Physicochemical inactivation of zoonotic viruses: Hemorrhagic fever with renal syndrome and lymphocytic choriomeningitis viruses

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Introduction

Hemorrhagic fever with renal syndrome virus (HFRSV), and lymphocytic choriomeningitis virus (LCMV) both being zoonotic viruses, have been transmitted from wild and laboratory rodents to humans (*Brummer-Korvenkontio et al.* 1980, *Lee et al.* 1979, *Lee et al.* 1980). The HFRSV groups is classified into Bunyaviridae, which have three segmented single-strand RNA, 5-structure proteins and a viral envelope, and are considered to be sensitive to lipid-solvents (*Martin et al.,* 1985). LCMV is also ethersensitive and belongs to arenaviridae (*Pfau et al.,* 1974). To destroy these viruses with disinfectants and/or physical treatments in biomedical animal research facilities, the virucidal effects of physicochemical means must be determined, especially to insure protection of laboratory personnel.

There have been several reports on the relative effectiveness of chemical disinfectants on conventional human viruses in hospital settings (*Kobayashi et al.* 1984, *Narang et al.* 1983, *Platt et al.* 1985, *Rodgers et al.* 1985, *Svedmyr et al.* 1980, *Spire et al.* 1984, *Tan et al.* 1981). However, the effectiveness of commonly used physicochemical treatments to zoonotic murine viruses has not yet been reported except for the effect of betapropiolactone on human serum in a suspected case of Lassa fever, an arenavirus infection (*Mitchell & McCormick,* 1984).

Recently established highly quantitative methods to titrate HFRSV and LCMV infectivity have made it possible for us to examine the virucidal activities of chemical and physical treatments, and to help determine the best choice for decontamination.

Materials and methods

Viruses: HFRSV (Hantaan 76-118 and B-1 strains) were obtained through the courtesy of Dr. T. Yamanouchi, Institute of Microbial Disease, Osaka University, Osaka. LCMV (WE strain) was used as one of arenavirus group (*Rivers & Scott,* 1936). HFRSV was propagated in Vero E6 cell (received from Dr. S. Morikawa, National Institute of Health of Japan) and LCMV in BHK-21 cells. Eagle's minimum essential medium (MEM) supplemented with 2% heatinactivated fetal calf serum (2% fcs) was employed as a maintenance medium. The viruses were harvested after 7-day and 3-day incubations at 37°C in atmosphere of 5% CO_2 air for HFRSV and LCMV, respectively. The virus stock was prepared by freezethawing three times, and by centrifugation at 2,000 rpm for 10 min at 4°C, and thereafter stored at -70°C until use. The average titers of HFRSV were in the range of 5.5 to 6.0 in $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$) per ml depending on virus lot, and of 7.0 of LCMV in $\log_{10}$ plaque forming units (PFU) per ml.

Assay procedures of virus infectivity: Viruses were assayed as follows: For HFRSV, 50 microliters of virus samples after tenfold dilutions in Eagle's MEM containing 10% fcs were inoculated to four wells each of the Vero E6 cells in 96-well microplates. After adsorption of the virus for 1 hr at 37°C, inoculum was aspirated off and cells were refed with the maintenance medium. After a 1-week incubation at 37°C in 5% CO_2, infected cells were harvested with a mixture of 0.05% trypsin and 0.02% EDTA in PBS. Washed cells in PBS from each well were were spotted on a 3-mm well of multispot slides (Fuji Glass Co., Fukuoka, Japan) and air-dried for 2 hr in a class II safety cabinet at room temperature. After a 15-min fixation with cold acetone, slides were stained with the indirect fluorescent antibody

(IFA) method (Sato & Miyata, 1986). An anti-Hantaan 76-118 rat serum (IFA titer, 1/15,000) bled after a 5-week postinfection was applied to both Hantaan and B-1 virus antigens as the primary antibody at a dilution of 1:500 (30 IFA units). Slides were observed under fluorescent microscope (Olympus Optical Co., Tokyo, Japan) and $TCID_{50}$ was calculated.

For LCMV, 0.2 ml of samples were inoculated after 10-fold dilutions in Vero cells in 35-mm petri dishes. After exposure to the virus at 37°C for 1 hr, the virus inoculum was removed and a maintenance medium containing 0.6% agarose (Seakem ME, Me, U.S.A.) was overlayed on each dish. LCMV plaques were counted under neutral red staining on day 4.

Physicochemical treatments: 1. Chemical inactivation. Eight commonly used commercial disinfectants; alcohols (ethanol-Amakas Chemicals, lot No. 601113, isopropanol-Kanto Chemicals, lot No. 009R5828), aldehyde (formaldehyde-Kanto Chemicals, lot No. 011B5008), quaternary ammonium chloride (Benzalkonium chlorideKennei Pharm., lot No. 13030), halogen compounds (iodophor-Taito Pfizer, lot No. 5LT29, sodium chlorite-Kanto chemicals, lot No. 010R1124), substituted phenolitics (chlorhexidine-Sumitomo Pharm. lot No. PS268, Sapopnated cresol-Fujimi Pharm. lot No. 53011) were tested.

Double-strength disinfectants diluted with ultra-pure water (10-18 megohm) were combined with the virus suspension including 2% FCS. In the case of ethanol, absolute ethanol was mixed with the virus suspension at a ratio 7:3. Mixtures were allowed to react for 10 min at 20-25°C and were detoxified by the gelfiltration method with a minor modification using Sephadex LH-20 (Pharmacia Biotechnology International AB, Uppsala, Sweden) as previously described by Blackwell (*Blackwell & Chen,* 1970; *Blackwell et al.,* 1975). An assembly consists of inner and outer tubes. An 11% slurry of 50-ml gel in PBS was packed into the inner tube. By centrifugation (Hitachi Koki; 05PR-22, Ibaraki, Japan) at 1,000 rpm for 1 min (instead of 10 min with the original method) at 4°C, the void volume in the column was discarded and the assembly was stored at 4°C until use. The virus-disinfectant mixture (4 ml) was applied to the top of the gel, then centrifuged immediately at 1,00 rpm for 1 min at 4°C. As a consequence, the virus was sieved from the gel and collected in the 50-ml outer tube while the disinfectant was trapped in the gel. In a short-term exposure to the Halogen compounds, the mixture was diluted immediately after addition of an equal volume of 20% FCS in Eagle's MEM to neutralize the virucidal activity, instead of using the gel-filtration method.

2. Physical inactivation. Viruses were examined for physical inactivation with a heat treatment. The inactivation was conducted as follows: Pre-chilled 10-mm test tubes which contain 1 ml of virus samples were immersed in water baths at 45°C, 60°C, or 80°C. At the appropriate time, these tubes were transferred immediately to an ice-water bath.

Quantitative analysis of sodium hypochlorite) Total active chlorine was quantified by orthotoluidine after the method of APHA (*APHA,* 1985).

Results

Characterization of the gel filtration method: With the gelfiltration method, virus suspensions were diluted by at least 2-fold (virus-disinfectant mixture). Furthermore, 50 µl of HFRSV or 0.2 ml of LCMV was inoculated to Vero cells for infectivity assays. In these procedures, the dilution factor, and the small volume of inoculum, were impossible to measure in initial virus titers. Both viruses were measurable at maximum titer to 3.3 $\log_{10}$ $TCID_{50}$/0.5 ml for HFRSV and 4.5 $\log_{10}$ PFU/2 ml for LCMV in the experiments. Nonspecific trapping of viruses was neglected by puncturing a small hole on a top of the outer tube.

We examined the 10-min-centrifugation methods of the assembly originally recommended by Blackwell et al. (*Blackwell et al.* 1970, *Blackwell et al.* 1975). Centrifugation for 1 min was equivalent in efficiency to 10 min (data not shown). In this study, we modified the filtration assembly to 4°C for 1 min to be centrifuged.

Exposure to various disinfectants for 10 min: Disinfectants of alcohol (70% ethanol, 50% isopropanol), formaldehyde (2% formalin), 0.05% benzalkonium chloride and sodium hypochlorite (>50 ppm) show significant results with high inactivation as effective disinfectants (HFRSV; >3.1 $\log_{10}$ virus reduction, <0.08% survival, LCMV; >4.5 $\log_{10}$ virus reduction, <0.0003% survival). One percent saponated cresol also has strong virucidal activity over 3.1 $\log_{10}$ $TCID_{50}$ reduction, >99.92% reduction, in infectivity of HFRSV and 4.5 $\log_{10}$ PFU of LCMV (>99.9997% reduction) by the treatment for 10 min, although manufacturers give some cautions against an application to virus pathogens. Representative results are shown in Table 1. In contrast to the above disinfectants, the efficacy of sodium hypochlorite is differenciable, when 10 or 50 ppm of final concentration of the substance in applied. Ten ppm, which has been recommended for drinking water for laboratory animals sustained in a barrier area, was an ineffective concentration on two viruses. In the same manner, iodophor, one of two halogen compounds, was less effective on HFRSV than LCMV at a concentration of 26 ppm as compared with 52 ppm. HFRSV is slightly inactivated at 26 ppm (0.7-1.1 $\log_{10}$

Table 1. Effect of 10-min exposure to chemical disinfectants on HFRSV (Hantaan & B-1) and LCMV (WE) infectivity.

Compound tested	Conc.	Reduction of virus infectivity*		
		HFRSV		LCMV
		Hantaan	B-1	WE
Alcohols				
Ethanol	70%	>3.3	>3.1	>4.5
Isopropanol	50%	>3.3	>3.1	>4.5
Aldehyde				
Formalin	2%	>3.3	>3.1	>4.5
Quaternary ammonium				
Benzalkonium chloride	0.05%	>3.3	>3.1	>4.5
Halogen compounds				
Iodophor	26ppm	0.7	1.1	>4.5
	52ppm	>3.3	>3.1	NT**
Sodium hypochlorite	10ppm	0.3	0.3	<1.7
	50ppm	>3.3	>3.1	>4.5
Substituted phenolitics				
Chlorhexidine	0.02%	-0.3	NT	0.3
	0.05%	1.5	2.1	1.4
Saponated cresol	1.0%	>3.3	>3.1	>4.5

Fifty microliters of HFRSV or 0.2 ml of LCMV-disinfectant mixture was inoculated to Vero cells for infectivity assays. These procedures, the dilution factor plus the small size of the inoculum, made it impossible to recover initial virus titers. Both viruses are measurable at maximum titer to 3.3 $\log_{10}$ $TCID_{50}$/0.5 ml for HFRSV and 4.5 $\log_{10}$ PFU/2 ml for LCMV.

* Reduction was given by a reciprocal number which was calculated by dividing the $\log_{10}$ virus titer (treated with disinfectant) by the $\log_{10}$ virus titer (treated with water; control).

** Not tested.

$TCID_{50}$), although LCMV is inactivated almost completely (>4.5 $\log_{10}$ PFU).

Thirty-second treatment of HFRSV with halogen compounds: The viruses were inactivated using halogen compounds for a short-term period. As shown in Table 2, 52 ppm of iodophor and 100 ppm of sodium hypochlorite also caused a marked virus reduction over 3.3 $\log_{10}$ $TCID_{50}$, <0.05% virus survival, on HFRSV infectivity.

Heat treatment: No significant reduction was seen in HFRSV infectivity at 45°C after 45 min (Table 3). On the other hand, LCMV infectivity was reduced to 99.37% after 45 min at 45°C. However, incubation at 60 or 80°C for 1 min caused a significant reduction of virus infectivity of both HFRSV and LCMV.

Discussion

Both zoonotic HFRSV and LCMV contain lipids in viral envelopes and are spread by contact or aerosol transmission from infected animals to humans. HFRSV is classified into Bunyaviridae at present time. However, it has been reported that there are some clear distinctions between common Bunyavirus and Hantavirus using negative-stain electron microscopy (*Martin et al.*, 1985). In general, Bunyaviridae might be susceptible to many disinfectants including detergents and heating. This presumption was certainly supported in this study. Results given in this study differed from Reovirus type 3 and murine encephalomyelitis virus which show high resistance against various physicochemical treatments (unpublished da-

Table 2. Effect of 30 seconds exposure to Halogen compounds on HFRSV (Hantaan & B-1) infectivity.

		Reduction of virus infectivity*	
Compound tested	Final conc. (ppm)	Hantaan	B-1
Halogen compounds			
Iodophor	52	>3.3	>3.1
Sodium hypochlorite	50	2.7	>3.1
	100	>3.3	>3.1

* See Table 1.

Table 3. Heat treatment on HFRSV and LCMV infectivity.

		Reduction of virus infectivity*		
Temperature	Time (min.)	Hantaan	B-1	LCMV(WE)
	10	0.2	NT**	0.5
45°C	30	0.9	-0.6	1.5
	45	0.8	NT	2.2
	1	>3.3	>3.1	3.4
60°C	2	>3.3	>3.1	NT
	3	>3.3	>3.1	NT
	1	>3.3	>3.1	>4.5
80°C	2	>3.3	>3.1	NT
	3	>3.3	>3.1	NT

The treated samples were transferred immediately to ice-water bath, and titrated for their infectivity.

* See Table 1.

** Not tested.

ta). No significant difference between Hantaan and B-1 strain was found in the susceptibility to 8 commonly used chemical disinfectants and/or physical treatments. Our results indicate that HFRSV shows a higher degree of resistance than LCMV (arenaviridae) against physicochemical treatment (Table 1, 3). An LCMV contamination case in transplantable murine tumors was recently reported in the U.S.A. which is very similar to the cases of HFRSV infection in Japan (*Bhatt et al.* 1986; *Yamanishi et al.* 1983).

Mechanisms of chemical and physical inactivation of HFRSV and LCMV are unclear. However, it is believed that chemical inactivation may be due to the degeneration of specific proteins, lipoproteins, virion surfaces and/or an aggregation of virus particles (*Drayna & Fields,* 1982a, 1982b).

Iodophor (>52 ppm), 2% formalin, alcohols (70% ethanol, 50% isopropanol), 0.05% benzalkonium chloride (one of quaternary ammonium chloride), sod. hypochlorite (>100 ppm), and 1% saponated cresol significantly reduced the infectivity of two zoonotic murine viruses. Substituted phenolitics, chlorhexidine (without detergent) showed less virucidal effects in comparison to saponated cresol against both viruses as in the case of respiratory syncytial virus which is an RNA virus with an outer lipoprotein coat derived from the plasma of an infected cell (*Platt & Bucknall,* 1985). This data suggests that detergent containing these disinfectants may be responsible for virus inactivation, especially of enveloped RNA viruses. Furthermore, repeated observations that sod. hypochrolite does not inactivate HFRSV and LCMV at 10 ppm concentration, which is generally considered to be a reasonable level for protection against most water-borne pathogens, is of sufficient interest to merit a change in many animal laboratory practices. In this study, we used 2% FCS for virus stabilization, the medium of virus stocks. However, this concentration of FCS did not show any interference to with the virucidal effect of Halogens (unpublished data). A 30 second treatment with a sufficient concentration of halogen compounds (iodophor; 52 ppm, sodium hypochlorite; 100 ppm) was effective in inactivating two strains of HFRSV which demand use of a virucide, although these disinfectants usually promote rust formation.

Laboratory animal cages, wood shavings, and small equipment should generally be autoclaved. However, quaternary ammonium chloride or saponated cresol, especially diluted with hot water over 60°C also seems to be a good choice for daily use in animal rooms, floors and racks. Furthermore, a 70% ethanol, 50% isopropanol solution could be used for spraying, since the route for natural infection of the two viruses is considered to be direct contact or aerosol from animal excrement.

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Summary

To seek the most effective and practical methods of disinfecting and for inactivating two zoonotic viruses, hemorrhagic fever with renal syndrome virus (HFRSV) and lymphocytic choriomeningitis virus (LCMV), we analysed inactivation of the viruses by 8 commonly-used chemical disinfectants and physical treatments. The results demonstrated that alcohols (70% ethanol, 50% isopropanol), formaldehyde (2% formalin), quaternary ammonium chloride (0.05% benzalkonium chloride) and 1% saponated cresol were virucidal on more than 99.9% of the infectious virus particles. HFRSV was more resistant than LCMV on several treatments. No significant difference was observed in strains of HFRSV.

Quaternary ammonium chloride and saponated cresol diluted with hot water (>60°C), are considered to be the reagents of choice for disinfecting the two zoonotic viruses because of their long-term effectiveness and mild corrosive action to metals. Spraying of alcohols has proven to be a suitable means for decontaminating instruments, and experiments tools in limited areas of animal laboratories.

Sammendrag

Virus, der forårsager hæmorrhagisk feber med nyresyndrom (HFRSV, Hantaan virus) og lymfocytær choriomeningitis virus (LCMV) udgør to vigtige zoonotiske virus, der kan overføres fra laboratorie gnavere til menneske. For at finde den mest effektive metode til at inaktivere HFRS-virus og LCM-virus blev den virus-inaktiverende effekt af en række kemiske og thermiske desinfektionsmetoder undersøgt på to stammer af HFRS-virus (Hantaan 76-118 og B-1) og en stamme af LCM-virus (WE). Virus blev exponeret for en af 8 almindeligt brugte desinfektionsmidler eller udsat for forskellige varmebehandlinger. Resultaterne viste at 99.9% af de infektiøse virus partikler blev inaktiveret ved behandling med alkoholer (70% ethanol, 50% isopropanol), formaldehyd (2% formalin), en kvarternær ammoniumforbindelse (0.05% benzalkonium chlorid) eller 1% cresol sæbe. HFRS-virus viste sig mere resistent end LCM-virus ved de anvendte behandlinger. Der blev ikke påvist nogen signifikant forskel mellem de to stammer af Hantaan virus.

Det konkluderes, at kvarternære ammonium forbindelser og cresol sæbe opløst i varmt vand (>60C) må anses for de mest velegnede desinfektionsmidler til inaktivering af de HFRS- og LCM-virus, på grund af disse desinfektionsmidlers langtidsvirkende effekt og ringe korroderende virkning på metaller. Ved dekontamination af instrumenter og apparatur i begrænset omfang i laboratoriet betragtes sprøjtning eller aftørring med alkohol som særdeles anvendeligt.

Yhteenveto / K. Pelkonen

Työssä selvitettiin kahdeksan yleisesti käytetyn kemiallisen desinfektioaineen ja fysikaalisen käsittelyn tehoa kahden zoonoottisen viruksen (HFSRV ja LCMV) inaktivoimiseen. Tulokset osoittavat, että alkoholit (70% stanoli, 50% isopropanoli), formaldehydi (2% formaliini), kvaternaarinen ammoniumkloridi (0.05% benzalkoniumkloridi) ja 1% saippuoitu kresoli inaktivoivat 99.9% infektoivista viruspartikkeleista. HFSR V oli vastustuskykyisempi monelle käsittelylle kuin LCMV. HFSR V:n eri kontojen välillä ei ollut eroja.

Kvaternaarinen ammoniumkloridi ja saippuoitu kresoli liuotettuna kuumaan veteen (>60°C) todettiin teholtaan parhaiksi, koska niillä on pitkä vaikutusaika ja ne ovat vain heikosti metalleja syövyttäviä. Alkoholien sumuttaminen on osoittautunut sopivaksi menetelmäksi instrumenttien ja koevälineistön dekontaminoimiseen eläinlaboratorioiden rajoitetuissa tiloissa.

HFSR V = hemorrhagic fever with renal syndrome virus

LCMV = lymphocytic choriomeningitis virus

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