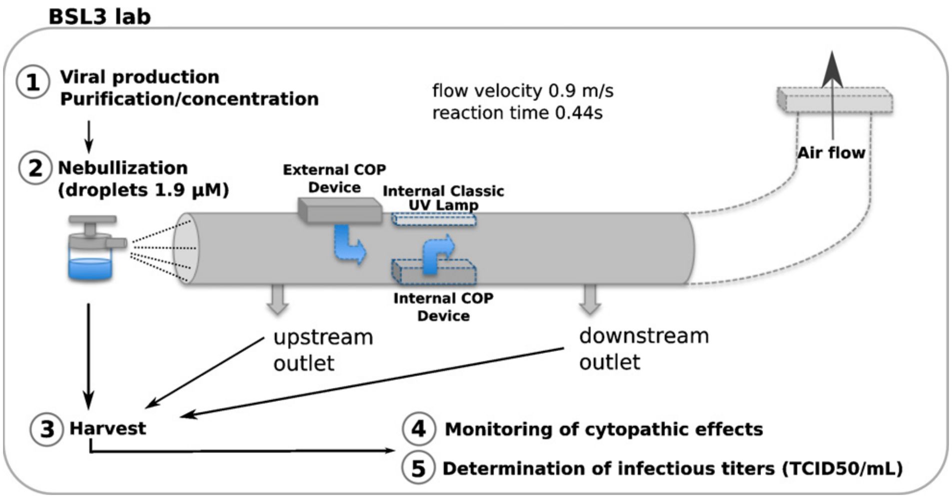

Fig. 1. Schematic representation of the experimental device and strategy used in this study.

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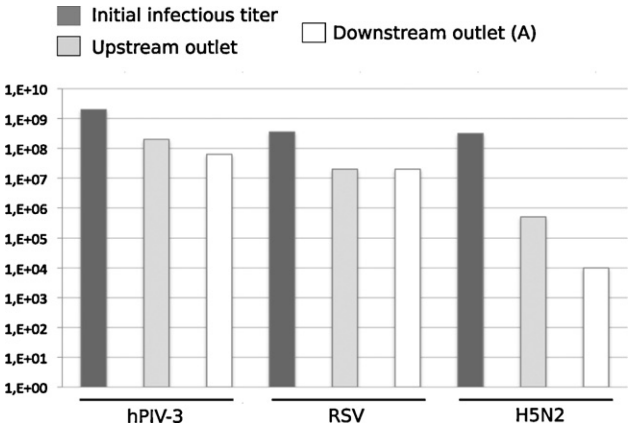

Fig. 2. Evaluation of the viral loss due to the nebulization in the experimental device. The infectious titers (TCID50/mL) of the suspensions harvested at the upstream and the downstream outlets are compared with the initial infectious titer of the viral suspensions, which have been nebulizated, for hPIV-3, RSV and A (H5N2) influenza.

lized and fixed at 0.9 m/s. The virus flow was sampled during 3 min using a sampling pump and then focused onto 3 mL of collection fluid (phosphate buffer saline) in 50 mL sterile plastic tube.

Three different experimental conditions were tested four times (Fig. 3): (B) the internal classic UV germicide UV-C light lamp, (C) a COP from an external device (gas plasma only) and (D) a COP from an internal device (gas plasma and UV light).

3.4. Determination of viral infectious titers

The amount of infectious virus in each batch was performed by limit-dilution titration test and determination of the dilution of virus required to infect 50% of inoculated cells (TCID50/mL).[18] For this purpose, virally induced cytopathic effects (CPE) were checked until 96–120 h post-infection.

4. Results

4.1. Evaluation of viral load reduction due to the nebulization in the experimental system (Fig. 2)

We first evaluated the load reduction of infectious particles during the nebulization in our experimental device. The first experiment was a blind test with no UV-C light or ozone produced into the virus stream. The Collison nebulizer was filled with 30 mL of influenza A (H5N2) or RSV or hPIV-3 purified viral suspensions, with infectious titers of, respectively $10^{8.5}$, $10^{8.55}$ and $10^{9.3}$ TICD50/mL.

After 30 min of stabilisation, four samples were taken alternatively at the upstream outlet and at the downstream outlet, and this operation has been repeated four times to check the reproducibility. The objective was to check that infectious titers, in the suspensions harvested at upstream and downstream outlet (Fig. 1), were high enough to further evaluate the effect of different experimental conditions (e.g. UV-C/COP/COP + UV). First preliminary results have shown that the initial infectious titers had to be very high before nebulization, which implicated concentration/purification steps after viral production.

The loss of infectious particles between the initial infectious titer and the upstream outlet for hPIV-3, RSV and influenza virus A (H5N2) was respectively of the order of 0, 1.25, and 2.8 log(10) TCID50/mL (Fig. 2). The decrease of amount of virus was very important (more than 99.8% for influenza virus A (H5N2)) but the upstream outlet infectious titers still represented non-negligible values ($10^{9.3}$, $10^{7.3}$, and $10^{5.7}$ respectively for hPIV-3, RSV and influenza virus A (H5N2), Fig. 2). We then measured the viral loss between upstream and downstream outlets. Surprisingly, there was no measurable loss for RSV between the two outlets. The loss of infectious particles between the upstream and the downstream outlet for hPIV-3, RSV and influenza virus A (H5N2) was respectively of the order of 0.5, 0, and 1.7 log(10) TCID50/mL (Fig. 2). We also observed marked loss rates at this step, but the downstream outlet infectious titers still represented non-negligible values ($10^{7.8}$, $10^{7.3}$, 10^4 respectively for hPIV-3, RSV and influenza virus A (H5N2), Fig. 2).

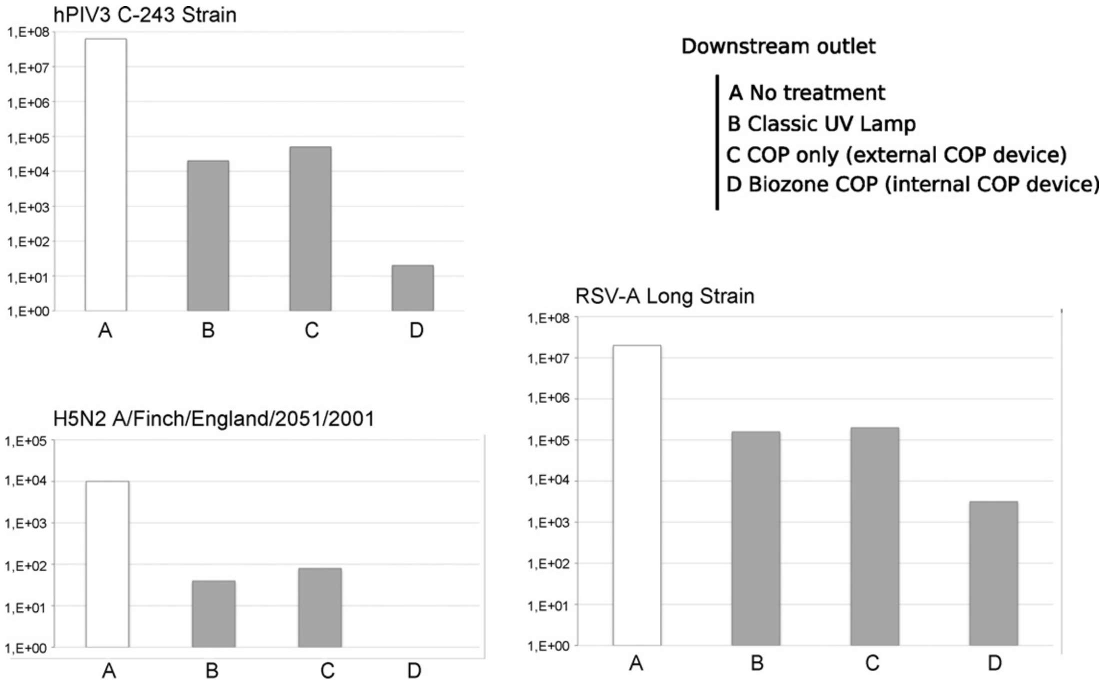

Fig. 3. Determination of downstream outlet infectious titers in different conditions for hPIV-3, RSV and A (H5N2) influenza.

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Table 1
Percentage efficiency of inactivation in the different experimental conditions (B–D, Fig. 3).

	% efficiency		
	Classic UV lamp (B)	COP only (C)	Biozone COP (D)
H5N2	99.60	99.20	>99.99
hPIV-3	99.97	99.92	>99.99
RSV	99.20	99.00	99.98

Altogether, these results have shown that it was possible to harvest, after nebulization of a highly concentrate viral suspension, significant quantities of infectious viruses despite important loss rates.

4.2. *Determination of downstream outlet infectious titers in different conditions (Fig. 3)*

The downstream outlet infectious titers for each virus, without treatment, previously determined were used as reference values (see Fig. 3A).

We first determined the effect of the classic UV-C light without gas plasma production. The germicidal effects of classic UV-C light lamp allowed a loss of infectious titers, for hPIV-3, RSV and influenza virus A (H5N2) of respectively 3.5, 2.1 and 2.4 log(10) of TCID50/mL (A *versus* B, Fig. 3). We then determined the effect of gas plasma (external COP device) into the virus flow. The ozone concentration was measured to be 0.05 ppmv in this stream complying with all certification levels. When the external COP device was tested, the loss of infectious particles for hPIV-3, RSV and influenza virus A (H5N2) was respectively of the order of 3.1, 2 and 2.1 log(10) TCID50/mL (A *versus* C, Fig. 3). The results between the two experimental conditions A and C were quite comparable. We finally determined the effect of both gas plasma and UV light (external and internal COP devices) into the virus flow. This configuration allowed a more important loss of infectious titers, for hPIV-3, RSV and influenza virus A (H5N2) of respectively 6.5, 3.8 and 4 log(10) TCID50/mL (A *versus* D Fig. 3).

All these results have been expressed as percentage efficiency using the following formula. *Percentage efficiency* = (*infectious titer in A* − *infectious titer in B, C or D*)/(*infectious titer in A*) × 100. The results are shown in Table 1.

4.3. *Monitoring of cytopathic effects: an illustration (Fig. 4)*

The infectious titers were determined for the observation of infected cell monolayers. In order to illustrate the results presented in Fig. 2, we have monitored the cytopathic effect on MDCK or LLC-MK2 cells, infected with samples harvested at upstream or downstream outlets, when the internal COP device was switched on. From the 3 mL harvested at each outlet, 500 μL was used to infect cell monolayers in 3.5 cm dishes. Representative photographs taken at 72 h post-infection are shown in Fig. 4. Marked cytopathic effects were observed in cell monolayers infected with samples harvested at upstream outlet, for the three viruses. The cell monolayers were totally destructed for influenza virus A (H5N2) (rounded and non-adherent cells, Fig. 4) and only partially for hPIV-3 and RSV, with multiple small characteristic foci (Fig. 4). We have not observed evident cytopathic effect in dishes infected with influenza virus A (H5N2) samples harvested at the downstream outlet; cell monolayers were similar to non-infected ones (MOCK, see Fig. 4). For hPIV-3 and RSV downstream outlet samples, only discrete cytopathic effects were visualised, with small foci (Fig. 4). Our following observations (up to 96 h post-infection) revealed that these foci were probably early syncytial structures (data not shown).

5. Discussion

The aim of this study was to evaluate the efficiency of a cold oxygen plasma generated by the Biozone scientific technology against different respiratory viruses. The main struggle consisted to set up an experimental device, which allowed us to test different treatments of nebulizated viral suspensions. The objective was not to precisely mimic human-produced droplets but the size range appeared to be important. Only limited data are available regarding the size distribution of human-produced droplets. For influenza virus, the average diameter of droplets is of the order of the micrometer,[19,20] which corresponds to the average diameter of droplets generated in our study.

The first set-up experiment (Fig. 2) showed that it was possible to harvest, after nebulization of a high concentrate viral suspension, significant quantities of infectious viruses, despite important loss rates. The important loss rates could be partially explained by a rapid aggregation and consecutive particles settling between the upstream and the downstream outlets and also the liquid impingement samplers have been used to sample the air. The loss rates could be explained by a probable high relative humidity of our experimental condition that is known to affect the infectivity of airborne influenza virus, for example.[21] This feature can be compensated by high initial viral titers.

The UV-C light irradiation capacity to inactivate airborne viruses was not extensively studied in litterature.[11] In early works, Jensen[22] has shown that the inactivation rate of UV-C on influenza (WSN strain) was greater than 99.99%. In our experimental conditions, we have found UV-C inactivation rates for A (H5N2) influenza virus, hPIV-3, and RSV, were respectively of 99.60%, 99.97% and 99.20% (Table 1). These percentages could first appear to be very close but represent lower efficiency considering the infectious titers. These differences could be explained by the number of UV-C lamp used in these two studies; only one in our study *versus* six lamps in the early works by Jensen[22] and also by differences of initial viral titers experimentally used.

In our experiment, gas plasma generated by the Biozone UV lamp is responsible for an important decrease of the viral titer for all the three respiratory viruses. One important element in the composition of a cold gas plasma is the ozone. It is well documented in the scientific literature that ozone–oxygen mixtures inactivate microorganisms including bacteria, fungi and viruses.[23,24] A recent study suggests that ozone inactivation of viruses occurs primarily by peroxidation of both lipid and protein.[24] Enveloped viruses in a thin liquid layer showed extreme sensitivity to ozone using concentrations ranging from 800 ppmv to 1500 ppmv.[24] The Biozone scientific COP only allows the production of 0.04 ppmv of ozone. The effect of such low ozone concentration on nebulizated viral suspensions will be further examined.

Our results showed a slightly lower effect of the gas plasma *versus* UV-C on viral air decontamination (B and C, Fig. 3 and Table 1). When the Biozone COP was tested, percentage efficiencies were significantly higher for influenza virus A (H5N2) and RSV (0.8–0.98% enhancement). The combined effects of gas plasma and internal UV, in the Biozone device brought a high level of inactivation rate. These particular features have never been described before. Future investigations will explore the efficiency of Biozone COP on contaminated surfaces.

Altogether, the results of this study revealed marked differences in inactivation rates amongst A (H5N2), hPIV-3 and RSV. The higher inactivation rates, in the three experimental conditions, were always obtained for hPIV-3. Lower inactivation rates were obtained for influenza virus A (H5N2) and RSV (Table 1). Because of the initial infectious titers and the sensitivity of the viral assays varied, it is difficult to determine if these differences represented a specific susceptibility to the disinfection processes or just reflect

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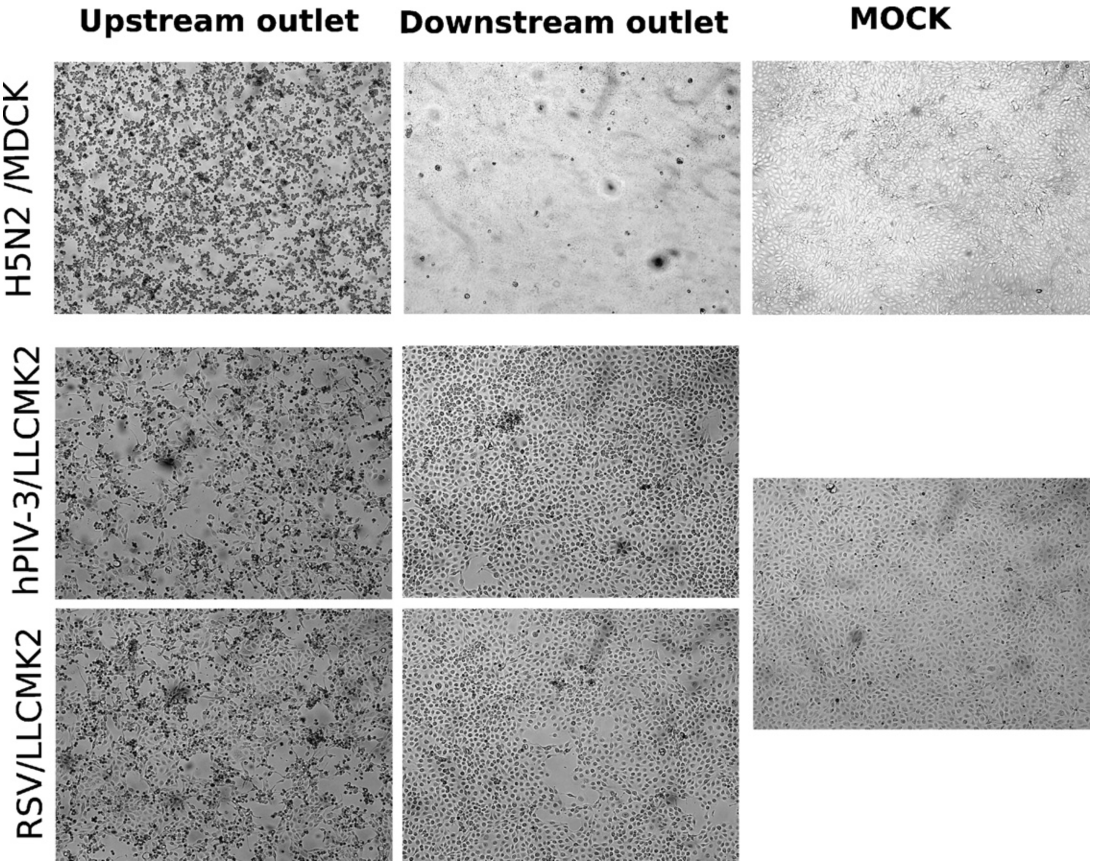

Fig. 4. Monitoring of the cytopathic effect obtained with infection of MDCK or LLC-MK2 cells with samples harvested at the upstream and downstream outlets when the internal Biozone COP was switched on.

variations or our experimental conditions. However, initial infectious titers for hPIV-3 and RSV were quite similar and the same cellular system was used. Differences of inactivation rates could be explain by viral features like the protein and lipid composition of the particle or the relative importance of the viral matrix, for example. The possible link between structural characteristics and susceptibility to UV and or plasma will be further investigated. The efficiency against non-enveloped virus, e.g. adenovirus will be also explored.

Cold oxygen plasma technology appears to be an efficient air decontamination tool to protect human population against airborne infections. The Biozone COP commercial apparatuses are already used to prevent dissemination of multiresistant bacteria in hospital, for example. In a similar way, this new-engineered method could be used to control the airborne transmission of viruses in high-risks settings, like hospital wards for example. With the recent emergence of viral respiratory pathogens such as avian influenza virus A (H5N1), the COP technology could constitute a precious tool for the reduction of influenza transmission in the various consultation settings implemented for the management of cases during a pandemic.

Conflict of interest

The authors declare that they have no conflict of interests.

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