Dermatology

Dermatology 2006;212(suppl 1):119–123
DOI: 10.1159/000089211

Inactivation of SARS Coronavirus by Means of Povidone-Iodine, Physical Conditions and Chemical Reagents

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Key Words
Severe acute respiratory syndrome · Coronavirus · Povidone-iodine · Infection control

Abstract
The efficacy of several povidone-iodine (PVP-I) products, a number of other chemical agents and various physical conditions were evaluated for their ability to inactivate the severe acute respiratory syndrome coronavirus (SARS-CoV). Treatment of SARS-CoV with PVP-I products for 2 min reduced the virus infectivity from 1.17×10^6 $TCID_{50}$/ml to below the detectable level. The efficacy of 70% ethanol was equivalent to that of PVP-I products. Fixation of SARS-CoV-infected Vero E6 cells with a fixative including formalin, glutaraldehyde, methanol and acetone for 5 min or longer eliminated all infectivity. Heating the virus at 56°C for 60 min or longer reduced the infectivity of the virus from 2.6×10^7 to undetectable levels. Irradiation with ultraviolet light at 134 $\mu W/cm^2$ for 15 min reduced the infectivity from 3.8×10^7 to 180 $TCID_{50}$/ml; however, prolonged irradiation (60 min) failed to eliminate the remaining virus, leaving 18.8 $TCID_{50}$/ml.

Introduction

Severe acute respiratory syndrome (SARS) was first reported as an atypical pneumonia in Gangdong, China, in November 2002 [1]. The epidemic expanded rapidly to 26 regions and countries [2–4], and by the end of July 2003, 8,098 probable cases had been reported and more than 774 people had died [5]. Although the epidemic seemed to be controlled during the summer of 2003, laboratory-associated infections appeared in Singapore in September 2003, in Taiwan in December 2003 and in Beijing in April 2004. At the end of 2003, a new series of probable SARS cases, which may have been unrelated to laboratory sources of infection, re-emerged in Gangdong. Therefore, it must be considered possible that SARS outbreaks could start at any time, anywhere in the world.

In March 2003, a distinct coronavirus was identified as the causative agent of SARS and designated as SARS coronavirus (SARS-CoV) [6–8]. SARS-CoV is an enveloped virus with a single positive-stranded RNA genome, for which the genome organization has been characterized [9]. Because SARS-CoV was isolated or identified in Himalayan palm civets *(Paguma larvata)* and raccoon dogs *(Nyctereutes procyonoides)*, SARS appears to have a zoonotic origin [10]. However, the definitive animal

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reservoir for human cases of SARS-CoV has not yet been determined, and no effective vaccines or antiviral drugs have yet been developed.

Given this situation, the initial infection control effort in healthcare, home and community settings is crucially important to minimize the occurrence and spread of epidemics. The WHO laboratory network for SARS diagnosis reported preliminary data concerning the stability and resistance of the virus [11]. The virus seems to be susceptible to inactivation by heating and exposure to organic chemicals that disrupt the virus envelope, such as acetone and ethanol, as well as fixatives such as formalin. However, additional information on inactivation of SARS-CoV is imperative for the establishment of effective infection control protocols in a variety of settings. As several SARS outbreaks may have originated from laboratories, the guidelines for handling the virus in the laboratory must be established according to accurate information on the stability of SARS-CoV.

Povidone-iodine (PVP-I) products have been used for the disinfection of various bacteria and viruses for years because of their strong bactericidal and antiviral activities. If reliable data confirm the efficacy of PVP-I for the elimination of SARS-CoV infectivity, these products will become extremely useful for the destruction of the virus in various settings.

In this paper we evaluated the antiviral efficacy of PVP-I against SARS-CoV; we also evaluated the antiviral efficacy of various physical and chemical inactivation conditions.

Materials and Methods

Virus and Cells

The Hanoi strain of SARS-CoV was kindly provided by Dr. Koichi Morita, of Nagasaki University. The virus was propagated in Vero E6 cells, cultured in minimum essential medium (MEM), containing 10% fetal bovine serum. Virus stocks were prepared by collecting the culture supernatants from infected cells 48 h after infection, centrifuging the fluid at 2,000 rpm for 10 min and storing the clarified supernatants at –80°C until use.

Evaluation of the Antiviral Activity of PVP-I Products against SARS-CoV

Aliquots of stock virus (0.1 ml) were mixed with an equal volume of various PVP-I products (Meiji Seika Kaisha Ltd., Tokyo, Japan), including Isodine® solution, Isodine Scrub®, Isodine Palm®, Isodine Gargle® and Isodine Nodo Fresh®. The mixtures were incubated for 1 min at room temperature and then diluted tenfold with sodium thiosulfate (0.5%) to neutralize the cytotoxicity and antiviral activity of PVP-I. The mixtures were serially diluted in MEM and 0.1-ml aliquots were inoculated onto Vero E6 monolayers in 96-well plates. The cells were incubated for 48 h in a CO_2 incubator and the cytopathic effect was observed under a microscope. The 50% tissue culture infectivity dose ($TCID_{50}$/ml) remaining in the virus-disinfectant mixture was determined by the method of Reed and Muench [12]. The antiviral efficacy of ethanol (70%) was evaluated in the same manner, except for the addition of MEM to dilute the mixture, rather than sodium thiosulfate.

Inactivation of Infectivity of SARS-CoV-Infected Vero E6 Cells by Chemical Reagents

Vero E6 cells grown in a 75-cm^2 flask were infected with SARS-CoV and cultured for 48 h in a CO_2 incubator. By 48 h after infection, most of the cells had detached from the substrate, due to the development of a strong cytopathic effect. These floating cells were collected with the medium and centrifuged at 2,000 rpm for 10 min. The cell pellet was resuspended in 2 ml of MEM and 0.5-ml aliquots of this cell suspension were distributed into cryogenic vials. After an additional centrifugation, the supernatant was removed and the cell pellets were stored at –80°C to be used as the stock of infected Vero E6 cells.

The infected cells were thawed and suspended in 100% acetone, 100% methanol, 3.5% paraformaldehyde or 2.5% glutaraldehyde for various times. The cells suspended in acetone were held at –10°C in a freezer. The cells suspended in the other reagents were held at room temperature. After the treatment, the cells were collected by centrifugation, washed with phosphate-buffered saline and suspended in 1 ml MEM. The serially diluted cells were inoculated onto Vero E6 cells grown in flat-bottom 96-well plates, and the remaining infectivity was determined by the $TCID_{50}$ method. Normal uninfected Vero E6 cells were also incubated with the fixatives and inoculated onto Vero E6 monolayers to see the cytotoxicity caused by the fixed cells.

Physical Inactivation of SARS-CoV

For the evaluation of heat inactivation, aliquots of the virus stock were placed in 50-ml tubes and heated at 56°C in a water bath for various times. The temperature in the tubes was monitored with a thermometer placed in the same amount of MEM in a separate tube. To evaluate the efficacy of ultraviolet (UV) irradiation, 2-ml aliquots of stock virus were placed in open 3-cm plastic Petri dishes, positioned under the UV light source in a biosafety cabinet and irradiated with 134 $\mu W/cm^2$ for various times. The treated virus stocks were serially diluted in U-bottom 96-well plates, and 100-μl aliquots of diluted virus were inoculated onto monolayers of Vero E6 cells in 96-well plates and cultured for 48 h in a CO_2 incubator. The cytopathic effect was observed under a microscope.

Results

Efficacy of PVP-I on SARS-CoV

We tested the efficacy of several PVP-I products for the inactivation of SARS-CoV, including Isodine, Isodine Scrub, Isodine Gargle, Isodine Palm and Isodine Nodo Fresh, all of which are used for disinfection in various settings. Treatment of SARS-CoV for 1 min with Isodine

Table 1. Efficacy of PVP-I products to SARS-CoV

Reagent or treatment	Final PVP-I concentration, %	Virus titer after treatment, $TCID_{50}$/ml	
		60 s	120 s
Control	0	1.17×10^6	n.d.
Isodine	1	95.1	u.d.
Isodine Gargle	0.47	190	u.d.
Isodine Scrub	1	u.d.	n.d.
Isodine Palm	0.25	u.d.	n.d.
Isodine Nodo Fresh	0.23	u.d.	n.d.
Ethanol (final 35%)	–	u.d.	n.d.

n.d. = Not done; u.d. = under the detectable level.

Table 2. Inactivation of Vero E6 cells infected with SARS-CoV by various reagents

Reagent	Infectivity of cells infected with SARS-CoV after treatment with reagents, $TCID_{50}$/ml					
	0 min	5 min	15 min	30 min	60 min	90 min
Methanol	2.1×10^7	n.d.	n.d.	<20	<20	<20
Acetone	1.3×10^7	<20	<20	<20	<20	n.d.
2.5% glutaraldehyde	2.2×10^6	<160	<80	<80	<80	n.d.
3.5% paraformaldehyde	1.6×10^6	<320	<320	<320	<320	n.d.

n.d. = Not done.

Scrub, Isodine Palm and Isodine Nodo Fresh strongly reduced the virus infectivity from 1.17×10^6 $TCID_{50}$/ml to below the detection limit, <40 to <160 (table 1). In contrast, 1-min treatment with Isodine and Isodine Gargle did not completely eliminate the virus infectivity; the reduction rates were 8.1×10^{-5} and 1.6×10^{-4}, respectively. However, treatment with all the PVP-I products for 2 min completely inactivated the virus. The treatment of 70% ethanol for 1 min also reduced the virus infectivity under the detectable level (<10). These results strongly indicate that PVP-I products and 70% ethanol are effective for the inactivation of SARS-CoV.

Fixation of Cells Infected with SARS-CoV by Chemical Reagents

To determine the stability of SARS-CoV treated with several chemical reagents usually used as laboratory fixatives, Vero E6 cells infected with SARS-CoV were suspended in fixatives including formalin, glutaraldehyde, methanol and acetone. The cells were treated with the fixatives, washed with phosphate-buffered saline and overlaid onto Vero E6 monolayers to evaluate the remaining infectivity. After treatment with any of the fixatives for 5 min or longer, no infectivity remained in the cells (table 2). Therefore, SARS-CoV-infected cells can be effectively inactivated by these chemical reagents.

Physical Inactivation of SARS-CoV

We tested the resistance of SARS-CoV to physical treatments such as heating and UV irradiation. Aliquots of virus were heated at 56°C for various times and the kinetics of virus inactivation were analyzed. Heating for 5 min rapidly inactivated the virus infectivity from 2.6×10^7 to 40 $TCID_{50}$/ml (fig. 1). Only low infectivity (<10 $TCID_{50}$/ml) remained after heating for 30 min. After 60-min and 90-min treatments with heat, no virus infectivity was detected. When SARS-CoV was irradiated under normal biosafety cabinet UV lights, the virus titer was reduced from 3.8×10^7 to 180 $TCID_{50}$/ml in 15 min, but the virus was still detected (18.8 $TCID_{50}$/ml), even after 60 min of irradiation (fig. 2). These results indicate that SARS-CoV is relatively resistant to UV irradiation.

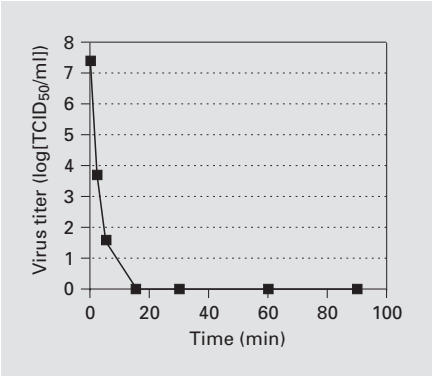

Fig. 1. Kinetics of SARS-CoV inactivation by heating. Aliquots of SARS-CoV were heated in a 56°C water bath for the indicated times. The heated virus aliquots were serially diluted and inoculated onto monolayers of Vero E6 cells grown in 96-well plates. After incubation for 48 h, the cytopathic effect in the cells was observed and the infectivity was determined by the $TCID_{50}$ method.

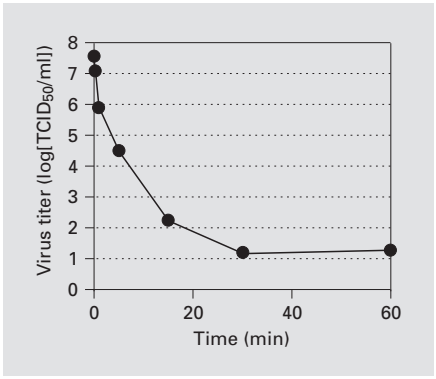

Fig. 2. Kinetics of SARS-CoV inactivation by UV irradiation. Aliquots of SARS-CoV were irradiated with UV light for the indicated times. The irradiated virus aliquots were serially diluted and inoculated onto monolayers of Vero E6 cells grown in 96-well plates. After incubation for 48 h, the cytopathic effect in the cells was observed and the infectivity was determined by the $TCID_{50}$ method.

Discussion

We tested the efficacy of several PVP-I products, including Isodine, Isodine Scrub, Isodine Palm, Isodine Gargle and Isodine Nodo Fresh, all of which cover a variety of disinfection applications; these applications include disinfecting medical instruments and skin as well as hand-washing, gargling and spraying the throat. The results clearly indicate that all the PVP-I products tested have strong virucidal activities against SARS-CoV. Because SARS-CoV is believed to be transmitted mainly through the airborne route [13], PVP-I products for gargling and spraying the throat may have a prophylactic effect on SARS during outbreaks. Since the virus is also shed into feces and urine [14, 15], scrubbing hands with PVP-I may be effective in hospitals and households, especially after contact with SARS patients and after handling the clothes or linen of the patients.

Chemical reagents, heating and UV irradiation are the common means of inactivating pathogens. SARS-CoV-infected cells were effectively inactivated by treatment with cold acetone, methanol, formalin and glutaraldehyde for 5 min or longer. Therefore, infected cells can be safely handled after fixation with these common reagents. The results of indirect fluorescent antibody assays indicated that the antigenicity of SARS-CoV in infected cells is restored after treatment with acetone and methanol (data not shown). SARS-CoV was completely inactivated by heating at 56°C for 60 min or longer. The virus nucleocapsid protein was detected in the supernatant of infected cells by Western immunoblotting after heating at 56°C for 90 min (data not shown). Therefore, prolonged heating would be a useful method of safely preparing samples for laboratory diagnostic tests. Residues of virus on laboratory benches can be inactivated by UV irradiation, but the efficacy is incomplete. The combination of spraying and wiping the bench with 70% ethanol, followed by UV irradiation may completely inactivate any virus on the bench.

We believe that the results of this study will be useful in the control of SARS and for establishing guidelines for the safe handling of live virus in the laboratory, thus preventing SARS outbreaks of laboratory origin.

Acknowledgments

We thank Dr. Koichi Morita for kindly providing the Hanoi strain of SARS-CoV. This work was supported by a grant of the 21st Century COE Program, 'Program of Excellence for Zoonosis Control', Ministry of Education, Science, Sports and Culture.

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