Ultraviolet inactivation of Influenza A viruses from different species

Official Master's Degree in Zoonoses and One Health

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Xavier Abad Morejón de Girón

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Abstract

Influenza virus type A is of great concern in human, domestic and wild animal health. The ability of this agent of moving from one species to another confirms the “one world” concept of influenza or “one flu”. For this reason, the necessity of a “One Health” approach is required for the control and prevention of this disease. The establishment of certain guidelines and protocols for viral inactivation of Influenza virus would be highly advisable in terms of control and prevention of transmission. Ultraviolet radiation has been demonstrated to be an effective method to inactivate microorganisms through damage of their genome. This investigation wants to use the germicidal range of ultraviolet wavelength (UV-C) to determine the kinetic decay of influenza virus and at the same time to evaluate if there are any differences between four influenza type A strains from different origins (avian, swine and human) in terms of viral inactivation. The results showed that this method is effective to inactivate Influenza virus from different species. At the last time point (10 min.), an inactivation of approximately half of the initial concentration of viruses was observed. The utility of this investigation is to translate these results of inactivation to more pathogenic viruses of the same family or of the same genome size, especially concerning the management of infectious sample in BSL-3 containment.

1. Introduction

Over the last 20 years, public health interest has had a great deal of focus on emerging or re-emerging infectious diseases, especially on zoonoses with a pleiotropic effect on animals, the environment and human health, both directly and indirectly (Brown, 2000; Ippolito & Rezza, 2017). Influenza viruses should never be ignored when dealing with emerging disease problems or the possibility of an agent moving from one species to another (Brown, 2000). Since 2011, zoonotic influenza is one of the three priority topics of the tripartite commitment formed by the World Organization For Animal Health (OIE), the Food and Agriculture Organization of United Nation (FAO) and the World Health Organization (WHO), which requires the necessity of a "One Health" approach for the control and prevention of diseases (FAO *et al*., 2017).

Influenza is a respiratory disease of humans, swine, birds, horses, mink, and some sea mammals caused by infection with influenza viruses of the family Orthomyxoviridae (Swayne, 2000). Influenza is endemic in humans, swine, horses, and some birds, but sporadically epidemics occur in influenza-naive populations or in the presence of the introduction of new subtypes (Swayne, 2000). In general, influenza viruses are adapted to a single host species and are transmitted freely between individuals of the same species. But, occasionally, influenza viruses may jump the species barrier with the capability of infecting a new host (Donatelli *et al*., 2016). Wild aquatic birds are considered the primordial genetic reservoir of influenza viruses (Donatelli *et al*., 2016).

There are three recognized influenza virus types: A, B and C. Only type A viruses are found in a number of mammalian and bird species (OIE, 2015; WHO, 2018). Influenza A viruses are further classified into subtypes according to the presence of different surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). At present 18 H subtypes (H1-H18) and 11 different N subtypes (N1-N11) have been identified (Donatelli *et al*., 2016). Influenza type A viruses are of most importance to public health due their potential of transmission to humans and the possibility of a human to human transmission causing an influenza pandemic (WHO, 2018).

Depending on the original host, influenza A viruses can be classified as avian influenza "bird flu", swine influenza "swine flu" with different symptomatology (WHO, 2018). In poultry, they can cause a severe disease with high death rate, with highly pathogenic avian influenza

(HPAI) subtypes, or a mid-disease with a mid-course, if the animals are infected with low pathogenic avian influenza (LPAI) subtypes (OIE, 2015). Avian Influenza viruses have been shown to cross the species barrier and infect mammalian species, most commonly swine and humans, with an important impact in public health. First documented case of human cross species transmission was during 1959 in the United States (Brown, 2000).

Influenza A viruses are generally classed by the OIE, at least, in risk group 2 for human and animal infection and must be handled with appropriate biosafety and biosecurity measures. The potential zoonotic viruses that cause HPAI and H5/H7 LPAI belong to risk group 3-4, and they have the capability to spread from the laboratory if adequate levels of biocontainment barriers and biosafety practices are not put in place. For these subtypes, a higher level of biocontainment is necessary (OIE, 2015).

Cases of laboratory workers infected from the incorrect inactivation or from mishandled biological samples from high pathogenic microorganisms, have been previously reported (Abad, 2012). A recent example was given in 2014 by the US Centers for Disease Control and Prevention (CDC) laboratories, when even with the high control measures of biosecurity, an inadvertent cross-contamination of one sample of a LPAI H9N3 virus with HPAI H5N1 was reported; revealing a likely departure from best practices and putting public health in a severe threat (CDC, 2014). The development of new strategies to increase biosecurity is extremely necessary. The establishment of certain guidelines and protocols for viral inactivation with a good studied efficacy, and the creation of a bank of potential viruses to be tested, would be highly advisable (Abad, 2012).

Inactivation through chemical, heat and radiation procedures are currently used to remove infectious and suspicious samples from these biocontainment units (Shurtleff *et al*., 2012). UV treatment has its most common use in laboratories in biological safety cabinets as a typical procedure to disinfect the working surface, after using the cabinet. In biosafety level 3 laboratories (BSL-3), UV treatment is also often used for passing out of the laboratory potentially contaminated materials through pass boxes in ultraviolet bathed portals (Turnbull *et al*., 2008).

It has been demonstrated that microorganisms exposed to UV light prove an exponential decrease in population similar to other inactivation methods (Cutler & Zimmerman, 2011; Kowalski *et al*., 2000). Ultraviolet light is a portion of the electromagnetic spectrum with the

wavelength range of 100-400 nm. This wavelength range is generally divided into four bands based on the interaction with the molecules: UV-A (320-400 nm), UV-B (320-280 nm), UV-C (200-280 nm) and the vacuum UV (100-200 nm) (Sastry *et al*., 2000; Yin *et al*., 2013). The UV-C band is also known as the germicidal part of the UV light, for the capability of this wavelength of being absorbed by nucleic acids, with the highest effect achieved using 254 nm (Cutler & Zimmerman, 2011). Unsaturated organic compounds of the nucleic acid have the capability to absorb wavelengths between 200 and 280 nm. The principal unsaturated organic compounds in cells are pyrimidines, purines and flavins, essential for reproduction and metabolism (Cutler & Zimmerman, 2011). This absorption by the nucleic acids leads to the formation of photoproducts, most common pyrimidine dimers, that block the DNA or RNA transcription and replication compromising the normal function of the cells leading to cell death (Cutler & Zimmerman, 2011; Sastry et al., 2000).

The first experimental studies of the inactivation effects with UV254 were conducted to attempt to control airborne spread of microbial infections. The first reported study of the effect of UV-C treatment on influenza viruses was carried out by Jensen in 1964. Since then, several studies were carried out about UV-C sensitivity of various microorganisms including parasites (Chang *et al*., 1985), spores and fungi (Green *et al*., 2004) and it has been shown that viruses are the most resistant organisms to Monochromatic (MC) UV254 nm radiation, especially those from the *Adenoviridae* family, followed by bacterial spores and the *Acanthamoeba spp.* protozoa (Chang *et al*., 1985; Hijnen *et al*., 2006). The highest UV sensitivity in a virus with veterinary importance was shown for bovine, canine and feline Caliciviruses and for human health, for Hepatitis A virus (Hijnen *et al*., 2006).

Concerning viruses, studying the inactivation kinetics of different types, it was demonstrated that the inactivation by UV-C light depends basically on the genome size of the virus and the type of genome (DNA-RNA, ss-ds) would be of secondary importance (Schmidt & Kauling, 2007;Thurston-Enriquez *et al*., 2003). Regarding the medium in exam, higher susceptibility in viral aerosol than in viral liquid suspension was observed (Walker & Ko, 2007).

Influenza A are negative-sense, single stranded segmented RNA (8 segments – 13.6 kb) viruses (Swayne, 2000; Donatelli *et al*., 2016); they are prone to change genetically with antigenic drift and antigenic shift, with a high mutation rate (Donatelli *et al*., 2016). This characteristic enables the virus to evolve from a LPAIV to a HPAIV form and to continually be highly variable, increasing the difficulty to prevent events increasing the risk for human health (EFSA, 2005). Moreover, that HPAI viruses arise from LPAI viruses through mutation

has been phylogenetically studied (Banks *et al*., 2000; Röhm *et al*., 1995), this is one of the reasons why they share similar physical and chemical characteristics (Thomas & Swayne, 2007).

The intention of this investigation is to profit of the similarities between this virus type, assaying four different influenza type A viruses with low pathogenicity as a surrogate to extrapolate as a possible information to HPAI virus characteristics of sensitivity to UV-C exposure inactivation.

2. Objective

The objective of this study was to determine the efficacy and kinetics of inactivation, of four different Influenza A virus isolated from different species (avian, swine and human), using UV-C (254 nm).

3. Materials and Methods

3.1. Viruses

Four different viral strains were used in the present study. Two strains were from avian-origin: A/Duck/Italy /281904/06 H1N1 (named avH1N1) and A/Mallard/Italy /239453/05 H4N6 (named avH4N6), both from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Italy). One swine strain A/Swine/Spain/SF11131/2007 H1N1 (named swH1N1) from the CReSA virus repository and a one human-origin IAV strain A/Catalonia /NSVH 10076691/2018 Pandemic H1N1 (named huH1N1) from Hospital de la Vall d'Hebron (Barcelona).

3.2. Virus propagation in embryonated Specific-Pathogen-Free (SPF) eggs

Virus stocks were produced in 11 day-old SPF eggs. Several dilutions from the original inoculum were used: 10^{-1}, 10^{-2}, 10^{-3} for avH1N1 and huH1N1 and 10^{-1}, 10^{-2}, swH1N1 and avH4N6, respectively. For each dilution, 5 eggs were used and 5 negative controls were also included.

The eggs with the air space up were placed inside a biological safety cabinet. The eggshell of each egg above the air space was disinfected with 70% ethanol. A sterile 18G needle was used

to punch a small hole in the shell; volumes of 0.1 ml of each of the diluted influenza virus suspensions were injected (carefully in a vertical position) into the allantoic cavity using a 25G needle insulin syringe. The hole was sealed with a drop of glue. After inoculation the eggs were placed back into the egg incubator with the air space pointed upwards and without turning.

At 24 hours p.i., eggs were candled and all eggs were viable. Briefly, after an incubation period of 72 hours, the eggs were chilled at -20 ºC for 20 minutes to kill the embryos. The eggs in an egg holder with the air sac facing up were transferred to a biological safety cabinet. The egg surface was cleaned with 70% ethanol. Sterile scissors were used to open the eggshell above the air sac, being careful not to leave traces of shell and not to destroy the chorioallantoic membrane. The chorioallantoic membrane was exposed with sterile blunt forceps and the sterile scissors. The embryo and the yolk sac were gently moved aside with a small spatula, taking care not to rupture the yolk. Using a Pasteur pipette, the allantoic fluid was collected. The allantoic fluids from the eggs spiked with same dilution of a virus were combined into a 50 ml sterile conical tubes. The different collection of the allantoic fluid was based on the time of death of the embryo and the dilution. Collection 50 ml tubes were keep on ice at all times during virus harvest. Once the harvest off the fluid from all of the eggs was done they were left to decant at 4 ºC. After then, they were centrifuged to pellet debris. The clear fluid from each virus harvest and from negative samples, was transferred into new 50 ml conical tubes kept on ice and 150 µl of each were aliquoted in sterile eppendorf microtubes. The 50 ml tubes and eppendorf microtubes were both frozen in a -75 °C ultra-freezer for long-term storage. Eggs were properly disposed into a BSL-2 group III waste container.

3.3. Viral detection by quantitative Real-Time Polymerase chain reaction (qRT-PCR)

In order to rapidly assess the viral loads of the different propagated viruses, a qRT-PCR was done with the allantoic fluids. Viral RNA was extracted using NucleoSpin® RNA Virus kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Negative and positive controls were added in each extraction batch.

A qRT-PCR to detect and quantify influenza type A virus was used using protocol already described that targets the M gene of influenza A virus (Busquets *et al*., 2010). Briefly, qRT-PCR reagent mix was prepared in laminar flow hood: in one eppendorf tube 0.8 µl of "RT-PCR Enzyme Mix 25x", 1 µl of "Probe +64 (6 µM) (Fam-Tamra)", 10 µl of "RT-PCR Buffer 2x", 0.8 µl of "Primer +25 (10 µM)", 0.8 µl of "Primer -124 (10 µM)", 1.37 µl of "Internal

Positive Control (IPC)" and 2.23 µl of "H20 Rnase Free" were added vortexing between each addition. A 96 well plate was prepared in a laminar flow hood, each well was numbered and filled up with 17 µl of the RT-PCR mix (previously calculated). A volume of 3 µl of each RNA sample (previously numbered on the extraction sheet) was subsequently added to each well, resulting in 20 µl for each well. The plate was sealed with PARAFILM® M Sealing Film (Merck KGaA, Darmstadt, Germany), very carefully to avoid contamination during the RT-PCR. Later, the plate was spun to homogenize the content of each well, by slow centrifugation, to eliminate the bubbles that might exist between both solutions. The plate was placed in the RT-PCR Applied Biosystems 7500 Fast Instrument. Using the "7500 software v. 2.3", each well was named and numbered according to the samples and specific conditions (cycles, duration and temperature) were chosen before the start. The routine consisted in several steps: 48 °C - 10 minutes; 95 °C - 10 minutes; 40x (97 °C - 2 seconds; 61 °C - 30 seconds). Positive and negative controls were added to each batch of samples. Results were expressed as Ct values.

Allantoic fluids inoculated with the same viruses were pooled. For this process, 3 different filters were used in the following sequence: 0.8 µm filter (FP 30/0.2), the 0.45 µm filter (FP 30/0.45) and lastly 0.2 µm filter (FP 30/0.8). The resulting liquid from each virus was collected using a 5 ml syringe and transferred to 50 ml Falcon tubes, and 150 µl were aliquoted in sterile eppendorf tubes (one for each virus). The obtained 50 ml tubes were frozen in a -75 °C ultra-freezer for long-term storage and the eppendorf tubes were used for the second RNA Extraction.

3.4. Experimental design of viral inactivation by UV exposure

A homogeneous volume of each virus strain was pipetted in a six well sterile cell culture plate (SPL Cell Culture Plate). The plate was exposed to a 254 nm UV Lamp and a power of 8W (Vilber Lourmat® 4.C) fixed at a height of 10 cm to the liquid layer to be inactivated. At 0 seconds, 30 seconds, 2 minutes, 5 minutes and 10 minutes, 100 µl of the irradiated viral suspension were withdrawn. At the last time point (10 minutes) all of the remaining volume was collected. This procedure was repeated twice for each virus strain, except for the 0 time point that was used for both repetitions. The obtained samples were kept in the -75 ºC ultra-freezer until further titration.

3.5. Titration in MDCK (Madin-Darby canine kidney) cells

MDCK cells were prepared in 96 well plates (SPL Life sciences Tissue Culture Test Plate) 24 or 48 hours before titration, following internal standard protocols. Briefly, cell monolayer was separated by the action of trypsin, and cells were suspended in 10% fetal bovine serum culture media to achieve a final concentration of 2 x 10-6 cells / ml. This solution was homogenized by gentle pipetting and then mixed with BioWhittaker® Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech Inc., Manassas, VA, USA) supplemented with 2 mM L-Glutamine and 100 UI/100 ug/ml of Penicillin + Streptomycin. To seed the microplates 100 µl of the aforementioned cell suspension was spiked in each well. Each plate was fully identified and incubated in a HERA cell CO_2 incubator at 37.0 ± 1.5 ºC. After 24 or 48 hours, the cell growth was checked with the Nikon Eclipse T5100 inverted microscope to see if confluent monolayers were achieved; titration was only performed if this criteria was met. Before titration, plates were washed for at least 10 minutes with PBS.

Initial inoculum as well as UV-treated samples at the different time points (0 seconds, 30 seconds, 2 minutes, 5 minutes and 10 minutes) were taken from the -75 ºC ultra-freezer and were left to defrost at room temperature inside a BCS. Microtubes containing 450 µl of sterile PBS supplemented with 10 µg/ml trypsin from porcine pancreas type IX (Sigma Chemical Co., St Louis, MO, USA) were prepared. Dilutions of samples for titration were done (from 10^{-1} to 10^{-6}, depending on the on-going results) and incubated in the cell CO_2 incubator at 37.0 ± 1.5 ºC for 30 minutes, for the trypsin to enhance virus infection. To proceed to titrate each sample, 20 µl of the previously prepared dilution, were inoculated at each well (from the most diluted to the original sample). The plates were left to incubate in a CO_2 incubator at 37ºC for 60 minutes. After incubation, 150 µl of p.i. media DMEM Lonza supplemented with Glutamine and Penicillin+Streptomycin and containing 1 µg/ml of trypsin from porcine pancreas type IX (Sigma Chemical Co., St Louis, MO, USA) was also added to each well. The plates were incubated in the CO_2 incubator at 37 ± 1.5 ºC for no more than 10 days p.i.

As a current schedule, the first reading of microplates was done 5 days and the final reading at 7 days p.i. A Nikon inverted microscope was used at a 40x magnification to read the cytopathic effect (CPE) on the cells. The results were recorded and the $\log_{10}$ $TCID_{50}$ was calculated following the formula shown below:

$$\log_{10} TCID_{50} = 1_r + 1/2d - d \Sigma p_i$$

Where:

1_r = logarithm of the highest dilution that has 100% cytopathic effect, this means, destruction of all of the replicas.

d = logarithm of the dilution ratio (in decimal dilutions it will always be 1)

p_i = positive response to dilution "i".

This equation was applicable when throughout the dilution series we find a range of infectivity from 100% to 0%. When there was no 0% associated to the dilution, the next dilution was given the value of 0%.

4. Results

4.1. Influenza virus propagation in SPF chicken eggs

All viruses replicated in SPF eggs. Egg mortality varied among the different viruses and dilutions. It was higher in eggs inoculated with higher dilutions of avH1N1 and avH4N6 (Table 1). At 48 hours p.i. , eggs were candled and 2 eggs were not viable, one from avH1N1 at 10^{-3} dilution and one from swH1N1 at 10^{-1}. In the case of huH1N1, 2 eggs of each dilution were left until 96 hours, they were still viable after this time. In general, a volume of 5-7 ml of a slightly yellowish allantoic fluid was obtained from each egg.

	Viruses			
Dilution	***avH1N1***	***avH4N6***	***swH1N1***	***huH1N1***
10^{-1}	80%	100%	50%	0%
10^{-2}	60%	50%	80%	0%
10^{-3}	50%	*nd*	*nd*	0%

Table 1. *Accumulated mortality of embryonated SPF eggs observed with different dilutions of each viral strain. eggs. nd: not determined.*

Regarding viral loads, no major differences were observed in the different dilutions and at different hours p.i. for each viral strain, neither between viable and non-viable eggs.

Differences in viral loads were observed when comparing the different viral strains (Table 2). The results of qRT-PCR were expressed as Threshold Cycle (Ct) values used to identify the amounts of target nucleic acid. Lower Ct values indicate high amounts of the target RNA while higher Ct values mean lower amounts of target RNA.

	Viruses			
Dilution	***avH1N1***	***avH4N6***	***swH1N1***	***huH1N1***
10^{-1}	15	20	18.5	28
10^{-2}	15	19	18	24.5
10^{-3}	15	*nd*	*nd*	28

Table 2. *Means of Ct values from viable and non-viable inoculated SPF eggs at different hours p.i. nd: not determined.*

Finally, viral loads (Ct) for each virus strain pool to be used in the inactivation study were: avH1N1= 16; avH4N6= 22; swH1N1= 20; and huH1N1= 31.

4.2. Inactivation kinetics

All viruses were able to infect the confluent monolayer MDCK cells in the presence of trypsin, with significantly different capacities. AvH1N1 and swH1N1 and avH4N6 infection caused a complete MDCK cell monolayer destruction; instead, huH1N1 pandemic virus infection produced only small foci of infected cells. The first titration of each virus was done with the aliquot of the frozen samples to calculate the starting $\log_{10}$ $TCID_{50}$**.** No remarkable differences in terms of viral titre were observed among the four different influenza virus, with a range of means from 5.5 ± 0.7 (avH4N6) to 6.8 ± 0.3 (avH1N1) (Table 3).

Viruses

	avH1N1	*avH4N6*	*swH1N1*	*huH1N1*
Titration	7.0 ± 0.3; 6.6 ± 0.4	5.0 ± 0.3; 6.0 ± 0.4	6.2 ± 0.7; 5.6 ± 0.5	6.3 ± 0.6; 5.8 ± 0.6
Mean	6.8 ± 0.3	5.5 ± 0.7	5.9 ±0.4	6.1 ± 0.4

Table 3. *log_{10} $TCID_{50}$ per ml values of Avian-Pandemic-Swine H1N1 and avH4N6 viruses in initial titration.*

Detection limit was established considering the dilution without any positive wells in a range between 0 and 1.8 log_{10} $TCID_{50}$. Value of 1.8 was calculated considering the inoculated volume for each wells respect 1 ml. Thus, in this study, the limit of detection for the microtitre plate assay was 1.8 log_{10} $TCID_{50}$. For statistical and data analysis the working value was 1.8 considering the worst effect of the treatment at this situation.

The Residual Factor (RF) values were calculated to reveal the difference between the starting dose at time 0 from the calculated dose at each time point. All the inactivation kinetics reveal a decreasing in $log_{10}TCID_{50}$, shown by the RF values that increase proportionally to the time of exposure (Table 4). The most sensitive viruses to the UV-C treatment were swH1N1 and huH1N1. They started to be sensitive yet after 30 sec of exposure; and also at the last point time (10 min.) they showed a major value of RF, representing a loss of more of the half initial concentration of each virus.

RF	***30 sec.***	***2 min.***	***5 min.***	***10 min.***
avH1N1	0.1	0	2.3	2.6
avH4N6	0	1.4	2.6	2.6
swH1N1	1.3	1.7	3.3	3.2
huH1N1	1.0	2.0	2.7	3.5

Table 4. *Reduction factor (RF) values sorted in relation between viruses and time of exposure.*

The linear regressions showed that the general tendency of the variable of the mean and the time of each experiment were in an inverse correlation as shown on Figures 1 to 5. Negative results of the slope of the linear regression were clearly shown by each inactivation kinetics graph of each virus. This is numerically reported with their negative slope values: -0.0049 for avH1N1; -0.046 for avH4N6 virus; -0.047 for swH1N1; and -0.0051 for huH1N1. Other calculated data of the linear regression were the coefficient of determination "R^2" and the intercept value. The R^2 calculated values were: 0.84 for avH1N1; 0.76 for avH4N6; 0.72 for swH1N1; and 0.84 huH1N1. Estimated intercept values were: 5 for avH1N1; 4.7 for avH4N6; 4.4 for swH1N1; and 4.8 for huH1N1 (Fig.1 to 5).

To complete the comparison of the different effects of UV radiation between viruses, a Kruskal-Wallis test was effectuated using the slope values. The selected alpha value was of 0.05 and the resulting p-value was 0.39, so there were no statistically significant differences among the viruses.

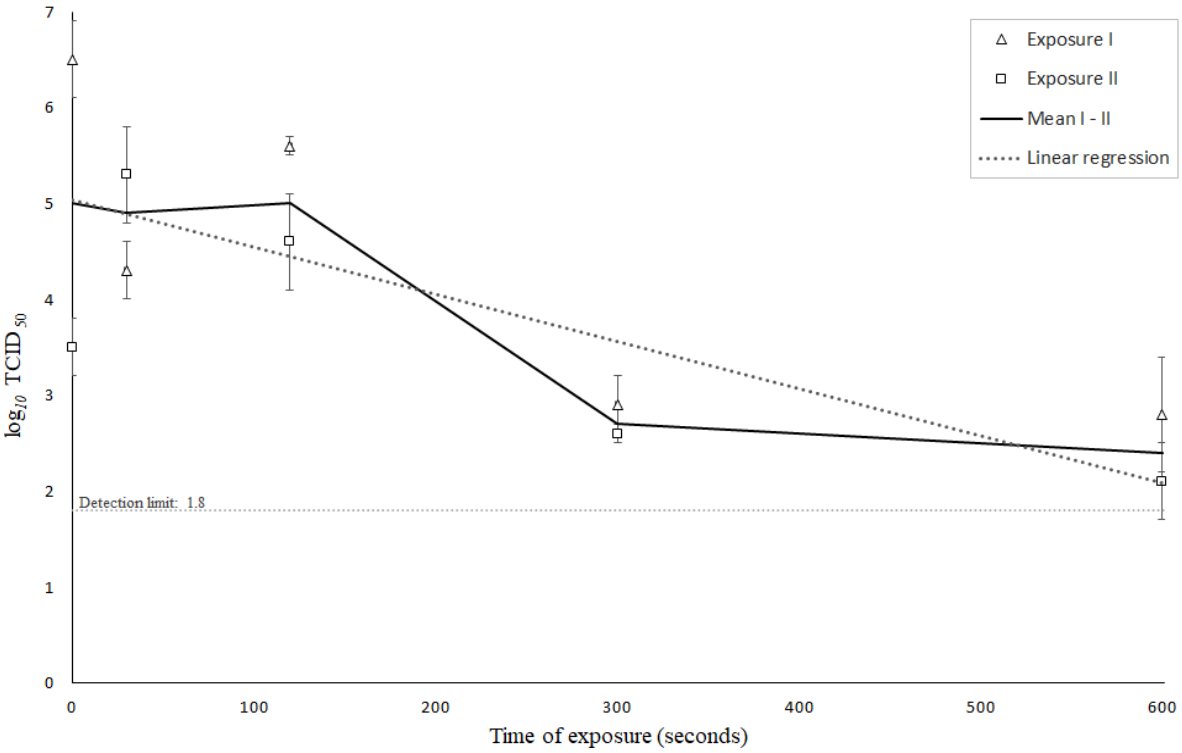

Figure 1. *avH1N1 virus: Kinetic and linear regression of log_{10} $TCID_{50}$ per ml values at different times of exposure.*

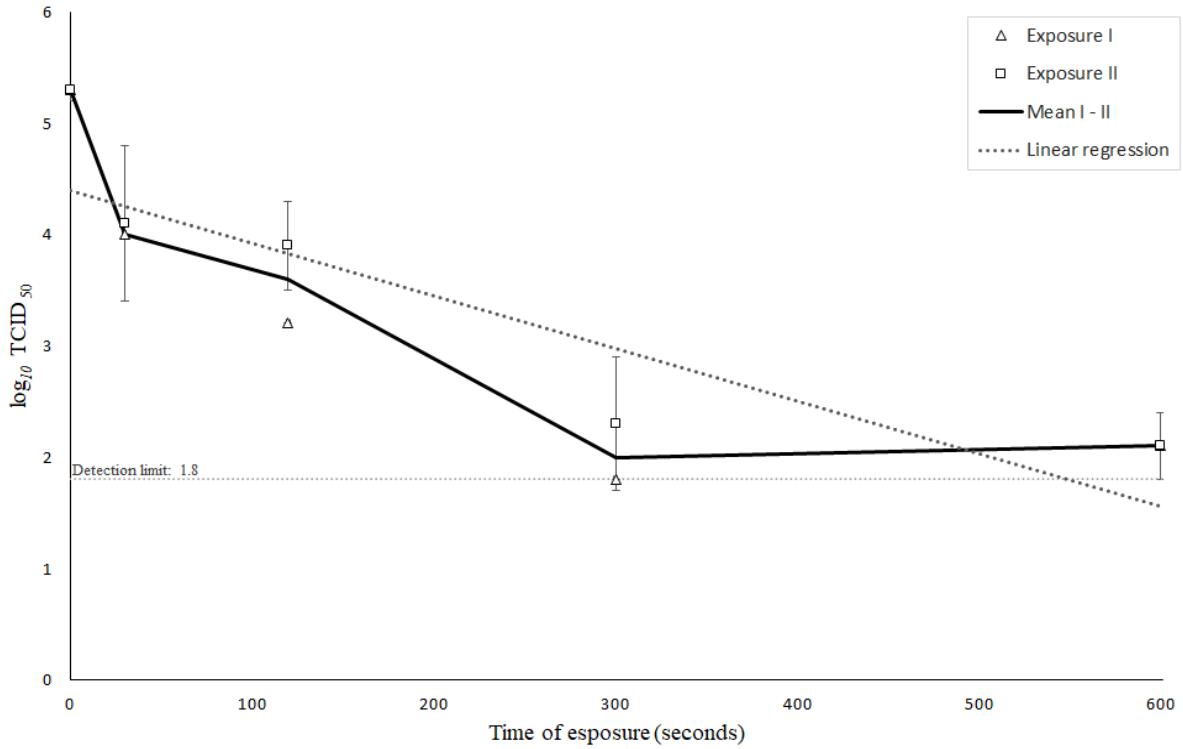

Figure 2. *swH1N1 virus: Kinetic and linear regression of log_{10} $TCID_{50}$ per ml values at different time of exposure.*

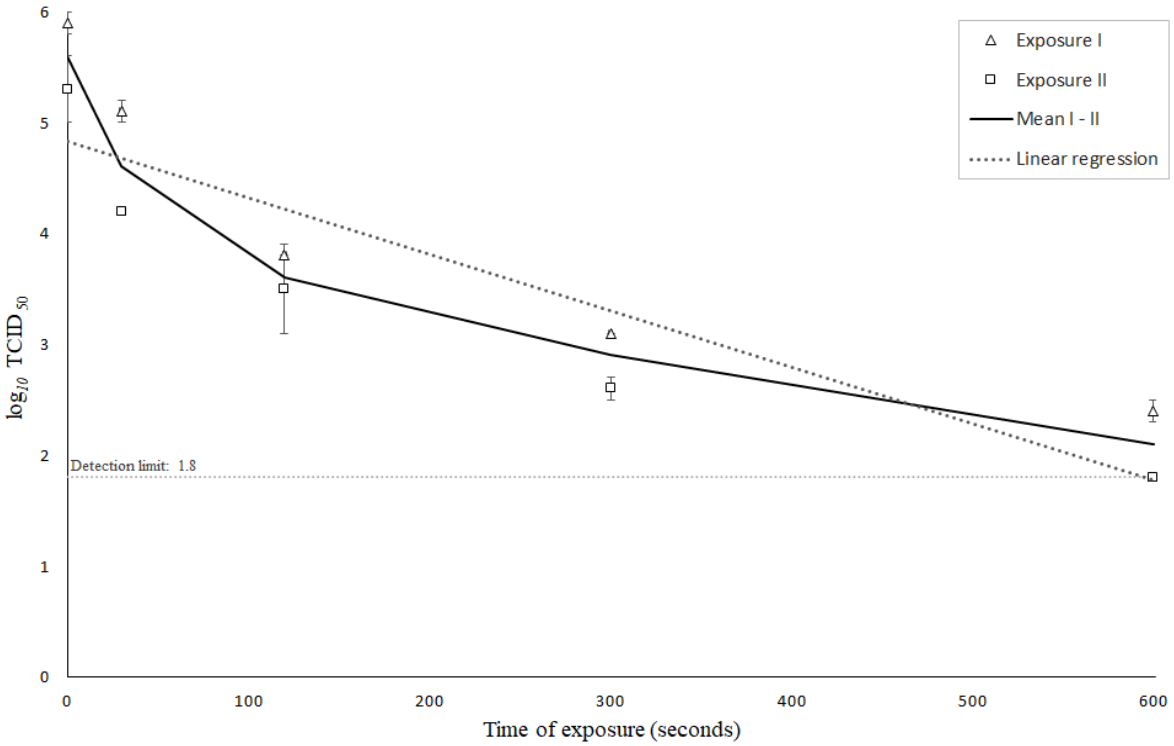

Figure 3. *huH1N1 virus: kinetic and linear regression of log_{10} $TCID_{50}$ per ml values at different times of exposure.*

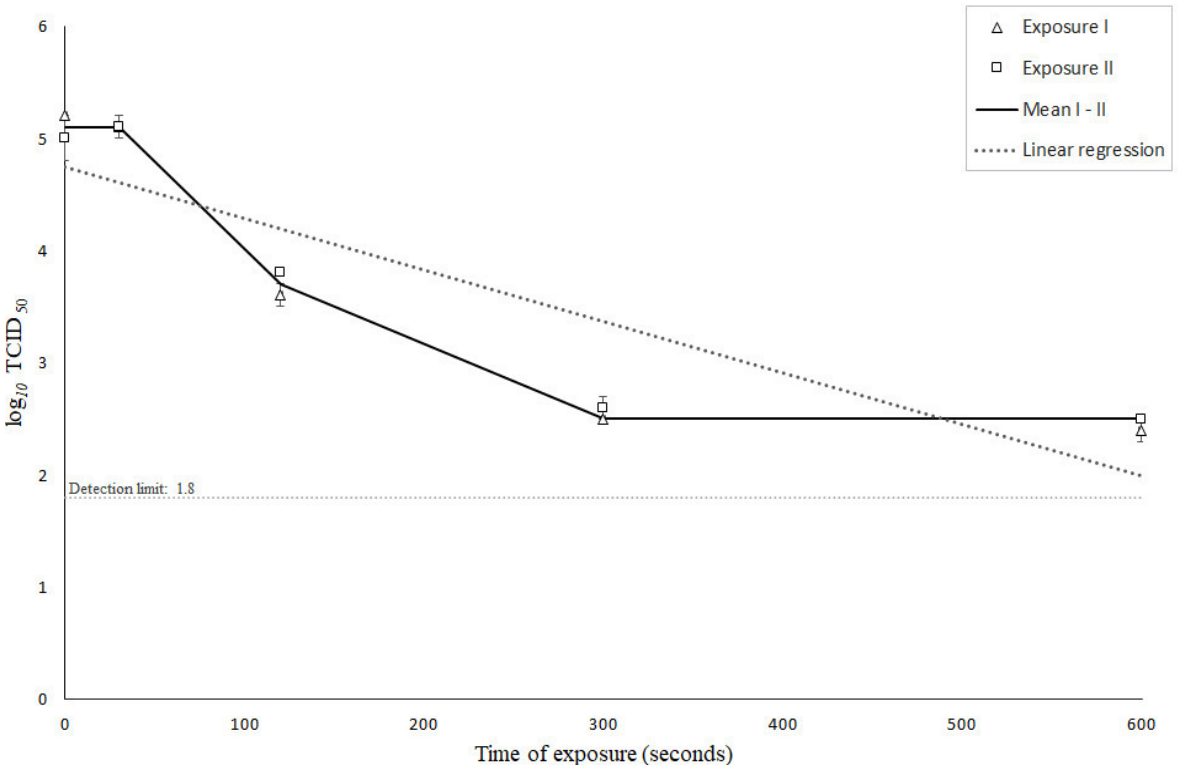

Figure 4. *avH4N6 virus: kinetic and linear regression of log_{10} $TCID_{50}$ per ml values at different times of exposure.*

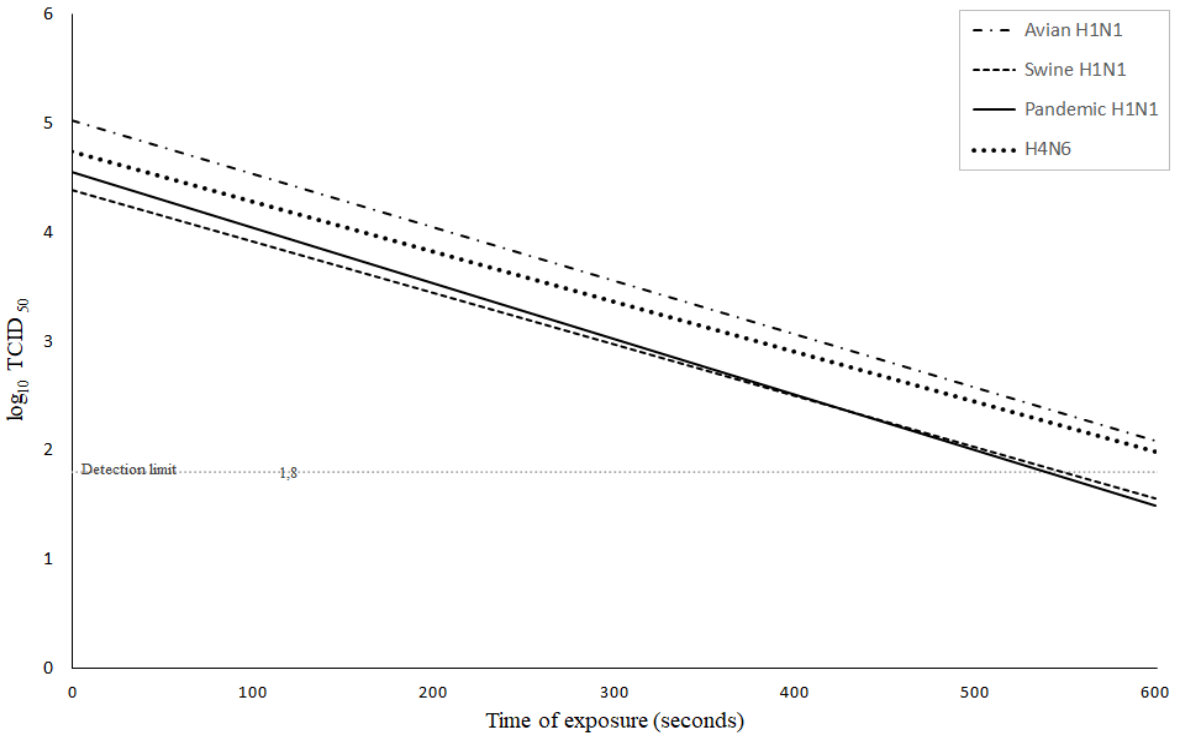

Figure 5. *Comparison between the linear regression of each virus.*

6. Discussion

When working with highly pathogenic influenza viruses in biocontainment facilities, one of the main challenges is the inactivation of samples for further studies in biocontainment or out of biocontainment. To do so, several systems are available, some are physical and others chemical. The choice among them will be driven by the final destination of the sample. For immunological studies, the target should be the nucleic acid, allowing proteins to keep these natural conformations while for molecular biology studies (as PCRs) the target will be the proteins and lipid envelopes. In both cases, it should be assessed if all these processes are able to completely inactivate infectivity from the samples as they will be handled in less stringent biosafety conditions (X. Abad, personal communication, July 2, 2018).

As UV treatment acts on nucleic acids, it has been used thoroughly in research centres as a way to inactivate viral suspensions keeping immunological properties intact (Schmidt & Kauling, 2007; Turnbull *et al*., 2008). However, when working with highly pathogenic viruses, in BSL-3 containment, the need to assure that this procedure gives completely inactivated viral suspensions is more evident, taking into account that sometimes such inactivations are done to transfer the samples outside for further processing (Abad, 2012). The establishment of certain guidelines and protocols for viral inactivation with good and reliable efficacy studies, and the creation of a bank of potential viruses to be tested, would be highly advisable (Abad, 2012).

For biological, sanitary and economic reasons, studying the most pathogenic viruses directly, is not always possible. Therefore, taking into account the similarities of biological and physical characteristics of viruses, it is useful to work in parallel using similar, less pathogenic viruses as surrogates. This is a common practice used in laboratories as showed by the studies of Steinmann (2004), Casanova & Weaver (2015) and Takada & co-workers (1999).

In our study, we have worked with four different influenza viruses isolated from the three main hosts of these viruses: birds, pigs and humans. This gives a more “One health” approach to the study and at the same time tries to cope with viral diversity.

The results on initial viral replication and viral loads in our study showed many differences, especially between avian and human pandemic virus. This event could have occurred because of the facility of the virus to replicate in the same species of provenience of the

cells. Furthermore, the different CPE in MDCK cells could suggest that pandemic viruses possesses limitations in this cell line, with no particular predilection for this cell line to replicate. This characteristic of different tropism of pandemic influenza viruses has been previously described (Ilyushina *et al*., 2012).

There are not many available studies specifically focused on resistance of Influenza A viruses to UV-C rays. Moreover, in most of these studies, the results are based on the "*UV dose (mJ/cm²)*" that represents the intensity or potency of the lamp on the sample for a given time (Cutler *et al*., 2011; Jensen, 1964). Indeed, the results are shown as rate of inactivation, named *"k"*, which is calculated based on the "*UV dose*". The rate of inactivation is sensitive to numerous secondary factors, such as: type of microorganism, composition of suspending medium, temperature, humidity and depth of the irradiated solution (Cutler *et al*., 2011; Jensen, 1964; Kowalski *et al*., 2000). Unfortunately, in the present experiment the evaluation of the intensity value of the lamp during the inactivation trial was not possible, making it difficult to compare the numerical results.

Regarding the exposure to ultraviolet light in our work, all viruses showed an inverse correlation between the infectivity values (expressed as mean of $\log_{10}$ TCD_{50} of survival viruses) and exposure times, as it is demonstrated with the negative slope values from 0 to -1 of the linear regressions. So, any increase in the UV-C irradiation time corresponds to a decrease of viral infectivity, obtaining at the last time point (10min.) an inactivation of approximately more of half of the initial doses of the viruses. This is a typical representation of an inactivation model of many other germicidal methods (Cutler & Zimmerman, 2011; Kowalski *et al*., 2000), confirming the lethal effect of the UV-C on the viruses (Cutler *et al*., 2011; Harris *et al*., 1987; Jensen, 1964; Schmidt & Kauling, 2007).

The inactivation model was first studied by Hiatt (1964) that described the rapidity of decay by the rate of inactivation "k" which expresses algebraically the relationship between concentration and time. This model was called the first order one-stage model and its principal limitation was that it considered the sample as a homogenous sample. Later on, other authors observed that many inactivation reactions did not follow this one-stage model, probably due to the fact that not all samples are homogeneous or for other reasons. Based on this, a two-stage inactivation model with two different phases with respectively different rates of inactivation was proposed (Cutler *et al*., 2011; Thurston-Enriquez *et al*., 2003). Nevertheless, the two-stage approach has not been widely used to describe the kinetics of microbial inactivation and most of the studies still base the results on the one stage model

(Cutler *et al*., 2011). A clear example of the limitation of the one-stage model is given by what we found in our results. In the initial inactivation, 3 out of 4 viruses showed around 2 $\log_{10}$ inactivation at 2 minutes of UV-C treatment with best values of RF for swH1N1 and huH1N1. Following this linear process it would be expected that at 10 minutes the treatment would be able to totally inactivate the infectivity of the assayed viruses (as their viral titers slightly surpass 6 $\log_{10}$ $TCID_{50}$). However, for 4 out of 4 assayed viruses residual infectivity was detected in final samples, a bit major of the detection limit value ($\leqslant$1,8). Moreover, the measure of goodness of the linear regression was important to perform by the evaluation of R^2 values, for each kinetic decay equation. Best values were calculated for avH1N1 and huH1N1 kinetics, for these viruses we can affirm that the one-stage inactivation model is more representative respect to swH1N1 and avH4N6 viruses in which a two stages model decay could be supposed. So, we can conclude that generally in influenza A viruses, the one-phase model does not fit perfectly to the calculated data of doses. This evaluation strengthens Cutler's theories of inactivation studied with only one strain of influenza virus.

The difference in velocity of decay observed among the four different viruses is a common problem in kinetics of inactivation studies effectuated with UV-C treatment. This event could be related to the fact that a homogenous sample was not used and also to the possibility of clumping, dormancy or other factors (Thurston-Enriquez *et al*., 2003; Kowalski *et al*., 2000).

The results of the Kruskal-Wallis test give us two important pieces of information to take into consideration. One is the fact that there are not differences between the response of each virus to the UV-C treatment, confirming the theory that though from different origins, they maintain similar biological and physical characteristics (Thomas & Swayne, 2007). Secondly, is that we could combine the results of each virus as a single result, obtaining a generic result for inactivation response for a generic influenza type A virus. This data could also contribute to adding more information in the response to physical treatment with UV-C rays, extending the results to the viruses with the same genetic characteristics (ss-RNA and 13.6 kb).

Finally, we can conclude that the inactivation treatment tested in this laboratory conditions was not able to inactivate the totality of the viruses in the samples. Although viral infectivity was clearly diminished, infectious viruses still remain in the samples. So, we can conclude that further assays should be performed, increasing exposure time, to ensure that such viral suspensions are really safe to be handled in lower biosafety levels.

7. Conclusions

With this study of the inactivation of four different Influenza A type viruses at five different time points, we can conclude that:

- The inactivation treatment with UV-C (254 nm) is germicidal for the four influenza viruses tested (AvH1N1, avH4N6, swH1N1 and huH1N1).
- The inactivation kinetics of the treatment with UV-C (254 nm) does not differ among the four influenza viruses used in the present study and follows a first-order reduction tendency, with differences in one or two-stage reduction.
- The inactivation treatment with UV-C (254 nm) was not able to totally inactivate the viruses in the samples after 10 minutes of exposure.

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8. Anexos

avH1N1	*0*	*30 sec*	*2 min*	*5 min*	*10 min*
Exp. I	6.2 ± 0.0; 6.8 ± 0.3	4.3 ± 0.3	5.5 ± 0.3; 5.7 ± 0.4	3.1 ± 0.2; 2.7 ± 0.4	2.3 ± 0.3; 3.2 ± 0.0
Exp. II	3.7 ± 0.4; 3.3 ± 0.2	5.5 ± 0.3; 5.0 ± 0.3	4.9 ± 0.4; 4.2 ± 0.5	2.6 ± 0.4; 2.5 ± 0.3	2.3 ± 0.3; 1.8
Mean I	6.5 ± 0.4	4.3 ± 0.3	5.6 ± 0.1	2.9 ± 0.3	2.8 ± 0.6
Mean II	3.5 ± 0.3	5.3 ± 0.5	4.6 ± 0.5	2.6 ± 0.1	2.1 ± 0.4
Total Mean	5.0 ± 2.7	4.9 ± 0.9	5.0 ± 0.9	2.7 ± 0.4	2.4 ± 0.9

Table 4. *Single and mean values of log_{10} $TCID_{50}$ per ml of avH1N1 virus inactivation treatment. Classification sorted by titration, time of exposure.*

swH1N1	*0*	*30 sec*	*2 min*	*5 min*	*10 min*
Exp. I	5.3 ± 0.2; 5.2 ± 0.3	3.5 ± 0.3; 4.3 ± 0.2	3.2 ± 0.5; 3.2 ± 0.0	1.8; 1.8	2.3 ± 0.3; 1.8
Exp. II	5.3 ± 0.5	3.6 ± 0.4; 4.6 ± 0.4	3.8 ± 0.4; 4.0 ± 0.3	1.8; 2.7 ± 0.4	2.3 ± 0.3; 1.8
Mean I	5.3 ± 0.1	4 ± 0.6	3.2 ± 0	1.8	2.1 ± 0.3
Mean II	5.3 ± 0.0	4.1 ± 0.7	3.9 ± 0.4	2.3 ± 0.6	2.1 ± 0.3
Total Mean	5.3 ± 0.1	4.0 ± 0.8	3.6 ± 0.6	2.0 ± 0.6	2.1 ± 0.5

Table 6. *Single and mean values of $log_{10}TCID_{50}$ per ml of swH1N1 virus inactivation treatment. Classification sorted by titration, time of exposure. RF value calculated for each time of exposure.*

huH1N1	*0*	*30 sec*	*2 min*	*5 min*	*10 min*
Exp. I	5.8 ± 0.4; 6.0 ± 0.5	5.0 ± 0.4; 5.1 ± 0.5	3.8 ± 0.4; 3.8 ± 0.4	3.1 ± 0.5; 3.1 ± 0.5	2.5 ± 0.3; 2.3 ± 0.3
Exp. II	5.5 ± 0.5; 5.1 ± 0.5	4.2 ± 0.5; 4.2 ± 0.5	3.7 ± 0.4; 3.2 ± 0.0	2.7 ± 0.4; 2.5 ± 0.3	1.8; 1.8
Mean I	5.9 ± 0.1	5.1 ± 0.1	3.8 ± 0.0	3.1 ± 0.0	2.4 ± 0.1
Mean II	5.3 ± 0.3	4.2 ± 0.0	3.5 ± 0.4	2.6 ± 0.1	1.8 ± 0.0
Total Mean	5.6 ± 0.5	4.6 ± 0.8	3.6 ± 0.4	2.9 ± 0.4	2.1 ± 0.6

Table 5. *Single and mean values of $log_{10}TCID_{50}$ per ml of huH1N1 virus. Classification sorted by titration, time of exposure.*

avH4N6	*0*	*30 sec*	*2 min*	*5min*	*10min*
Exp. I	5.2; 5.2 ± 0.6	5.2 ± 0.5; 5.0 ± 0.4	3.6 ± 0.4; 3.5 ± 0.3	2.5 ± 0.3; 2.5 ± 0.3	2.3 ± 0.5; 2.5 ± 0.3
Exp. II	5.1 ± 0.4; 4.8 ± 0.4	5.0 ± 0.4; 5.2 ± 0.5	3.8 ± 0.4; 3.8 ± 0.4	2.6 ± 0.4; 2.5 ± 0.3	2.5 ± 0.3; 2.5 ± 0.3
Mean I	5.2 ± 0.0	5.1 ± 0.1	3.6 ± 0.1	2.5 ± 0.0	2.4 ± 0.1
Mean II	5.0 ± 0.2	5.1 ± 0.1	3.8 ± 0.0	2.6 ± 0.1	2.5 ± 0.0
Total Mean	5.1 ± 0.2	5.1 ± 0.2	3.7 ± 0.2	2.5 ± 0.1	2.5 ± 0.2

Table 7. *Single and mean values of $log_{10}TCID_{50}$ per ml of avH4N6 virus. Classification sorted by titration, time of exposure.*