Research Paper

Investigating the Stability of Enterovirus 71 in Antiseptics and Environmental Variables

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Ji *et al.*: To Evaluate Virucidal Activity of Disinfectants

The model virus is crucial for evaluating virucidal activity of disinfectants. However, the utilization of poliovirus is only temporary because of the global polio eradication program. Enterovirus 71 has the advantages of high virus titer, convenient treatment and little harm, and can be used as a potential model virus for evaluating virus inactivation activity. To investigate resistance of enterovirus 71 to environmental (dry surfaces and hard water) and 10 hand disinfectants, compared with poliovirus-I virus. On dry surface, two viruses had shown reduction in activity with the increment of treating time and the activity of <4 $log_{10}TCID_{50}$ (log) at 4 h-treated time. However, neither poliovirus-I or enterovirus 71 in virus activity had maintained >4 log in hard water after treatment for 14 d. Six of 10 disinfectants reach the 4-log reduction requirement. Enterovirus 71 compared with poliovirus-I, exhibited the similar resistance to dry surface, hard water and disinfectants. Enterovirus 71 can be considered a suitable and important alternative model virus in the replacement of poliovirus-I to support the claims of virucidal activity. The model virus is crucial to evaluate the virucidal activity of disinfectants. However, the use of poliovirus is only temporary due to the global polio eradication program. Enterovirus 71 has the advantages of high viral titer, convenient treatment and little damage, and can be used as a potential model virus to evaluate virus inactivation activity. To investigate the resistance of enterovirus 71 to the environment (dry surfaces and hard water) and 10 hand sanitizers, compared to the poliovirus-I virus. On dry surfaces, two viruses showed a reduction in activity with increasing treatment time and an activity of <4 $log_{10}TCID_{50}$ (log) at 4 h of treatment time. However, neither poliovirus-I nor enterovirus 71 virus activity remained >4 log in hard water after treatment for 14 d. 6 out of 10 disinfectants meet the 4 log reduction requirement. Enterovirus 71, compared to poliovirus-I, showed similar resistance to dry surfaces, hard water and disinfectants. Enterovirus 71 may be considered a suitable and important alternative model virus replacing poliovirus-I to support claims of virucidal activity.

Key words: Enterovirus 71, environmental factors, poliovirus-I, disinfectants, virucidal activity

As a result of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) pandemic, the interest and demand for virucidal disinfectants have increased. Therefore, the evaluation of virucidal activity of these chemical disinfectants has been received high attention[1]. An appropriate model virus is essential for assessing the virucidal activity, which has a high titer in culture and high resistance to chemical disinfectants and environmental factors[2].

Poliovirus (PV) is a non-enveloped Ribonucleic Acid (RNA) virus classified into human Enterovirus 71 (EV71) and is neurotropic, causing severe neurological diseases in humans[3]. PV is a causative agent of poliomyelitis, resulting in flaccid paralysis[4]. Getting benefit from successful global vaccination efforts of the past few decades, poliomyelitis has been nearly eradicated from the world[5]. PV-I, especially, have many characteristic which are as follows; the virus exhibits simple virus propagation, safe operation and a high level of resistance to disinfectants and environmental conditions[2]. Therefore, PV-I, is used as a model virus by technical standards for disinfection and EN 14476 (phase 2/step 1)[6,7]. In 1988, The World

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Health Organization (WHO) launched the global polio eradication program, bringing on poliomyelitis significantly decreased. Nevertheless, the use of PV-I will require a higher level of biosafety, so the use of PV-I is only temporary[8]. On the other hand, vaccine derived PV-I cases were sometimes reported[9]. Therefore, it is recommended to substitute PV-I with an alternative model virus. For this reason, human EV71 such as the EV71 can be used. EV71 is ideal because this positive-sense RNA virus are cultivable on continuously growing cells with high titer and operability that it can be easily managed in a standard laboratory setting. Meanwhile, it poses little potential risk to employees performing the tests, because of vaccination[10]. Thus, EV71 can be chosen as a potential model virus for evaluating virucidal activity.

Our research has two objectives, both of which are crucial for evaluating EV71 as a model virus. The first aim was to evaluate EV71 resistance to dry surface and suspension (hard water) with lasting for different duration. The second aim was to evaluate EV71 resistance to different ingredients hand disinfectants, which widely used commercial disinfectants in China.

MATERIALS AND METHODS

Virus propagation and cell culture:

EV71 and PV-I were obtained from Guangdong Provincial Center for Disease Prevention and Control, China. Hep-2 cells and Vero cells were used to EV71 and PV-I virus propagation, respectively. The viral growth medium for Hep-2 cells and Vero cells was Modified Eagle Medium (MEM, GIBCO), supplemented with 2 % Fetal Calf Serum (FCS, GIBCO), 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO). The culture flasks were maintained at 37° in a 5 % Carbon dioxide (CO_2) atmosphere and monitored daily until the appearance of cytopathic effects. By repeatedly freezing and thawing infected cells three times, the virus is released through the infected cells. Centrifuge the suspension at 4000 rpm for 30 min to eliminate cell debris, and collect the upper culture medium for storage at -70°.

Infectivity assay:

For titers of EV71 and PV-I by the Tissue Culture Infectious Dose 50 ($TCID_{50}$) assay, cells were grown to 95 % confluence in flat-bottom 96-well plates (Corning 96-well plates). Thereafter, virus samples were prepared by a 10-fold dilution series with MEM including 2 % FCS. The supernatant from each well was removed and replaced with 150 µl of the appropriately diluted virus sample. Following incubation for 7 d, inverted microscopy was employed to distinguish infected wells from non-infected ones. The highest dilution of the virus suspension that induced a cytopathic effect in 50 % of cell monolayers was determined through microscopic observation. The $TCID_{50}$ value was calculated using the Reed and Muench method[11].

Resistance test to environment factors (wet and dry):

Resistance test on dry surface: The test was performed according to EN 14476:2013+A2:2019 with a modification (European Committee for Standardization)[12]. The cleaning of the stainless steel discs (20 mm diameter) was performed as already described[2]. The discs were prepared by autoclaving. A total of 50 µl of the virus was added to each pre-treated discs surface and dried at room temperature. Then, the discs were transferred into sterile petri dish and placed for 0.5, 1, 2, 4, 12, 24, 48 and 72 h. At the end of the every treating time, the discs were transferred into 950 µl of culture medium. Vials were vortexed for 60 s to collect residual viruses and immediately dilute the eluent 10 times to determine virus infectivity.

Resistance test in hard water: A total of 50 µl of the virus was added into 450 µl of sterile hard water. Then, the mixture was placed at room temperature for 1, 2, 3, 4, 5, 6, 7 and 14 d, respectively. At the end of the every exposure time, the 50 µl mixture were transferred into 950 µl of culture medium. Vials were vortexed for 30 s followed by 10-fold dilution for determining viral infectivity.

Chemical disinfectants:

There are 10 commercially and commonly used chemical disinfectants for testing. Determination of active ingredient according to technical standards for disinfection in China[6]. The disinfectants include A (sodium hypochlorite, 0.037 % effective chlorine), B (75 % ethanol and 0.4 % triclosan), C (70 % ethanol and 0.5 % Polyhexamethylene Biguanide Hydrochloride (PHMB)), D (70 % ethanol and 0.5 % chlorhexidine glucanate), E (0.25 % chlorhexidine glucanate and 0.25 % PHMB), F (70 % ethanol and 0.105 % PHMB), G (60 % ethanol and 10 % isopropanol), H (55 % ethanol and 0.4 %

PHMB·HCl), I (70 % ethanol) and J (77 % ethanol and 0.12 % Hydrogen peroxide (H_2O_2)). Treatment times and concentrations used were according to instructions on these disinfectants.

Neutralization validation:

The neutralization validation was performed according to technical standards for disinfection 6 with a modification by means of the dilution-ultracentrifugation method[13]. The mixture were vortexed for 15 s, followed by incubation at 20° for 60 s, and then centrifuged with 85 000 g at 4° for 2.5 h. Discarding the supernatant and suspending the precipitates with 1 ml MEM, following diluting the mixture by serial 10-fold with MEM including 2 % FCS. The virus titers were determined by $TCID_{50}$.

Virus inactivation test:

Mix the virus suspension (0.2 ml) with 0.8 ml of the test disinfectant. Vortex the virus disinfectant mixture for 10 s and incubate at 20° for 1 min. As mentioned earlier, after the end of the exposure period, neutralize 0.1 ml of the mixture by ultracentrifugation dilution method. The virus titer was determined by $TCID_{50}$. A reduction of infectivity of ≥4 $log_{10}TCID_{50}$ (4-log) steps (inactivation 99.99 %) was considered evidence of sufficient antiviral activity against the tested virus[6]. Take the logarithm of the difference between the virus titers of each disinfectant and the virus titers of the virus control as the average, and calculate the average log10 reduction factor attenuation coefficient.

RESULTS AND DISCUSSION

There have been few reports on EV71, which was used as model viruses to evaluate virucidal activity. Two viruses are applied to the stainless steel surface and then dried, which more accurately imitates the actual situation. Surfaces play an important role in viral transmission directly from contaminated surfaces to susceptible individuals[14]. In order to evaluate the relation between virus's activity and time on the dry surface, the treating time of discs were set as 0.5, 1, 2, 4, 12, 24, 48 and 72 h, respectively. When placing dried viruses on stainless steel carriers with lasting for different consecutive time intervals. The results displayed the relations of vitality decreased over time. It could result in >4-log reduction at 4 h-treated time for both viruses. This observations are also supported by Eggers' study which exert 4-log reduction of the PV-I[15]. Based on the present study, both of two viruses have displayed a similar resistance, which are sensitive to the drying environment. It should be noted that EV71 may persist on dry surface for approximately 2 h-4 h. However, several virucidal carrier testes (made of various materials, such as plastic, glass and fabrics) may need to confirm this concept and further understand (Table 1 and fig. 1).

Both of PV-I and EV71 exhibit the similar time-dependent activity pattern. That is, the lower virus activity are shown when the mixture (virus and hard water) have longer exposure time. In present test, both viruses and sterile hard water were kept in contact with each other in a liquid phase with different exposure durations varying from (1-14) d. Although both viruses reduced over time, neither of viruses showed the 4-log reduction requirements. That is, EV71 displayed a very high resistance to be in hard water. Similar results were also observed by previous study which this virus maintain activity in wastewater over several months (Table 2 and fig. 2)[16].

TABLE 1: CHANGES IN VIRUS ACTIVITY ON DRY SURFACE

Time (h)	PV-I		EV71	
	Virus activity	Reduction activity	Virus activity	Reduction activity
0	5.67	0	5.5	0
0.5	5.33	0.22	5.5	0
1	5	0.56	5	0.5
2	4.67	0.83	4.33	1.17
4	1.33	4.34	1	4.5
12	0.5	5.17	0	5.5
24	0	5.67	0	5.5
48	0	5.67	0	5.5
72	0	5.67	0	5.5

Note: (*) the average $log_{10}TCID_{50}$ of the untreated PV-I control was 5.67±0.28 and the average $log_{10}TCID_{50}$ of the untreated EV71 control was 5.50±0.55. The virus activity showed the average in $log_{10}TCID_{50}$. The reduction activity showed the average reduction in $log_{10}TCID_{50}$ from the controls

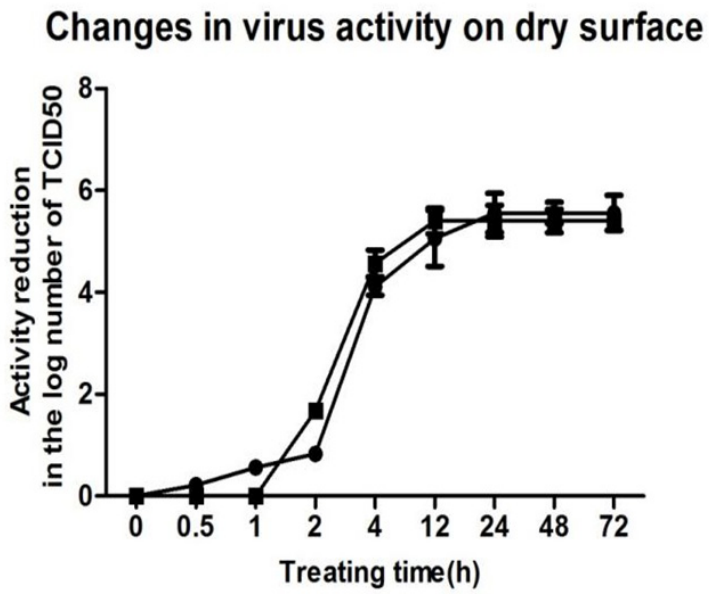

Fig. 1: Changes in virus activity on dry surface
Note: ( ): PV-1 and ( ): EV71

TABLE 2: CHANGES IN VIRUS ACTIVITY IN SUSPENSION

Time (d)	PV-I		EV71	
	Virus activity	Reduction activity	Virus activity	Reduction activity
0	6.00*	0	6.28*	0
1	5.56	0.44	5.61	0.67
2	5.44	0.56	5.61	0.67
3	5.44	0.56	5.61	0.67
4	5.28	0.72	5.5	0.78
5	5.16	0.84	5.44	0.84
6	5.05	0.95	5.28	1
7	4.83	1.17	5.06	1.22
14	4.78	1.22	4.86	1.42

Note: (*) The average $log_{10}TCID_{50}$ of the untreated PV-I control was 6.00±0.37 and the average $log_{10}TCID_{50}$ of the untreated EV71 control was 6.28±0.41. The virus activity showed the average in $log_{10}TCID_{50}$. The reduction activity showed the average reduction in $log_{10}TCID_{50}$ from the controls

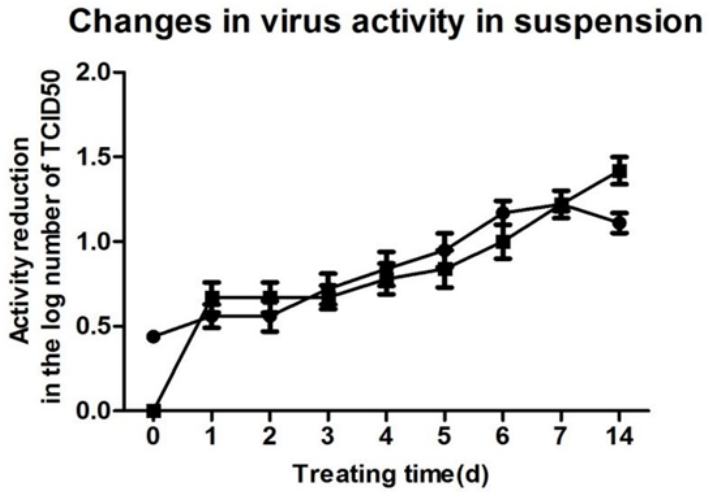

Fig. 2: Changes in virus activity in hard water
Note: ( ): PV-1 and ( ): EV71

The main aim of this study was to investigate both viruses resistance to chemical disinfectants. The tested EV71 revealed similar log reductions to that of PV-I. Sodium hypochlorite (NaClO), as a high-level disinfectants, which is a strong oxidizing agent[17] and recommended by the WHO[18], displayed high virucidal activity against EV71 (Table 3). Conversely, the intermediate-level disinfectants, such as formulations containing chlorhexidine glucanate-PHMB or ethanol and PHMB·HCl can exhibit less activity against EV71. The disinfectant formulation based on alcohol has broad-spectrum antimicrobial activity against bacteria, fungi and enveloped viruses[19]. In this study, ethanol and ethanol-based disinfectants (70 % and 75 %) showed the effective virucidal activity against EV71, which was consistent with previous observations showing ethanol's effective inactivation abilities against various viruses[20,21].

However, in this study, neither ethanol-only preparations (70 %, v/v) nor ethanol-isopropanol preparations (60 %:10 %, v) can provide high activity against EV71. The result is consistent with the conclusion from previous reports[5]. Conversely, addition of other active ingredients to alcohol preparations can improve significantly virucidal activity against EV71. Resistance to ethanol and other ingredients formulated preparations has also been demonstrated previously in tests against EV71 21, confirming our data (fig. 3).

According to present study, EV71 was similarly resistant to surface (suspension) and commonly used disinfectants with PV-I. Considering its similar properties in EV71, it is well accepted that the effective measures applicable to PV-I also apply to EV71. Moreover, EV71, as a clinically relevant virus, belong to the member of EV71, which have similar characteristics to PV-I (standard strains currently in use). Taken together, these results demonstrate that EV71 can be considered a suitable and important alternative model virus in there placement of PV-I to support the claims of virucidal activity to PV-I. We hope that the results of this study can be used to select the most suitable mode of virus for virucidal activity testing experiments, provide proven efficacy to the end user and play an important role in preventing and controlling virus outbreaks and transmission in medical institutions and communities.

TABLE 3: VIRUCIDAL ACTIVITY OF DISINFECTANTS AGAINST PV-I AND EV71

Disinfectants	Active ingredients	Treatment	PV-I		EV71	
		Time (min)	Virus activity	Reduction activity	Virus activity	Reduction activity
A	(NaClO, 0.037 % effective chlorine)	1	0	6.33	0	6.67
B	Ethanol (75 % v/v) and triclosan (0.4 % w/v)	1	2	4.33	2.11	4.56
C	Ethanol (70 % v/v) and PHMB (0.5 % w/v)	1	1.61	4.72	1.44	5.23
D	Ethanol (70 % v/v) and chlorhexidine glucanate (0.5 % v/v)	1	1.89	4.44	2.56	4.11
E	Chlorhexidine glucanate (0.25 % w/v) and PHMB (0.25 % w/v)	1	4.22	2.11	4.89	1.78
F	Ethanol (70 % v/v) and PHMB (0.105 % w/v)	1	2	4.33	2.56	4.11
G	Ethanol (60 %,v/v) and isopropanol (10 %, v/v)	1	4.56	1.77	5.39	1.28
H	Ethanol (55 % v/v) and PHMB·HCl (0.4 % w/w)	1	5.22	1.11	4.89	1.78
I	Ethanol (70 % v/v)	1	5.22	1.11	4.67	2
J	Ethanol (77 % v/v) and (H_2O_2, 0.12 % v/v)	1	2.45	3.88	2.61	4.06

Note: (*) the average $\log_{10}$ $TCID_{50}$ of the untreated PV-I control was 6.33±0.52 and the average $\log_{10}$ $TCID_{50}$ of the untreated EV71 control was 6.67±0.29. The virus activity showed the average in $\log_{10}$ $TCID_{50}$. The reduction activity showed the average reduction in $\log_{10}$ $TCID_{50}$ from the controls

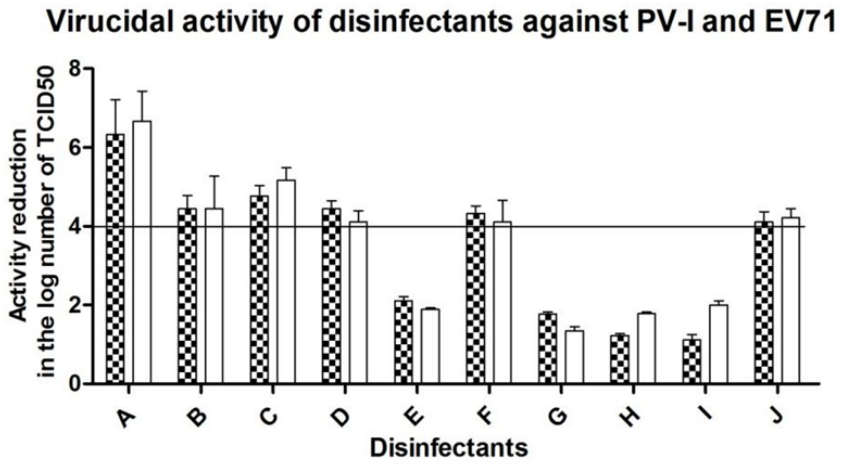

Fig. 3: Virucidal activity of disinfectants against PV-I and EV71
Note: (▩): PV-1 and (▭): EV71

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Author's contributions:

Xunmin Ji and Wei Xiao have contributed equally to this work.

Conflict of interests:

The authors declared no conflict of interests.

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