Journal of Virological Methods 304 (2022) 114527

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Inactivation efficacy of H5N1 avian influenza virus by commonly used sample preparation reagents for safe laboratory practices

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ARTICLE INFO

Keywords:
Inactivation
Biosafety
Avian influenza
Influenza virus
Lysis buffer
Fixation

ABSTRACT

The objective of this study was to determine the inactivation efficiency of common sample preparation reagents against highly pathogenic avian influenza A (HPAI) H5N1 virus. HPAI H5N1 virus has caused infections in humans with a mortality rate of over 50%. Due to the high mortality and the risk of aerosol transmission of that virus to humans and birds, infectious HPAI H5N1 viruses are contained in a biosafety level 3 laboratory. However, many procedures for further molecular analyses would be easier in lower biosafety conditions. To ensure the laboratory safety the successful inactivation procedures should be demonstrated before the samples are transferred to a lower containment facility. We tested the inactivation capacity of commonly used cell lysis buffer radio-immuno precipitation assay (RIPA) buffer for protein samples, cell fixatives methanol (MeOH) and paraformaldehyde (PFA) and guanidine isothiocyanate-containing lysis buffer for RNA isolation (RLT, Qiagen) in H5N1-infected cells. Based on our results RLT buffer, 90% MeOH (20 min, −20 °C) and 4% PFA (30 min, RT) all completely inactivated the HPAI H5N1 virus. However, RIPA buffer alone was not sufficient to inactivate the HPAI H5N1 virus in infected cell samples but, instead, combining RIPA lysis buffer and boiling for 10 min the samples in Laemmli buffer led to complete inactivation of the virus.

1. Introduction

Influenza viruses are one of the most significant human pathogens causing annual epidemics and at irregular intervals worldwide pandemics like the devastating 1918 H1N1 “Spanish influenza” pandemic. So far, H1N1, H2N2 and H3N2 influenza A viruses have caused pandemics and epidemics in humans. In addition, H5, H6, H7, H9 and H10 hemagglutinin subtype viruses have caused sporadic infections in humans. Aquatic birds function as a natural reservoir for influenza A viruses and H5 and H7 subtypes continue to cause infections among wild birds and outbreaks in poultry. According to The World Organisation for Animal Health and The Food and Agriculture Organization of the United Nations during the season 2020–2021 highly pathogenic avian influenza (HPAI) H5 viruses have been observed to an increasing extent among birds in Europe, Africa and Asia (The Food and Agriculture Organization of the United Nations, 2021; World Organisation for Animal Health, 2021). Transmission of avian influenza viruses (AIVs) from human to human has been limited and typically humans get infected via a close contact to infected poultry and thus a high incidence of the virus in poultry also increases the probability of human infections. So far H5N1 and H7N9 viruses have caused nearly 900 and 1600 reported human infections with a mortality rate of around 50% and 40%, respectively (World Health Organization, 2021). Although most of the human infections have been caused by H5N1 and H7N9 AIV subtypes, also human infections with other H5 subtypes have been increasing recently (World Health Organization, 2021). Human infections with H5N1 or H7N9 viruses have been associated with severe pneumonia and acute respiratory distress syndrome (ARDS) (Yu et al., 2008; Gao et al., 2013a, 2013b). Avian influenza viruses are a major public health threat and a risk to cause a pandemic due to their high ability to recombine and mutate, and because humans lack pre-existing immunity against influenza A virus strains of zoonotic origin. Indeed, all influenza pandemics in the 20th century have been of avian origin (Horimoto and Kawaoka, 2005; Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team et al., 2009).

To be prepared for a potential pandemic caused by an avian influenza virus several laboratories and scientists are working with the avian influenza viruses for diagnostics and research purposes. The potential risk associated with the work with these viruses can be minimized by

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https://doi.org/10.1016/j.jviromet.2022.114527
Received 2 February 2022; Received in revised form 22 March 2022; Accepted 24 March 2022
Available online 27 March 2022

adopting proper biosafety principles and practices. HPAI H5N1 virus and other AIV strains which cause a severe disease in humans but are not efficiently transmitted between humans are normally handled in biosafety level (BSL)-3 laboratories. However, with inactivated specimens many of the downstream analyses could be conducted in BSL-2 or -1 laboratories. In diagnostics of high-risk pathogens usually the genetic material (DNA/RNA) needs to be extracted from the specimen in a higher biosafety level facility before the material is brought for subsequent PCR analysis. However, the DNA/RNA isolation could be performed more easily and with lower costs in a lower BSL laboratory if the lysis buffer for the DNA/RNA extraction can be demonstrated to be sufficient to inactivate the pathogen. In addition, there is a demand for conducting many downstream studies in a lower containment level with inactivated samples. Completing the downstream studies at lower containment level has many advantages: the availability of high-level equipment and devises, like flow cytometers, real time PCR machines and microscopes, easier methodological measures, infectious waste minimization and less stressful and less laborious working practices with lighter personal protective equipment. Generally, working in a BSL-3 laboratory is more expensive and laborious than in BSL-1 or -2 laboratories which warrants the need for transferring the work to a lower containment level after the samples are verifiably inactivated.

In our previous study, we have shown that HPAI H5N1 virus is able to spread and replicate in human immune cells with such an efficacy that the whole cell culture becomes infected even from an extremely low virus dose (Westenius et al., 2018). Thus, it is very important that H5N1 virus-containing samples are confirmed to be fully inactivated before they are transferred into a lower biocontainment level. Normally, BSL-3 or BSL-4 facilities are needed for handling the high-risk microorganisms like many of the emerging viruses. Each country (or region) makes their own national classification of different microorganisms into four biological risk groups according to the risk they present to the laboratory workers and to the community or environment if the microbes would escape from the laboratory. AIV strains that cause severe disease in humans, like H5N1 and H7N9 subtypes, are classified in risk group 3 in many countries as those viruses can pose a high individual risk, but a rather low community risk. As microbes are classified based on their biological risks, traditionally, laboratories have been classified to different biosafety level laboratories (1–4) according to the laboratory biosafety manual (World Health Organization, 2004) published by World Health Organization. However, the risk group of a microbe does not directly determine the biosafety level at which it should be handled. Instead, the decision on the safety level is based on a risk assessment where the nature of the work, materials and safety measures are assessed case by case. In addition, National Institutes of Health of the United States has given guidelines for research involving recombinant or synthetic nucleic acid molecules (Meyer et al., 2019) which includes guidelines for handling of pandemic potential-posing influenza A viruses generated by reverse genetics. Thus, the risk assessments for emerging or unnatural microorganisms where the clinical knowledge is limited tend to be more cautious than those for microbes that occur in nature.

There are studies where typical means of inactivating influenza virus stock cultures propagated in embryonated chicken eggs or cell cultures with different chemicals or physical treatments have been tested (Zou et al., 2013; Shahid et al., 2009; Pawar et al., 2015; Jeong et al., 2010; Wanaratana et al., 2010). However, inactivation studies with infected cells and treatment protocols to the cells, instead of virus stocks, are much rarer. In the present study we tested the sample inactivation capacity of radio-immuno precipitation assay (RIPA) lysis buffer for lysing cells to extract whole cellular proteins, guanidine isothiocyanate-containing lysis buffer for RNA isolation (RLT, Qiagen) and commonly used fixatives methanol (MeOH) and paraformaldehyde (PFA). Inactivation treatments were done to H5N1 virus-infected human primary monocyte-derived dendritic cells (moDCs) and widely used A549 human lung epithelial cells. Testing the inactivation of these typical sample preparation reagents enables the downstream analyses of H5N1-infected cells for protein expression studies (flow cytometry, immunofluorescence microscopy or Western blotting) or for gene expression analyses (qRT-PCR). We demonstrated that sample fixation with 90% MeOH for 20 min and with 4% PFA for 30 min as well as RLT lysis buffer for RNA extraction are efficient to fully inactivate HPAI H5N1 virus. Instead, our results indicates that RIPA lysis buffer alone was not sufficient to inactivate the H5N1 virus in virus-infected cells while combining RIPA buffer with Laemmli buffer followed by boiling fully inactivated the H5N1 virus.

2. Materials and methods

2.1. Ethics statement

The permission to import the human isolate of avian influenza virus strain A/Vietnam/1194/2004 (H5N1) for research purposes was obtained from the Finnish Food Safety Authority (permission no 8634/0527/2012). Infective H5N1 virus was handled in a Biosafety Level (BSL) 3 laboratory, audited by Det Norske Veritas in 2013, at the Finnish Institute for Health and Welfare (THL), Finland. Adult human blood was obtained from anonymous healthy blood donors through the Finnish Red Cross Blood Service (permission no 36/2018, renewed annually).

2.2. Virus and cells

A human isolate of a highly pathogenic avian influenza virus A/Vietnam/1194/2004 (H5N1, clade 1) (originates from Molecular Virology, Erasmus MC - Department of Viroscience, Rotterdam, Netherlands) was grown in the allantoic cavity of 10-day-old embryonated chicken eggs at +36 °C for 2 days. The titers of virus stock were determined as described and reported previously (Westenius et al., 2018).

The buffy coat fractions were obtained from healthy blood donors (Finnish Red Cross Blood Service, Helsinki, Finland). Monocytes were purified from buffy coats and moDCs were differentiated from monocytes as described previously (Westenius et al., 2018; Veckman et al., 2004). In brief, human peripheral blood mononuclear cells were isolated by Ficoll-Paque (Pharmacia Biotech) gradient centrifugation. From mononuclear cells the monocytes were fractioned by centrifugation on a Percoll gradient (Amersham Biosciences) and further purified by lymphocyte depletion with anti-CD3 and anti-CD19 magnetic beads (Dynal). Immature moDCs were generated by cultivating adherent monocytes in RPMI 1640 (Sigma-Aldrich) supplemented with 0.6 μg/ml penicillin, 60 μg/ml streptomycin, 2 mM L-glutamine, 20 mM HEPES, 10% fetal calf serum (FCS) (Sigma), 10 ng/ml human recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) (Gibco) and 20 ng/ml human recombinant interleukin-4 (rIL-4) (GenScript). The cells were cultivated for 6 days, and fresh medium was added every 2 days.

The human lung epithelial cell line (ATCC CCL185) (A549) and Madin-Darby canine kidney cell line (ATCC CCL34) (MDCK) were cultured in Eagle minimal essential medium (E-MEM) (Sigma Aldrich) supplemented with 0.6 μg/ml penicillin, 60 μg/ml streptomycin, 2 mM L-glutamine and 20 mM HEPES and 10% FCS.

moDCs were cultivated in 12-well plates at a density of 0.5×10^6/well and the cells were infected with A/Vietnam/1194/2004 (H5N1) virus with a MOI of 5 at +37 C, 5% CO_2 for 24 h. The semi-adherent moDCs were harvested by gently scraping and the cells were washed with PBS (Fig. 1A). Confluent A549 cells in 12-well plates were infected with A/Vietnam/1194/2004 (H5N1) virus with a MOI of 2 at +37 C, 5% CO_2 for 24 h. Cells were harvested with a cell scraper and washed with PBS (Fig. 1B). The infected cells were then treated with different sample preparation reagents and the efficiency of inactivation of the virus was analyzed by further cultivating the samples (Fig. 1). As a negative and positive control uninfected and infected cells, respectively, were treated with PBS.

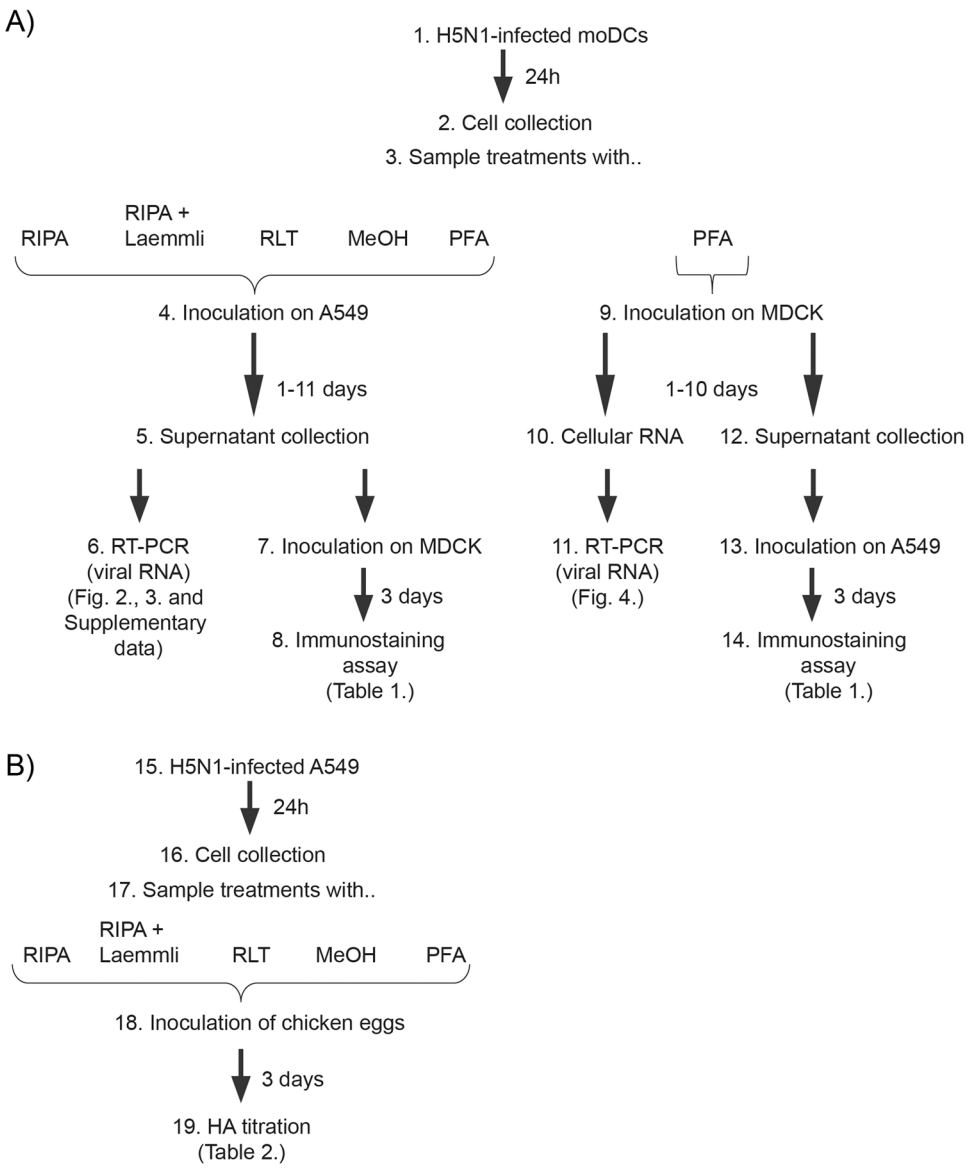

Fig. 1. Inactivation testing protocol. A) 1. Human monocyte-derived dendritic cells (moDCs) were infected with A/Vietnam/1194/2004 (H5N1, clade 1) virus at MOI 5. 2. Cells were harvested 24 h after infection. 3. Cells were treated with RIPA, RIPA+Laemmli buffer, RLT, MeOH or PFA. 4. Different amounts of treated samples were inoculated on A549 cells. 5. Supernatant samples were collected during the incubation period (1 h-11 days). 6. Viral RNA expression was analyzed by RT-PCR from supernatant samples. 7. Supernatant samples from A549 cells were inoculated on MDCK cells. 8. After 3 days of incubation MDCK cells were fixed and immunostained with mouse anti-influenza A NP antibody, secondary goat anti-mouse HRP and 3-amino-9-ethylcarbazole – (AEC) substrate. 9. PFA-treated moDC samples were inoculated on MDCK cells. 10. Total cellular RNA was isolated from cells collected after different incubation times (1 h-10 days). 11. Viral RNA expression was analyzed by RT-PCR from total cellular RNA samples. 12. Supernatant samples were collected from MDCK cells during the incubation period (1 h-10 days). 13. Supernatant samples from MDCK cells were inoculated on A549 cells. 14. After 3 days incubation A549 cells were fixed and immunostaining with AEC substrate was done as described above. B) 15. A549 cells were infected with A/Vietnam/1194/2004 (H5N1) virus. 16. Cells were harvested 24 h after infection. 17. Cells were treated with RIPA, RIPA+Laemmli buffer, RLT, MeOH or PFA. 18. Treated samples were inoculated in embryonated chicken eggs. 19. After 3 days incubation allantoic fluid was collected and hemagglutination titration was performed using guinea pig red blood cells.

2.3. Protein lysis buffer treatment and passaging of treated samples on A549 cells

Infected moDCs were lysed to RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2) containing 0.5 mM DTT, 1 mM Na_3VO_4 and protease inhibitor mixture (Complete, Roche) (2.5×10^6 cells/ 1 ml RIPA). As a positive control infected moDC and as a negative control uninfected moDCs were treated in a similar way but with PBS instead of RIPA treatment. For testing virus inactivation efficiency in combination of RIPA and Laemmli sample buffer, 335 μl 4XLaemmli buffer was added per 1 ml RIPA-treated sample and samples were boiled for 10 min

In order to demonstrate the inactivation of the virus after the sample treatments, different amounts (100 μl, 30 μl and 5 μl) of RIPA-treated and RIPA and Laemmli buffer-treated samples were inoculated over confluent A549 cells in 12-well plate in five replicate wells. The same amounts of the positive control sample and 100 μl of the negative control sample were also inoculated. Fresh media (1.5 ml/well) was changed after 2 h incubation. On day 4 the supernatant from one replicate well was collected, clarified and transferred (200 μl/well) to a fresh culture of A549 cells in a 12-well plate in three replicate wells. Supernatant samples were collected from one of the replicate wells as follows: on day 1 before and after the media change, 2nd, 3rd, 4th, 8th and 11th days. From the supernatant samples the loss of infectivity was determined on MDCK cells with an immunostaining assay and the viral RNA amounts were analyzed by RT-PCR.

2.4. Guanidine-isothiocyanate

Guanidine-isothiocyanate (GITC) is a commonly used protein denaturation agent in lysis buffers for RNA and DNA extractions, like in RLT buffer in RNEasy mini kit (Qiagen). 10 μl β-mercaptoethanol per 1 ml RLT buffer was added. Infected moDCs were lysed to RLT buffer (2.5×10^6 cells/ 1 ml RLT). As a positive control H5N1 virus-infected moDC and as a negative control uninfected moDCs were collected by scraping and the cell pellet was resuspended in PBS instead of RLT treatment.

In order to demonstrate the inactivation of the virus after the sample treatment, different amounts of samples were blind passaged on A549 cells and supernatant samples were collected up to 11 days after inoculum. The existence of viruses was tested with immunostaining assay and RT-PCR as described below.

2.5. Inactivation with methanol fixation

Infected moDCs were fixed with ice cold 90% methanol (MeOH) (0.5×10^6cells/1 ml 90% MeOH) for 20 min at −20 °C with gently rotation and cells were washed with PBS and resuspended in 200 μl PBS. As a positive control the infected moDC and as a negative control uninfected moDCs were treated with PBS instead of MeOH treatment.

Passaging of MeOH-treated samples on A549 cells were performed as described above for the protein lysis buffer-treated samples. Briefly, 100 μl, 30 μl and 5 μl of samples were added to confluent A549 cells in 12-well plates. Fresh media (1.5 ml/well) was changed after 2 h. On day 4 supernatant samples were collected, clarified and inoculated on fresh A549 cells in 12-well plates for a new passage. Supernatant samples were collected during the 11 days incubation and the potential infectivity was determined on MDCK cells using the immunostaining assay. In addition, from supernatant samples viral RNA amounts were analyzed by RT-PCR.

2.6. Paraformaldehyde fixation and serial passaging of fixed cells on A549 cells

Infected moDCs were fixed at room temperature with 4% paraformaldehyde (PFA) (0.5×10^6cells/1 ml 4% PFA) for 30 min with a gentle rotation and cells were washed with PBS and resuspended in 200 μl PBS. Positive and negative controls were prepared as described above for the MeOH-treated samples.

Different amounts of samples (100 μl, 30 μl and 5 μl) were incubated and blind passaged on A549 cells and supernatant samples were collected up to 11 days after incubation. The viral RNA amounts were analyzed by RT-PCR as described below.

2.7. Paraformaldehyde fixation and serial passaging of fixed cells on MDCK cells

Infected moDCs were fixed with 4% PFA (10^6cells/1 ml 4% PFA) for 30 min at room temperature with a gentle rotation and cells were washed with PBS and 10^6cells were resuspended in 400 μl of PBS. As a positive control the infected moDC and as a negative control uninfected moDCs were resuspended in PBS without PFA treatment.

For testing the inactivation of the virus in PFA-fixed cells, different amounts (100 μl, 30 μl and 5 μl) of samples were inoculated on confluent MDCK cells in 12-well plates in replicate wells followed by collection of supernatant samples at different time points. Positive and negative samples were also inoculated. Fresh media (2 ml/well) was changed after 2 h and cells were left to incubate for 4 days. On day 4, and again on day 8, supernatant samples (200 μl) were inoculated on fresh MDCK cells in 12-well plates for a new passage. Total cellular RNA and supernatant samples were collected on days 1, 2, 3, 4, 7, 8, 9 and 10. The supernatant samples were subjected to an infectivity test on A549 cells using an immunostaining assay. The total cellular RNA samples were tested by RT-PCR for detecting any increase in viral RNA.

2.8. Immunostaining assay

Immunostaining assay with 3-amino-9-ethylcarbazole (AEC) substrate was used for testing any remaining infectivity in the supernatant samples collected from A549 (in RIPA, RIPA and Laemmli, RLT and MeOH cases) or MDCK cells (in case of PFA) after inoculation of treated samples. Tested samples which are described in Table 1, were diluted 1:10 in E-MEM and inoculated onto MDCK or A549 cells, respectively. Fresh E-MEM medium with 5% FCS was changed after 2 h incubation. After 3 days cells were fixed with ice cold 80% acetone for 10 min at +5 °C. Acetone was removed and the wells were washed with PBS before immunostaining. Cells were stained with mouse monoclonal antibodies against influenza A virus nucleoprotein (NP) (Medix Biochemica, 7307 SPRN-5). Secondary antibody was HRP-conjugated polyclonal goat anti-mouse immunoglobulins (Dako). After antibody staining cells were treated with AEC substrate with 0.03% hydrogen peroxide and washed with PBS. The cells were monitored under Olympus CK2 microscope.

For supernatant samples collected from A549 cells, as with RIPA, RIPA + Laemmli buffer, RLT and MeOH inactivation testing, immunostaining assay was done in MDCK cells (canine) instead of A549 cells (human) to avoid any antiviral effects of possible interferons in the supernatant samples. Vice versa, if supernatant samples were collected from MDCK cells the immunostaining assay was done in A549 cells.

2.9. RT-PCR

After RIPA and RIPA+Laemmli buffer, RLT, MeOH and PFA treatments, the infectivity of samples was tested on fresh A549 cells. Supernatant samples were collected at different time points for the detection of any residual viral RNA expression into the supernatants. 100 μl of supernatant sample was lysed in 350 μl of RLT buffer. RNA was isolated using RNEasy Mini kit (Qiagen) with Qiacube device (Qiagen). cDNA was synthetized with RevertAid H Minus Reverse Transcriptase (Thermo Scientific) kit according to the manufacturer's instructions with RiboLock RNase inhibitor (Thermo Scientific) and random

Table 1
Immunostaining of MDCK cell cultures with anti-nucleoprotein antibodies for detecting any remaining infectivity in re-culturing H5N1-infected moDCs after different sample treatments.

			Passage 1				Passage 2			
	Treatment[a]	μl tested	before wash day 1	after wash day 1	day 2	day 3	day 4	day 8	day 11	
H5N1 infected moDCs	RIPA	100 μl	neg	neg	neg	neg	neg	neg	neg	
		5 μl	neg	pos	pos	pos	pos	pos	pos	
	RIPA + Laemmli	100 μl	neg	neg	neg	neg	neg	neg	neg	
	RLT	100 μl	neg	neg	neg	neg	neg	neg	neg	
	MeOH 90%	100 μl	neg	neg	neg	neg	neg	neg	neg	
	PBS	5 μl	pos	pos	pos	pos	pos	pos	pos	
Uninfected moDCs	PBS	100 μl	neg	neg	neg	neg	neg	neg	neg	

			Passage 1		Passage 2		Passage 3			
			after wash day 1	day 2	day 3	day 4	day 7	day 8	day 9	day 10
H5N1 infected moDCs	PFA 4%	100 μl	neg	neg	neg	neg	neg	neg	neg	neg
	PBS	5 μl	neg	pos	pos	pos	pos	pos	pos	pos
Uninfected moDCs	PBS	100 μl	neg	neg	neg	neg	neg	neg	neg	neg

[a] RIPA, radio-immuno precipitation assay buffer; MeOH, methanol; PFA, paraformaldehyde

hexamers (Roche) as primers. RT-PCR was performed using QIAGEN® QuantiTect™ Multiplex PCR NoRox Kit (Qiagen) with primers and probes specific for influenza A virus M1 gene (Arilahti et al., 2014). The data is shown as threshold cycle (Ct) values, which is the cycle number at which the fluorescent signal of the reaction crosses the threshold.

For PFA-treated samples the sample inactivation was also tested by inoculation on MDCK cells for different time periods. Cells were washed with PBS and lysed in RLT buffer and total cellular RNA was isolated using RNEasy Mini kit (Qiagen). DNase digestion was performed with RNase-free DNase kit (Qiagen). 0.5 μg of total cellular RNA was transcribed to cDNA using TaqMan Reverse Transcriptase kit (Applied Biosystems) with random hexamers as primers. cDNAs were amplified by PCR using TaqMan Universal PCR Master mix and with the same influenza A virus M1 primer–probe pair as above. The data is shown as threshold cycle (Ct) values, which is the cycle number at which the fluorescent signal of the reaction crosses the threshold.

2.10. Viral inactivation tests in chicken eggs

The efficiency of inactivation treatments was also tested by culturing the samples in chicken eggs (Fig. 1B). Infected A549 cells were fixed (1 well of 12-well plate/0.5 ml fixative) with 4% PFA for 30 min at room temperature or with ice cold 90% MeOH for 20 min at −20 °C, after which cells were washed with PBS. Cells from one well were resuspended in 500 μl of PBS, further diluted 1:20 in PBS and inoculated into fertilized chicken eggs in the volume of 100 μl/egg (1/100 of the cells in the well per egg). As a negative control uninfected A549 cells were prepared with the fixatives in a similar manner.

Infected A549 cells from one well of 12-well plate were lysed in 100 μl of RIPA buffer. The sample was diluted 1:100 in PBS and 100 μl of the sample was inoculated into eggs. 33 μl of 4 x Laemmli buffer was added to 100 μl RIPA-treated sample and boiled for 10 min. RIPA + Laemmli buffer-treated sample was diluted 1:75 in PBS and 100 μl of the sample was inoculated into eggs (1/100 of the cells in a well per one egg). As a negative control uninfected A549 cells were prepared with RIPA and RIPA + Laemmli buffer the same way. Infected A549 cells and uninfected negative control A549 cells both from one well of 12-well plate were lysed to 350 μl of RLT buffer (Qiagen). Samples were diluted 1:30 in PBS and 100 μl of sample was inoculated into eggs (1/100 of the cells in a well per egg).

As a positive control infected A549 cells from one well of 12-well plate were resuspended in 250 μl PBS, further diluted 1:400 in PBS and inoculated into eggs in the volume of 100 μl to get 1:1000 final dilution of the cells to be inoculated into the egg. A negative control was prepared the same way except that 1:100 dilution of the cells was inoculated in one egg.

Chemically inactivated/treated and control samples were inoculated into 10 day-old embryonated chicken eggs. The volume of the inoculations was 100 μl. All infected samples, either PBS-treated or inactivated with different reagents, were inoculated into six eggs and non-infected samples with different treatments into three eggs. 24 h after the inoculation the mortality of the embryos were determined and the death of an embryo at this point was considered not to be due to an infection but due to egg injection. 72 h after inoculation allantoic fluid was collected from each egg, and a hemagglutination (HA) titration was done with a standard protocol using 0.5% guinea pig red blood cells. Hemagglutination titer was considered as the sign of viral growth in the eggs.

3. Results

3.1. RIPA

Originally RIPA buffer is described as a cell lysis buffer for immunoprecipitation assay but it is also widely used for many other downstream applications, like for SDS-PAGE, Western blotting and ELISA. RIPA buffer lyses and solubilizes of cytoplasmic, membrane and nuclear proteins from cultured mammalian cells. RIPA buffer is commercially available but it is also often in-house made. Therefore, there may be some variation between different RIPA recipes. For Western blotting cells are lysed in RIPA buffer, Laemmli sample buffer is added and samples are boiled before the SDS-PAGE separation and immunoblotting.

To investigate the inactivation capacity of RIPA buffer against the HPAI H5N1 virus, we infected moDCs with H5N1 virus and lysed the cells in RIPA buffer. We inoculated different amounts of the RIPA-treated moDCs samples onto A549 cells. Supernatant samples were collected at different time points for viral RNA level analysis by RT-PCR and detection of infective viral particles by immunostaining. Viral RNA levels decreased in the A549 cell culture during the incubation period in the inoculations with 100 μl or 30 μl of RIPA buffer-lysed moDCs sample, but with 5 μl of the sample viral RNA increased after the 2nd day of culturing and continued to increase in the second passage (Fig. 2A). This suggests that with larger volumes of the RIPA-lysed sample the toxicity of the reagent prevents the re-culture of the sample but in low volume of the RIPA buffer-lysed sample still contained replication competent viral particles and the sample could still be infective after the RIPA treatment. Culturing the supernatant samples from inoculation with 100 μl of RIPA buffer-lysed moDCs sample on MDCK cells we did not reveal any infected cells with immunostaining (Table 1). However, the supernatant samples from 5 μl inoculation of RIPA buffer-lysed moDCs sample on MDCK cells showed infectivity with immunostaining (Table 1). Thus, immunostaining results are completely compatible with RT-PCR results. Based on these results, it seems that RIPA buffer alone is not sufficient to inactivate H5N1 virus in cell lysates. Next, we wanted to test the addition of the Laemmli sample buffer to the lysates and denaturation of the samples by boiling. With these treatments viral RNA levels decreased in all A549 cell cultures inoculated with H5N1-infected cellular lysates (Fig. 2B). The same supernatant samples showed no infectivity in cell cultures by immunostaining (Table 1). This suggests that a combination of RIPA, Laemmli buffer and boiling is sufficient to fully inactivate the H5N1 virus.

We also conducted inactivation experiments with another method. H5N1 infected A549 cells were collected in RIPA buffer alone or in RIPA combined with Laemmli buffer and boiling as previously described. Now we inoculated the treated samples in embryonated chicken eggs and performed HA titration for the allantoic fluid samples collected 3 days after inoculation. We noticed that all the six replicative eggs inoculated with RIPA-treated sample showed hemagglutination (HA) positivity indicating that RIPA buffer did not inactivate the H5N1 virus (Table 2). Instead, only one out of six eggs after the RIPA and Laemmli-treated sample inoculation showed HA titers. This confirms our results from RT-PCR and immunostaining.

This data suggests that combining the RIPA and Laemmli sample buffers followed by boiling the sample for 10 min inactivates the HPAI H5N1 virus from infected cell lysates, but RIPA buffer alone is not sufficient to kill the H5N1 virus.

3.2. RLT

Guanidinium thiocyanate or guanidine thiocyanate (GITC) is commonly used in RNA and DNA extraction protocols because GITC lyses cells and viruses and denatures proteins including RNases and DNases. It is generally considered that GITC inactivates viruses (Blow et al., 2004; Honeywood et al., 2021). To analyze whether GITC containing lysis buffer is sufficient to inactivate H5N1 virus from cell samples, we tested the RLT buffer from RNEasy kit (Qiagen) in the same way as previously described with RIPA buffer. We infected moDCs with HPAI H5N1 virus and after 24 h infection cells were lysed with GITC containing RLT buffer. 100 μl, 30 μl or 5 μl RLT-treated moDCs samples were inoculated on A549 cells and supernatant samples were collected on the 1st day before and after the media change, 2nd and 3rd day, and

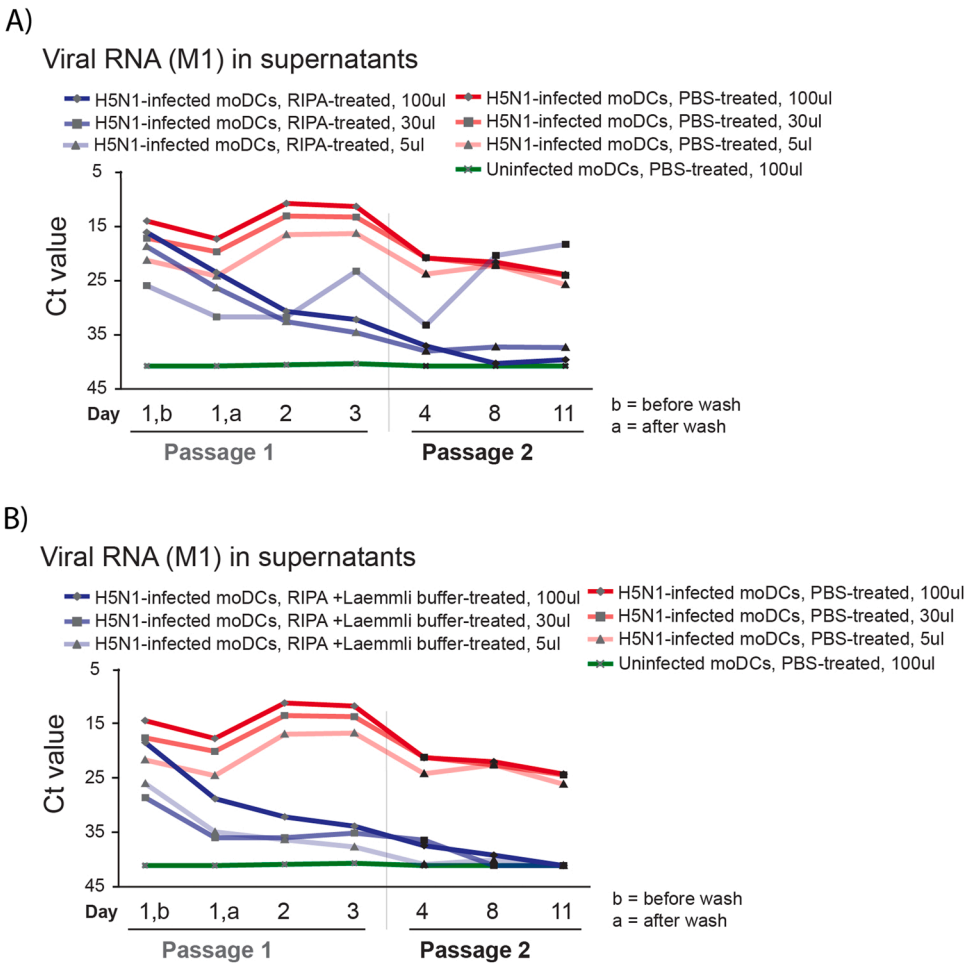

Fig. 2. Effect of RIPA or a combination of RIPA and Laemmli buffer and boiling on replication of H5N1 virus. The moDCs were infected with A/Vietnam/1194/2004 (H5N1) virus at MOI 5 and cells were harvested 24 h after infection. Cells were lysed to A) RIPA buffer or B) RIPA and Lemmli buffer and boiled for 10 min. Treated samples were inoculated on A549 cells and fresh medium was changed after 1 h. Supernatant samples were collected on day one before and after medium change, and on the 2nd, 3rd, 4th, 8th and 11th days. Supernatant samples were inoculated on fresh cells on the 4th day. Viral M1 RNA was analyzed from supernatant samples by RT-PCR. The experiment was repeated twice.

after passaging the culture to fresh A549 cells on 4th, 8th and 11th day. In RLT-treated samples influenza M1 gene-specific viral RNA decreased in culture supernatants during the incubation period while with PBS-treated samples viral RNA increased between 1st and 3rd days of cultivation and again between 4th and 8th days in the second passage (Fig. 3A). Further on, the same supernatant samples from the 100 µl inoculation of RLT-treated moDCs failed to show any infectivity after passaging them in MDCK cells (Table 1). This indicates that H5N1 virus was able to start the replication from the PBS-treated cell samples, but RLT-treatment fully inactivated the H5N1 virus. Similar experiment was also done in the embryonated chicken egg model. None of the six eggs inoculated with RLT-treated A549 samples showed positive HA titers while all six eggs from PBS-treated cell samples showed positive HA titers (Table 2). These results confirm that RLT is able to inactivate the H5N1 virus in cell samples.

3.3. MeOH

Methanol (MeOH) dehydrates the cells, precipitates proteins and removes lipids from the cell membrane (Hobro and Smith, 2017) and is a commonly used cell fixative. We tested the inactivation capacity of MeOH with a similar cultivation experiment as we used with RIPA and RLT buffers. H5N1 virus-infected moDCs were treated with MeOH, samples were washed, diluted to PBS and inoculated on A549 cells. Supernatant samples were collected on the 1st day before and after media change, 2nd and 3rd days and after second passage on the 4th, 8th and 11th days. Based on RT-PCR analysis of the supernatants, in MeOH-treated cell samples viral RNA decreased while viral RNA levels increased in the wells of PBS-treated infected cell samples (Fig. 3 B). With immunostaining we did not detect any infected MDCK cells on the wells with supernatants from MeOH-treated samples (Table 1). Also, virus inactivation test in chicken eggs confirmed these results, and none of six eggs inoculated with MeOH-treated samples showed positive HA titer (Table 2). These results show that MeOH treatment is sufficient to inactivate the H5N1 virus in infected cells.

3.4. PFA

Paraformaldehyde (PFA) and other aldehyde-based fixatives act by cross-linking proteins and other molecules in the cells (Hobro and Smith, 2017). Like organic solvents also aldehyde-based fixatives are widely and for long used fixatives. In this study we tested the efficiency of PFA to inactivate HPAI H5N1 virus from cell samples. We tested the inactivation capacity of PFA in the same way as RIPA, RLT and MeOH. H5N1 virus-infected moDCs were treated with 4% PFA, samples were washed, diluted to PBS and inoculated on A549 cells. Supernatant samples were collected on the 1st day before and after media change, 2nd and 3rd days and after second passage on 4th, 8th and 11th days. Viral RNA levels were analyzed by RT-PCR. Viral RNA levels seemed to increase during the passage one, but after the new passage of the samples in a fresh set of cells the increase in viral RNA level was weakly detectable only with the 5 µl sample (Supplementary data Fig. 1).

To analyze whether the detected viral RNA originated from the infectivity of the fixed moDC sample or whether the RNA was released from the fixed cells without virus replication, we conducted the cultivation experiment in a different way. We changed the cell model to MDCK cells which can resist longer cultivation time and we analyzed total cellular RNA levels instead of supernatant RNA levels. H5N1-

Table 2
Inactivation efficacy of sample treatments of the highly pathogenic avian influenza H5N1 virus-infected cells. A549 cells infected with H5N1 virus were treated with sample preparation reagents and inoculated into embryonated chicken eggs. Infectivity of the treated samples were determined from the allantoic fluid with hemagglutination titration.

	Treatment[a]	Score *
H5N1-infected A549 cells	RLT	0/6
	MeOH 90%	0/6
	PFA 4%	0/6
	RIPA	6/6
	RIPA + Laemmli	1/6
	PBS	6/6
Uninfected A549 cells	RLT	0/3
	MeOH 90%	0/3
	PFA 4%	0/3
	RIPA	0/3
	RIPA + Laemmli	0/3
	PBS	0/3

[a] MeOH, methanol; PFA, paraformaldehyde; RIPA, radio-immuno precipitation assay buffer
* Number of infected embryos / number of total embryos inoculated; n=6 or n=3

infected moDCs were treated with 4% PFA and cells were washed with PBS before the inoculation on MDCK cells. Total cellular RNA and supernatant samples were collected from MDCK cells at 1st, 2nd,3rd, 4th, 7th, 8th, 9th and 10th days. The cultures were blind passaged on fresh MDCK cells on the 4th and 8th days. Viral M1 RNA expression was analyzed by RT-PCR from total cellular RNA samples. H5N1-infected cells treated with PFA failed to produce increasing levels of viral M1 RNA after culturing in MDCK cells (Fig. 4), while H5N1-infected cells treated with PBS prior to inoculation on MDCK cells led to an increase in viral M1 RNA expression in cellular RNA samples (Fig. 4). Supernatant samples collected from MDCK cultures with H5N1-infected and PFA or PBS-treated moDCs were further cultivated on A549 cells for detecting any remaining infectivity. In immunostaining all the wells with supernatants from the 100 µl of PFA-treated samples were negative (Table 1.), whereas the positive control, H5N1 virus-infected moDCs in PBS led to an infection that was evident in immunostaining in A549 cells (Table 1). Both the results from RT-PCR and immunostaining indicate that 4% PFA inactivates HPAI H5N1 virus from infected cell samples.

The inactivation efficiency of PFA on H5N1 virus was tested in embryonated chicken eggs. The results showed that H5N1 virus was fully inactivated by PFA treatment since none of the eggs inoculated with PFA-treated cellular samples showed positivity in the HA titrations (Table 2.). Results from HA titrations confirm the results from RT-PCR and immunostaining and indicate that 4% PFA inactivates the HPAI H5N1 virus from cell samples.

4. Discussion

In many laboratories infectious samples are inactivated in a BSL-3 laboratory followed by further analyses in lower biosafety conditions. To ensure the biosafety at lower containment level the efficacy of the inactivation procedures should be properly demonstrated. Laboratory methods, circumstances, working practices, microbes etc. vary from one laboratory to another and consequently inactivation methods should be tested in each laboratory individually. Unfortunately, there have been many incidences of laboratory-acquired infections from improperly inactivated or mishandled microbes (Abad, 2012a; Centers for Disease Control and Prevention (CDC), 2002; Sewell, 1995. Thus far there are no reports on human exposure with avian influenza virus by a laboratory-acquired incident but there is one report on a cross-contamination of a low pathogenic avian influenza (LPAI) H9N2 virus culture with an HPAI H5N1 virus (CDC, 2014). The risk for a laboratory-acquired infection can be reduced using appropriate procedures and techniques, containment devices and facilities, and training of the personnel. Often, inactivation studies have been done directly for the virus culture, not for the cell samples containing potentially infective viruses. For efficient virus inactivation, the inactivation reagents must reach viral components inside the cells. The efficacy of a given inactivating chemical can vary between cell types, reagent types, and the type of virus used in the experiments. It has been shown that influenza virus infectivity is preserved when the virus is in a protein rich environment, such as dried mucus or culture medium which indicates that the infectivity of the virus is higher if the virus is stored in its original production environment, supernatant and cells (Thomas et al., 2008). In that aspect our approach to study the efficacy of inactivation methods in infected cell samples is crucial and can be adopted to other sample types with lower infectivity. In our previous study we have shown that the HPAI H5N1 virus infection is productive and the virus spreads extremely efficiently in human moDCs (Westenius et al., 2018) and it also readily replicates in A549 cells (Arilahti et al., 2014). Thus, we decided to use these cell models to study the efficacy of different inactivation methods to HPAI H5N1 virus. We tested the inactivation efficiency of commonly used reagents such as RIPA lysis buffer, a combination of RIPA and Laemmli denaturation buffers, RLT lysis buffer for RNA/DNA extraction, and MeOH and PFA that are used to fix cells.

Although RIPA buffer is a commonly used buffer for several protocols, there is no information on its inactivation efficiency against influenza viruses. We analyzed whether the HPAI H5N1 infected cells still maintain their infectivity after RIPA treatment. Surprisingly, based on our results it seems that RIPA buffer alone is not sufficient to inactivate the HPAI H5N1 virus in infected cells (Fig. 2A and Tables 1 and 2). Based on the viral RNA expression in the supernatant after inoculation of RIPA-treated samples, we noticed that in one out of the three samples viral RNA seemed to increase during the cultivation (Fig. 2A) and the same supernatant sample (5 µl of treated sample) showed positivity in immunostaining indicating infectivity. In addition, all six inoculated eggs showed positive HA titers from RIPA-treated samples (Table 2). Thus, based on our results from RT-PCR and immunostaining, it seems that only the culture supernatant samples from the inoculation with low volume (5 µl) of RIPA-lysed moDCs sample induced infectivity and those cultures from inoculation with 30 µl or 100 µl of RIPA-lysed moDCs samples could not start the infection. It seems that inoculation with 30 µl or 100 µl of RIPA-lysed moDCs samples with higher amount of RIPA buffer in those cultures, the A549 cells were lysed and killed by the reagent and thus the supernatant samples remained negative in RT-PCR and immunostaining tests.

For the Western blot analysis, protein lysates are further denatured in Laemmli sample buffer and boiling. Thus, we investigated whether these treatments would be sufficient to inactivate the infectivity of the HPAI H5N1 virus. Our results from RNA level analysis by RT-PCR (Fig. 2B) and detection of infective viral particles by immunostaining (Table 1) clearly indicate that the combination of RIPA and Laemmli buffers followed by boiling is effective in inactivating the H5N1 virus in human primary moDCs. However, one out of the six eggs showed hemagglutination positivity from the sample after these treatments (Table 2) which may indicate that the inactivation was still not complete and that there was some infective virus left. It may be that even very small amounts of non-inactivated H5N1 virus is sufficient to trigger virus growth since chicken embryos are highly susceptible for the HPAI H5N1 virus. However, our other infection model with the results from RT-PCR and immunostaining showed clearly that RIPA and Laemmli buffers and

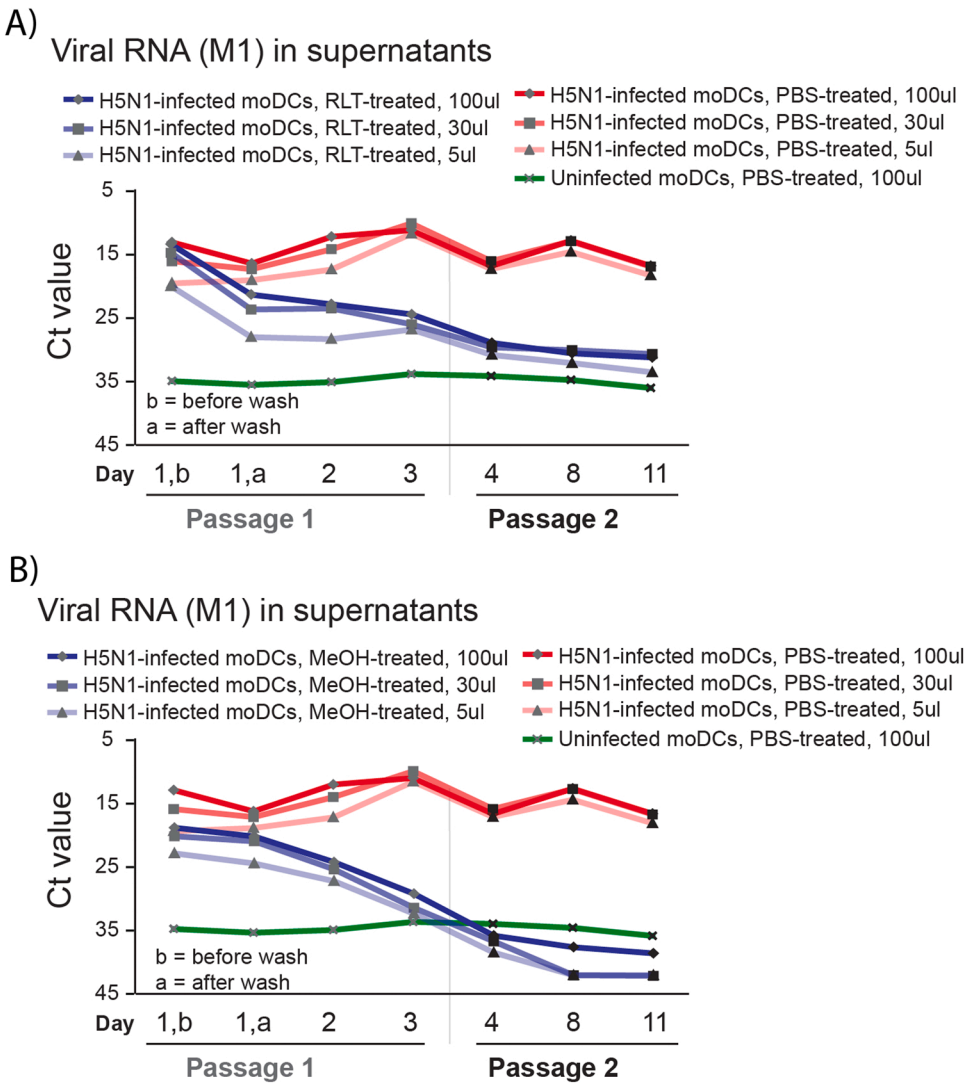

Fig. 3. Inactivation efficiency of RLT and MeOH on H5N1 virus. Influenza A/Vietnam/1194/2004 (H5N1) virus was used at MOI of 5 to infect moDCs for 24 h. After infection the cells were A) lysed to RLT buffer or B) fixed with 90% MeOH for 20 min at −20 °C. Treated samples were inoculated on A549 cells for 1 h and fresh medium was changed. Culture supernatants were inoculated on fresh cells on the 4th day as a second passage. Supernatant samples were collected at indicated time points for assessment of viral M1 RNA expression by RT-PCR. Results are mean Ct values from triplicates PCR tests. Both inactivation reagents were tested in two repeated experiments.

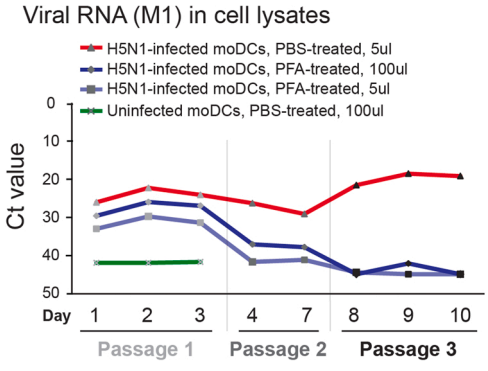

Fig. 4. H5N1 virus infectivity in PFA-fixed cells. After 24 h post-infection with influenza A/Vietnam/1194/2004 (H5N1) virus moDCs were fixed with 4% PFA for 30 min at room temperature and samples were inoculated on MDCK cells. The inoculation was changed to fresh medium at 1 h time point, and cultures were passaged onto fresh cells at day 4 and 8. Total cellular RNA samples were collected during the cultivation and virus replication was analyzed by RT-PCR using the M1 gene specific primers and probes. Mean Ct value from duplicates PCR reactions is shown. A representative experiment out of two is shown.

boiling inactivated the H5N1 virus. Our finding is in line with other studies showing that influenza viruses, including HPAI H5N1 viruses, are sensitive to heat inactivation albeit the ability to tolerate heat varied between the strains (Shahid et al., 2009; Wanaratana et al., 2010; Swayne, 2006). Thus, it is expected that boiling for 10 min would inactivate HPAI H5N1 virus as it has been reported that the treatment at 75 °C for 10 min is sufficient to do that (Wanaratana et al., 2010). Sodium dodecyl sulfate (SDS) is an antimicrobial/denaturing agent which is able to inactivate several viruses (Howett et al., 1999; Krebs et al., 1999). Usually RIPA buffers contain 0.1–0.5% SDS whereas the Laemmli sample buffer contains 2% SDS. 0.1% SDS does not inactivate Ebola virus in blood samples (van Kampen et al., 2017) while 0.5% SDS has been demonstrated to inactivate viruses like SARS-CoV-2 and HIV (Krebs et al., 1999; Patterson et al., 2020; Urdaneta et al., 2005). This indicates that the Laemmli sample buffer as such may be sufficient to inactivate the H5N1 virus before the samples are even boiled. We did, however, not test this option in our study.

A combination of GITC and phenol, like in Trizol®, is typically used for RNA or DNA isolation. These chemicals have been shown to efficiently inactivate enveloped viruses (Blow et al., 2004). However, presently many nucleic acid extraction kits are provided with commercial lysis buffers which contain only GITC due to the toxicity of phenol, like AVL (Qiagen), RLT (Qiagen) or easyMAG (bioMerieux). GITC-containing buffers have been proven to inactivate multiple

viruses, including enveloped viruses such as Middle East respiratory syndrome coronavirus (Kumar et al., 2015) and influenza B virus (Ngo et al., 2017). It is generally thought that enveloped viruses are more susceptible than non-enveloped viruses to physical and chemical inactivation treatments (Chandra et al., 1999; Watanabe et al., 1989). However, one should not assume that GITC-containing buffers would be efficient to inactivate all enveloped viruses. Indeed, there are studies showing impaired inactivation capacity of GITC against West Nile virus (Ngo et al., 2017; Abad, 2012b), and moreover, it has been shown that neither RLT or AVL buffer is able to inactivate Chikungunya virus (Ngo et al., 2017). Here we showed that HPAI H5N1 virus is efficiently inactivated by RLT buffer and this is well in line with reports with influenza B virus (Ngo et al., 2017).

In many research applications of life sciences the sample fixation is a fundamental step; after fixation samples are preserved and they can be stored and analyzed later. For highly infectious samples, if the fixation inactivates the infectious agents, samples could be analyzed in a lower biosafety level. Organic solvents, like MeOH, and aldehyde-based fixatives, like PFA, are used for this purpose. Although both MeOH and PFA are historical and widely used fixatives for different kind of sample materials, the virus inactivation efficiency of aldehydes has shown to be inconsistent and dependent on the incubation time (Kumar et al., 2015; Fischman and Ward, 1969) while the inactivation efficiency of MeOH is much less studied. Based on our results both MeOH and PFA inactivate the HPAI H5N1 virus and thus the samples can be safely transported to a lower biosafety level after MeOH or PFA fixation.

Several studies have investigated the inactivation efficiency of different chemical and physical conditions against influenza virus but the results appear to be somewhat variable (Zou et al., 2013; Pawar et al., 2015; De Benedictis et al., 2007). Also, many of these studies have been focusing on disinfection efficacy instead of sample preparation for further analysis. In the present study we evaluated some commonly used sample preparation reagents for biochemical laboratory analyses for inactivation of HPAI H5N1 virus. In conclusion, this study demonstrates that some of the cell lysis buffers which break the cell membranes are not fully sufficient to destroy the infectivity of the virus in its cellular context. Increasing the concentration of detergents to solubilize also the membrane proteins seemed to disrupt the formation of infectious particles from cell lysates. However, the common fixatives MeOH and PFA as well as the lysis buffer for RNA/DNA extraction kits, RLT buffer, were all effective in inactivating the H5N1 avian influenza virus in virus infected cell samples.

CRediT authorship contribution statement

Veera Avelin: Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing. **Susanna Sissonen:** Conceptualization, Funding acquisition, Writing – review & editing. **Ilkka Julkunen:** Conceptualization, Investigation, Resources, Funding acquisition, Writing – review & editing. **Pamela Österlund:** Conceptualization, Methodology, Investigation, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

We thank Professor Ron A. M. Fouchier for the HPAI H5N1 virus. Influenza A NP antibody was obtained from Medix Biochemica, we appreciate that. We are grateful to our technical assistants Hanna Valtonen, Riitta Santanen, Johanna Rintamäki, Marja-Liisa Ollonen and Tiina Sihvonen.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2022.114527.

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