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RESEARCH ARTICLE

Antiviral activity of povidone-iodine gargle and mouthwash solution against Enterovirus A71, Coxsackieviruses A16, A10 and A6

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ABSTRACT

Hand, Foot and Mouth Disease (HFMD), a highly contagious viral disease common among infants and young children, is primarily caused by Enterovirus A71 (EV-A71) and Coxsackievirus A16 (CV-A16). Nonetheless, emerging enteroviruses, such as CV-A10 and CV-A6, have also caused widespread outbreaks globally, in part due to the absence of effective antiviral therapies, and the high person-to-person transmission rate. Person-to-person transmission is usually through fecal-oral or oral-oral routes, and sometimes via droplets. As the oral cavity is a primary site for early virus infection and replication, controlling oral viral shedding can mitigate the risk of transmission through this route. Povidone-iodine (PVP-I), a widely used antiseptic, has shown broad-spectrum antimicrobial properties but antiviral studies against HFMD-causing enteroviruses are limited, especially for CV-A10 and CV-A6. Our study demonstrated that a 1% PVP-I solution (final concentration of 0.5%) exhibited virucidal activity against EV-A71, CV-A16, CV-A10, and CV-A6. All seven EV-A71 isolates and five CV-A16 isolates showed a significant virus titer reduction after a 1-minute incubation, while five CV-A10 isolates and two CV-A6 isolates required a 5-minute incubation to achieve this. The virucidal activity was confirmed through the EN14476:2013+A2:2019 virucidal quantitative suspension test, wherein all four viruses were completely inactivated after a 30-minute incubation with PVP-I at 37°C under both clean and dirty conditions. Western blot analysis suggested that PVP-I could affect the VP1 structural proteins of EV-A71. Our results suggest that PVP-I could serve as a potential virucidal agent to reduce the risk of person-to-person transmission of HFMD.

Keywords: Coxsackievirus A16; coxsackievirus A10; coxsackievirus A6; enterovirus A71; povidone-iodine.

INTRODUCTION

Hand, foot and mouth disease (HFMD) is a common infectious disease caused mainly by enterovirus A71 (EV-A71) and coxsackievirus A16 (CV-A16), which may be complicated by serious neurological complications such as acute flaccid paralysis and encephalomyelitis. More recently, coxsackievirus A10 (CV-A10) and coxsackievirus A6 (CV-A6) have also emerged to cause HFMD outbreaks (Blomqvist *et al.*, 2010; Wei *et al.*, 2011; Mirand *et al.*, 2012; Chen *et al.*, 2017; Bian *et al.*, 2019). HFMD is most prevalent in children younger than 5 years of age (Lei *et al.*, 2015), and typically presents with fever, mouth ulcers and rashes around the mouth, palms and feet (Repass *et al.*, 2014). However, CV-A10 and CV-A6 infection may occasionally be associated with atypical manifestations such as onychomadesis, widespread rashes and lesions at unusual skin sites such as the trunk and neck (Wei *et al.*, 2011).

Infectious viruses are shed via oral or fecal routes to the external environment (Klein & Chong, 2015). Recent studies revealed that EV-A71 and CV-A16 suspension droplets remain infective in a covered well plate for up to 11 days at room temperature. When dried on plastic surfaces, infectivity decreased within 2 to 4 hours but persisted longer on wood and stainless-steel surfaces, where viral RNA was detectable for up to 28 days (Sittikul *et al.*, 2023). Coxsackievirus B4 was also found to survive for up to 5 weeks on dried petri dishes (Firquet *et al.*, 2015). These findings highlighted the risk of enteroviral infection from contaminated surfaces and close contact with infected individuals. Adults or older children (6 years and above) with HFMD who often exhibit milder symptoms or remain asymptomatic may also unknowingly spread infection, especially within households where parents and children are in frequent close contact. Studies have shown that the transmission rates of HFMD in household settings are high (Chang *et al.*, 2004; Hoang *et al.*, 2019).

As a non-enveloped virus, enteroviruses are relatively stable (Solomon *et al.*, 2010) and cannot be easily inactivated by alcohol-based sanitizers and other common disinfectants such as hypochlorite, deoxycholate and ether (Chan & Abu Bakar, 2005). Large HFMD outbreaks that are still frequently reported pose important health concerns and economic burdens to affected countries (Wang *et al.*, 2016; Nhan *et al.*, 2019). Despite the

licensing of 3 monovalent vaccines against EV-A71 in China (Lin *et al.*, 2019), multivalent vaccines remain unavailable. Thus, having an effective antiviral agent is deemed necessary to contain and limit the transmission of HFMD.

Povidone-iodine (PVP-I) is an iodophore-based formulation that is well-known and widely used in the medical field for decades as an effective broad-spectrum antimicrobial agent, especially in wound care (Bigliardi *et al.*, 2017a). In PVP-I, iodine forms a complex with polyvinylpyrrolidone (PVP) which acts as a non-biocidal, synthetic carrier for the free iodine, the main germicidal component (Bigliardi *et al.*, 2017b). Compared to ordinary iodine solution, PVP-I is less toxic and irritating to the skin and oral cavity (Frank *et al.*, 2020). A range of PVP-I products has been formulated to cater to specific medical needs, including oral gargle and mouthwash solutions, eye drops, creams, throat sprays, gel and antiseptic solutions. PVP-I is irrefutably an excellent antimicrobial agent as it has antimicrobial activity against a wide range of bacteria and fungi (Reimer *et al.*, 2002; Eggers, 2019; Tan & Johari, 2021), and resistant mutants have not been reported (Eggers, 2019; Barreto *et al.*, 2020). It has been found to have good inactivating efficacy against some enveloped viruses including MERS-CoV, herpes simplex virus, human immunodeficiency virus, rubella virus, measles virus, mumps virus, influenza virus (H1N1), and Ebola virus (Kawana *et al.*, 1997; Eggers, 2019) and non-enveloped viruses such as adenovirus, polyomavirus, rotavirus and human rhinovirus (Kawana *et al.*, 1997; Reimer *et al.*, 2002; Sauerbrei & Wutzler, 2010). Recent studies have also shown that PVP-I demonstrated virucidal activity against SARS-CoV-2 (Bidra *et al.*, 2020; Hassandarvish *et al.*, 2020).

To our knowledge, investigations on PVP-I antiviral activity against enteroviruses that cause HFMD are scarce. PVP-I was only tested to be effective in reducing viral titers of EV-A71 and CV-A16 (1 viral isolate for each) in one previous publication (Tan & Johari, 2021). Antiviral activity of PVP-I on CV-A10 and CV-A6 remained unexplored. In addition, the target sites and mechanisms of action of PVP-I on EV-A71, and other enterovirus infections have hitherto remained unknown. Therefore, the primary objectives of this study were to confirm the antiviral activity of a commercial gargle and mouthwash solution (Betadine® Gargle and Mouthwash, Mundipharma, Germany) against various clinical isolates of EV-A71 and CV-A16, and to investigate the antiviral activity of PVP-I against CV-A10 and CV-A6, as well as the mechanism of action of PVP-I.

MATERIALS AND METHODS

Cells and viruses

Human primary oral fibroblast (HPOF) cells isolated previously from a 3-month-old infant's lip mucosa (Phyu *et al.*, 2017) were grown and maintained in 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, USA) and Ham's F-12 Nutrient Mixture (F12) (Sigma-Aldrich, USA), supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA) and 1 ng/ml fibroblast growth factor. Human scavenger receptor class B member 2 (hSCARB2)-expressing, L929 mouse cells (L-hSCARB2 cells) (courtesy of Dr. Satoshi Koike, Tokyo Metropolitan of Medical Science, Tokyo, Japan) were cultured in DMEM growth medium (DMEM-GM) supplemented with 5% FBS and 4 µg/ml puromycin, and maintained in maintenance medium (DMEM-MM) supplemented with 2% FBS and 4 µg/ml puromycin (Yamayoshi *et al.*, 2009). These cells were used to investigate if PVP-I affects virus attachment before cell entry via hSCARB2 receptors. African green monkey kidney (Vero) cells were cultured in DMEM-GM supplemented with 5% FBS and human rhabdomyosarcoma (RD) cells were cultured in DMEM-GM supplemented with 5% FBS and 5% horse serum (HS) (Gibco, USA). To prepare the virus stocks, all EV-A71 and CV-A16 isolates were propagated in Vero cells, and CV-A10 and CV-A6 in RD cells, at a multiplicity of infection (MOI) of 0.01. Infected Vero cells and RD cells were maintained in DMEM MM supplemented with 2% FBS and DMEM MM supplemented with 2% FBS and 2% HS, respectively. After incubation at 37°C and when 90% cytopathic effect (CPE) was obtained, the infected cells were subjected to 3 freeze-thawing cycles to release the intracellular virus and clarified by centrifugation at 4000 rpm for 10 mins at 4°C (WHO, 2004; Shingler *et al.*, 2014). The supernatant was collected for virus titer determination using a 50% cell culture infective dose ($CCID_{50}$) assay (WHO, 2004; Ong *et al.*, 2008).

All the clinical virus isolates were previously isolated from uncomplicated HFMD patients or fatal HFMD patients with CNS complications (Table 1). The representative viruses used in the experiments were EV-A71 (18435), CV-A16 (North), CV-A10 (S4/336/1) and CV-A6 (S1/1376/1) unless otherwise stated. The selection of these four viruses was based on their favorable characteristics, specifically their robust growth in HPOF and the ability to generate high viral titers.

Table 1. Clinical isolates of enteroviruses used in this study

Virus	Genotype	Virus ID	Origin	Year	Disease
EV-A71	B3	MY104	Malaysia	1998	Fatal HFMD with encephalomyelitis
	B4	A10/4	Malaysia	2000	HFMD
	B5	18435	Malaysia	2006	Fatal HFMD with encephalomyelitis
	C2	8/M	Australia	1999	Myelitis
	C3	001-KOR-00	Korea	2000	HFMD
	C4	VN5559	Vietnam	2005	HFMD
	C5	VN5784	Vietnam	2005	HFMD
CV-A16	B1	North	Malaysia	2006	HFMD
	B1	Central	Malaysia	2000	HFMD
	B1	South	Malaysia	2003	HFMD
	B2	New	Malaysia	1998	HFMD
	NA	19/501/1	Malaysia	2019	HFMD
CV-A10	F	S4/336/1	Malaysia	2005	HFMD
	F	19/177/2	Malaysia	2019	HFMD
	F	19/188/2	Malaysia	2019	HFMD
	F	19/297/2	Malaysia	2019	HFMD
	F	19/392/1	Malaysia	2019	HFMD
CV-A6	D	S1/1376/1	Malaysia	2005	HFMD
	D	15/028/3	Malaysia	2015	HFMD

*HFMD = Hand, foot and mouth disease.

Virus titration

A 50% cell culture infectious dose ($CCID_{50}$) assay was performed to determine the virus titer, and the $CCID_{50}$ was calculated using the Spearman-Karber method as described previously (Karber, 1931). Briefly, ten-fold serial dilutions of the virus supernatant (10^{-1} to 10^{-8}) were prepared and 100 µl of each dilution was inoculated into the cells in quadruplicate. After incubation for 5 days, the number of wells with CPE at each dilution was recorded. The virus titer of CV-A16 in all the antiviral assays was determined using Vero cells while the virus titer of EV-A71, CV-A10 and CV-A6 was determined using RD cells.

Povidone-iodine

A commercially-available oral gargle and mouthwash solution containing 1% PVP-I as active ingredient (Betadine® Gargle and Mouthwash, Mundipharma, Germany) was filtered through a syringe filter with a 0.22 µm pore-size hydrophilic polyethersulfone (PES) membrane (Sartorius, Minisart, Germany), aliquoted and stored at 4°C before use.

Virucidal activity of PVP-I against representative EV-A71, CV-A16, CV-A10 and CV-A6

The antiviral activity of PVP-I was evaluated using a virucidal assay. An equal volume of 1% w/v PVP-I was mixed with the virus ($2x10^5$ $CCID_{50}$) and incubated for 5 mins and/or 1 hr at 37°C. Having predetermined that 0.25% PVP-I was non-toxic to cells, the mixtures were then diluted with DMEM/F12 medium in a ratio of 1:1 before virus pre-absorption in HPOF cells at 37°C. After 2 hours, the virus-PVP-I mixture was removed, and the cells were washed twice with PBS before adding fresh DMEM/F12 medium and observed daily under an inverted light microscope. Cells inoculated with virus-DMEM/F12 mixture and DMEM/F12 were used as controls. At 5 days post-infection (dpi), the plate was freeze-thawed 3 times and viral-titrated using the $CCID_{50}$ assay as described.

Virucidal log reduction assay

A virucidal log reduction assay was performed to measure the log reduction of the representative EV-A71, CV-A16, CV-A10 and CV-A6 infectious virus titers using the $CCID_{50}$ assays after exposure to PVP-I at different incubation timepoints. Briefly, $2x10^5$ $CCID_{50}$ of EV-A71 and CV-A16 were incubated at 37°C with 1% PVP-I at equal volume for 30 secs, 1 min, 5 mins and 1 hr, respectively. CV-A10 and CV-A16 were incubated at 37°C with 1% PVP-I at equal volume for 30 secs, 1 min, 5 mins, 15 mins and 1 hr, respectively. After each specified contact time, the mixtures were then ten-fold serially diluted in DMEM-MM before inoculation into RD or Vero cells in a 96-well plate in quadruplicate. After incubation for 5 days, the $CCID_{50}$ was determined. This assay was repeated 3 times independently and the average virus titers were reported. The following formula was used to calculate the percentage of virus titer reduction after incubation with PVP-I at each incubation time point:

$$\text{The percentage of virus titer reduction} = \frac{Vi - Va}{Vi} \times 100\%$$

Where Vi = Virus titer unexposed to PVP-I
Va = Virus titer after exposure to PVP-I

Virucidal activity of PVP-I against other clinical isolates

The virucidal activity of PVP-I against other EV-A71, CV-A16, CV-A10 and CV-A6 clinical isolates (Table 1) was investigated using the virucidal log reduction assay as described with slight modifications. 1% PVP-I was incubated with EV-A71 and CV-A16 for 1 min and 5 mins, and with CV-A10 and CV-A6 for 5 mins and 15 mins. These modified time points were chosen based on the log reduction assay results as shown in Figure 2.

Virus binding and entry assays

To verify that PVP-I prevents EV-A71 from attaching to hSCARB2 and other receptors, thereby blocking viral entry, a virus binding and entry assay was performed on HPOF and L-hSCARB2 cells. HPOF or L-hSCARB2 cells were seeded in a 12-well plate and incubated overnight at 37°C. EV-A71 (strain 18435; $2x10^5$ $CCID_{50}$) and 1% PVP-I were mixed at equal volumes and incubated for 1 hour at 37°C. The EV-A71-PVP-I mixture was diluted into half before inoculation into the cells for virus pre-absorption. The plate was then incubated at 4°C for 2 hours for virus pre-absorption with a gentle shaking of the plate at 15-minute intervals. The cells were then gently washed twice with cold PBS to remove unbound viruses.

i. Quantitative real-time PCR (qRT-PCR)

To quantitate the viral RNA copies, total RNA was extracted using the easy-RED™ Total RNA Extraction solution (iNtRON Biotechnology, South Korea) according to the manufacturer's protocol. The viral RNA copies which correlate with the amount of virus that was attached to the surface of the cells were quantified using a SYBR-Green based, two step qRT-PCR, as described previously (Jonsson *et al.*, 2009), using a pair of qPCR Pan-Enterovirus forward primer (Pan-EV-RT-F: 5'-CCT GAA TGC GGC TAA TCC TA-3') and reverse primer (Pan-EV-RT-R: 5'-ACG GAC ACC CAA AGT AGT CG-3') that targets the 5'NTR region of the viral genome. To construct the standard curve, an EV-A71 plasmid containing the 5'NTR region of EV-A71 with a T7 promoter was linearized, purified and *in vitro* transcribed into RNA using the MAXIscript® T7 Transcription Kit (Ambion, USA) following the manufacturer's protocol. The *in vitro* transcribed EV-A71 RNA was purified, quantified and 10-fold serially diluted (0.707 ng to $7.07x10^{-8}$ ng). cDNA was synthesized using the Maxime™ RT PreMix (Random Primer) Kit (iNtRON Biotechnology, South Korea). The qRT-PCR was performed using the SYBR ExcelTaq 2x Fast Q-PCR Master Mix (SMOBIO Technology, Taiwan). The cycling conditions for qRT-PCR were shown in Table 2, using the Applied Biosystems StepOne Plus Real-Time PCR System (Applied Biosystem, USA). Results were analyzed using StepOne Software version 2.3. The EV-A71 RNA copy numbers were compared between the mock-treated cells and EV-A71-PVP-I mixture-treated cells. The results were presented as viral copy numbers per µl.

Table 2. Cycling conditions for qRT-PCR

qRT-PCR cycling conditions	Temperature	Duration	Cycles
Template denaturation and enzyme activation	95°C	2 mins	1
Denaturation	95°C	15 secs	40
Annealing/ Extension	60°C	60 secs	

ii. Virus titration

To measure the live virus titer, after virus pre-absorption and washing the cells with cold PBS, 500 µl of new DMEM-MM was added to each well and the cells were further incubated for 5 days at 37°C. At 5 dpi, the plate was freeze-thawed and viral titrated as described.

Effect of PVP-I on EV-A71 structural proteins using Western blot analysis

We speculated that PVP-I may affect viral structural proteins. To confirm this, the effect of PVP-I on EV-A71 VP1 protein was evaluated using Western blotting. PVP-I at 1% was mixed with 10% sodium thiosulfate (ST) stock solution or distilled water (DH_2O) and incubated for 30 mins at room temperature (rtp). The above respective

mixtures were aliquoted into two separate microcentrifuge tubes at equal volume before mixed with an equal volume of EV-A71/18435 virus (6.5 $\log_{10}$ $CCID_{50}$/ml). To prepare the unexposed virus control, DH_2O was mixed with an equal volume of undiluted EV-A71/18435 virus. After 30 mins incubation in at 37°C, each mixture was aliquoted and mixed with an equal volume of 2x Laemmli sample buffer (Bio-Rad, USA) containing 2-mercaptoethanol (Sigma, USA) and boiled for 10 mins to denature the samples. All the samples then underwent SDS-PAGE. The separated proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, USA) before blocking with 5% non-fat dry milk in Tris-buffered saline with 0.05% tween 20 (TBST), followed by overnight incubation with a polyclonal rabbit anti-enterovirus 71 VP1 primary antibody (1:1000 dilution) (GeneTex, USA) at 4°C. Secondary goat anti-rabbit IgG H+L (HRP) antibody (Abcam, UK) was sequentially added. VP1 protein was detected using the WesternBright Quantum Chemiluminescent HRP Substrate (Advansta, USA). The membrane was imaged by the ChemiDoc MP Imaging System (Bio-Rad, USA). The blot images and intensities of the protein bands were analyzed using Image Lab software (Version 6.0.1, Bio-Rad, USA). The background-adjusted volumes of the EV-A71/18435 VP1 protein were determined using the non-cropped blots. The fold change of the VP1 band intensities were calculated relative to DH_2O + 18435, by dividing the intensity of the protein band of each sample (PVP-I + ST + 18435 or PVP-I + DH_2O + 18435) by the intensity of the control (DH_2O + 18435). The average fold change ± SD was calculated from three biological replicates. This Western blot result was compared with the virus titer measured with a log reduction assay done under similar incubation conditions. Briefly, the samples were prepared similarly as described for Western blot. After each specified incubation time, 50 µl from each mixture was aliquoted and ten-fold serially diluted in DMEM-MM before being inoculated into RD cells in a 96-well plate at quadruplicate for virus titration.

European Standard Suspension Test EN14476

The European Standard Suspension Test EN14476:2013+A2:2019 is a standardized quantitative suspension test established to evaluate the virucidal efficacy of chemical disinfectants and antiseptics intended for use in the medical area (British Standard Institute, 2019). Following the EN14476 virucidal quantitative suspension test, the virucidal effect of 1% PVP-I against the representative EV-A71 (10^8 $CCID_{50}$/ml), CV-A16 (10^7 $CCID_{50}$/ml), CV-A10 (10^6 $CCID_{50}$/ml) and CV-A6 (10^6 $CCID_{50}$/ml) were tested at rtp (25°C ± 1°C) and 37°C ± 1°C under simulated clean condition and dirty condition. For the clean condition, 0.3 g/l bovine serum albumin (BSA) (Rockland, USA) was used as the interfering substance. For the dirty condition, a mixture of 3 ml/l sheep erythrocyte (Hardy Diagnostics, USA) and 3 g/l BSA was used as the interfering substances. Equal volumes of virus (50 µl) and interfering substances were mixed before adding with 400 µl of 1% PVP-I (final concentration of 0.8%). For unexposed virus control, PVP-I was replaced with distilled water instead. After the specified contact time (0.5 min, 1 min, 5 min, 30 min and 60 min) at rtp or 37°C, the mixture was immediately neutralized by adding 450 µl of ice-cold DMEM-MM. The mixture was then 10-fold serially diluted and viral titrated as described above. This assay was repeated thrice independently. The percentage of virus titer reduction was calculated using the formula mentioned above.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). All statistical analyses were performed using the MaxStat Lite Statistic Software (MaxStat, Germany). As data were normally distributed (Anderson-Darling Normality Test), a two-tailed unpaired t-test was used to determine the statistical significance between treated and untreated cells. A p-value<0.05 was considered statistically significant.

RESULTS

Virucidal activity of PVP-I against representative EV-A71, CV-A16, CV-A10 and CV-A6

PVP-I at a final concentration of 0.5% demonstrated virucidal activity against EV-A71, CV-A16, CV-A10 and CV-A6 (Figure 1). No viruses were detected in HPOF cells when infected with EV-A71 and CV-A16 that were exposed to 0.5% PVP-I for 5 min (Figure 1A, 1B). In contrast, 1 hour of exposure time was required to fully inactivate CV-A10 and CV-A6 required 1 hour incubation time for demonstrable virucidal activity (Figure 1C, 1D).

Virucidal log reduction assay

Figure 2 shows the viral titers of EV-A71, CV-A16, CV-A10 and CV-A6 after incubation with 1% of PVP-I (final concentration of 0.5%) at different incubation times. The virus titers of both EV-A71 and CV-A16 were significantly reduced following 30 secs and 1 min incubation with PVP-I (Figure 2A, 2B), and were completely inactivated at 5 min (Figure 2A, 2B). In contrast, CV-A10 and CV-A6 demonstrated significant virus titer reduction following 5 mins incubation and achieved complete inactivation at 15 mins of incubation with PVP-I (Figure 2C, 2D).

Virucidal activity of PVP-I against other clinical isolates

All the EV-A71, CV-A16, CV-A10 and CV-A6 isolates showed significant virus titer reduction after incubated with PVP-I (final concentration of 0.5%) for 1 and 5 min (Figure 3A, 3B). No EV-A71 and CV-A16 viruses were detected after incubated with PVP-I for 5 min (Figure 3A). For CV-A10 and CV-A6, virus titer was not detected after incubation with PVP-I for 15 min (Figure 3B).

Virus binding and entry assays

To examine the possible mechanism of antiviral action of PVP-I, we performed viral binding and entry assays, and Western blot. EV-A71 was used as the representative virus due to the availability of the L-hSCARB2 cells and primary antibody. These provides a basic foundation to study the basic mechanism of PVP-I antiviral action against EV-A71 in particular. The binding and/or entry of EV-A71 (18435) to HPOF cells and L-hSCARB2 cells was prevented after incubation with PVP-I (final concentration of 0.5%) as no infectious virus was isolated from these cells (Figure 4A, 4B) compared to the virus control (p<0.0001). As quantitated by qRT-PCR, the binding of EV-A71 (18435) to HPOF cells (Figure 4C) and L-hSCARB2 (Figure 4D) showed a significant reduction after incubation with PVP-I compared to the virus control. The amplification efficiency of the qRT-PCR was ~106%.

Effect of PVP-I on EV-A71 structural proteins

Western blot analysis showed a marked reduction in VP1 protein after EV-A71/18435 was exposed to PVP-I for 30 mins (PVP-I + DH_2O + 18435) as compared to the unexposed virus control (DH_2O + 18435) (Figure 5A). The VP1 protein band of EV-A71/18435 after incubation with PVP-I that was neutralized by sodium thiosulfate (PVP-I + ST + 18435) did not show a significant difference in the intensity as compared to the unexposed virus control (DH_2O + 18435). The mean fold change of the VP1 protein band of PVP-I + DH_2O + 18435 relative to the virus control (DH_2O + 18435) was 0.305 ± 0.11, and the difference between these two samples was statistically significant (p=0.0341) (Figure 5B). The Western blot results were confirmed by a virus log reduction assay. Similarly, as shown in the Western blot, the virus titer of EV-A71/18435 was significantly reduced after 30 mins incubation with PVP-I (3.25 $\log_{10}$ $CCID_{50}$ ± 0.25, p=0.0001). In contrast, the virus titer of EV-A71/18435 incubated with PVP-I neutralized by ST (5.58 $\log_{10}$ $CCID_{50}$ ± 0.14) did not show a significant difference compared with the virus control (DH_2O + 18435) (5.67 $\log_{10}$ $CCID_{50}$ ± 0.14) (Figure 5C).

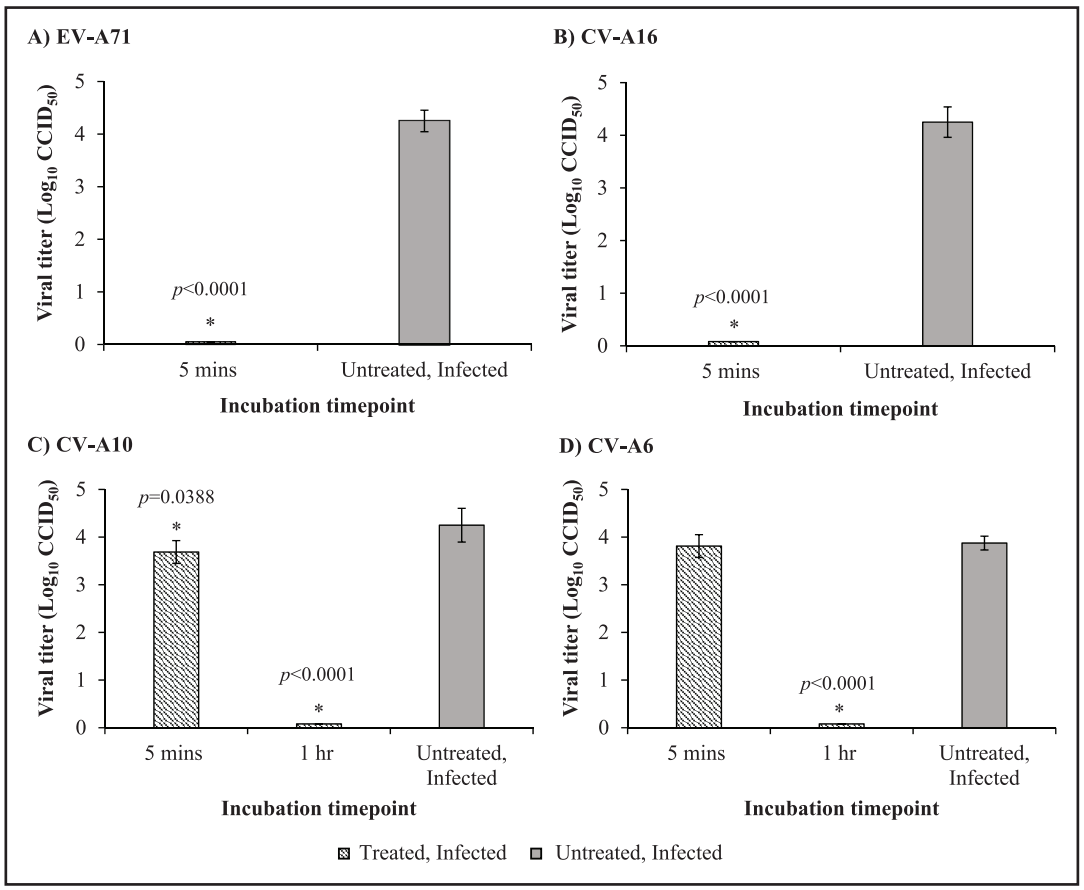

Figure 1. Virucidal activity of PVP-I against representative A) EV-A71, B) CV-A16, C) CV-A10 and D) CV-A6. The final concentration of PVP-I was 0.5% after 1:1 dilution. Virus titers were expressed as the mean $\log_{10}$ $CCID_{50}$ ± standard deviation. * = statistically significant (p< 0.05).

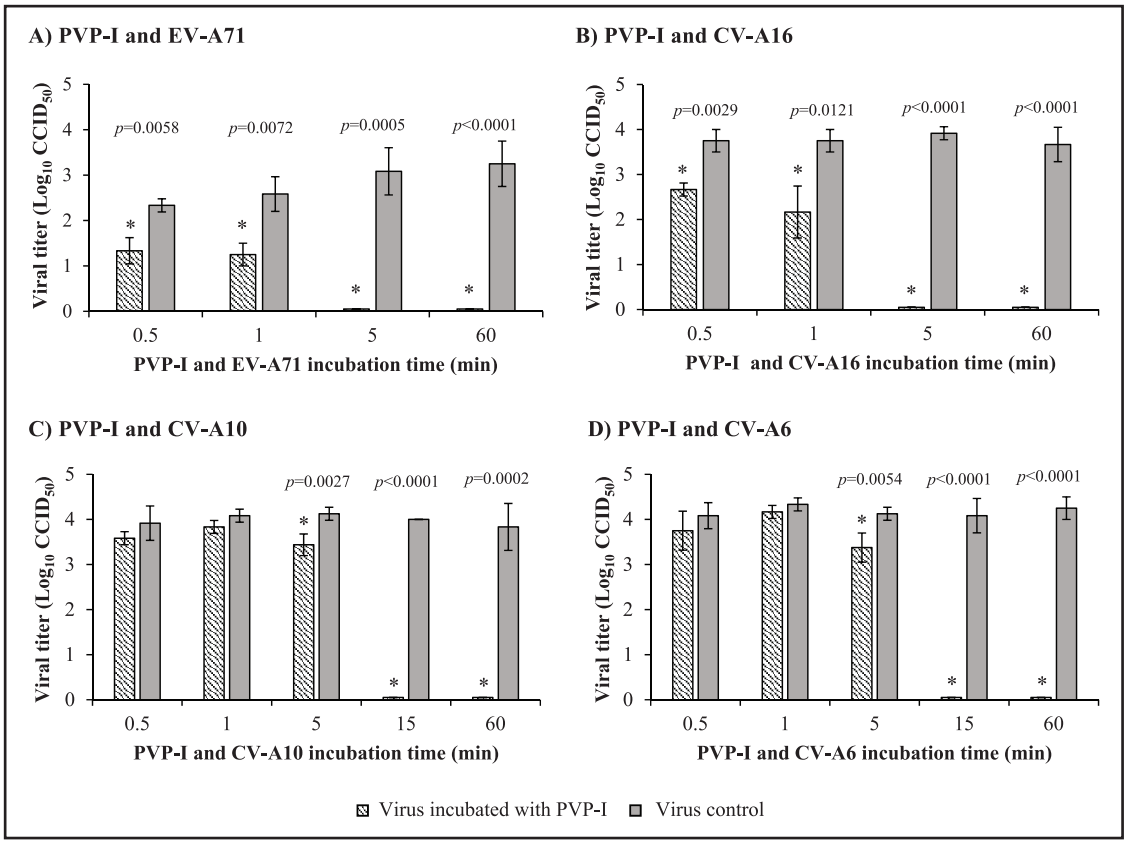

Figure 2. Virus log reduction assay for A) EV-A71, B) CV-A16, C) CV-A10 and D) CV-A6 after incubation with 1% PVP-I (final concentration of 0.5%) at various incubation times. Each timepoint was repeated 3 times and the average virus titers were reported. Virus titers were expressed as the mean $\log_{10}$ $CCID_{50}$ ± standard deviation. * = statistically significant (p< 0.05).

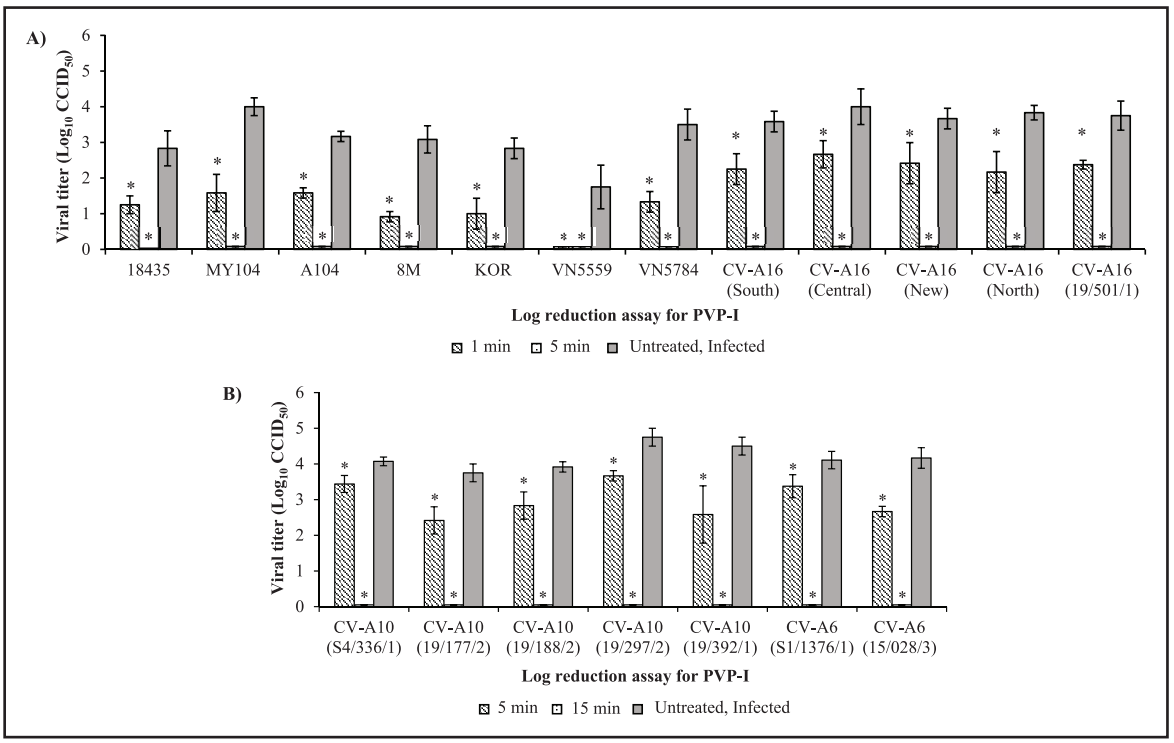

Figure 3. Virus log reduction assay of PVP-I against different A) EV-A71 and CV-A16; B) CV-A10 and CV-A6 clinical isolates. Virus titers were expressed as the mean $\log_{10}$ $CCID_{50}$ ± standard deviation. * = statistically significant ($p< 0.05$).

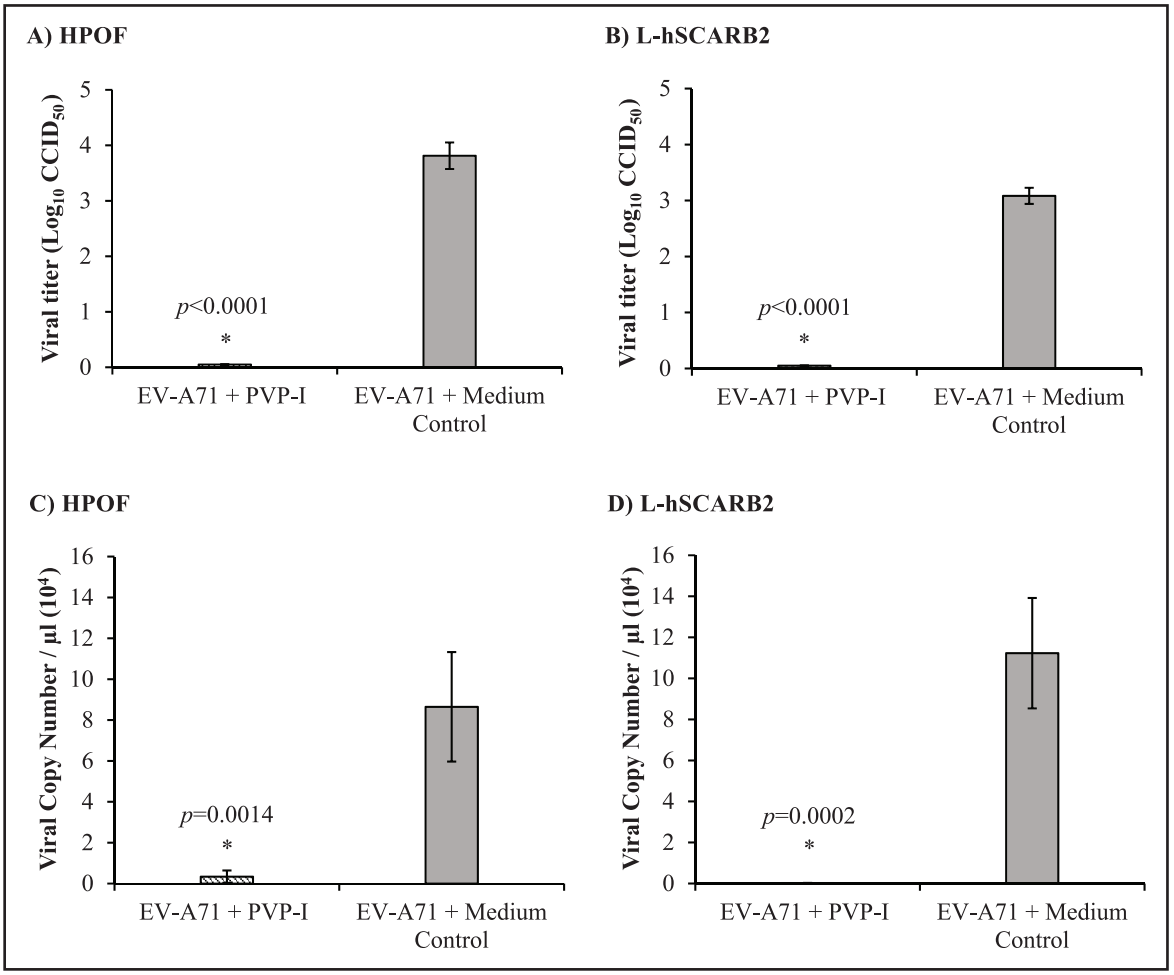

Figure 4. Virus binding and entry assays. HPOF cells (A, C) and L-hSCARB2 cells (B, D) were infected with EV-A71 + PVP-I and EV-A71 + medium (virus positive control). Viral RNA copies were expressed as the mean viral RNA copy number ± standard deviation. Virus titers were expressed as the mean $\log_{10}$ $CCID_{50}$ ± standard deviation. * = statistically significant ($p< 0.05$).

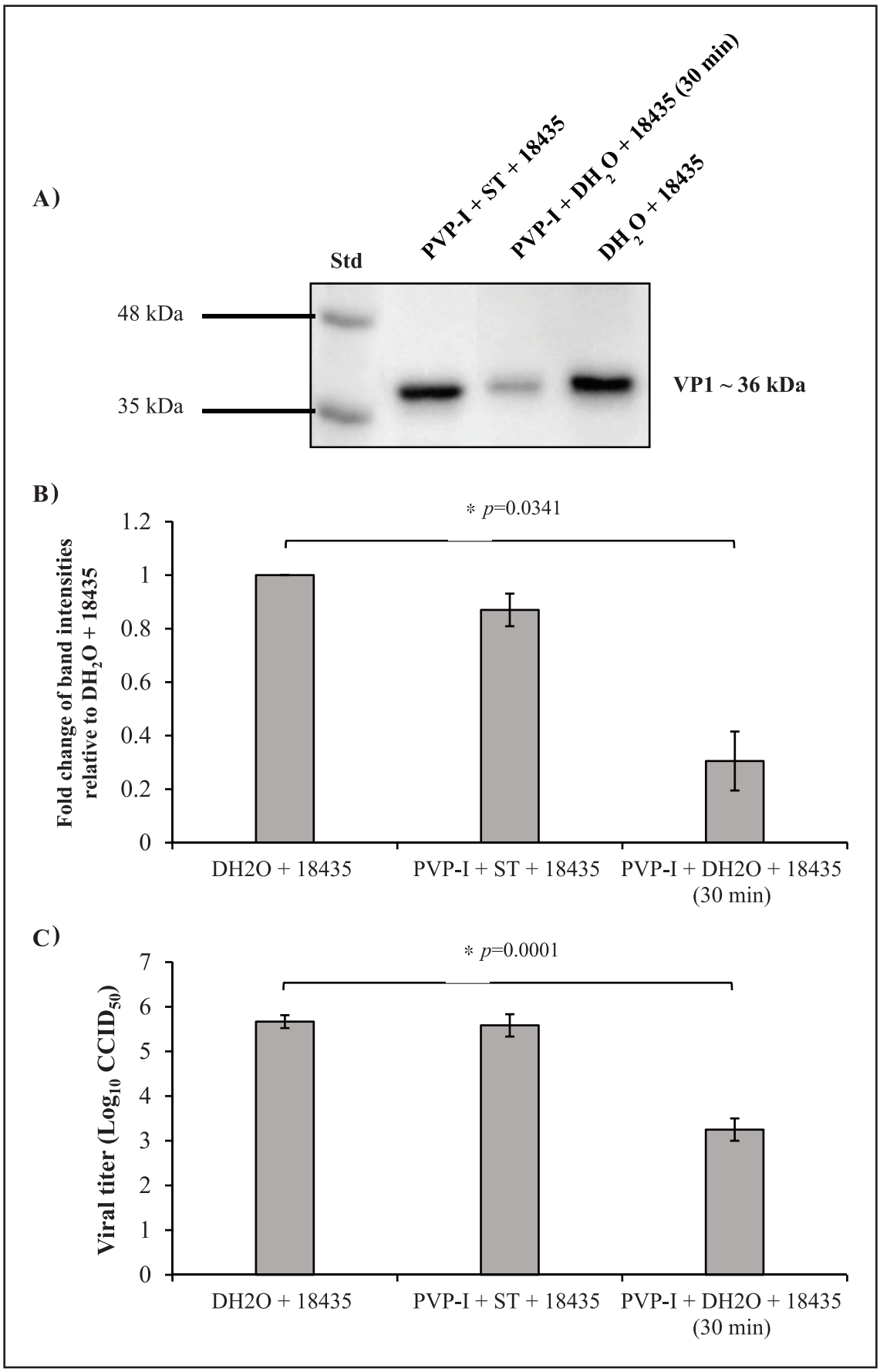

Figure 5. A) The representative Western blot image of VP1 protein after incubation with PVP-I + DH_2O + 18435, PVP-I + ST + 18435 or DH_2O + 18435. B) A graphic illustration of the densitometric analysis of the digital images of 3 independent Western blots. C) The correspondence virus titers of PVP-I + DH_2O + 18435, PVP-I + ST + 18435 or DH_2O + 18435. Virus titers were expressed as the mean log_{10} $CCID_{50}$ ± standard deviation. * = statistically significant (p< 0.05).

Table 3. Percentage reduction in virus titers by PVP-I based on the EN14476 test protocol

Virus	Test Temperature	Percentage of virus titer reduction									
		Clean condition					Dirty condition				
		Incubation time (min)					Incubation time (min)				
		0.5	1	5	30	60	0.5	1	5	30	60
EV-A71	rtp	15.4#	25.5*	19.2*	25.5*	28.8*	4.7#	14.1#	14.1#	20.3*	32.8*
	37°C	14.0#	26.9*	30.8*	**100**Δ	**100**Δ	23.9*	34.8*	39.1*	**100**Δ	**100**Δ
CV-A16	rtp	15.7#	2.1#	12.2#	20.6*	36.7*	0#	8.3#	10.4#	18.8#	35.4*
	37°C	8.3#	16.3#	25.5*	**100**Δ	**100**Δ	6.1#	6.1#	38.8*	**100**Δ	**100**Δ
CV-A10	rtp	5.0#	5.3#	8.8#	8.8#	25.0*	1.7#	3.4#	11.9#	16.9*	67.8*
	37°C	3.4#	10.7#	12.1#	**100**Δ	**100**Δ	9.4#	18.8#	23.4*	**100**Δ	**100**Δ
CV-A6	rtp	6.7#	9.3#	10.7#	15.5#	31.1*	3.4#	5.1#	15.3#	13.6#	45.8*
	37°C	18.5#	23.8#	7.0#	**100**Δ	**100**Δ	8.2#	13.1#	21.3*	**100**Δ	**100**Δ

* = Statistically significant virus titer reduction
# = No statistically significant virus titer reduction
Δ In bold = complete virus inactivation with no live virus detected

European Standard Suspension Test EN14476

The EN14476 suspension test was performed to confirm the virucidal activity shown in the log reduction assay and to study the virus inactivation kinetics of PVP-I against EV-A71, CV-A16, CV-A10 and CV-A6 over time. In brief, all EV-A71, CV-A16, CV-A10 and CV-A6 were completely inactivated after incubation with PVP-I for 30 mins at 37°C under both clean and dirty conditions (Table 3, highlighted in bold). Overall, the results suggested that the virucidal activity of PVP-I is unlikely to be significantly affected by the presence of interfering substances in either dirty or clean conditions, but rather by the incubation temperature. Table 3 summarizes the percentage of titer reduction of EV-A71, CV-A16, CV-A10 and CV-A6 after incubation with PVP-I.

DISCUSSION

Numerous HFMD outbreaks have been reported worldwide, especially in the Asia Pacific countries, causing enormous health challenges, morbidity and even mortality. Since there are no multivalent vaccines or broad-acting antiviral drugs available, prevention of viral transmission to curb outbreaks is a viable alternative. Indeed, personal hygiene, including handwashing and environmental sanitation remain important ways to curb the spread of infection by fecal-oral or oral-oral routes. The oral cavity and peri-oral skin represent a critical interface between the exterior and the host environment as it is an important primary site of viral infection and replication that leads to significant oral viral shedding (Ooi *et al.*, 2007). An antiviral mouth rinse that could rapidly reduce the viral load and shedding from the oral cavity in infected children and asymptomatic adults should be useful to impede viral transmission and contribute significantly to the control of HFMD outbreaks.

PVP-I has been long known as an effective antiseptic against a broad range of microbial species with good tolerability, high effectiveness and rapid action (Gmur & Karpiński, 2020). Previous studies have demonstrated the efficacy of PVP-I against a range of viruses, including the recent SARS-CoV-2 (Shet *et al.*, 2022) and some picornaviruses such as poliovirus, CV-A9, EV-A71 and CV-A16. In this study, the antiviral activity of the PVP-I gargle and mouthwash solution was tested against 4 enteroviruses (EV-A71, CV-A16, CV-A10 and CV-A6), in a total of 19 clinical virus isolates.

To our knowledge, this is the first study investigating the antiviral activity of PVP-I against CV-A10 and CV-A6. The 1% PVP-I gargle and mouthwash solution (final concentration of 0.5%) demonstrated virucidal activity against EV-A71, CV-A16, CV-A10 and CV-A6 in a virus dose-dependent and incubation time-dependent manner. CV-A10 and CV-A6 were more resistant compared to EV-A71 and CV-A16. All EV-A71 and CV-A16 clinical isolates were able to be completely inactivated after 5 mins incubation with 1% PVP-I, while all CV-A10 and CV-A6 only showed complete virus inactivation after 15 mins incubation with 1% PVP-I.

The virucidal results were further confirmed by the EN14476 virucidal quantitative suspension test. The rate of action of PVP-I was accelerated at 37°C compared to at rtp. All 4 tested viruses were completely inactivated after 30 mins incubation with PVP-I at 37°C in both simulated 'clean' and 'dirty' conditions (3.9 log or ≥4 log reduction). There was no discernible difference in the PVP-I virus inactivation rate under clean and dirty conditions, suggesting that the virucidal activity is unlikely to be significantly affected by the presence of the interfering substances in either clean or dirty conditions, but rather the temperature.

To the best of our knowledge, there is only one study that has reported the virucidal activity of PVP-I on one isolate of EV-A71 and CV-A16 (Tan & Johari, 2021), but none for CV-A10 and CV-A6. Tan *et al.* have reported that the tested oral PVP-I products (oral gargle solutions and throat spray) (final concentration of 6%, 0.8% and 0.36%) demonstrated a greater than 4 log reduction of the EV-A71 and CV-A16 virus titer after 0.5 min and 2 min of contact time, respectively (Tan & Johari, 2021). In contrast, our study required 30 minutes for a similar reduction in viral titers. These observed variations could be due to the utilization of different EV-A71 and CV-A16 isolates.

Poliovirus which is also from the same *Picornaviridae* family as enteroviruses, required more than 60 mins to achieve a ≥ 4 $\log_{10}$ reduction in virus titer (Poliovirus type 1; vaccine strain LSc-ab) (Sauerbrei & Wutzler, 2010). Coxsackievirus A9 was also rather resistant to PVP-I with a reduction factor not higher than 2.5 after 15 mins exposure (Reimer *et al.*, 2002). In contrast, enveloped viruses like the SARS-CoV-2 were completely inactivated after 15 secs (final PVP-I concentration of 0.75%, 0.5%, 0.25%) (Bidra *et al.*, 2020) and 30 secs (final PVP-I concentration of 7%, 5.25%, 0.7%, 0.35%, 0.315%) (Anderson *et al.*, 2020) of contact with PVP-I gargle and mouth rinse *in vitro*. Influenza virus, HIV, HSV and Ebola demonstrated a more than 4.5 $\log_{10}$ reduction in virus titer after 15 secs to 30 secs incubation with PVP-I (Kawana *et al.*, 1997; Eggers, 2019). Likewise, these results were consistent with our findings that non-enveloped viruses like enteroviruses generally require a longer contact time with PVP-I to achieve significant virus titer reduction or complete virus inactivation.

The primary contributor to the antimicrobial activity of PVP-I is reported to be the released free iodine. Free iodine readily penetrates the bacterial cell wall and oxidizes various components of the cell membrane and cytoplasmic proteins, nucleotides, and

fatty acids (Lachapelle *et al.*, 2013). Iodine binds and oxidizes the S-H bonds in amino acids such as methionine and cysteine and causes protein denaturation. Iodine also causes protein denaturation by reacting with N-H groups in arginine, histidine and lysine or the phenolic group of tyrosine, thereby impeding hydrogen bonding and further promotes protein denaturation (Cooper, 2007). Consequently, the alterations in cell walls, membranes, and cytoplasm induces extensive deleterious effects on microbial function and rapid cell death following exposure to iodine.

Interestingly, there is a scarcity of published data regarding the mechanism of action of PVP-I against both enveloped and non-enveloped viruses, with non-enveloped viruses having even less information available. Nevertheless, iodine may play a role in inactivating enveloped viruses by interacting with and/or destroying the viral envelope protein layer (Cooper, 2007), or glycoproteins found therein (Sriwilaijaroen *et al.*, 2009). In the context of enteroviruses which are non-enveloped viruses, iodine could inactivate the viruses by denaturing the viral structural protein(s) and/or viral genome. As shown by the Western blot results, iodine could possibly inactivate EV-A71 by denaturing the VP1 capsid protein, thus preventing virus attachment and/or entry as demonstrated in the viral binding and entry assays. The capsid proteins of EV-A71 consist of 60 copies of four viral structural proteins (VP1 to VP4), with VP1, VP2, and VP3 exposed on the surface and VP4 arranged internally. VP1 is the major capsid protein and plays a crucial role in viral particle assembly and cell entry via hSCARB2 receptor and/or other binding receptors such as P-selectin glycoprotein ligand-1, sialylated glycan, nucleolin, heparan sulfate, or annexin II (Kobayashi & Koike, 2020). Nevertheless, whether or not PVP-I denatured other viral capsid proteins (VP2 and VP3) or viral genome, remains unknown and requires additional studies. As these findings could be EV-A71 specific, further study is required to validate and extend this observation to CV-A16, CV-A10 and CV-A6. Further investigations are also needed to confirm whether differences in VP1 amino acid compositions affect the sensitivity of different isolates of EV-A71, CV-A16, CV-A10 and CV-A6 to PVP-I inactivation.

Based on our findings, it is evident that PVP-I demonstrated virucidal activity against the tested enteroviruses. However, the tested PVP-I oral gargle and mouthwash solution required at least 5 minutes of contact time with the virus to attain a significant decrease in virus titer. While this might not be an issue for certain medical applications, practical challenges arise when contemplating its daily use as an oral gargle. This issue is especially concerning for younger children (< 6 years old) as they might not comprehend and spit out the solution after gargling due to their limited swallowing reflexes and motor control. This may lead to accidental ingestion of the PVP-I solution, causing discomfort. Although iodine toxicity due to inadvertent ingestion of PVP-I is uncommon, it may cause transient hypothyroidism in rare instances (Martinez *et al.*, 2016; Swaminathan & Karunakar, 2023). Since gargling for an extended duration is impractical for most individuals, an oral spray may be a more feasible alternative. The spray format allows better control over the application and theoretically could enhance the contact time between the virus and PVP-I, thereby increasing the effectiveness of virus inactivation. However, further investigations are needed to assess its safety profile, especially concerning its application in young children.

Typically, PVP-I is used to inactivate cell-free pathogens, and its post-treatment effects are unclear. However, we cannot completely rule out the possibility of post-treatment activity. To our knowledge, only one study has demonstrated the post-treatment activity of PVP-I in reducing Zika and Chikungunya virus replication in corneal and retinal cells without causing cellular toxicity (Singh *et al.*, 2021). Therefore, in addition to inactivating cell-free viruses directly, PVP-I could potentially suppress viral replication within cells, leading to reduced viral shedding from the infected individual and possibly lowering transmission rates. Further studies are needed to confirm this hypothesis.

In conclusion, our results demonstrated that PVP-I is a potential antiviral agent that exhibits broad-spectrum antiviral activity against EV-A71, CV-A16, CV-A10 and CV-A6. Our study confirms that PVP-I inhibits EV-A71 entry into host cells, most likely through the denaturation of the VP1 capsid protein. Further investigations using an *in vivo* animal model or human clinical trial are needed to corroborate the effectiveness of PVP-I against HFMD enteroviruses before arriving at a definitive conclusion regarding its suitability as an antiviral agent for preventing or reducing HFMD transmission.

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Conflict of Interest

The author declares that they have no conflict of interests.

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