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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.

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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).
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% CO₂. Virus supernatants, quantified by real-time quantitative reverse transcriptase polymerase chain reaction and standardized to contain 5 × 10⁴ 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 × 10⁴ PUUV particle-associated virus genome equivalents per mL (5 × 10⁴ 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 Händelsdornermenging, Heythuisen, 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) 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 particles 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′-CTAAGAGAAGAATGGGATTGAACCTGA-3′) and reverse primer (PUUVSsegTqR 5′-CATTCACATCAAGGACATTTCCA-3′), 250 nM FAM-TAMRA probe (PUUVSsegTqF 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

### Table 1

| Product          | Active ingredients                           | Tested product concentrations | Titer before inactivation (geq/mL) | Titer after inactivation (geq/mL) |
|------------------|-----------------------------------------------|-------------------------------|-----------------------------------|----------------------------------|
| Bleach           | Sodium hypochloride                           | 1%                            | 1.2 × 10¹²                        | <10²                             |
| Clidox®          | Chlorine dioxide                              | 1–5%                          | 1.2 × 10¹²                        | <10²                             |
| Dettol®          | Peracetic acid                                | 1–5%                          | 1.2 × 10¹²                        | <10²                             |
| Ethanol (10 min) | Ethanol Absolute (70%)                        |                               | 1.2 × 10¹²                        | <10²                             |
| Ethanol (30 min) | Ethanol Absolute (70%)                        |                               | 1.2 × 10¹²                        | 1.7 × 10⁴ (3.6 × 10⁴)             |
| Halamid-d®       | Sodium-p-toluene-sulfonchloramide             | 1–5%                          | 1.2 × 10¹²                        | <10²                             |
| Methanol         | Methanol Absolute                             |                               | 1.2 × 10¹²                        | <10²                             |
| Peracetic acid   | Peracetic acid                                | 1%                            | 1.2 × 10¹²                        | <10²                             |
| Virkon®S         | Potassium peroxomonosulfate sulfamic acid     | 1–5%                          | 1.2 × 10¹²                        | <10²                             |

The inactivation procedure was done with an initial virus concentration of 5 × 10⁴ geq/mL, in a time frame of 10 min.
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 PUUV SsegTqF primer (PUUV SsegTqFT7 5’-TAATACGACTCACTATAGGGAGGTACAAGAGAAGAATGGCCAGATGCT-3’, with the T7-promotor sequence underlined) and the reverse primer PUUV SsegTqR 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 antiseras (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 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 Ct 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 Ct 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 × 10^5 focus forming units.

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 × 10^6 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

![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 Ct 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 C₀ value to detect one copy, was 45.805.](Image 358x643 to 600x773)
was $1.7 \times 10^5$ 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 $3.6 \times 10^5$ 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 $6.0 \times 10^3$ focus forming units and $1.2 \times 10^4$ 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 hypochlorite was complete with a 1% solution. Sodium hypochlorite can be used against a wide range of infections. 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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