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Original Article

Evaluation of the Anti-Adenoviral Activity of ALTANT, an Ozonated Alcohol Disinfectant

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SUMMARY: Seven human mastadenovirus (HAdV) species (A–G) are known with more than 100 reported types. HAdV is highly resistant to common hand sanitizers. Epidemic keratoconjunctivitis and pharyngoconjunctival fever are caused by HAdV, which can be explosively transmitted in a confined space, resulting in outbreaks, such as nosocomial infections. Given the absence of an antiviral agent against the HAdV infection, it is important to prevent the spread of the infection by using disinfectants. Ozone has already been well-known for its bactericidal and virucidal effects. ALTANT is an ozonated alcohol preparation developed by E-TECH Co., Ltd. (Kobe, Hyogo, Japan). In this study, we mixed ALTANT with different HAdV types at a ratio of 9:1 and determined HAdV viability after instantaneous reactions for varying periods (flash to 5 minutes) using the TCID50 assay. The assay results demonstrated that the HAdV viability decreased by 1/10 to 1/100 within 1 minute after the reaction; additionally, slight differences in the reactivity were observed among the HAdV types. HAdV viability decreased by a factor of > $4\log_{10}$, and the virus was eliminated within 3 minutes. This study demonstrated the potent HAdV disinfection effect of ALTANT.

INTRODUCTION

Human mastadenovirus (HAdV) is a non-enveloped DNA virus and is classified into seven species, i.e., A–G. Currently, more than 100 different HAdV types have been described (1). HAdV is reportedly highly resistant to disinfectants (2,3), particularly to alcoholic preparations (4,5). Chlorine is an effective HAdV disinfectant (2). Pharyngoconjunctival fever (PCF), also known as swimming pool fever, and epidemic keratoconjunctivitis (EKC) are typical acute and highly infectious adenoviral diseases. Relevant laws prescribe that swimming pool water must be disinfected with free residual chlorine at a concentration between 0.4 mg/L and 1.0 mg/L. Sodium hypochlorite is a recommended chlorine-based disinfectant. However, it is difficult to use it for the disinfection of hands, eyes, and metallic instruments due to the mucosal irritation and metal corrosion effect of chlorine gas.

Hemorrhagic cystitis-associated HAdV-B11 is a major concern in hematopoietic stem cell transplantation as it causes secondary infection due to the infection of the virus in the environment (6–9). Post-transplantation infection, caused by adenovirus species B or C, is a global challenge (10–16). Adenoviruses associated with these infections are known to be explosively transmitted in confined spaces, such as hospitals. Patients with HAdV have been reported to discharge infectious viruses in their saliva and urine for an extended period (17). Adenoviruses are highly infectious, and HAdV can propagate even when only one virion is present in a culture medium (17,18). Considering that no anti-HAdV drugs are currently in clinical use, disinfectants are important for preventing an HAdV outbreak (1,10) and it is important to evaluate the virucidal effects of disinfectants on HAdVs.

Ozone has already been well-known for its bactericidal and virucidal effects (19). Disinfection with ozonated water solutions has been used for water sterilization in water supply and sewerage systems (19). The efficiency of ozone was demonstrated against enteric adenovirus types 40 and 41 in sewage treatment (20,21). Ozone is relatively stable in acidic solutions, but it rapidly decomposes at high temperature or pH. Under such decomposing conditions, ozone is hydrolyzed to generate hydroxyl radical (•OH), hydroperoxyl radical (HO_2), or hydrogen peroxide (H_2O_2). In particular, hydroxy radicals have a higher oxidizing capacity than ozone molecules, and they contribute greatly to the

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inactivation of bacteria and viruses (19). Apart from its high antibacterial and antiviral effects, the advantages of using ozone include no generation of organochlorine compounds, such as trihalomethane; no persistence; easy iron and manganese removal; and deodorization effect. Its disadvantages include transient efficacy due to the lack of persistence, high cytotoxicity even at relatively low concentrations, and high corrosiveness, particularly against rubber and plastics.

ALTANT is an ozonated alcohol-based disinfectant, in which ozone is stably retained in alcohol (developed by E-TECH Co., Ltd., Kobe, Hyogo, Japan). ALTANT is just as effective as other disinfectants, such as peracetic acid, glutaral, and phytoral. Its safety has been evaluated in various tests, including acute oral toxicity, eye irritancy, skin irritancy, mutagenicity, and oral mucosal irritation tests (Japan Food Research Laboratories; No.17094693001-0101). ALTANT has been patented in several countries, including Japan, the United States, the EU, South Korea, and Taiwan. It reportedly (Kitakanhatu 2017_0026 and Hokuseihatu 2017_0161) exhibits antiseptic effects against *Bacillus subtilis* (ATCC 6633) and the norovirus surrogate *Feline calicivirus* (strain F-9; ATCC VR-782). ALTANT thus expectedly exhibits a broad antiviral effect.

Several disinfectant efficiency tests have been performed against adenoviruses. However, only a few types, including species B type 3 and species C type 5, have been used in these tests (22,23). Recently, recombinant adenoviruses have been prevalent worldwide (1). However, many recombinant adenoviruses have not yet been fully characterized, and their susceptibility to disinfectants remains unknown. Although there are several types of HAdVs worldwide, the prevalent ones differ among countries and regions (Infectious Agents Surveillance Report (IASR): https://www.niid.go.jp/niid/en/865-iasr/7390-449te.html). Moreover, HAdVs of different serotypes reportedly show different responses to a disinfectant (2,24). Disinfectants should be evaluated for their effects on specific HAdV types prevalent in Japan.

The present study evaluated the anti-adenoviral effects of ALTANT against 14 HAdVs types that cause respiratory system, ophthalmic, genito-urinary tract, and systemic infections.

MATERIALS ND METHODS

Virus preparation: A549 cells (CCL-185, American Type Culture Collection (ATCC)) were used as host cells for the adenovirus culture. A549 cells were grown with Eagle's Minimum Essential Medium with Earle's salts (Eagle's MEM, Sigma-Aldrich Japan, Tokyo, Japan) comprising of 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), 1% L-alanine/L-glutamine (200 mmol/L) (Wako Pure Chemical Industries (Wako, Osaka, Japan), 0.2% gentamicin sulfate solution (50 mg/mL) (Wako), and 0.1% amphotericin B (Wako).

HAdVs were introduced into the A549 cells with the same medium (except with 5% FBS instead of 10% FBS) and incubated until the cytopathic effects (CPE) could be visually observed using a WRAYCAM (WRAYMER Inc., Osaka, Japan) camera using on an inverted microscope CKX53 (Olympus Inc., Tokyo, Japan). Reference adenovirus types were either purchased from ATCC or reference strains as reported previously (25). Information regarding the endemic HAdV types in Japan was obtained from IASR. In the present study, HAdV types were selected based on the IASR data. A total of 14 adenovirus types were used as follows: HAdV-1, HAdV-2, HAdV-3, HAdV-4, HAdV-5, and HAdV-6 responsible for PCF; HAdV-7 responsible for ARDS; HAdV-11 responsible for HC; HAdV-37, HAdV-53, HAdV-54, HAdV-56, HAdV-64, and HAdV-85 responsible for EKC. HAdV-85 has been recently reported as a causative agent of conjunctivitis in Japan (25). For preparing the virus stock solution, intact adenovirus was roughly purified from the cell culture, and complete CPE was observed as follows: the virus culture supernatant was centrifuged at 1,500 × g for 2 minutes, the supernatant was collected, dispended into several tubes, and preserved at -80°C until further evaluation.

Virus quantification and TCID50 assay: The virus quantification was performed using real-time polymerase chain reaction (PCR) as described previously (26). HAdV titration, which was adjusted to 1×10^5 copies, was performed using microtiter plates by two-fold serial dilutions. The diluted virus sample was inoculated into each of the 96 wells comprising confluent A549 cells at 34°C with 5% CO_2, and the CPE appearance was observed daily for 20 days. The Spearman–Karber method was used to calculate the median tissue culture infective dose (TCID50)/mL (27,28).

The effects of ALTANT on the A549 cell line: ALTANT was provided by E-TECH Co., Ltd. To determine the effects of ALTANT on A549 cells, an ALTANT dilution series was established using the A549 cell growth medium. ALTANT was mixed with the medium at varying ratios as follows: 1 : 2, 1 : 5, 1 : 10, 1 : 50, 1 : 100, 1 : 1000, and 1 : 5000, respectively. The diluted solution was applied to confluent A549 cells in a 96-well plate and was incubated at 34°C for 7 days. Cell growth was recorded every day, and cell passage was performed on day 7 to evaluate the effects on cell growth. Floating A549 cells treated with trypsin were also evaluated using the same method. The effects of isopropyl alcohol, which is the base material of ALTANT, were also evaluated as described above.

ALTANT neutralization: In order to neutralize ALTANT, mixtures of ALTANT and the cell growth medium with 0.1%, 0.5%, and 1% sodium thiosulphate were investigated. Each solution was applied to confluent A549 cells and incubated at 34°C for 7 days. Cell growth was analyzed as described above.

Evaluation of the anti-adenoviral activity of ALTANT: Based on the European Committee for Standardization (CEN: https://www.cen.eu/Pages/default.aspx) EN 14476 (Chemical disinfectants and antiseptics) and previously described methods (24,29), a modified assay was performed as follows: the evaluation assay was performed using the "endpoint" method. A549 cells were propagated in 96-well cell culture plates to quantify the amount of virus. To investigate whether ALTANT has a HAdV stabilizing or disinfection effect, the HAdV mixture of was neutralized with ALTANT, diluted to 1^{-0}–10^{-20} by two-

Table 1. Adenovirus reduction efficacy of ALTANT[1)]

HAdV type	HAdV titer[2)]	(< 3")		10"		30"		1'		3'		5'	
	Log_{10} TCID50 / ml	Log_{10}	%	Log_{10}	%	Log_{10}	%	Log_{10}	%	Log_{10}	%	Log_{10}	%
1	4.67	1.20	93.75	1.8	98.44	2.71	99.80	3.61	99.98	<4.67	<99.99	<4.67	<99.99
2	4.67	1.81	98.44	1.8	98.44	2.71	99.80	3.31	99.95	<4.67	<99.99	<4.67	<99.99
3	4.06	0.90	87.50	1.2	93.75	2.11	99.22	3.61	99.98	<4.06	<99.99	<4.06	<99.99
4	4.06	2.11	99.22	2.4	99.61	2.71	99.80	<4.06	<99.99	<4.06	<99.99	<4.06	<99.99
5	4.36	1.20	93.75	1.5	96.88	1.81	98.44	3.61	99.98	<4.36	<99.99	<4.36	<99.99
6	4.67	0.90	87.50	1.8	98.44	2.71	99.80	4.21	99.99	<4.67	<99.99	<4.67	<99.99
7	4.06	1.51	96.88	2.4	99.61	3.01	99.90	<4.06	<99.99	<4.06	<99.99	<4.06	<99.99
11	4.36	2.11	99.22	2.4	99.61	2.71	99.80	3.61	99.98	<4.36	<99.99	<4.36	<99.99
37	4.67	0.90	87.50	1.2	93.75	3.61	99.98	<4.67	<99.99	<4.67	<99.99	<4.67	<99.99
53	4.67	0.30	50.00	0.3	50.00	3.01	99.90	<4.67	<99.99	<4.67	<99.99	<4.67	<99.99
54	4.06	0.60	75.00	1.2	93.75	2.71	99.80	<4.06	<99.99	<4.06	<99.99	<4.06	<99.99
56	4.97	0.90	87.50	1.2	93.75	3.31	99.95	<4.97	<99.99	<4.97	<99.99	<4.97	<99.99
64	4.67	0.30	50.00	0.6	75.00	4.21	99.99	<4.67	<99.99	<4.67	<99.99	<4.67	<99.99
85	4.67	0.90	87.50	0.9	87.50	2.41	99.61	<4.67	<99.99	<4.67	<99.99	<4.67	<99.99

[1)]: Reaction times are indicated as follows: 3" means 3 seconds, and 1' means 1minute.
[2)]: 1×10^5 copy of HAdV was inoculated and incubated until determined titer at day 14.
Virus titer was calculated with CPE observation on day 17 because of the slow growth phenotype of HAdV54.

fold serial dilution, inoculated into A549 cell-containing 96-well plates, then incubated at 34°C for 21 days. The time (day) and rate of CPE appearance were determined (18,24,29). One row, including 8 wells of the 96 well plate, was used for one dilution. The detection of CPE-positive rows was determined when CPE was observed in ≥ 5/8 wells. To calculate the TCID50 of the CPE observation endpoint, all CPE-positive line was regarded as 8/8 among all the tests. This assay was performed as follows: 900 µL of ALTANT and 100 µL of the virus stock solution (1×10^5 copies) was mixed by vortexing for 2 seconds; then this mixture was kept standing at room temperature for the following periods: flash (within 3 seconds), 10 seconds, 30 seconds, 1 minute, 3 minutes, and 5 minutes; 10 µL of the reaction solution was neutralized by 990 µL of 0.5% sodium thiosulphate. Subsequently, 100 µL of the neutralized mixture was diluted to 2^{-1}–2^{-20} by two-fold serial dilution with 100 µL fresh medium. Then 100 µL of the diluted solution was added to confluent A549 cells filled with 100 µL fresh medium in each well in the 96-well plate; this plate was then incubated at 34°C for 21 days, and CPE was observed regularly. All the assays were performed twice independently. Eagle's MEM medium was used instead of ALTANT for untreated control.

RESULTS

Cytotoxicity and neutralization of ALTANT: ALTANT did not affect cell growth at a 1/1000 dilution in the MEM cell growth medium but inhibited cell growth at a 1/500 dilution. In the ALTANT neutralization test using sodium thiosulfate, the ALTANT cell toxicity on the A549 cell growth was abolished when mixed at a ratio of 1 : 100 with the MEM cell growth medium, comprising 0.5% or 1% sodium thiosulfate. These results indicate that the MEM cell growth medium comprising 0.5% or 1% sodium thiosulfate has an activity at 10-fold lower dilution compared to the case when only MEM cell culture medium is used.

HAdV infectious titer determination: HAdV infectious titers (TCID50) were determined using a fixed virus copy number of 1×10^5 for inoculation. After approximately 14 days of culture, the cultures were inspected to observe the CPE around the dilution limit (at line 16) to determine TCID50. The results are presented in Table 1. For HAdV-54, the CPE observed on day 17 of culture was used for calculation as this type showed a significant growth delay. Although there was some variability among the strains, the infectious titers were within a range of $4.06\log_{10}$–$4.67\log_{10}$ TCID50/mL (Table 1).

ALTANT antiseptic activity against several HAdV types: The results did not differ considerably between the two independent assays. The antiseptic activities were determined based on EN-14476 of CEN, and the results are summarized in Table. On day 14, after HAdV inoculation, the complete virucidal effect was observed for all adenovirus types when they were allowed to react with ALTANT for 3 minutes. Even in 1 minute, ALTANT satisfied the EN-14476 criterion for the antiviral effect (> $4\log_{10}$) for all HAdV types tested in the present study. For PCF-related HAdV types, i.e., HAdV-1, HAdV-2, HAdV-3, HAdV-4, HAdV-5, and HAdV-6), ALTANT showed 80.5% – 99.22% virucidal effects even in a short reaction time (flash; within 3 seconds from ALTANT-HAdV reaction to neutralization). HAdV-7, associated with severe respiratory disease, showed high susceptibility to the ALTANT treatment. HAdV-11 associated with HC also showed a high susceptibility even in short reaction time. Among EKC-associated HAdV types, HAdV-37, HAdV-53, HAdV-54, HAdV-56, HAdV-64, and HAdV-85 were less susceptible than PCF in flash, with

only 50% and 75% virucidal effects being observed for HAdV-53 and HAdV-64, respectively, even after 10 seconds of reaction. However, ALTANT showed approximately 90% virucidal effects on HAdV-37, HAdV-54, HAdV-56, and HAdV-85, which increased to ≥ 99% in 30 seconds for all the types tested.

DISCUSSION

The drug susceptibilities for different types of HAdV varies according to the type of HAdV (22,24) and should be tested using clinically important types of HAdV. HAdV is highly resistant to common hand sanitizers, such as ethanol (2,3,22–24). Ozone is a strong oxidizer and exhibits a very high potency and disinfecting efficacy. While the stable use of ozone has been difficult, ALTANT is a formulation wherein ozone is stabilized in an alcoholic solution for more than 3 years (http://www.e-teck.co.jp/). ALTANT is cytotoxic to cultured cells, but we demonstrated that its cytotoxicity could be neutralized by 0.5% sodium thiosulfate. In the evaluation of disinfectants against bacteria according to the standardized reference EN 1276 of CEN, common neutralizers are shown; however, in the case of viruses, many of these culture medium components affect the growth of host cells and could not be used (data not shown). Therefore, sodium thiosulfate alone was used to test the neutralizing effect, as described in the previous reports (24).

After the drug was allowed to react with the virus for varying lengths of time, the reaction mixtures were subjected to 2-fold serial dilutions, and the neutralizing effect was determined by measuring the virus decrease rates using the CPE occurrence as an indicator after culturing for 7–21 days. The assay used in this study allowed us to determine the endpoint of the complete HAdV-disinfecting effect by comparing the result with that of the control group. Generally, TCID50, PFU, and virus copy numbers are used to indicate infectious titers of viruses. When a single type of virus is used in the assay, one method is usually enough to determine the infectious titer depending on a condition. When multiple types of viruses are used in the assay, it is difficult to set a common infectious titer because their growth conditions and capacities vary. Different HAdV types grow at varying rates (30) and differ in the drug susceptibility (2,23,24), and thus it is difficult to determine infectious titers of such viruses. Accordingly, the observation was performed up to day 21, and the infectious titer of each HAdV type was determined during this period. For the evaluation of ALTANT, the percentage of viral CPE decrease for each HAdV type was calculated in reference to the control group (no drug reaction). Moreover, as the reacted virus solution in this method is subjected to limiting dilution, and CPE caused by the diluted virus is observed, the virus decrease rate at the highest dilution indicates the virucidal effect. The sterilizing antiviral effect was determined using the longest possible length of the observation period. In CPE assays for adenoviruses in 96-well plates, the CPE in one well equals 1 plaque forming unit (PFU), particularly for HAdV-5. Mathematically, lanes 16–17 correspond to 1 virus copy. For virtually all strains, lanes 14–16 were limits of CPE detection. In this study, the virus nucleic acid copy number and infectious titer were approximately in agreement for some trains or differed by about 10-fold for some other strains. This finding is consistent with previous reports (30). The isopropanol solution, which is the base of ALTANT, showed no anti-adenovirus effect (data not shown). ALTANT showed efficacy levels below $4\log_{10}$, which is regarded as the efficiency limit by EN-14476. 70% ethanol or isopropanol with/without 0.5% chlorhexidine-digluconate showed no disinfection efficacy for HAdV with reduction levels below $4\log_{10}$ (24).

In general, disinfectants are known to show large activity changes, decreases in most cases, in actual settings where viruses or bacteria of interest present, because of influences from many coexisting substances, such as proteins and salts (22). In this study, the drug effect was evaluated in the presence of many serum components which reduce the effectiveness of disinfectants (a condition closer to the actual environment). However, the effects in actual foods and environments were not evaluated and remain to be tested in the future. ALTANT is not approval usage to the mucous membrane, however, there is no corrosion or discoloration of the surface of human skin or environmental substances as with alcohol sterilization preparation (http://www.e-teck.co.jp/). ALTANT is used by direct-spray or indirectly using a soft cloth, such as cotton gauze, for the surface of hands and environmental disinfection(http://www.e-teck.co.jp/?page_id=171).

In this study, among PCF-related HAdV types, HAdV-3 was found to be slightly less susceptible to ALTANT. EKC-associated strains showed characteristic responses for low sensitivity within 3–10 seconds, presumably because of a unique mechanism that they use to enter host cells (5,31). Since all HAdV types tested were highly susceptible to ALTANT, the sufficient anti-HAdV effect can be expected in simple hand sanitization and environmental disinfection using ALTANT.

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