

Assay of Several Inactivation Steps on West Nile Virus and H7N1 Highly Pathogenic Avian Influenza Virus Suspensions

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Abstract

Objectives: In any research laboratory, precautionary measures must be taken in order to reduce or eliminate the potential risk of accidental infection by biosafety level 3 (BSL3) pathogens, as Highly Pathogenic Avian Influenza Virus (HPAI) or West Nile Virus (WNV). Appropriate virus inactivation procedures have to be set up to allow necessary further processing of specimens outside BSL3 facilities.

Methods: To study the elimination of WN and HPAI virus infectivity, the effect of different chemical and physical inactivation procedures on viral suspensions were investigated. A proper cell culture assay for each virus was performed to verify several treatments, which are commonly performed preceding transfer of materials outside of biocontainment, still allowing further investigations like sequencing or genome amplification.

Results: Chemical inactivation with AVL buffer (Qiagen), Trizol[®] Reagent or Phenol chloroform: isoamyl alcohol treatment, as well as physical treatment (heat at two temperatures and three contact times) reduced viral infectivity in the viral suspension below the detection limit.

Conclusion: Thermal treatments, but also Trizol[®] Reagent and AVL buffer (Qiagen), are suitable to produce non-infectious specimens for further use in molecular biology techniques.

Keywords: Biosafety laboratory; West Nile Virus; Highly Pathogenic Avian Influenza Virus; Virus inactivation; Heat; Nucleic acid extraction

Introduction

Over the last few years, there has been an important increase in research on viral emerging diseases. This has led to rise in the numbers of BSL3 facilities handling those emerging viruses (Avian Influenza Virus, SARS Coronavirus, and West Nile Virus). However, the set up and maintenance of huge BSL3 areas is expensive. If proper inactivation procedures are evaluated and performed in a standardized way, the BSL3 areas could be smaller, and especially devoted to initial handling and inactivation treatment of a BSL3 pathogen, while further technical processing (immunology, molecular biology) could be performed on the bench of a BSL3 area or indeed, areas of lower biosafety level (BSL2).

West Nile virus (WN virus), belonging to the genus *Flavivirus* in the *Flaviviridae* family, are spherical with a lipid envelope, 40-60 nm in diameter, enclosing a single linear positive sense RNA molecule [1]. This virus can cause human diseases as meningitis and encephalitis and for its handling and propagation, a BSL3 environment is mandatory.

HPAI viruses belong to *Orthomyxoviridae* family. Virions are pleomorphic, 80-120 nm in diameter, enclosing a negative strand RNA genome constituted by 8 segments [1]. They can cause a severe human disease and also require containment level 3.

Swine Vesicular Disease Virus (SVDV) is a species in the genus *Enterovirus*, in the *Picornaviridae* family. It is a non-enveloped virus, with icosahedral capsids of 22-30 nm in diameter surrounding a RNA genome [1]. They were included in these studies as a representative of non-enveloped viruses.

Thus, the objective of the present study was to verify the ability of several treatments on the reduction of infectious titres of WN and HPAI viral suspensions.

The threshold standards of efficacy ($>4 \log_{10}R$) to be achieved

has been taken from the guidelines of viral safety standard of blood derivative products [2,3], an issue that became especially important with the rise of AIDS, as well as with episodes of transmission of hepatitis C virus to recipients. In case of total inactivation, a further use of those treated materials in research assays on the bench could be allowed.

Material and Methods

Cells and viruses

MDCK cells (Madin Darby Canine Kidney) were used for infection with highly pathogenic H7N1 avian influenza (HPAI) virus (*A/Instituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy*). Cells were grown in 96-well plates using Earle's MEM supplemented with 2 mM L-Glutamine (Biochrom AG), 10% Foetal calf serum (FCS, Euroclone Ref. ECS0180L), and Penicillin/Streptomycin/Nystatin (100 U/100 µl/100 U per ml, Biochrom AG and SIGMA, respectively).

Vero cells (kindly provided by Catherine Cetre-Sossah from CIRAD, France) were used for infection with WNV (NY-99 strain). Cells were grown in 96-well plates using Earle's MEM supplemented

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with 2 mM L-Glutamine, 5 % FCS (Euroclone Ref. ECS0180L) and Penicillin/Streptomycin/Nystatin.

IBR-2 cells grown in Earle's MEM supplemented with 2 mM L-Glutamine (Biochrom AG), 5% FCS (Euro-Clone Ref. ECS0180L) and Penicillin/Streptomycin/Nystatin (100 U/100 µg/40 U per ml, from Biochrom AG and SIGMA, respectively), were used for swine vesicular disease virus (SVDV; UK-72 strain, kindly provided by Institute for Animal Health, Pirbright, UK). SVDV was included as a model of non-enveloped virus, with a higher tenacity to inactivation treatments than enveloped viruses [4-6].

Inactivation treatments

Chemical treatments: The following treatments were evaluated: viral suspension mixed with Phenol:chloroform:isoamyl alcohol (1:9, v:v) for 10 minutes; a mixture of Trizol® reagent and viral stock (9:1, v:v) for 10 minutes and a mixture of AVL buffer and viral stock (9:1, v:v), also for 10 minutes. Immediately after treatment, treated and non-treated samples were serially diluted and titrated.

Heat treatments: Viral stocks diluted 1/10 in PBS were heated at 70°C for 5 or 15 minutes or at 100°C for 8 minutes, by using a thermoblock (VLM Bio1, VLM GmbH, Germany). Treated samples were transferred to an ice bath immediately after the treatment. Next, samples were diluted and titrated with the non-treated samples.

Experimental assays

The initial titres of all viral suspensions were up to 7 log₁₀ Tissue Culture Infectious Dose or TCID₅₀/ml. For all viruses, two different viral stocks were used. All viral suspensions (WNV, H7N1 and SVDV) were obtained from supernatants of the corresponding infected cell cultures (Vero, MDCK and IBR-2, respectively). All samples (non-treated and treated after the corresponding contact times) were 10-fold serially diluted with PBS and titrated twice in the corresponding cell lines grown in 96 well-plates, using at least eight wells for each dilution. All treatments were performed in duplicate and samples titrated twice.

After four to seven days of incubation at 37°C in a CO₂ incubator, cells were microscopically examined for virus-specific Cytopathogenic Effects (CPE). The results were read on the basis of microscopically visible virus specific CPE. The virus titres were calculated according to

and Emmons RW [7], and expressed as log₁₀ TCID₅₀/ml.

In all cases, cytotoxicity assays were performed in order to ascertain the first non cytotoxic dilution. In those assays, reaction mixtures in which viral suspensions were replaced by the corresponding volumes of cell culture medium were 5-fold serially diluted with PBS, and titrated twice in the corresponding cell lines grown in 96 well-plates using 8 replicates by dilution. Any destruction of cell monolayers should be assigned to toxicity, as viruses (and its hypothetical cytopathic effects) were not present in the reaction mixture. Only the first non cytotoxic dilution of the reaction mixtures was assayed to look for residual infectivity.

The reduction factor (RF) was calculated as the difference in the quotient of the infectious titre before (initial titre or before treatment, BT) and after incubation of the virus with the reagent, or thermal treatment (remaining or residual virus, after treatment, AT), expressed on a log₁₀ scale.

Results

Results of the different inactivation steps are depicted in Table 1. Initial virus titres in all assays were currently higher than 6.5 log₁₀ TCID₅₀/ml (with the sole exception of one HPAI trial). With respect to thermal treatment, Reduction Factors (RF) up to 4.5 log₁₀ was achieved in all cases, regardless of the virus type or the assayed temperature. As Phenol: chloroform: isoamyl alcohol, AVL or Trizol® resulted in higher toxicity for the aforementioned cell lines; the final RF achieved was lower, between 2.5 and 4.5 log₁₀. Moreover, in most of the cases, total inactivation (or inactivation below the detection threshold of the experimental assay) was reached. The symbol "≥" before each figure of viral titres at RF column indicated that the threshold of detection has been achieved, the first non cytotoxic dilution able to be assayed was completely free of viruses.

Discussion

When assaying viral inactivation, two different approaches can be followed: to assay a defined step from a well-known and standardized procedure, or to include an extra step to assure an additional viral inactivation. In our case, the approaches evaluated in this study were chosen because they represent component parts of widely accepted protocols (or commercial kits) in molecular biology.

	Contact time	Trial number	WNV			H7N1 AIV			SVDV		
			BT	AT	RF	BT	AT	RF	BT	AT	RF
Phenol:chloroform:isoamyl alcohol	10 min	1	ND	ND	ND	6.58	≤ 3.44	≥ 3.14	8.44	≤ 3.74	≥ 4.70
		2	ND	ND	ND	6.58	≤ 3.44	≥ 3.14	8.44	≤ 3.74	≥ 4.70
AVL lysis buffer	10 min	1	6.82	≤ 3.63	≥ 3.19	7.02	≤ 3.44	≥ 3.58	8.00	≤ 3.63	≥ 4.37
		2	6.66	≤ 3.73	≥ 3.03	7.64	≤ 4.44	≥ 3.21	8.38	≤ 3.73	≥ 4.65
Trizol	10 min	1	ND	ND	ND	6.83	≤ 4.44	≥ 2.39	7.57	≤ 4.73	≥ 2.84
		2	7.07	≤ 4.73	≥ 2.34	6.89	≤ 4.44	≥ 2.45	7.01	≤ 4.73	≥ 2.28
Heat treatment (70°C)	5 min	1	6.44	≤ 1.73	≥ 4.76	7.33	≤ 1.73	≥ 4.89	7.19	≤ 1.73	≥ 5.46
		2	6.63	≤ 1.16	≥ 5.47	5.76	≤ 1.16	≥ 4.60	7.63	≤ 1.16	≥ 6.47
Heat treatment (70°C)	15 min	1	≤ 8.13	≤ 2.24*	≤ 5.89	≤ 7.52	≤ 2.18*	≤ 5.34	≤ 8.76	≤ 2.49*	≤ 6.27
		2	6.75	≤ 1.16	≥ 5.59	5.88	≤ 1.16	≥ 4.72	7.75	≤ 1.16	≥ 6.59
Heat treatment (100°C)	8 min	1	8.25	≤ 0.74	≥ 7.51	6.39	≤ 1.73	≥ 4.66	8.38	≤ 0.74	≥ 7.64
		2	8.00	≤ 1.16	≥ 6.84	6.95	≤ 1.16	≥ 5.79	9.25	≤ 1.16	≥ 8.09

All data are expressed as log₁₀ TCID₅₀/ml (each value is the mean of two titres). WNV: West Nile Virus; AIV: Avian Influenza virus; SVDV: Swine vesicular disease virus; BT: Before treatment; AT: After treatment; RF: Reduction Factor; ND: Not done; *: residual infectivity detected.

When symbol ≤ appears before a viral titre at AT column, it means that all wells of the first non cytotoxic dilution of the reaction mixture were free of viruses, CPE was not observed; however, the inactivation could be higher.

Table 1: Efficacy of viral inactivation treatments for WNV, HPAI virus and SVDV.

Phenol: chloroform: isoamyl alcohol is an old and well-known step in many *in-house* nucleic acid (NA) extraction procedures. AVL lysis buffer is included in a commercial nucleic acid extraction kit (QIAamp Viral RNA Kit, Qiagen) and it contains guanidine thiocyanate. Also, Trizol[®] treatment is in the basis of several *in house* NA extraction procedures. Finally, the thermal treatments are also part of standardized protocols (70°C for 5 minutes in Nucleospin[®]RNA virus kit or 10-15 minutes in Nucleospin[®] Blood kit, both from Macherey-Nagel, Germany) and 100°C treatments for 8 minutes is classically used in our facility in bacterial NA extraction protocols for further processing outside.

SVDV was chosen as a control of non-enveloped virus, opposite to BSL3 enveloped viruses as HPAI virus or WNV. As a general rule, non-enveloped viruses are more resistant than enveloped viruses to physical and chemical inactivation treatments [4-6]. In the conditions, no significant differences in inactivation between SVDV and WNV or HPAI virus were observed, probably due to the strength of the applied procedures.

Inactivation of WNV in a detergent-containing buffer has been previously demonstrated [8]. Reductions of 3 log₁₀ WNV pfu's, by treatment with AVL or Trizol have been previously described [9]. As those effects have been observed for several other enveloped viruses (bunyavirus, alphavirus, filovirus), it could be generalized for the majority of enveloped viruses (as Avian Influenza). In this context, the inactivation results (but also the cytotoxicity results; cell death was reported until 1/100 dilution for AVL and 1/100-1/1000 for Trizol) for HPAI virus, are in total agreement with previous studies [9].

As expected, thermal treatments gave a strong safety margin in terms of robust inactivation of all tested temperatures and viruses. Classically, pasteurization treatments have given excellent results in terms of viral infectivity reduction [10,11]. These deleterious effects of higher temperature have been previously reported for BSL2 viruses as Hepatitis A virus, Reovirus, Bovine Parvovirus, Pseudorabies virus [12], or Encephalomyocarditis virus, but also for BSL3 viruses [13-16]. Frankfurt-1 strain of SARS Coronavirus suffered 4-5 log₁₀ reduction (log₁₀R) in infectious titres, when spiked in several plasma products and submitted to 60°C for 1 hour [17]. WNV is easily inactivated by pasteurization (60°C for 10 hours) [18,19]. With respect to HPAI virus, effective inactivation at 70°C to 73.9°C for less than 1 s has been reported [16]. Indeed, around 4 log₁₀ R has been recorded at the end of ramp-up from room temperature to 70°C (lasting 40 seconds) in thermoblock for two HPAI isolates [15], and predicted D-values for H5N1 in chicken meat at 70°C have been reported as short as 0.3-0.5 s [19]. In the given conditions, all trials fulfilled the general commitment of 4 log₁₀ R, but one single trial (70°C for 15 minutes) failed in the *gold standard* rule: no infectivity in final samples. The explanation for this could be related to inadequate mixing of the viral suspension, air bubbles or viral aggregation. Incomplete poliovirus inactivation, depending on mixing has been reported [20,21]. Air bubbles poorly conduct heat, and viral aggregation is an old and well-known explanation, when deviations in normal inactivation slopes were reported.

Since, the efficacy of the aforementioned inactivation procedures for the assayed viruses has been verified, the question arises whether these methods are suitable also for other BSL3 pathogens be handled in the facility in the near future (Rift Valley fever virus), but also for non human pathogenic viruses with a well know profile of resistance against inactivation treatments (as canine, bovine or porcine parvovirus), that could be used as model viruses [22,23].

In conclusion, different inactivation procedures (sometimes adapted from steps of commercial kits) for BSL3 pathogens (WNV and HPAIV) were evaluated. The RF obtained allows the use of treated samples in subsequent molecular biology assays on the bench. In fact, nowadays those inactivation procedures have been implemented in the laboratory with good reproducibility and accuracy.

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References

1. Mahy BWJ, Rowson KEK (1997) A dictionary of Virology (2nd edition), Academic Press, The University of Michigan.
2. Note for guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses (1996) The European Agency for the Evaluation of Medicinal Products Human Medicines Evaluation Unit, London.
3. The European Agency for the Evaluation of Medicinal Products Human Medicines Evaluation Unit (2001) Note for guidance on plasma-derived medicinal products, London.
4. Chandra S, Cavanaugh JE, Lin CM, Pierre-Jerome C, Yerram N, et al. (1999) Virus reductions in the preparation of intravenous immune globulin: in vitro experiments. *Transfusion* 39: 249-257.
5. Watanabe Y, Miyata H, Sato H (1989) Inactivation of laboratory animal RNA-viruses by physicochemical treatment. *Jikken Dobutsu* 38: 305-311.
6. The European Agency for the Evaluation of Medicinal Products, Evaluation of Medicines for Human Use (2001) EMEA workshop on viral safety of plasma-derived medicinal products with particular focus on non-enveloped viruses, London.
7. Schmidt NJ, Emmons RW (1989) General principles of laboratory diagnostic methods for viral, rickettsial and chlamydial infections. In: Diagnostic procedures for rickettsial and chlamydial infections (6th Edition) Schmidt NJ and Emmons RW (eds). American Public Health Association, Washington DC.
8. Mayo DR, Beckwith WH 3rd (2002) Inactivation of West Nile virus during serologic testing and transport. *J Clin Microbiol* 40: 3044-3046.
9. Blow JA, Dohm DJ, Negley DL, Mores CN (2004) Virus inactivation by nucleic acid extraction reagents. *J Virol Methods* 119: 195-198.
10. Biesert L, Lemon S, Goudeau A, Suhartono H, Wang L, et al. (1996) Viral safety of a new highly purified factor VIII (OCTATE). *J Med Virol* 48: 360-366.
11. Kempf C, Stucki M, Boschetti N (2007) Pathogen inactivation and removal procedures used in the production of intravenous immunoglobulins. *Biologicals* 35: 35-42.
12. Dichtelmüller H, Rudnick D, Breuer B, Kotitschke R, Kloft M, et al. (1996) Improvement of virus safety of a S/D-treated factor VIII concentrate by dry heat treatment at 100 degrees C. *Biologicals* 24: 125-130.
13. Isbarn S, Buckow R, Himmelreich A, Lehmacher A, Heinz V (2007) Inactivation of avian influenza virus by heat and high hydrostatic pressure. *J Food Prot* 70: 667-673.
14. Kraus AA, Priemer C, Heider H, Kruger DH, Ulrich R (2005) Inactivation of Hantaan virus-containing samples for subsequent investigations outside biosafety level 3 facilities. *Intervirology* 48: 255-261.
15. Swayne DE (2006) Microassay for measuring thermal inactivation of H5N1 high pathogenicity avian influenza virus in naturally infected chicken meat. *Int J Food Microbiol* 108: 268-271.
16. Thomas C, King DJ, Swayne DE (2008) Thermal inactivation of avian influenza and Newcastle disease viruses in chicken meat. *J Food Prot* 71: 1214-1222.
17. Yunoki M, Urayama T, Yamamoto I, Abe S, Ikuta K (2004) Heat sensitivity of a SARS-associated coronavirus introduced into plasma products. *Vox Sang* 87: 302-303.

18. Kreil TR, Berting A, Kistner O, Kindermann J (2003) West Nile virus and the safety of plasma derivatives: verification of high safety margins, and the validity of predictions based on model virus data. *Transfusion* 43: 1023-1028.
19. Remington KM, Trejo SR, Buczynski G, Li H, Osheroff WP, et al. (2004) Inactivation of West Nile virus, vaccinia virus and viral surrogates for relevant and emergent viral pathogens in plasma-derived products. *Vox Sang* 87: 10-18.
20. Thomas C, Swayne DE (2007) Thermal inactivation of H5N1 high pathogenicity avian influenza virus in naturally infected chicken meat. *J Food Prot* 70: 674-680.
21. Tierney JT, Larkin EP (1978) Potential sources of error during virus thermal inactivation. *Appl Environ Microbiol* 36: 432-437.
22. Borovec S, Broumis C, Adcock W, Fang R, Uren E (1998) Inactivation kinetics of model and relevant blood-borne viruses by treatment with sodium hydroxide and heat. *Biologicals* 26: 237-244.
23. Roberts PL, Hart H (2000) Comparison of the inactivation of canine and bovine parvovirus by freeze-drying and dry-heat treatment in two high purity factor VIII concentrates. *Biologicals* 28: 185-188.