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Study on virus killing efficiency and related properties of a composite hand & skin antiseptics containing chlorhexidine

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ABSTRACT: Objective To verify the virus killing efficiency, stability and clinical safety of a composite hand & skin antiseptics containing chlorhexidine. **Methods** The suspension inactivation test, physical & chemical analysis, accelerated aging study and animal toxicity test were executed to evaluate the virus killing efficiency, stability and clinical safety of the composite hand & skin antiseptics. **Results** The composite hand & skin antiseptics containing 0.45 % gluconic acid gluconate, 75 % ethanol and synergists was used for 1 min, the average inactivation of poliovirus type I (PV-I) and EV71 enterovirus in suspension were both >4.00. The sealed package was stored at 37 °C for 3 months, and its active ingredient decline rate was <10%. The composite hand & skin antiseptics is actually non-toxic; it has no micronucleus effect on mouse bone marrow polychromatic red blood cells; it has no irritation to white rabbit in multiple intact skin test. **Conclusion** The composite hand & skin antiseptics has a good killing performance on viruses with good stability. It is safe to use for skin and hand disinfection.

KEY WORDS: Composite hand antiseptics; Chlorhexidine; Ethanol; virus; Inactivation efficiency; Stability, Clinical safety

People with post-COVID conditions can have a wide range of symptoms that can last weeks, months, or even years after infection. Sometimes the symptoms can even go away or come back again. China suffered a wide infection of COVID-19 in the past couple of months. Up to January 2023, US CDC has reported total 102, 171, 644 COVID-19 infected cases, which caused total 1, 103, 615 deaths. And its post effect is worthy of continued attention. In July 2021, "long COVID," also known as post-COVID conditions, was added as a recognized condition that could result in a disability under the Americans with Disabilities Act (ADA). Some people who have been infected with the virus that causes COVID-19 can experience long-term effects from their infection, known as post-COVID conditions (PCC) or long COVID. People with post-COVID conditions may experience health problems from different types and combinations of symptoms happening over different lengths of time. For some people, post-COVID conditions can last weeks, months, or years after COVID-19 illness and can sometimes result in disability. However, infection prevention is the most important and effective way to avoid hurt of COVID-19 and other virus. So, it is very helpful to know the hand & skin sanitizers and its virus killing efficiency.

Poliovirus type I vaccine strain, as a representative of hydrophilic virus, is recognized as an indicator microorganism for the evaluation of medium-level disinfection effects, and its resistance to antiseptics is generally considered to be the strongest among viruses. Enterovirus EV71 is the main pathogenic virus of hand, foot and mouth disease, and it has also attracted great attention in recent years. Therefore, it is of great practical value to explore the killing efficiency of this virus [1-4]. It has been reported [5] that composite of ethanol and hydrogen peroxide or peracetic acid has a certain efficiency of inactivating viruses, but these antiseptic ingredients lack long last disinfection and the per-

oxide itself is irritating to the skin, so not suitable for routine frequent use of surgical hands and skin antiseptics.

This paper studied the virus killing efficiency, stability and clinical safety of a composite hand & skin antiseptics containing chlorhexidine, alcohol, and synergist.

1 Materials and methods

1.1 Materials

Test virus strains: poliovirus type I (PV-I) vaccine strain and EV71 type enterovirus, host cells are HepG-2 cells and Vero cells respectively.

The composite skin and hand antiseptics used in the test is BiSteri® antiseptics, which contains 0.45% chlorhexidine gluconate, 75% ethanol and synergist, and is independently developed by Hangzhou Hygen Health Biotechnology Co., Ltd.

Ultrafiltration tube for testing: PALL ultrafiltration tube with a filter diameter of 30KD (PALL®Nanosep®)

Neutralizer for test: 3% Tween, 1% sodium thiosulfate cell culture medium

1.2 Killing efficiency test method

1.2.1 Preparation of virus suspension

Preparation of virus suspension: The virus suspension was prepared according to the provisions of the "Disinfection Technical Specifications" (2002 edition), and the logarithmic value of the average virus titer (TCID50) was determined to be between 5 and 7.

1.2.2 Residual antiseptics removal identification test

According to the "Disinfection Technical Specifications" (2002 edition), the test groups are: (1) antiseptics + virus suspension, (2) (antiseptics + virus suspension) + ultrafiltration, (3) virus suspension + ultrafiltration, (4) (antiseptics + ultrafiltration > + virus suspension, (5) deionized water + virus suspension, (6) HepG-2 cells, cultured after adding cell maintenance solution, and the experiment was repeated 3 times.

1.2.3 Neutralizer identification test

According to the "Disinfection Technical Specifications" (2002 edition), the test groups are: (1) (2) Antiseptics + virus suspension; (3) Antiseptics + neutralizer; (4) Neutralizer; (5) PBS buffer Solution + virus suspension, mix well and let stand at room temperature for a period of time, then add PBS buffer in (1), add neutralizer in (2), add virus suspension in (3) and (4), After mixing, place it at room temperature for a period of time; and set normal Vero cell control as (6). The experiment was repeated three times.

1.2.4 Virus killing efficiency test

According to the "Disinfection Technical Specifications" (2002 Edition) suspension quantitative inactivation test method, the action time was 1 min, 3.0 min and/or 5 min, and the test was repeated 3 times.

1.3 Stability test

Store the packaged antiseptics in a 37°C incubator for 3 months. According to the analysis method of the active ingredient content of ethanol and chlorhexidine gluconate in the "Disinfection Technical Specifications" (2002 edition), the active ingredient content in the antiseptics before and after storage was measured, the decline rate of the active ingredient was calculated, and the test was repeated twice.

1.4 Toxicity test

1.4.1 Acute oral toxicity test

Select 20 KM mice (half male and half female, weighing 18-22g, purchased from China Institute for Food and Drug Control, SPF animals), and carry out according to item 2.3.1 of "echnical Specifications for Disinfection" (200 edition).

1.4.2 Mouse bone marrow polychromatic erythrocyte micronucleus test

Select 50 KM mice (half male and half female, weighing 25-30 g, purchased from SPF (Beijing) Biotechnology Co., Ltd., SPF grade animals), according to item 2.3.8.4 of "Technical Specifications for Disinfection" (200 edition) conduct.

1.4.3 Multiple complete skin irritation test

Select 3 Japanese big-eared white rabbits (female, weighing 2.0-3.5 kg, purchased from Changyang Xishan Farm, Beijing, ordinary animals), and

carry out according to Item 2.3.3.3.1 of "Disinfection Technical Specifications" (200 Edition) .

1.5 Statistical analysis

SPSS 22.0 software was adopted to the statistical analyze. The count data were described as numbers and percentages, and differences between groups were analysed using the Chi-square test. The continuous data were described as means ± standard deviations, and comparisons between groups were performed using an independent samples t test. A two-tailed P-value < 0.05 was considered statistically significant.

2 Results

2.1 Identification of Residual Antiseptics Removal

The experimental results show that the poliovirus type I (PV-I) vaccine strain is used as the indicator virus, and the ultrafiltration tube with a filter diameter of 30KD can effectively remove the disinfection effect of the sample residue. The titers of each group met the specified requirements.

Table 1 Residual antiseptics removal identification test results

Group	Log value of virus titer for each test ($TCID_{50}$)			Average log value of virus titer ($TCID_{50}$)
	1	2	3	
1	Cytotoxicity			
2	＜0. 50	1. 50	＜0. 67	＜0. 89
3	6. 50	6. 33	5. 67	6. 17
4	5. 33	6. 67	6. 33	6. 11
5	6. 33	5. 33	5. 67	5. 78
6	cells grow well			

Note: Negative control cells grow well

2.2 Neutralizer identification test results

Test result shows, take EV71 type enterovirus as indicator virus, the cell culture medium of 3% tween-80 and 1% sodium thiosulfate as neutralizing agent, can effectively neutralize this compound hand/skin containing chlorhexidine For the residual effect of antisepticss on EV71 enterovirus, the titers of each group met the specified requirements, and the neutralizer and its neutralization products had no effect on EV71 enterovirus and cell growth (Table 2).

There was no significant difference in the logarithmic value of virus titer (TCID50) in each test between the results of the residual disinfectant removal identification test and the structure of the neutralizer identification test (P>0.05).

Table 2 Neutralizer identification test results

Group	Log value of virus titer for each test ($TCID_{50}$)			Average log value of virus titer ($TCID_{50}$)
	1	2	3	
1	4. 33	4. 00	4. 33	4. 22
2	4. 76	4. 88	4. 56	4. 73
3	5. 67	5. 67	5. 50	5. 61
4	6. 23	6. 00	6. 00	6. 08
5	6. 00	6. 00	5. 00	6. 00
6	cells grow well			

2.3 Results of virus inactivation test

After 3 repeated tests, the compound hand/skin antiseptics containing chlorhexidine acted for 1 minute, and the average inactivation logarithm value to poliovirus type I (PV-I) vaccine strain and EV71 type enterovirus was 6.02 respectively. and 5.33 (Table 3, Table 4), which meet the requirements of the inactivation logarithmic value ⩾ 4.00 required by the "Disinfection Technical Specifications" (2002 edition).

Table 3 Killing efficiency of composite hand & skin antiseptics on poliovirus type I vaccine strains

The average inactivation log value of different time (min)			The logarithmic value of the mean viral titer (TCID50) of the positive control group
1	3	5	
5. 98	5. 86	6. 48	6. 11

Table 4 Killing efficiency of composite hand & skin antiseptics on EV71 enterovirus

The average inactivation log value of different time (min)		The logarithmic value of the mean viral titer (TCID50) of the positive control group
1	3	
5. 89	6. 00	5. 95

2.4 Stability test results

The experimental results show that the complete package of the compound hand/skin antiseptics containing chlorhexidine is stored at 37°C for 3 months, and the decline rate of the active ingredients is less than 10%, which is in line with the "Disinfection Technical Specifications" (2002 edition) Require.

Table 5 Stability test results

Test group	ethanol concentration%		Chlorhexidine concentration %	
	1	2	1	2
Before aging	82. 8	82. 5	0. 46	0. 45
After aging	82. 3	81. 6	0. 43	0. 42
Decrease rate	0. 6%	1. 09%	6. 5%	6. 7%

2.5 Toxicity test results

2.5.1 Results of acute oral toxicity test

The results of the acute oral toxicity test showed that no abnormal symptoms and signs were found in the mice after continuous observation for 14 days, the growth and development were not affected, and no death or other abnormal manifestations occurred. The acute oral toxicity LD50 in mice is more than 5000 mg/kg (body weight), which belongs to the practical non-toxic level.

2.5.2 Micronucleus test results of mouse bone marrow polychromatic erythrocytes

The results of mouse bone marrow polychromatic erythrocyte micronucleus test showed that, compared with the negative control group, each dose group had no significant difference in the animal bone marrow polychromatic erythrocyte micronucleus rate (P>0.05). Compared with the control group, there is a very significant difference (P< 0.01). The ratio of bone marrow PCE/NCE in each dosage group was more than 0.1, indicating that the test sample had no obvious inhibitory effect on bone, and the ratio of PCE/NCE in each dosage group of samples had no significant difference compared with the negative control group (P>0.05) (table 6). During the administration period, no obvious abnormalities were seen in animals in each dose group, and it was determined that the compound hand/skin antiseptics had no micronucleus-induced effect on mouse bone marrow polychromatic erythrocytes.

2.5.3 Multiple complete skin irritation test results

The results of multiple complete skin irritation tests showed that the local skin reaction was observed 24 hours after each administration, and within 14 days, no abnormalities were found in the skin of the 3 Japanese big-eared white rabbits on the poisoned side and the skin on the control side. 0, the stimulation intensity is non-irritating.

3 Discussion

Chlorhexidine has been used to disinfect hands, skin and mucous membranes as early as the 1950s. Because of its good bactericidal effect, stable performance, low toxicity to the human body, and convenient use, it has always occupied a certain position among antiseptics [6-8]. And because of its cationic properties, it can be adsorbed on the surface of objects and skin to maintain its bactericidal effect [9], and it has been widely used in surgical hand disinfection and preoperative skin disinfection at home and abroad. However, the traditional chlorhexidine or chlorhexidine/alcohol system can only have a good inactivation effect on common pathogenic bacteria, but it has a good inactivation effect on viruses, especially hydrophilic viruses, such as hand-foot-mouth virus, poliovirus, etc. bad.

The skin antisepticss and hand antisepticss currently on the market are not effective in inactivating viruses. It has been reported [10] that the synergist was compounded into triclosan ethanol anti-

Table 6 Micronucleus test results of mouse bone marrow polychromatic erythrocytes

Group	dose (mg/kg)	Number of animals	Number of PCEs	Number of PCEs with micronuclei	Percentage of PCEs with microkernels (‰,$\bar{x}\pm s$)	PCE/NEC ($\bar{x}\pm s$)
Test samples	500	10	10000	9	0. 90±0. 74	1. 855±0. 539
	2000	10	10000	8	0. 80±0. 92	2. 430±0. 461
	5000	10	10000	11	1. 10±0. 99	2. 638±0. 364
negative control group	0	10	10000	10	1. 00±0. 82	2. 293±0. 436
positive control group	40	10	10000	197	19. 70±3. 80**	1. 893±0. 990

Note: The micronucleus cell rate (‰) and PCE/NEC are calculated in mice, expressed as mean ± standard deviation. ** means P<0. 01 compared with the negative control group.

septics, and the compound system was studied against poliovirus. and EV71 virus inactivation effect, the results showed that the triclosan ethanol compound antiseptics stock solution acted for 1min, and the inactivation logarithms of poliovirus and EV71 virus in the suspension were all ⩾4.0. However, due to the toxicity and bioaccumulation of triclosan, as early as 2013, the FDA issued a final rule on OTC, requiring antibacterial washing products containing triclosan and triclocarban (including liquids, foams, handmade soap gels) gels, soaps and bath products) shall not enter the market. The European Chemicals Agency (ECHA) announced in 2015 that triclosan would be phased out in hygienic applications and replaced by more suitable alternatives. In 2019, the National Health Commission of China released the "List of Active Ingredients of Antibacterial (Antibacterial) Agents (Draft for Comment)", which removed triclosan and should not be used in antibacterial agents. Therefore, whether triclosan can be used in skin hand antisepticss is still controversial.

It is also reported [11] that the antiseptics solution containing 500 mg/L available chlorine acts on poliovirus for 20 minutes, and the logarithmic value of killing is >4.00. Because hypochlorous acid or sodium hypochlorite itself is irritating to the skin and lacks the persistence of sterilization, it is not suitable for disinfection of surgical hands and preoperative skin.

As representatives of non-enveloped viruses, poliovirus and EV71 hand-foot-mouth virus have a simple structure, consisting only of a protein capsid structure and RNA genetic nucleic acid genes within it. Viral proteins and host cell receptors are in a certain suitable environment Easy to combine to cause infection[12-13]. Under appropriate pH conditions, virions and cell surfaces can form a combination through electrostatic attraction, and changes in pH will cause changes in the charge of virions and cell surface groups, thereby affecting the adsorption of virions and cells. For example, rhinovirus mainly proliferates in the upper respiratory tract and is unstable when the pH is lower than 5-6. [14-15]

Since cell receptors and VAPs tend to be negatively charged, virus particles can only be adsorbed on the cell surface by electrostatic attraction in a cationic environment[16]. The working principle of a compound hand/skin disinfectant containing chlorhexidine in this study is to use a synergist to change the surface electrostatic attraction of the virus, and at the same time provide a cationic environment through chlorhexidine, thereby taking the lead in changing the protein capsid structure of the virus , so that the conventional disinfectant ethanol can effectively destroy the genetic genes inside the virus, thereby inactivating the virus. [17]

Therefore, in this study a compound hand/skin antiseptics containing chlorhexidine uses a synergist to first change the protein capsid structure of the virus, so that ethanol and chlorhexidine can effectively destroy the genetic genes inside the virus, thereby inactivating The role of the virus. It can achieve rapid inactivation effect on poliovirus type I (PV-I) and EV71 enterovirus, and the average inactivation logarithms of poliovirus and EV71 enterovirus in 1 minute are both > 4.00, and have Certain bactericidal persistence. The stability test proves that the system has good stability. Toxicological tests have proved that the antiseptics is an actual non-toxic substance, has no irritation to the skin, and has no micronucleus effect on polychromatic erythrocytes in mouse bone marrow.

4 Conclusion

The ethanol/chlorhexidine/synergist composite hand/skin antiseptics prepared by formulation technology can effectively kill viruses represented by poliovirus and EV71 type enterovirus, and has certain bactericidal persistence, And the composite formulation is stable and safe to use.

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