Thermal inactivation of avian and swine H1N1 Influenza viruses

Official Master's Degree in Zoonoses and One Health

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Universitat Autònoma de Barcelona

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Index

1. Abstract

Influenza is a single stranded RNA virus that belongs to the Orthomyxoviridae family, influenza virus type A is responsible for the pandemics that have occurred in the past years. Influenza A are very complex and wide range viruses as they can occasionally jump between species. Improper disinfection can lead to a public health risk of a new influenza outbreak, for this reason it is important that correct sanitation processes are included in the control of these viruses. Viral inactivation is also used to remove infectious and suspicious samples from biosafety units and proper protocols for this are needed. This investigation aims to determine differences between two diferent influenza virus strains regarding their heat inactivation using different conditions of temperature and time. In this study a H1N1 influenza strain from avian origin and a H1N1 influenza strain from swine origin were used. Each virus was exposed to two different temperatures, 56 and 70 ºC, for five different time points (1 min, 5 min, 10 min and 15 min) using a dry bath. The results show that at 70 ºC both Swine and Avian H1N1 influenza strains were completely inactivated after 5 minutes. At 56 ºC Swine influenza virus H1N1 reached inactivation after 15 minutes but Avian influenza virus H1N1 did not reach inactivation. We could also determine that higher temperature increases the virus inactivation rate, when comparing 56 and 70 ºC. Both viruses at both temperatures were seen to follow a mono-phasic reduction tendency.

2. Introduction

Influenza is a single stranded RNA virus that belongs to the Orthomyxoviridae family (Treanor, 2014). There are four types of influenza viruses: A, B, C and D (CDC, 2017), however, only influenza A viruses cause pandemics (Monto & Webster, 2013). Even though influenza A viruses can affect an extended variety of hosts, including pigs, humans, horses, dogs and cats (Fouchier & Guan, 2013), aquatic birds are the natural hosts, and are thought to be the reservoirs of influenza A viruses. Effective and applicable tactics to control these viruses are still not clear, even after many years of research (Treanor, 2014). Influenza A viruses are divided into subtypes on the basis of two proteins on the surface of the virus: hemagglutinin (HA) and neuraminidase (NA). There are 18 known HA subtypes and 11 known NA subtypes. Avian influenza A viruses are designated as highly pathogenic avian influenza virus (HPAIV) or low pathogenicity avian influenza virus (LPAIV) based on their molecular characteristics and their ability to cause disease and mortality in chickens in a laboratory setting (CDC, 2017).

Almost all subtypes of influenza A virus can cause disease in susceptible birds (CDC, 2017). The disease usually has a low mortality rate and presents with minor respiratory symptoms (Benedictis et al., 2007). These birds can transmit the virus to domestic poultry and this can cause severe outbreaks, as it has been seen with H1N1, H2N2 and H2N2 subtypes (Treanor, 2014). All known influenza A viruses found in mammals and terrestrial birds have originated, either directly or indirectly, from waterfowl viruses. In swine, influenza does not have a great impact on the production but it has a potential impact on the global trade of pork meat, because of the public fear of swine influenza infecting people (Jernigan & Cox, 2013). Antigenic drifts and virus reassortment can be the cause of epidemics and even pandemics of influenza virus (Potter, 2001). Influenza A is a very complex and wide range virus as it can occasionally jump between species (Jernigan & Cox, 2013). It has been shown that avian influenza viruses are capable of infecting human hosts (Benedictis et al., 2007), this means they pose a public health risk that can be even more severe because of the current world globalization. From the human health point of view, patients can have acute respiratory symptoms, high fever, headache, nausea, malaise and myalgia. Severe cases are associated with secondary contamination with other viruses or bacteria (Klenk & Garten, 2013).

The correct application of biosafety measures is considered fundamental for the control of the introduction of influenza viruses (EFSA, 2005). Biosafety levels (BSLs) describe the correct practices, control measures and safety procedures required for a laboratory, it also states the biological agents which can be safely handled within the aforementioned laboratory. There are 4 levels of biosafety, BSL-1 are teaching and training labs, BSL-2 contain moderate risk agents, BSL-3 contain agents that are known to cause severe to lethal infections in humans or animals but poor or moderate spreading capability to the community and BSL-4 contain agents that pose a higher risk for fatality and that have no effective immunization therapies (Shurtleff et al., 2012). Bio-exclusion is an element of biosafety that describes the prevention actions taken to exclude biological agents from uninfected locations (EFSA, 2005). Control and containment measures are implemented to protect researchers and technicians and to ensure biological agents are not released to the exterior, this could potentially be a risk to the environment and to global public health (Berns, 2014). Bio-containment is another element of biosecurity that describes the prevention of biological agents spreading from an infected location. These measures aim to prevent secondary spread of the biological agents, and disinfection plays an important role in this process (EFSA, 2005). Inactivation through heat, radiation and chemical procedures are currently used to remove infectious and suspicious samples from these bio-contained units (Shurtleff et al., 2012).

Influenza A virus is generally known to transmit by airborne (Klenk & Garten, 2013) and waterborne routes (Sooryanarain & Elankumaran, 2015). Moreover, secondary transmission can occur through various routes, including many human activities. Vehicles, equipment, movement of staff and the restocking of animals after an influenza event without the proper disinfection (Benedictis et al., 2007; Sooryanarain & Elankumaran, 2015), can all lead to a public health risk of a new influenza outbreak, for this reason it is important that correct sanitation processes are included in the control of this virus (Benedictis et al., 2007). Physical and chemical characteristics of influenza viruses are very similar, therefore thermal sensitivity among them are not anticipated to be very variable (Thomas & Swayne, 2007). Different strains of influenzas viruses are expected to behave in the same way because of their similar properties (Swayne, 2006).

During the last 15 years, there have been many biosafety breach events (Henkel et al., 2012; Weiss et al., 2015). The most important recent incidents have occurred in the United States and include the mishandling of *Bacillus anthracis* by the Center for Disease Prevention and Control (CDC),

the cross contamination of LPAIV with HPAIV in the National Institutes of Health (NIH) and the lack of inventory follow-up of vials including some from variola virus by the Food and Drug Administration (FDA) (Weiss et al., 2015). Consequently, special attention should be given to implementing effective inactivation protocols. In the case of outbreaks of influenza in farms, a further decontamination of the premises is required to be able to safely reuse the facilities, the processes that are usually performed can be very costly and the use of heating or thermal inactivation of the virus could be a good option (Stephens & Spackman, 2017).

Temperature, or thermal treatment, is one of the methods applied in food industries because it is one of the main factors determining virus inactivation in the environment (Bertrand et al., 2012). High temperature inactivation includes pasteurization and dry heat methods. These treatments destabilize the intermolecular interactions between virus capsid proteins and this means the virus loses its integrity and infectivity. These processes are irreversible (Boschetti et al., 2004). Heat inactivation has a considerable stronger effect on virus infectivity than on viral genome integrity (Baert et al., 2008).

Avian influenza virus (AIV) is an enveloped virus, heat treatment has been used on other enveloped viruses as flaviviruses. Infectivity of Alkhumra hemorrhagic fever virus was completely lost (reduction of 7.8 $\log_{10}$) when heating at 60 °C for 3 minutes and at 56 °C for 30 minutes (Madani et al., 2014). Yellow fever virus was completely inactivated by heating at 60 °C for 5 minutes and at 56 °C for 30 minutes (Remington et al., 2004). For Hepatitis C virus, 10 min at 60 °C or 4 min at 65 °C was sufficient to achieve a reduction of 4 $\log_{10}$, but incubation for 40 min at 56 °C was required to achieve this same reduction (Song et al., 2010). An example of a non-enveloped virus is polyomavirus, heating in solution at temperatures below 60 ºC, for periods of time as long as 30 min, has little effect on infectivity. Partial inactivation, 3 $\log_{10}$, of mouse polyomavirus has been observed at 65 ºC for 30 min, while increasing the temperature to 70 ºC for the same amount of time gives a 6 $\log_{10}$ reduction (Brodsky et al., 1959). Another example is Hepatitis A Virus (HAV), this virus required longer exposure to heat than Murine Norovirus 1 for complete inactivation in soft-shell clams, complete inactivation (reduction of 5.47 $\log_{10}$) of HAV was obtained with a treatment of 90 ºC for 180 seconds (Sow et al., 2011).

In the case of AIV, to inactivate HPAIV and reduce the viral titer in 5 $\log_{10}$ in fat free eggs, 93 minutes were needed at 55 ºC, meanwhile at 57 ºC and 58 ºC, only 13 and 2 minutes were needed respectively (Chmielewski et al., 2011). It has been seen that 2.7 minutes at 55.6 ºC and 3 minutes at 56.7 ºC gives a reduction of 4.9 $\log_{10}$ (Swayne & Beck, 2004). Chmielewski and co-workers (2011), found that to inactivate 5 $\log_{10}$ of LPAIV, 5 minutes at 55 ºC were needed, but it took less than 2 minutes to achieve complete inactivation at 58 and 59 ºC in fat free eggs. For a strain of H1N1, after exposure to 56 ºC for 30 minutes a 2 $\log_{10}$ reduction was achieved and at 73 ºC it took 30 seconds for the same reduction to be obtained (Tuladhar et al., 2012). All these references suggest that temperature has a strong influence on AIV survival; a slight change of few Celcius degrees could have a big effect on their inactivation times.

In this study a H1N1 strain of influenza virus from avian origin and a H1N1 strain from swine origin were exposed to 56 and 70 ° C for 0 to 15 minutes. With the collected information we will be able to expand the knowledge on the inactivation of influenza viruses and help with this global public health issue.

3. Objectives

3.1 General objective

This investigation aims to determine differences between two diferent influenza viruses regarding their heat inactivation using different conditions of temperature and time.

3.2 Specific objectives

- To determine if there is a diference between a H1N1 influenza virus from avian origin and a H1N1 influenza virus from swine origin, regarding heat treatment.
- To compare the differences that may exist between a H1N1 influenza virus from avian origin and a H1N1 influenza virus from swine origin, regarding temperature and time.
- To determine the kinetics of inactivation as mainly bi-phasic or mono-phasic.

4. Materials and methods

4.1 Viruses

1) Avian H1N1: A/Duck/Italy/281904/06 H1N1
2) Swine H1N1: A/Swine/Spain/SF11131/2007 H1N1

4.2 Influenza virus propagation in embryonated chicken eggs

4.2.1 Specific pathogen free (SPF) eggs

SPF eggs were incubated (Masalles egg incubator*)* at 37.5 °C (35-39 °C) and 45% relative humidity (RH) with an automatic egg turner for the first 6 days in a BSL-2 laboratory at the "*Animal Health Research Centre* (from now CReSA)". After 6 days of incubation, the eggs were removed from the incubator and they were examined with an egg candler, one at a time, against a bright light in a dark room to check for infertility. Thin blood vessels leading to a bean-shaped embryo were clearly visible in viable eggs. Unfertilized eggs were discarded and the viable eggs were return to the incubator. Eggs were not left outside of the incubator for more than 30 min.

4.2.2 Dilution method

Working in a Class II Biological Safety Cabinet, the virus stocks were tenfold diluted using 900 µl sterile BioWhittaker® Phospate Buffered Saline (PBS), 0,0067 M (PO_4) without Ca and Mg, Lonza #BE17-516F, and 100 µl of each virus at the starting dilution in Eppendorf microtubes. At all-time, all viral dilutions were kept on ice. The dilutions assayed were 10^{-1}, 10^{-2}, 10^{-3} for Avian H1N1 and 10^{-1} and 10^{-2} for Swine H1N1.

4.2.3 Influenza inoculation in eggs.

The viral propagation in eggs by inoculation through the allantoic cavity or chorion allantoic sac (SAC) was performed using internal SOP (IT-A4-EVI 215 (Ex PE ML 015)). Briefly, on the day of virus inoculation (day 11), eggs were candled again. The position of the embryos and the air sac were marked with a permanent marker, and they were placed with the air sac up, any dead embryos were eliminated. For each dilution, 5 eggs were used; and 5 negative control eggs 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 an insulin syringe attached to a 25G needle. 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.

4.2.4 Egg viability post-inoculation

The protocol states that the optimum time to harvest the virus is between 72 and 96 hours post inoculation. At 24 hours post inoculation eggs were candled and all eggs were viable. From 48 to 96 hours, allantoic fluids were collected from non-viable eggs, this implied virus propagation.

4.2.5 Influenza virus harvest

The harvesting of influenza virus from embryonated eggs was performed by using the internal SOP PE VI 018-06 C. After the 72 hours incubation period, the eggs were chilled at -20ºC for 20 minutes to kill the viable 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. The shell around the air sac was removed while 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 pipette, the allantoic fluid was carefully collected. The allantoic fluids from the eggs with the same dilution of virus were combined into 50 ml sterile Corning™ Falcon™ Conical Centrifuge Tubes. The different collection of the allantoic fluid was based on the time of death of the embryo and the dilution. The Falcon tubes were keep on ice at all times during virus harvest. Once the harvest of the fluid from all of the eggs was done they were left to decant at 4ºC. After, they were centrifuged using Eppendorf Centrifuge 5810R at 1,500 x g for 10 min at 4 °C to pellet debris. The clear fluid from each virus harvest was transferred into new 50 ml Corning™ Falcon™ kept on ice and 150 µl of each were aliquoted in sterile eppendorf microtubes. The 50 ml Falcon 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.

4.3 Viral titration by molecular techniques

4.3.1 RNA extraction

The eppendorf microtubes containing the 150 µl aliquot of allantoic fluid of each virus and 4 negative aliquots (from the negative eggs) were taken from the -75 ºC ultra-freezer and defrosted. The extraction procedure was performed inside a biological safety cabinet (BSC) by using a MACHEREY-NAGEL, Düren, Germany - 07/2014, Rev. 11, NucleoSpin® RNA Virus protocols/Viral RNA isolation from cell - free biological fluids.

4.3.2 First reverse transcription quantitative polymerase chain reaction (RT-qPCR)

This first RT-qPCR was done to compare the viral loads of the different dilutions of each virus and the viral load of the infected non-viable eggs before the 72h. To detect and quantify influenza viruses, RT-qPCR was done using a specific influenza virus probe, and an Internal Positive Control (IPC) to monitor for false negative results. The selected probe consisted of oligonucleotides with the 5' reporter dye 6-carboxyfluorescein (FAM) and the 3' quencher dye 6-carboxytetramethylrhodamine (TAMRA), active for influenza A virus (Elden & Nijhuis, 2001).
The RT-qPCR reagent mix was prepared in a laminar flow hood. Briefly, 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.

Once the RT-qPCR mix was ready, a 96 well plate was prepared in a laminar flow hood. Each well was numbered and 17 µl of the RT-qPCR mix (previously calculated) were added to each one. A volume of 3 µl of each RNA sample (previously numbered on the extraction sheet) was next added to each well, resulting in 20 µl for each well. The plate was sealed with PARAFILM® M Sealing Film, very carefully leaving as little air inside as possible and sealing the edges around of each well to avoid contamination during the RT-qPCR. Later, the plate was spun to homogenize the content of each well, by slow centrifugation with the Eppendorf Centrifuge 5810R, to eliminate the bubbles that might exist between both solutions. The plate was placed in the RT-qPCR 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 (number of cycles, duration and temperature) were

chosen before starting. The routine consisted in several steps: 48 °C - 10 minutes; 95 °C - 10 minutes; 40x (97 °C - 2 seconds; 61 °C - 30 seconds).

4.3.3 Sample filtration

After analysing the results from the first RT-qPCR and as there was not much difference in viral loads among the different dilutions for each viral strain (see Results), the samples from each virus were pooled together in 2 sterile falcon tubes (one for each virus).

For this process 3 different Whatman® filters were used, starting from the one with greatest pore size to the one with smallest pore size. First the 0.8 µm filter (FP 30/0.8), then the 0.45 µm filter (FP 30/0.45) and lastly the 0.2 µm filter (FP 30/0.2). The resulting liquid from each virus was collected using a 5 ml syringe until it was all transferred to 50 ml Falcon tubes, and 150 µl were aliquoted in sterile eppendorf tubes. The obtained 50 ml tubes were aliquoted and frozen in a -75 °C ultra-freezer for long-term storage and the eppendorf tubes were used for the second RNA extraction.

4.3.4 Second RNA extraction

The same procedure of the first RNA extraction was followed using the previous eppendorf microtubes. The obtained samples were used for the second RT-qPCR.

4.3.5 Second RT-qPCR

A second RT-qPCR was done to quantify the definitive viral load of the 2 viral samples. The same procedure of the first RT-qPCR was followed.

4.4 Thermal exposure

Two vials of each influenza virus strain were taken from the -75 ºC ultra-freezer and defrosted. In a Class II Biosafety cabinet (Biostar, Telstar, Spain), a 1:10 dilution of each virus was prepared to achieve 20 ml of total viral suspension. Subsequently, a volume of 1 ml was aliquoted in eppendorf microtubes (18 for each virus). Each virus was subjected to 2 different temperatures (56 ºC and 70 ºC) for 5 different time points (1 min, 5 min, 10 min and 15 min) using an AccuBlock™ Digital Dry Bath. Two replicates for each time point and virus strain were performed, except for the 0 time point that was used for both repetitions.

According to the instructions of use on the Dry Bath, the temperature must be programmed 2 ºC higher than the temperature to be achieved. For this reason, in the case of 56 ºC, the set up was at 58 ºC; for 70ºC it was set at 73 ºC (the temperature ranged from 72.9 and 73.6 ºC). For each virus, 8 eppendorf microtubes (at one time) containing 1 ml were placed in the thermoblock, and a couple of replicates were withdrawn at the mentioned time points (1 min, 5 min, 10 min and 15 min) and immediately placed in an ice bath to stop thermal effect. This process was carried out with all of the samples and then they were kept in the -75 ºC ultra-freezer until titration.

4.5 Titration in MDCK (Madin-Darby Canine Kidney) cells

4.5.1 Preparation of MDCK cells.

MDCK cell were prepared in 96 well plates (SPL Life sciences Tissue Culture Test Plate) 24 or 48 hours before titration, MDCK cells were prepared following CReSA internal standard protocols. Briefly, the cell monolayer was separated by 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) supplemented with 2 mm L-Glutamine and 100 UI/100 ug/ml of Penicillin + Streptomycin. To seed the microplates, 100 µl of the mentioned cell suspension was pipetted into each well. Each plate was fully identified and incubated in a HERAcell 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.

4.5.2 Serial dilution

The samples were taken from the -75º C ultra-freezer and were left to defrost at room temperature inside a Class II biosafety cabinet. Inside the biosafety cabinet, microtubes containing 450 µl of sterile PBS supplemented with 5 µl (1 mg/ml) of trypsin from porcine pancreas type IX (T-0303 SIGMA) were prepared. Subsequently, a tenfold dilution of the samples using these eppendorf microtubes was performed. After all of the dilutions were done, the eppendorf microtubes were incubated in the cell CO_2 incubator at 37.0 ± 1.5 ºC for 30 minutes. At this temperature, the trypsin helps the efficiency of the virus infection.

4.5.3 Titration

This procedure was carried out inside a Class II biosafety cabinet in the CReSA BSL2 Laboratories, following the internal SOP IT-A4-EVI 010.

The culture medium present in the plates contained serum that can inhibit the virus infectivity, for this reason the plates were washed with PBS by putting at least 100 µl in each well and subsequently leaving them for at least 10 minutes before disposing of this solution. Once the plates had been washed, 20 µl of the previously prepared dilutions of the virus samples were inoculated in each well (beginning with the most diluted sample). In each plate, 2 replicas of the titration were performed. The plates were left to incubate in a CO_2 incubator at 37 ºC for 60 minutes. After incubation, 150 µl of post infection media DMEM Lonza supplemented with Glutamine and Penicillin + Streptomycin and containing 1 µg/ml of trypsin from porcine pancreas type IX (T-0303 SIGMA) were 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 post-infection (PI).

4.6 Calculations

4.6.1 Results reading

The first reading of microplates was done 5 days PI and the final reading at 7 days PI. A Nikon inverted microscope was used at a 40X magnification to read the cytopathic effect on the cells. The results were recorded and the $TCID_{50}$% was calculated.

4.6.2 log_{10} $TCID_{50}$% calculations

The calculations of the viral titers of the samples generated along the inactivation studies were performed following an internal SOP (PE VI 017-01). Briefly, the following formula was applied:

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

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

4.6.3 Further calculations

The results obtained after thermal exposure were plotted in graphs alongside the linear regression of the mean results. To evaluate the effect of heat on inactivation of the viruses, the D_t value was calculated using the data from the linear regressions by the following equation:

$$D_t = \left(\frac{1 - I_t}{S_t}\right) - \left(\frac{2 - I_t}{S_t}\right) = -\frac{1}{S_t}$$

Where t is temperature, I_t is the y-axis intercept and S_t is the slope from the linear trendline equation. The D_t value represents the time to reduce the infectious titer by 1 $\log_{10}$ $TCID_{50}$ at a specified temperature.

5. Results

5.1 Egg viability post-inoculation

At 48 hours post inoculation eggs were candled and 2 eggs were non-viable, one from Avian H1N1 at 10^{-2} dilution and one from Swine H1N1 at 10^{-1}, in the case of these eggs the harvest was done at 48 hours following the protocol described above. At 72 hours post inoculation eggs were candled; in the case of Avian H1N1, at 10^{-1} dilution 4 eggs were still viable and 1 was non-viable, at 10^{-2} dilution 3 eggs were non-viable and 2 were viable and at 10^{-3} dilution 2 were viable and 2 were non-viable (one was found not viable at 48h). For Swine H1N1, at 10^{-1} dilution 2 were viable and 2 were non-viable (one was found not viable at 48h), at 10^{-2} dilution 4 eggs were viable and 1 was non-viable. All of the negative controls were viable.

5.2 Influenza Virus Harvest

In general, 5-7 ml of a slightly yellowish fluid was obtained from each egg.

5.3 First RT-qPCR

The viral loads of the different dilutions of each virus and the viral loads of the infected non-viable eggs before the 72h did not present much differences (Table 1 and 2). This can be seen by comparing at the Cycle Threshold (C_t) values. Avian H1N1 virus samples are faster to start to replicate (C_t mean= 15) followed by Swine H1N1 virus (C_t mean=18).

Avian

Dilution	Status	C_t
-1	Non-viable	14,85
-1	Viable	14.58
-2	Not-viable	14,94
-2	Viable	14,77
-3	Non-viable at 48h	14,85
-3	Non-viable	14,52
-3	Viable	14,71

Table 1. Avian Influenza H1N1, C_t in the case of each dilution and status of egg viability.

Swine

Dilution	Status	C_t
-1	Non-viable at 48h	17,97
-1	Non-viable	18,75
-1	Viable	18,74
-2	Non-viable	17,79
-2	Viable	17,62

Table 2. Swine Influenza H1N1, C_t in the case of each dilution and status of egg viability.

5.4 Sample Filtration

For each virus, the allantoic fluid resulted in 18 aliquots of 1.25 ml each, plus a 50 ml falcon tube of filtered fluid and a 50 ml falcon tube on non-filtered fluid.

5.5 Second RT-qPCR

The second RT-qPCR was done to quantify the definitive viral load of the 2 viral samples. The C_t values for this RT-qPCR correspond with the first RT-qPCR, the most rapid samples to start to replicate are from the Avian H1N1 with a mean C_t of 16, followed by Swine H1N1 with a mean C_t value of 20.

5.6 Titration

Both of the investigated viruses were able to infect the confluent monolayer of MDCK cells in the presence of trypsin. To investigate the ability of Avian H1N1 and Swine H1N1 influenza viruses to survive different temperatures, first the initial viral load was examined. The titration of the initial virus, before temperature exposure and before dilution, gave $\log_{10}TCID_{50}$ per ml values of 6.95 ±

0.3 and 6.6 ± 0.4 (mean: 6.8 ± 0.3) in the case of Avian H1N1 and 6.2 ± 0.7 and 5.6 ± 0.5 (mean: 5.9 ± 0.4) for Swine H1N1.

Thermal inactivation was evaluated at 56 and 70 ºC for 5 different time points (0, 1 min, 5 min, 10 min and 15 min). Dilution without any positive wells are considered in a range between 0 and 1.8 of $log_{10}TCID_{50}$. The limit of detection was 1.8 $log_{10}TCID_{50}$ per ml. This 1.8 value was calculated considering the inoculated volume for each well respect to 1 ml. The results obtained after thermal exposure can be seen in Tables 3, 4, 5 and 6 and are plotted in graphs in Figures 1, 2, 3 and 4, alongside the linear regression of the mean results. In these graphs we can see that for 56 ºC Avian influenza virus H1N1 did not reach inactivation and Swine influenza virus H1N1 reached inactivation at the 15 minute time point. In the case of 70 ºC both Avian and Swine influenza viruses reached inactivation after 5 minutes. The linear regression shows that the general tendency of the variables is an inverse correlation. Figures 5 and 6 show graphs comparing the linear regressions of both viruses for each temperature, here we can see a higher viral load for Avian H1N1 influenza virus throughout the experiment. To evaluate the effect of heat on inactivation of the viruses, the D_t value was calculated using the data from the linear regressions. For 70 ºC, the D_{70} values for Avian a Swine viruses were 80.65 and 131.58 seconds respectively. For 56 ºC, the D_{56} values for Avian a Swine viruses were 400 and 344.83 seconds respectively. The time for inactivation of the viruses, based on Dt values, was inversely related to temperature, this means that at higher temperature, less time is needed to reduce the infectious titer by 1 $log_{10}TCID_{50}$. The Residual Factor (RF) value exposes how far the value at a certain time point is from time 0. In the case of Avian H1N, the viral load after 1 minute of exposure to heat increased both cases (positive RF). Avian H1N1 has at least a 3.3 log_{10} difference when inactivated at 70 ºC, Swine H1N1 has at least 2.5 and 2.2 log_{10} difference when inactivated at 56 ºC and 70 ºC respectively.

Avian 56ºC	*0 min*	*1 min*	*5 min*	*10 min*	*15 min*
Titration 1	5.3 ±.0.3, 5.5 ± 0.2	5.7 ± 0.4, 5.7 ± 0.4	4.8 ± 0.4, 5.2 ± 0.0	4.5 ± 0.4, 4.3 ± 0.2	3.2 ± 0.0, 3.2 ± 0.0
Titration 2	5.6 ± 0.2, 5.6 ± 0.3	5.8 ± 0.4, 6.0 ± 0.4	4.7 ± 0.4, 5.1 ± 0.2	4.6 ± 0.4, 4.6 ± 0.4	3.7 ± 0.4, 3.2 ± 0.0
Mean 1	5.4 ± 0.1	5.7 ± 0.0	5.0 ± 0.2	4.4 ± 0.1	3.2 ± 0.0
Mean 2	5.6 ± 0.0	5.9 ± 0.1	4.9 ± 0.3	4.6 ± 0.0	3.3 ± 0.3
Mean	5.5 ± 0.1	5.8 ± 0.1	5.0 ± 0.2	4.5 ± 0.1	3.3 ± 0.3
RF		+ 0.5	- 0.5	- 1.0	- 2.2

Table 3. Avian Influenza H1N1, results of $log_{10}TCID_{50}$ per ml at 56 ºC. Titration 1 and 2 refer to the repetition, each has 2 replicas. Mean values are shown for each titration and the mean value of both is also shown. RF refers to Residual Factor.

Avian 70ºC	*0 min*	*1 min*	*5 min*	*10 min*	*15 min*
Titration 1	5.2 ± 0.5, 3.6 ± 0.4	5.3 ± 0.2, 5.5 ± 0.3	≤ 1.8	≤ 1.8	≤ 1.8
Titration 2	5.9 ± 0.5, 5.8 ± 0.5	5.7 ± 0.5, 5.7 ± 0.4	≤ 1.8	≤ 1.8	≤ 1.8
Mean 1	5.6 ± 0.5	5.5 ± 0.3	≤ 1.8	≤ 1.8	≤ 1.8
Mean 2	4.7 ± 1.6	5.6 ± 0.1	≤ 1.8	≤ 1.8	≤ 1.8
Mean	5.1 ± 1.0	5.60 ± .2	≤ 1.8	≤ 1.8	≤ 1.8
RF		+ 0.5	≥ 3.3	≥ 3.3	≥ 3.3

Table 4. Avian Influenza H1N1, results of $\log_{10}TCID_{50}$ per ml at 70ºC. Titration 1 and 2 refer to the repetition, each has 2 replicas. Mean values are shown for each titration and the mean value of both is also shown. RF refers to Residual Factor.

Swine 56ºC	*0 min*	*1 min*	*5 min*	*10 min*	*15 min*
Titration 1	4.2 ± 0.5, 4.3 ± 0.4	4.2 ± 0.3, 4.5 ± 0.3	4.0 ± 0.4, 3.8 ± 0.4	2.3 ± 0.2, 2.5 ± 0.3	≤ 1.8
Titration 2	4.1 ± 0.5, 4.5 ± 0.4	4.0 ± 0.4, 4.2 ± 0.2	3.8 ± 0.4, 3.8 ± 0.4	2.7 ± 0.4, 2.5 ± 0.3	≤ 1.8
Mean 1	4.3 ± 0.1	4.4 ± 0.2	3.9 ± 0.1	2.4 ± 0.1	≤ 1.8
Mean 2	4.3 ± 0.3	4.1 ± 0.1	3.8 ± 0.0	2.6 ± 0.1	≤ 1.8
Mean	4.3 ± 0.2	4.2 ± 0.2	3.9 ± 0.1	2.5 ± 0.2	≤ 1.8
RF		- 0.1	- 0.4	- 1.8	≥ 2.5

Table 5. Swine Influenza H1N1, results of $\log_{10}TCID_{50}$ per ml at 56ºC. Titration 1 and 2 refer to the repetition, each has 2 replicas. Mean values are shown for each titration and the mean value of both is also shown. RF refers to Residual Factor.

Swine 70ºC	*0 min*	*1 min*	*5 min*	*10 min*	*15 min*
Titration 1	4.3 ± 0.5, 4.0 ± 0.3	3.6 ± 0.4, 3.8 ± 0.4	≤ 1.8	≤ 1.8	≤ 1.8
Titration 2	3.6 ± 0.4, 4.0 ± 0.3	3.7 ± 0.4, 4.2 ± 0.3	≤ 1.8	≤ 1.8	≤ 1.8
Mean 1	4.1 ± 0.2	3.8 ± 0.3	≤ 1.8	≤ 1.8	≤ 1.8
Mean 2	3.8 ± 0.3	3.7 ± 0.1	≤ 1.8	≤ 1.8	≤ 1.8
Mean	4.0 ± 0.3	3.8 ± 0.3	≤ 1.8	≤ 1.8	≤ 1.8
RF		- 0.2	≥ 2.2	≥ 2.2	≥ 2.2

Table 6. Swine Influenza H1N1, results of $\log_{10}TCID_{50}$ per ml at 70ºC. Titration 1 and 2 refer to the repetition, each has 2 replicas. Mean values are shown for each titration and the mean value of both is also shown. RF refers to Residual Factor.

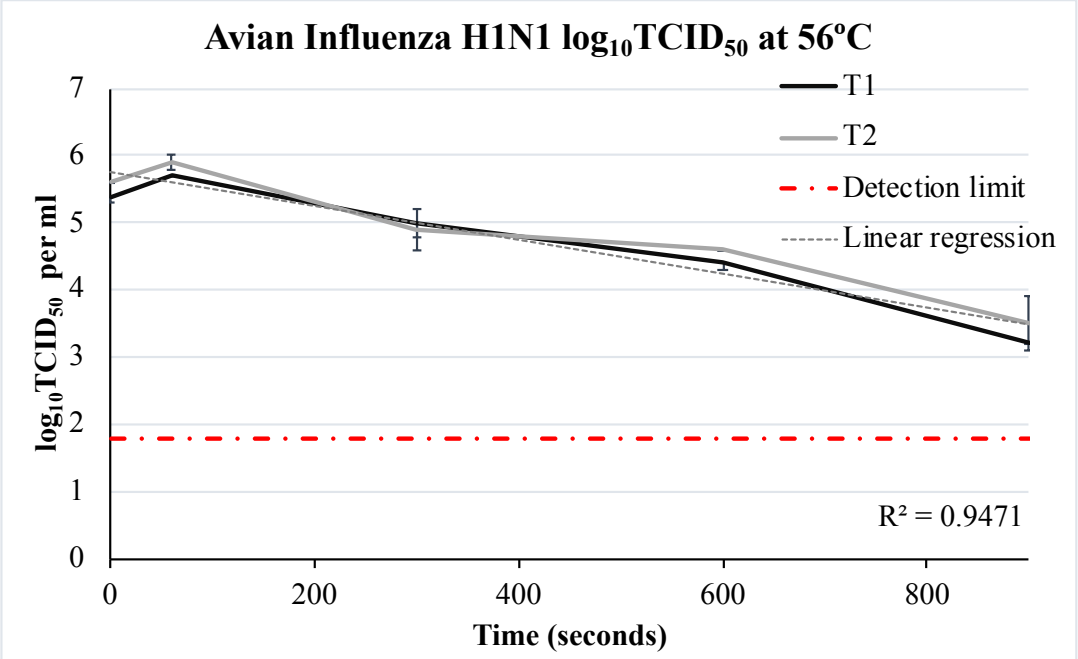

Figure 1. $\log_{10}TCID_{50}$ per ml of Avian Influenza H1N1 at 56 ºC at the different time points. R^2 is the coefficient of determination of the linear regression.

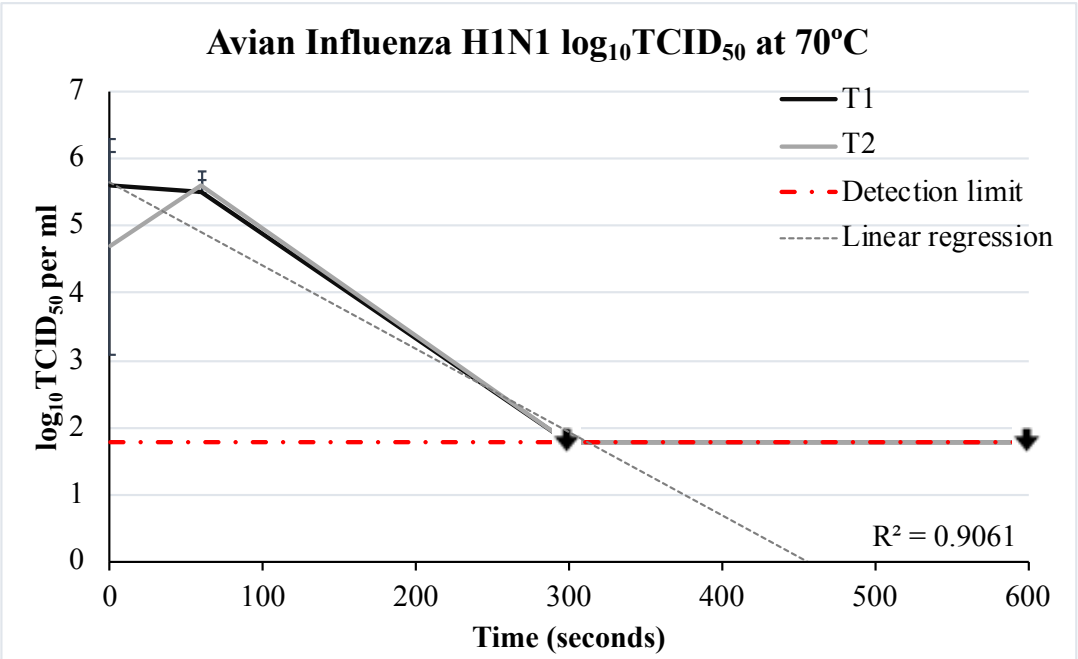

Figure 2. $\log_{10}TCID_{50}$ per ml of Avian Influenza H1N1 at 70 ºC at the different time points. R^2 is the coefficient of determination of the linear regression.

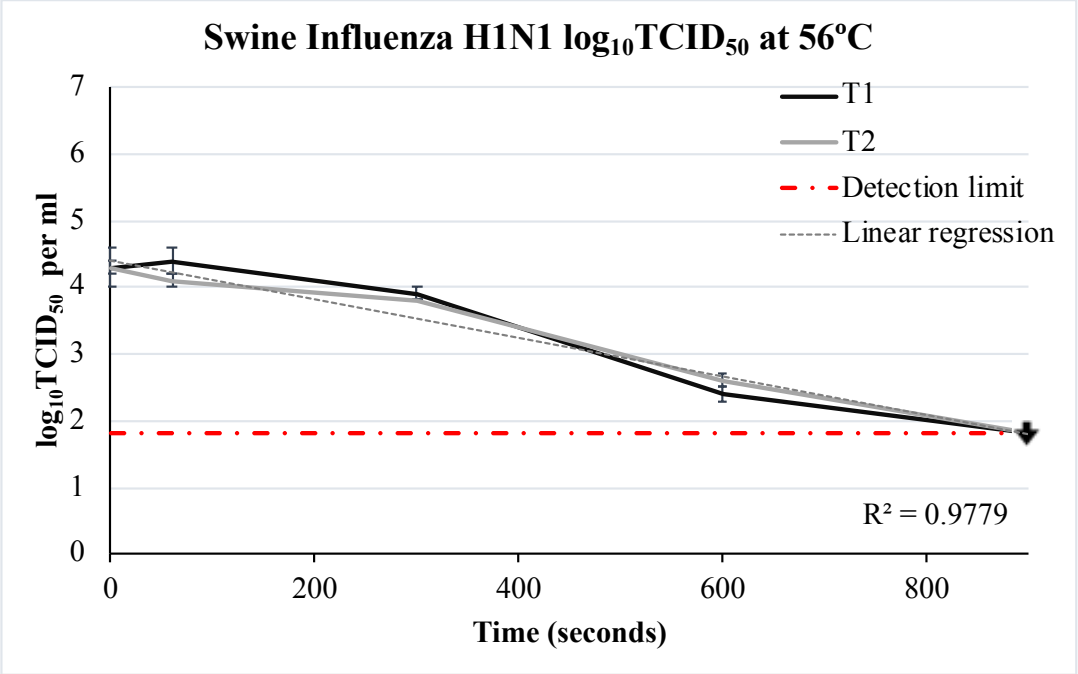

Figure 3. $\log_{10}TCID_{50}$ per ml of Swine Influenza H1N1 at 56 ºC at the different time points. R^2 is the coefficient of determination of the linear regression.

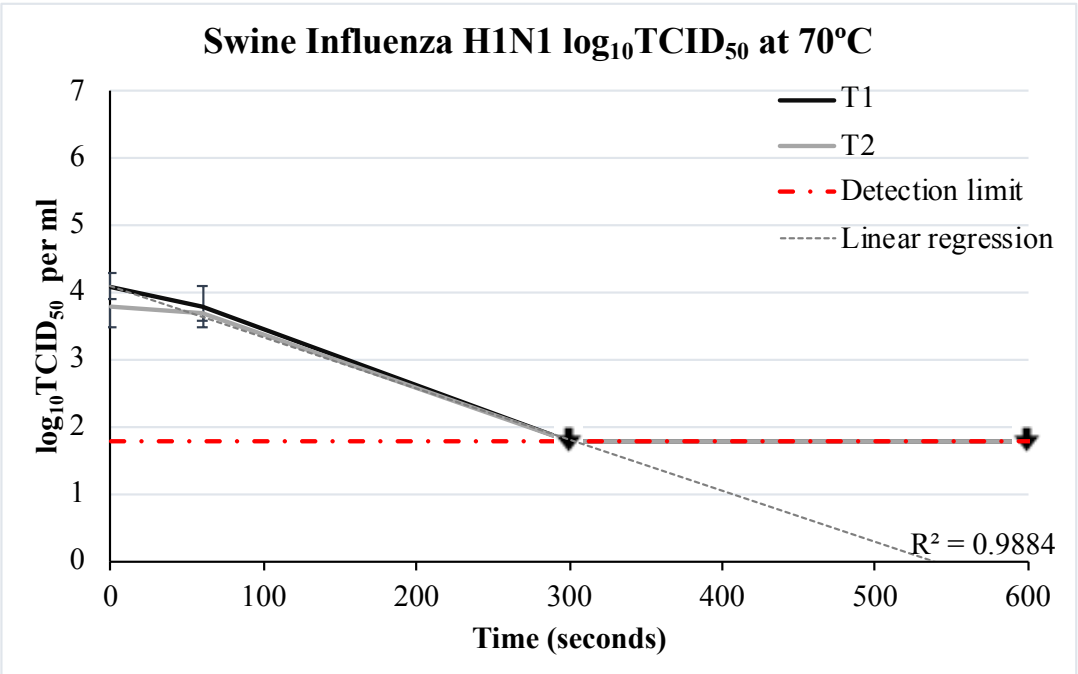

Figure 4. $\log_{10}TCID_{50}$ per ml of Swine Influenza H1N1 at 70 ºC at the different time points. R^2 is the coefficient of determination of the linear regression.

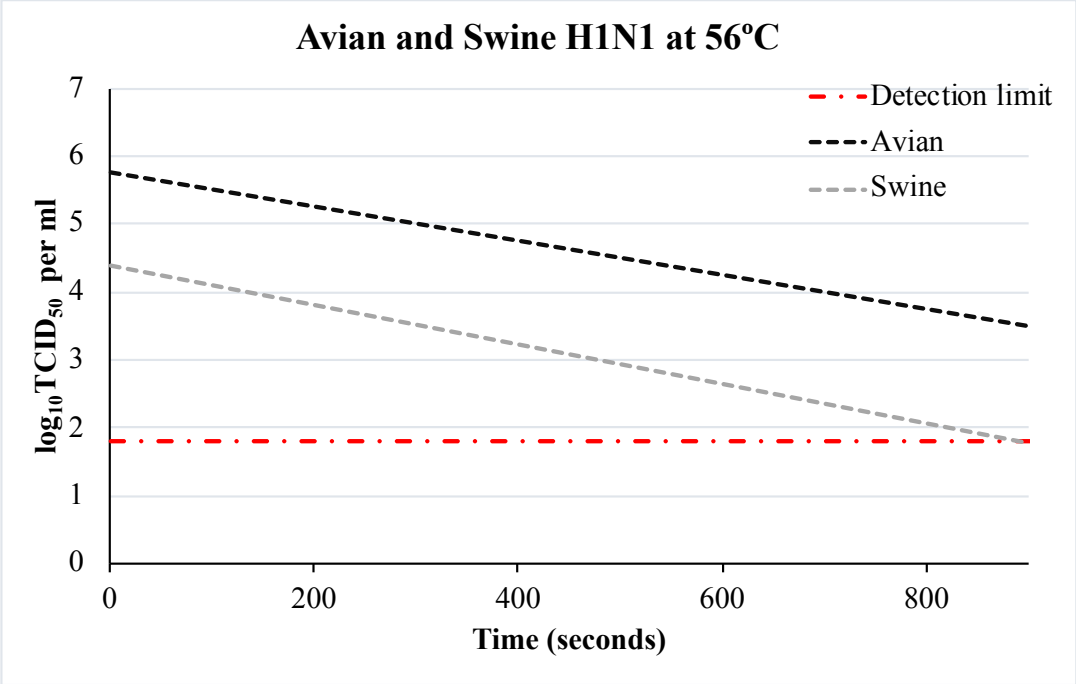

Figure 5. Linear regression of $\log_{10}TCID_{50}$ per ml of Avian and Swine Influenza H1N1 at 56 ºC. Avian linear regression: y = -0.0025x + 5.7617; Swine linear regression: y = -0.0029x + 4.4023

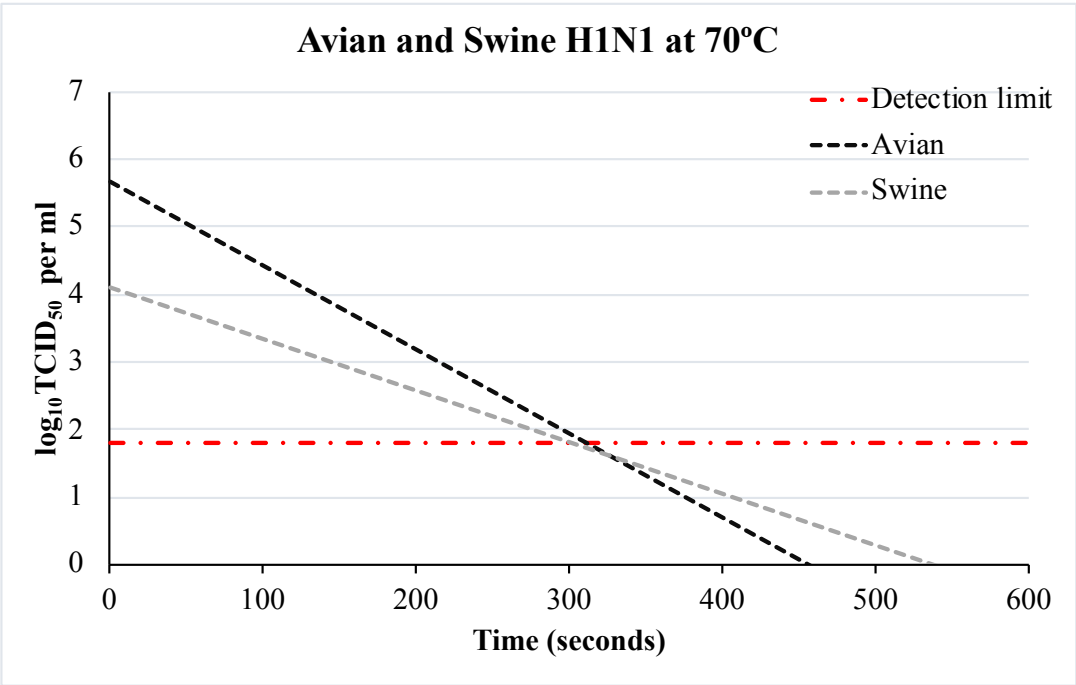

Figure 6. Linear regression of $\log_{10}TCID_{50}$ per ml of Avian and Swine Influenza H1N1 at 70 ºC. Avian linear regression: y = -0.0124x + 5.6524; Swine linear regression: y = -0.0076x + 4.1143

6. Discussion

Influenza viruses have few restrictions, circulating within species and occasionally jumping between them and causing infections all around the world. These viruses have a wide-spread impact and the growing complexities of globalization offer a greater challenge to the prevention and control of this disease. The greatest impact of influenza from swine and birds is their vital role as sources of novel influenza viruses capable of causing pandemics that affect human health (Jernigan & Cox, 2013). Resistance of influenza viruses to environmental factors outside the host, is influenced by many factors including viral strain, host origin, environmental matrix and environmental conditions like temperature, salinity, pH and protein content, among others (Brown et al., 2009; EFSA, 2008). Knowledge of the impact of these factors could be very useful in risk assessments concerning the spread and transmission of influenza during outbreaks (EFSA, 2008). International networks of laboratories and public health agencies that incorporate new technologies, new regulations, and new information, sharing tools and platforms have been established to try to manage influenza virus control (Jernigan & Cox, 2013). When working with highly pathogenic influenza viruses in bio-containment facilities one of the main challenges is the inactivation of samples for further studies in bio-containment or out of bio-containment. To do so several systems are available, some physical others chemical. The choice among them will be driven by the final destination of the sample. If the sample is destined to be used for immunological studies the inactivation target should be the nucleic acids, allowing proteins to keep there natural conformations while for molecular biology studies (as PCRs) the target should be the proteins and lipid envelopes. In both cases, the fact that all these processes are able to completely inactivate infectivity from the samples should be assessed, as they will be handled in less stringent biosafety conditions (X, Abad, personal communication, July 2, 2018).

The use of PCR techniques for virus detection and quantification has high sensitivity and reproducibility, quantitative virus testing has therefore become an indispensable diagnostic instrument in many situations (Watzinger et al., 2004). In this case, the mean Ct values for the first and second RT-qPCR are very similar but slightly higher for Avian H1N1 than for Swine H1N1. Ct values are predictive for quantity of input target (Heid et al., 1996), in this case nucleic acid. The Ct value refers to the number of cycles were the PCR curve reaches the stated threshold (Luu-

The et al., 2005). For the first RT-qPCR, as each sample gives a similar result, this demonstrates the reproducibility of this technique and method, so we can assume that the number of target nucleic acids present in the sample corresponds to the viral load (Heid et al., 1996). Assuming that the PCR reactions were equally efficient for both viruses, the avian strain reached the threshold 3 cycles before the swine strain. 3 cycles approximately correspond to a factor of 8-10; this means that there are about 10 times more avian genomes than swine genomes. This corresponds to the 1 $\log_{10}$ difference observed in the results of the titration of the initial virus (6.8 ± 0.3 and 5.9 ± 0.4). This suggests that the ratio genome to infectious particle is not dependent on origin.

Thermal inactivation of influenza viruses has been studied since the 1960s (Woese, 1960). This method has long been recognized as an effective method of inactivating viruses (Swayne & Beck, 2004) and it has been used in the food industry to inactivate microorganisms (Bertrand et al., 2012), and more recently it has been proposed to prevent outbreaks cause by biosafety accidents (Stephens & Spackman, 2017). Heat disinfection method is easy to use, readily available, and cost-effective (Jeong et al., 2010). Temperatures such as 56 ºC, are also used to inactivate virus from sera samples, currently coupled with detergents as Tween 20 (Cutts et al., 2016). To know if this temperature alone can cope with influenza virus infectivity it is convenient to perform assays, that should be considered as a worst case scenario (for instance if Tween is wrongly absent in an inactivation treatment) and must follow inactivation guidelines (EMEA, 1996, 1997). Regarding our study, at 56 ºC, Avian H1N1 did not reach complete inactivation (2.2 $\log_{10}$ reduction, from now $\log_{10}$R) and Swine H1N1 reached complete inactivation (more than 2.5 $\log_{10}$R) after 15 minutes. Similar results have been previously reported: Avian Influenza H7N3 can withstand 56 ºC for 45 min (Muhmmad et al., 2001), or H5N1 virus can tolerate 15 minutes at 56 ºC but is inactivated after 30 minutes (initial titer of 8.3 $\log_{10}$) at this same temperature (Shahid et al., 2009); and H1N1 achieved a 2 $\log_{10}$R in 30 minutes at 56 ºC (Tuladhar et al., 2012). Additionally, Chmielewski and co-workers, in 2011 stated that to inactivate HPAIV and achieve a 5 $\log_{10}$R in fat free eggs, 93 minutes were needed at 55 ºC, 43 minutes at 56 ºC, 18 minutes at 56.7 ºC and at 57º C and 58 ºC, 13 and 2 minutes respectively. However, in this same study by Chmielewski et al. they concluded that to inactivate 5 $\log_{10}$ of LPAIV 5 minutes at 55 ºC were needed, 4 minutes at 57 ºC and that it took less than 2 minutes to achieve complete inactivation at 58 and 59 ºC in fat free eggs. This differs from what we found and with the other strains examined in their study but is similar to another study by Swayne & Beck in 2004, who reported that to achieve a 4.9 $\log_{10}$R they needed to apply

55,6 and 56.7 ºC for 2.7 and 3 minutes respectively. All this data points that at this temperature range, below 60 ºC, each influenza viral strain acts in its own way, further studies would be needed to see how they differ or harmonize.

High temperatures can cause the denaturation of structural and functional proteins, unwinding of nucleic acids, and destabilization of surface structure of viral envelopes, causing loss of viral infectivity. As the temperature increases further, proteins and other molecules precipitate, leading to further loss of viral structure and function (Boschetti et al., 2003; Jeong et al., 2010). In this study we observed that both Avian H1N1 and Swine H1N1 are inactivated after 5 minutes at 70 ºC heat treatment achieving at least 3.3 and 2.2 $\log_{10}$R respectively. These results resemble those found in another report on a H1N1 A/NWS/33 strain (Jeong et al., 2010). In this study the virus achieved a 0.55 $\log_{10}$R after 1 minute at 70 ºC, a secondary 2.51 $\log_{10}$R after 2.5 minutes and then was completely inactivated. This is also consistent with published literature that shows that some strains (Korea/03) of influenza are inactivated (4.6 $\log_{10}$R) after 1 minute al 70 ºC (Swayne, 2006); a H1N1 strain showed a 2 $\log_{10}$R in 30 seconds at 73 ºC (Tuladhar et al., 2012), some H5N1 viruses were completely inactivated (initial viral titer of 9 $\log_{10}$) after the treatment at 70 ºC treatment for 60 min or at 75 ºC for 45 min (Wanaratana et al., 2010) and for HPAIV H7N7 which, after a 90 seconds incubation at 63 ºC achieved a 4 $\log_{10}$R and after 30 seconds at 65 ºC, the initial infectivity was no longer detectable (around 5 $\log_{10}$R) (Isbarn et al., 2007). This data is important in terms of biosafety procedures; some nucleic acid extraction kits include this thermal step (70 ºC for 5 minutes) in its protocols (MACHEREY-NAGEL GmbH & Co. KG, 2014); others include similar ones after lysis buffer addition. The knowledge that this step alone is able to inactivate virus without participation of the lysis buffer (again a worst case scenario by human mistake) gives an extra-safety margin to these procedures.

Many factors can affect the thermal inactivation time. These include the presence of salts and organic compounds, the pH and the density of the suspension, and the presence of other microorganisms (Mitchell & McCormick, 1984). The European Medicines Agency warns that laboratory strains of virus may differ in their sensitivity to the same treatment (EMEA, 1996). When preparing the high-titer it is very important to avoid aggregation which may enhance physical removal and decrease inactivation (EMEA, 1997). This last point is what could have happened in the cases with avian influenza where at time 0 there seems to be a lower viral load than after 1

minute of thermal treatment. Another possibility is that as this virus was the first one we worked with, the technique was not yet mastered and all the components might not have been handled in the best way. Small differences in, for example, buffers, media, or reagents, can substantially affect the results (EMEA, 1997).

Regarding inactivation kinetics, thermal inactivation of viruses has often been observed to proceed as a first-order reaction until approximately 90% or more of the infective particles have perished (Hiatt, 1964). Swayne & Beck in 2004 found that, in their study, when plotting the $\log_{10}$ titer versus time they generated different inactivation curves, 2 bi-phasic and a mono-phasic curves. The first bi-phasic curve began with an immediate decline from time zero with two inactivation curves: the first curve having a steeper slope than the second curve. For the other bi-phasic curve, the 0 min, 1 min and, sometimes, 2 min time points were a plateau that was followed by a linear decline in titer. The mono-phasic curve had a single linear curve beginning with the zero time point. In another study at 56 °C inactivation took place in a bi-phasic fashion, each following first-order kinetics (De Flora & Badolati, 1973). In this same study, the authors stated that this bi-phasic inactivation pattern could be caused because of various factors including inhomogeneity of the virus population and formation of virus aggregates. The kinetics of the inactivation of a virus suspension invitro in the 50 to 60 °C temperature range are usually determined by the inactivation of the most thermolabile proteins that are essential for infection (Mitchell & McCormick, 1984).

In the case of our study, both viruses at both temperatures seem to follow a mono-phasic trend with different characteristics. If we calculate the coefficient of determination of the linear regression (R^2), shown in Figures 1-4, we can see that at 70 ºC they are 0.91 and 0.98 for Avian and Swine Influenza, respectively. At 56 ºC they were 0.95 and 0.98 for Avian and Swine Influenza, respectively. All of the R^2 values are close to 1, meaning they adapt well and are very close to a straight line, implying they show a tendency to fit to a mono-phasic line., conclusion that has been also reported previously; Tuladhar and co-workers (2012) also found that influenza virus had a mono-phasic reduction tendency.

For the lineal regression graphs, the D_t values (time to reduce the infectious titer by 1 $\log_{10}TCID_{50}$ or by 90% at a specified temperature) were calculated giving results of D_{70} values for Avian and Swine viruses of 80.65 and 131.58 seconds respectively. The D_t values at 56 º, D_{56}, for Avian and Swine viruses were 400 and 344.83 seconds respectively. We can see that both results are similar

and that in one case (at 70 ºC) the avian origin virus has a lower D_t value and in the other case (at 56 ºC) the swine origin virus has a lower D_t value. We could think that if this experiment was to be performed again with more number of samples and time points or at another sublethal temperature (50ºC for example), the results could give a greater difference, but this will remain as a hypothesis until further work. We could also conclude, that higher temperature increases the virus inactivation rate, as can also be seen in previous literature (Isbarn et al., 2007; Tuladhar et al., 2012).

Besides thermal treatment, there are several physical and chemical methods that can be used to inactivate influenza viruses. It was observed that H5N1 subtype lost its viability when exposed to pH 1, 3, 11 and 13 after 6 hours while it remained viable at pH 7 for 24 hours. This same virus retained its virulence at pH 5 for 18 hours but was inactivated after 24 hours. It also kept its infectivity at pH 9 for more than 24 hours (Shahid et al., 2009). The available literature states that H1N1 virus can be inactivated from an initial titer of 7.44 $\log_{10}$ $TCID_{50}$ to an undetectable level in 1 minute with 70% ethanol treatment (Jeong et al., 2010). Another study reveals that H5N1 isolates lost approximately 50% of their infectivity when exposed to quaternary ammonium compound and formalin regardless of the temperature and storage time. Shahid et al. in 2009 also reported that H5N1 was inactivated with formalin (0.2, 0.4 and 0.6%) after 15 minutes.

The limitations of this study, that focuses on the capabilities of 56 ºC and 70º C treatment as safe treatments to inactivate influenza virus, were that only two viral strains were investigated, meaning we cannot actually confirm if there is a difference between both influenza virus strains, and if the reported behaviour could be considered as general of all influenza strains. Another restriction could be that only two different temperatures were evaluated, it could be interesting to investigate other temperatures in the range from 56 to 70 ºC to see if these viruses could be inactivated with a lower temperature and maybe a similar amount of time. However, it could be concluded that, for the assayed Avian and Swine influenza viruses, 70 ºC for 5 minutes is a safe temperature to take samples out of biocontainment units, to process a contaminated area after an outbreak or to assure complete inactivation in different procedures without adding another component (in case of a human mistake), since no infectious virus was detected after this process. More studies are need in this field to be able to evaluate new or better methods to inactivate influenza virus and try to prevent possible outbreaks and make a different in this global public health issue.

7. Conclusions

In this study, the effect of two different temperatures were evaluated in two influenza virus strains at various time points.

- We can conclude that at 70 ºC both Swine and Avian H1N1 influenza strains were completely inactivated after 5 minutes.
- At 56 ºC Avian influenza virus H1N1 did not reach inactivation and Swine influenza virus H1N1 reached inactivation only after 15 minutes.
- We could also determine, that higher temperature increases the virus inactivation rate when comparing 56 and 70 ºC.
- Both viruses at both temperatures were seen to follow a mono-phasic reduction trend.

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