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Short communication

Evaluation of the efficacy of disinfectants against Puumala hantavirus by real-time RT-PCR

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Abstract

Puumala virus, a hantavirus belonging to the *Bunyaviridae* family, causes a human disease known as nephropathia epidemica, a mild form of hemorrhagic fever with renal syndrome. The implementation of effective decontamination procedures is critical in hantavirus research to minimize the risk of personnel exposure. This study investigated the efficacy of Clidox®, Dettol®, ethanol, Halamid-d®, peracetic acid, sodium hypochloride and Virkon®S for inactivating Puumala virus. A real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to quantify Puumala virus before and after treatment with these products. Inactivation of Puumala virus was effective after 10 min with all products except ethanol. Inactivation with absolute ethanol was effective only after 30 min. Using the qRT-PCR method, this study has shown that the commercially available products Clidox®, Halamid-d® and Virkon®S in particular represent a rapid and safe way to decontaminate surfaces with possible Puumala virus contamination. These products can be used in solutions of 1–2%, with contact times greater than 10 min, for inactivating effectively Puumala virus.

Keywords: Decontamination; Taqman PCR; Quantitative PCR; Real-time PCR; Puumala virus

1. Introduction

Hantaviruses belong to the family of the *Bunyaviridae*, and are a group of closely related negative-stranded RNA viruses, with a trisegmented genome (Schmaljohn et al., 1985). They are lipid-enveloped, spherical viruses of 80–110 nm in diameter. In contrast to other *Bunyaviridae* genera that are transmitted by arthropods, hantaviruses are rodent-borne viruses that are transmitted to humans, probably through inhalation of infected rodent excreta (Maes et al., 2004). Hantaviruses produce persistent non-pathogenic infections in rodents, but give rise to several severe clinical diseases in humans (Avsic-Zupanc et al., 1999; Clement et al., 1997; Peters and Khan, 2002). On the Eurasian continent, two murine subfamilies, *Arvicolinae* and *Murinae*, are the natural rodent hosts of hantaviruses, which are the causative agents of hemorrhagic fever with renal function (HFRS). Hantaviruses of the American continent, are responsible for hantavirus pulmonary syndrome and are carried by the murine subfamily *Sigmodontinae* (Chu et al., 1994; Hjelle et al., 1995; Monroe et al., 1999). Each hantavirus is associated with a specific host species, but occasional spillover infections in related rodents have been reported (Vincent et al., 2000; Wang et al., 2000). In Europe, two hantaviruses, Puumala (PUUV) and Dobrava-Belgrade viruses, are known to cause HFRS (Clement et al., 1997; Plyusnin et al., 2001). PUUV, which is carried by bank voles (*Clethrionomys glareolus*) and found almost throughout Europe, generally causes a mild form of HFRS, called nephropathia epidemica, characterized mainly by fever and renal dysfunction, sometimes with hemorrhagic manifestations (Kanerva et al., 1998; Mustonen et al., 1994). The case fatality rate of nephropathia epidemica varies from 0.1 to 0.3%, and with approximately 5000 cases annually, PUUV is responsible for a significant morbidity in Europe (Brummer-Korvenkontio et al., 1999; Valtonen et al., 1995).

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Table 1
Antiseptic/disinfectant solutions used in this study with their efficiency for PUUV inactivation

Product	Active ingredients	Tested product concentrations	Titer before inactivation (geq/mL)	Titer after inactivation (geq/mL)
Bleach	Sodium hypochloride	1%	1.2×10^{12}	$<10^2$
Clidox®	Chlorine dioxide	1–5%	1.2×10^{12}	$<10^2$
Dettol®	Parachlorometaxylenol	1–5%	1.2×10^{12}	$<10^2$
Ethanol (10 min)	Ethanol	Absolute (70%)	1.2×10^{12}	1.7×10^5 (3.6×10^5)
Ethanol (30 min)	Ethanol	Absolute (70%)	1.2×10^{12}	$<10^2$ ($<10^2$)
Halamid-d®	Sodium-*p*-toluene-sulfonchloramide	1–5%	1.2×10^{12}	$<10^2$
Methanol	Methanol	Absolute	1.2×10^{12}	$<10^2$
Peracetic acid	Peracetic acid	1%	1.2×10^{12}	$<10^2$
Virkon®S	Potassium peroxomonosulfate sulfamic acid sodium alkylbenzene sulfonate	1–5%	1.2×10^{12}	$<10^2$

The inactivation procedure was done with an initial virus concentration of 5×10^4 geq/mL in a time frame of 10 min.

Laboratory work with hantaviruses requires a biosafety level three containment laboratory due to the hazardous nature of these viruses. As hantaviruses can be transmitted to humans by direct contact or by aerosolized infectious material, it is very important to have effective decontamination procedures. This study examined the inactivation of PUUV in suspension by treatment with a variety of chemical solutions, often used in laboratories as standard decontamination procedures. Quantitation of the virus was done by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) by using Taqman® technology.

2. Methods

2.1. Virus and cell culture

Vero E6 cells (American Type Culture Collection, C1008) were cultured in minimum essential medium (MEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum. The maintenance medium for virus propagation was identical but contained 2% (v/v) fetal calf serum. The PUUV strain CG1820 (Stohwasser et al., 1990) was propagated for 10 days on monolayers of Vero E6 cells. Cells and virus were cultured at 37 °C with 5% CO_2. Virus supernatants, quantified by real-time quantitative reverse transcriptase polymerase chain reaction and standardized to contain 5×10^4 particle-associated virus genome equivalents per mL (geq/mL), was used in the inactivation experiments.

2.2. PUUV inactivation experiments

Eight antiseptic/disinfectant solutions were tested (see Table 1). Dilutions were made in distilled water and used within 24 h. 5×10^4 PUUV particle-associated virus genome equivalents per mL (5×10^4 geq/mL) were exposed to different concentrations of Clidox® (1–5% consecutive solutions) (Tecnilab-BMI, Helmont, The Netherlands), ethanol (absolute and 70% solution), Dettol® (1–5% consecutive solutions) (Reckitt Benckiser, Brussels, Belgium), Halamid-d® (1–5% consecutive solutions) (Veip bv, Wijk bij Duurstede, The Netherlands), peracetic acid (1% solution) (Sigma–Aldrich, Bornem, Belgium), Sodium hypochloride (1% solution) and Virkon®S (1–5% consecutive solutions) (Antec International, Erika Handelsonderneming, Heythusen, The Netherlands), all at room temperature (20–25 °C). The active constituents and concentrations tested of the disinfectant solutions are shown in Table 1. After 10 min, samples were centrifuged at 30,000 × *g* for 30 min, and resuspended in 2% MEM. To exclude undetectable levels of infectious virus particles, the solutions were incubated on confluent Vero E6 monolayers on 96-well plates before qRT-PCR. After 10 days, RNase digestion was carried out with 60 units of Ribonuclease T1 (Sigma–Aldrich) and 20 units proteinase K (Sigma–Aldrich) for 30 min at 37 °C to degrade non-protected RNA strains in the virus supernatants (Nuanualsuwan and Cliver, 2002). In this way, only particle-associated RNA strains will be detected. Viral RNA was extracted immediately after RNase digestion by using the QIAamp viral RNA kit (Qiagen, Leusden, The Netherlands). The titers of infectious virus were determined by qRT-PCR. A virus control was included in the experiments, treated with sterile PBS.

2.3. Real-time RT-PCR (qRT-PCR)

qRT-PCR was carried out using the Eurogentec One Step RT qPCR kit (Eurogentec, Seraing, Belgium) with the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The reaction was conducted in a 25 μL volume containing 5 μL of extracted RNA, 12.5 μL of One step RT qPCR MasterMix (Eurogentec), 900 nM forward (PUUVSsegTqF 5′-TACAAGAGAAGAATGGCAGATGCT-3′) and reverse primer (PUUVSsegTqR 5′-CATTCACATCAAGGACATTTCCA-3′), 250 nM FAM-TAMRA probe (PUUVSsegTqTP FAM 5′-CTGACCCGACTGGGATTGAACCTGA-3′ TAMRA) and 0.125 μL Euroscript/RNase inhibitor (Eurogentec). A complete hantavirus genome alignment (data not shown) was screened for primer and probe target sides that would be compatible with TaqMan PCR requirements (ABI 7700 User Manual), using the Primer Express v2.0 software. A compatible region, specific for Puumala virus strain CG1820 located in the S segment, was found and used to select the primers and probe sequences. Reverse transcription was initiated at 48 °C for

30 min, followed by PCR activation at 95 °C for 10 min and 45 cycles of a two-step incubation at 95 °C for 15 s and 60 °C for 1 min. The threshold cycle was defined as the fractional cycle number at which the reporter fluorescence, generated by cleavage of the probe, reaches a threshold defined as 10 times the standard deviation of the mean baseline emission.

2.4. Quantitation of PUUV

A PUUV cRNA standard was used to calculate the exact PUUV particle-associated virus genome equivalents in the samples. The PUUV cRNA standard was made using the MEGAshortscript™ T7 High Yield Transcription kit (Ambion, Austin, Texas, USA) as described previously (Vijgen et al., 2005). Briefly, PCR products amplified with the modified PUUVSsegTqF primer (PUUVSsegTqFT7 5′-TAATACGACTCACTATAGGGAGGTACAAGAGAAGAATGGCAGATGCT-3′, with the T7-promotor sequence underlined) and the reverse primer PUUVSsegTqR were quantified spectrophotometrically at 260 nm. After *in vitro* transcription and purification, quantitation of the cRNA standards was done spectrophotometrically at 260 nm. The measurements of cRNA concentration were undertaken in duplicate and then converted to the molecule number (Fronhoffs et al., 2002).

2.5. Focus assay

The focus assay was carried out as described previously (Niklasson et al., 1991). Briefly, different virus dilutions quantified previously by using qRT-PCR, were incubated for 60 min and inoculated subsequently into six-well tissue culture plates containing confluent Vero E6 cell monolayers. After adsorption for 60 min, wells were overlaid with a mixture of 1% agarose and basal Eagle's medium, and plates were incubated for 12 days. Virus-infected cells were detected with hantavirus-specific rabbit polyclonal antisera (kindly provided by Dr. D.H. Krüger, Institut für Virologie, Charité, Berlin, Germany), followed by peroxidase-labelled goat anti-rabbit antibodies and substrate.

3. Results and discussion

cRNA standards were used for the generation of the standard curve (Fig. 1). Serial dilutions of the standards were made and corresponding copy numbers of specific transcripts from 10^2 up to 10^9 were used. Equal volumes of standard and sample were used for PCR amplification. The Sequence Detector v1.9 software (Applied Biosystems) was used for the analysis of the copy numbers and linear regression curve. The dynamic range of the assay was determined by testing 10-fold serial dilutions of the cRNA standard ranging from 10 to 10^{12} molecules. The results were analyzed in terms of C_t value (the cycle in which a target sequence was detected). The dynamic range of the assay spans 6 logs ranging from 2 to 8 log molecules PUUV per reaction, corresponding to C_t values ranging from 38.74 ± 0.27 to 14.32 ± 0.01.

The concentration as measured by qRT-PCR was scored by a focus assay. A PUUV dilution of 5×10^4 PUUV geq measured after RNase/proteinase K digestion was confirmed with focus assay and contained 1.6×10^3 focus forming units.

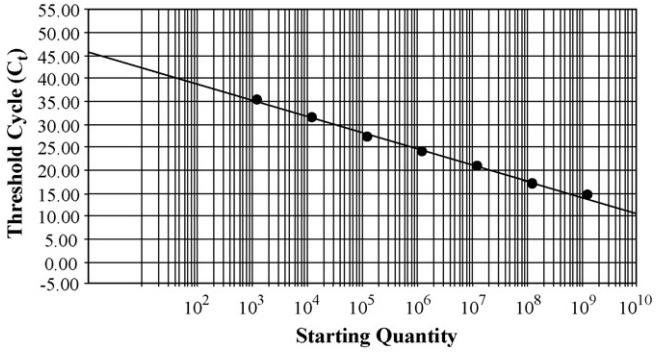

Fig. 1. Ten-fold serial dilutions ranging from 10^2 to 10^9 copies of cRNA were tested in duplicate in real-time RT-PCR. A standard curve graph is made by plotting the C_t values on the *y*-axis and the log of the input amounts on the *x*-axis. The slope was −3.525 and the correlation coefficient 0.998. The *Y*-intercept, the C_t value to detect one copy, was 45.805.

In order to ensure that the inactivation procedure itself was not inactivating the virus, the inactivation procedure was carried out with PBS and different dilutions of PUUV (from 10 to 1×10^4 PUUV particle-associated virus genome equivalents). After the complete inactivation procedure, samples were examined for the presence of PUUV with qRT-PCR. For all dilutions, the differences in virus titers recovered were within 0.5 log of the virus stock controls. Mock samples containing no virus but treated with disinfectant were also tested for their effect on Vero E6 cell monolayers. These mock samples had no adverse effect on the Vero E6 cell monolayers. The ultracentrifugation washing step was thus considered to have removed the disinfectants prior to virus titration and no additional steps to neutralize the disinfectants during the procedure were taken.

Hantaviruses represent a hazard for hospital and laboratory workers and thus it is important to determine the virucidal power of various disinfectant agents under strict experimental conditions. With the Klein–Deforest scheme, human viruses can be divided into groups A, B, and C with respect to inactivation of lipophilic (enveloped, lipid membrane) and hydrophilic (naked) viruses (Klein and Deforest, 1983). The *Bunyaviridae* are a family of negative-stranded, lipid-enveloped, spherical RNA viruses, and thus are classified into the lipid-enveloped virus containing group A. Group A viruses are susceptible to a wide variety of detergents and alcohols attaining the lipid-envelope. But there is little data available specific about hantavirus inactivation. Chloroform, β-propiolactone, sodium hypochloride, ethanol and phenol have been described as good disinfectant agents for hantaviruses (Schmaljohn et al., 1999). However, chloroform, β-propiolactone and phenol can induce cancer in humans, and are therefore not recommended as disinfectant agents in laboratories. Recently, effective inactivation of Hantaan virus with methanol and paraformaldehyde has been demonstrated (Kraus et al., 2005). This study confirmed complete inactivation of PUUV with methanol after 10 min (see Table 1), but in contrast to expectations, inactivation of PUUV with absolute ethanol was completed only after 30 min. After a 10 min inactivation with ethanol, the concentration of PUUV

was 1.7×10^5 geq/mL (Table 1). The inactivation of PUUV with a 70% solution of ethanol, also took 30 min to be completed. After an inactivation of 10 min, the concentration of PUUV was 3.6×10^5 geq/mL (Table 1). These findings were confirmed with focus assay. After inactivation with absolute ethanol or with a 70% solution of ethanol during 30 min, virus could not be detected by using focus assay. Applying focus assay after 10 min of inactivation, however, gave 6.0×10^3 focus forming units and 1.2×10^4 focus forming units for inactivation with absolute ethanol and a 70% ethanol solution, respectively. Peracetic acid is a strong oxidizing biocide with rapid biocidal action at relatively low concentrations. PUUV was inactivated by peracetic acid at a concentration of 1% in a timeframe of 10 min but due to the hazardous nature of this product, caution has to be taken when using this product in standard laboratory practice. Inactivation of PUUV by sodium hypochloride was complete with a 1% solution. Sodium hypochloride can be used against a wide variety of microbial and viral organisms (Sopwith et al., 2002), but it can be corrosive for stainless steel surfaces. Dettol® or parachlorometaxylenol, a common household disinfectant, was effective against PUUV, in a 1% (v/v) solution. Successful inactivation with Dettol® of several enveloped and non-enveloped viruses has been described (Wood and Payne, 1998). In solution, Virkon®S is activated to form hypochlorous acid, which has been shown to be a strong oxidizing agent (Spickett et al., 2000). This study has demonstrated that Virkon®S is effective for inactivating PUUV at the lowest (1%) concentration tested. Moreover, a 1% solution of Virkon®S is classified as a non-irritant to the skin and eyes (McCormick and Maheshwari, 2004). Clidox® (chlorine dioxide) is an alternative to chlorine as a primary disinfectant. It is a strong oxidant reported to be effective against several bacteria and viruses (Eleraky et al., 2002; Li et al., 2004; Thurston-Enriquez et al., 2005; Wang et al., 2005). Clidox® was shown to be effective for inactivating PUUV at the lowest concentration tested (1% solution). Although the use of chlorine dioxide has some disadvantages including the oxidation of iron surfaces and the formation of organic halides, its use can be recommended since, like Virkon®S, it is effective against several bacteria and viruses including PUUV. Halamid-d® is a disinfectant used against bacteria, viruses and fungi. It is proven to be effective against SARS coronavirus (www.halamid.com). A 1% solution of Halamid-d® was found to be effective against PUUV in this study.

The same study layout was used to test the efficacy of these disinfectants for the inactivation of Hantaan virus (HTNV), a pathogenic hantavirus endemic in Asia. Identical results to PUUV were obtained (data not shown).

One of the goals of the study was to determine whether qRT-PCR could be used as a way to detect viral inactivation. A recognized problem with RT-PCR and qRT-PCR detection of viral RNA, however, is that the presence of RNA does not necessarily indicate the presence of infectious virus (Gassilloud et al., 2003; Suarez et al., 2003). Applying an RNase/proteinase K digestion before RNA extraction, allows the detection of intact viral particles only, i.e. infectious virus. The concentration of virus found by using qRT-PCR correlated with the focus forming units found by focus assay. The qRT-PCR detects specific viral RNA strains, whereas a focus assay is based on the ability of a single infectious virus particle to give rise to a macroscopic visual amount of virus particles. Specifically, a single virus particle, infecting a single cell, will give rise to new virus particles, which can infect surrounding cells. During the initial incubation of a focus assay, the surrounding cells can also be infected. If new virus particles are produced by the surrounding cells, the different foci will be recognized as one. This can explain possibly the lower concentration of focus forming units found, in comparison with the particle-associated virus genome equivalents as found with the qRT-PCR.

In conclusion, the commercially available products Clidox®, Halamid-d® and Virkon®S represent a rapid and relatively safe way to decontaminate surfaces of possible contamination by Puumala virus and probably also other hantaviruses. The results of this study suggest that these products can be used in solutions of 1–2%, with contact times greater than 10 min, for inactivating effectively Puumala virus.

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