Disinfectant potential in inactivation of epidemic keratoconjunctivitis (EKC)-related adenoviruses by potassium peroxymonosulfate

Mei Hashizume[1,2], Koki Aoki[1,3,4], Shigeaki Ohno[3,4], Nobuyoshi Kitaichi[3,4], Nobuyo Yawata[5], Gabriel Gonzalez[6], Hirotaka Nonaka[1,2], Seiichi Sato[1,2] and Akinori Takaoka[1,2]

[1] Division of Signalling in Cancer and Immunology, Institute for Genetic Medicine, Hokkaido University, Sapporo, Hokkaido, Japan

[2] Molecular Medical Biochemistry Unit, Biological Chemistry and Engineering Course, Graduate School of Chemical Sciences and Engineering, Hokkaido University, Sapporo, Hokkaido, Japan

[3] Department of Ophthalmology, Health Sciences University of Hokkaido, Sapporo, Hokkaido, Japan

[4] Department of Ophthalmology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Hokkaido, Japan

[5] Department of Ocular Pathology and Imaging Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

[6] Division of Bioinformatics, Research Center for Zoonosis Control, Hokkaido University, Sapporo, Hokkaido, Japan

Corresponding author:

Akinori Takaoka

takaoka@igm.hokudai.ac.jp

Tel; +81 11-706-5020

Fax; +81 11-706-7541

Short title: Inactivation of EKC-related HAdV

ABSTRACT

Purpose: To be tested the antiviral effectivity of potassium peroxymonosulfate (RUBYSTA®, KYORIN) against five EKC-related types of *Human adenovirus D* (HAdV-D) *in vitro*.

Methods: Five types of HAdV-D (8, 37, 53, 54 and 56) were incubated with 1% potassium peroxymonosulfate, 0.1% sodium hypochlorite (NaClO) or alcohol-based disinfectant for 30 sec or 1 min. These solutions were subjected to measurements of viral titers by infection assays in A549 cells. At day 6 post-infection, both, supernatants and cells, were collected and the viral genome was assessed by real-time PCR analysis.

Results: Treatments with 1% potassium peroxymonosulfate led to significant reduction of all tested HAdV-D types comparable to disinfecting effects by 0.1% NaClO. Overall, potassium peroxymonosulfate demonstrated sufficient inactivation of the major EKC-causing HAdV-D to be considered for disinfection and prevention purposes in ophthalmological clinics and hospitals.

Conclusions: This study demonstrated that potassium peroxymonosulfate is a promising disinfectant for the prevention of EKC nosocomial infections in ophthalmological clinics.

Keywords: HAdV, EKC, disinfectants, prevention, RUBYSTA, Virkon

Introduction

Human adenoviruses (HAdVs) are currently classified into over 85 accepted human adenovirus types which can be divided into seven species (A to G). These types are classified by phylogenetic analysis of three genes encoding capsid proteins, which have large difference among species or types. HAdV infections are associated with respiratory diseases, conjunctivitis, obesity, gastroenteritis and even cancer.[1] HAdVs are highly contagious and resistant to multiple disinfectants due to physical properties of their capsid proteins.[1,2] Since HAdVs lack envelopes like other viruses such as norovirus, they are highly resistant to lipid disinfectants including alcohol and soap.[3,4] The resistance of adenoviral capsid proteins varies according to environmental conditions such as temperature and relative humidity; furthermore, virions have been shown to survive for many days on plastic or metal surfaces and eyedrop bottles.[5-7] These HAdV features are closely related to the spread of infections such as nosocomial outbreaks and outbreaks in schools, military bases, and swimming pools.[1,8,9]

HAdVs cause multiple clinical diseases, including pneumonia, conjunctivitis, gastroenteritis, hepatitis, and myocarditis. Among such HAdV-associated diseases, epidemic keratoconjunctivitis (EKC) is an ocular infection, which is caused mainly by HAdV-D types and commonly transmitted from hands to eyes. In particular, infections by HAdV-D8, -D19a, -D37, -D53, -D54 and -D56 are more frequently reported with the etiology of EKC, which can develop into severe cases with impaired vision.[2,10] A number of adenoviral EKC outbreaks are seen in ophthalmological clinics and hospitals worldwide.[11-13] In this regard, disinfection against viable HAdVs is critical for the prevention of such outbreaks. The most frequently used disinfectant solutions against HAdVs in clinic are ethanol (80%), popidone iodine (0.2%), and sodium hypochlorite (NaClO; 0.1%).[14] Although NaClO has been reported as the most effective disinfectant against HAdV-D,[14] it also presents problems such as metal corrosion and irritating smell, which hinder its usage in healthcare facilities.[15] Recently a disinfectant including potassium peroxymonosulfate “Multi-purpose disinfectant cleaner RUBYSTA®” (KYORIN Medical Supply, Tokyo, Japan) , which is also known as Virkon® in other countries, has been officially approved for various pathogenic agents including non-enveloped virus such as norovirus:[16] it is odourless and less metal corrosive

than NaClO because of the potassium peroxymonosulfate monohydrogen as oxidizing agent.[17] In the United States, it is registered as antimicrobial products for cleaning and disinfecting hard surfaces in hospital by the US Environmental Protection Agency (EPA). Moreover, a report showed that potassium peroxymonosulfate can inactivate HAdV-C5 and -C6,[17] however, it remains unclear whether potassium peroxymonosulfate also shows effectiveness against EKC-related HAdVs. In this study, to assess the potential of potassium peroxymonosulfate as a disinfectant against EKC-related HAdV-D strains, we compared the effects of potassium peroxymonosulfate and NaClO on the viability of these HAdVs, by measuring the viral titers after treatments with them.

Methods

Reagents

Potassium peroxymonosulfate solution (RUBYSTA®) was obtained from KYORIN Medical Supply (Tokyo, Japan). Sodium hypochlorite (NaClO) and sodium thiosulfate were obtained from Wako (Osaka, Japan). Antiseptic Solution WELPAS® for Hand 0.2% (alcoholic chlorbenzarconium) was purchased from Maruishi pharmaceutical (Osaka, Japan).

Cell culture

A549 cells were purchased from ATCC and cultured in DMEM medium supplemented with 4 mM L-glutamine and 10% heat-inactivated fetal bovine serum (FBS) (Gibco). Also, DMEM medium containing 5% FBS was used for culture of infected A549 cells.

Virus

HAdV-B3 and HAdV-D8 were provided from the Department of Medicine, Ophthalmology, Fukuoka Dental College. HAdV-D37, -D53 and -D56 were obtained from Kumamoto Institute of Public-Health and Environmental. HAdV-D54 was provided from the Department of Ophthalmology, Hokkaido University. For virus propagation, A549 cells were infected with each virus solution and incubated. After observation of cytopathic effects (CPE) in the infected culture, cells and culture media were collected followed by 3 times of freeze and thaw cycles. The supernatant including virus was collected by centrifuge at 3,000 rpm for 10 min. All viral titers were determined by $TCID_{50}$.

Examination of HAdV replication

A549 cells cultured in 24 well-plate were infected with HAdV at different concentrations (multiplicity of infection, MOI = 0.1, 1, 10). Then, infected cells were collected at 1, 3, 6 and 9 days after infection and the copy number was determined using real-time PCR.

Effects of disinfectants against HAdV were assessed using real-time polymerase chain reaction

A 0.02%, 0.2%, and 2% (w/v) potassium peroxymonosulfate solution and 0.002%, 0.02%, and 0.2% (w/v) NaClO solution were prepared. HAdV solutions were incubated with 0.01%, 0.1%, and 1% (w/v) potassium peroxymonosulfate solution and 0.001%, 0.01%, and 0.1% (w/v) NaClO solution at final concentration (the effective chlorine concentration is 10 ppm, 100 ppm and 1,000 ppm, respectively) for 30 sec or 1 min. After incubation, 0.1% (w/v) sodium thiosulfate at final concentration was added. The alcoholic chlorbenzarconium also reacted with HAdV for 1 min, then diluted 260 times with DMEM. Next, the HAdV solutions after treatment by three different disinfectants were used to infect A549 cells. After 6 days, viral DNAs were isolated from culture cells and their medium. Real-time PCR was performed using SYBR Premix Ex Taq (TOYOBO) and analysed on a StepOnePlus real-time PCR system (Applied Biosystems). Primers for amplification of HAdV DNA by real-time PCR were as follows. Forward primer: 5'-TTCCCCATGGC(A/T/C/G)CACAA(C/T)AC-3', reverse primer: 5'-TGCC(T/G)(A/G)CTCAT(A/G)GGCTG(A/G)AAGTT-3' (product length: 554 bp, product Tm: 88.0 °C). The real-time PCR condition included a first step at 98 ℃, followed by 45 cycles of 10 sec at 98 ℃, 10 sec at 50 ℃ and 45 sec at 68 ℃. Copy numbers were calculated using a standard curve method.

Cell growth assay

A549 cells seeded in 96 well-plate were treated with potassium peroxymonosulfate at the concentration with 1%, 0.1% and 0.01%. After 24 h we used Cell Counting Kit-8 (DOJINDO, Japan) and measured absorbance following the manufacturer's protocol.

Statistical analysis

Values are shown as mean ± standard deviation. Statistical significance between two samples was determined with Student's *t*-test.

Results

To determine an appropriate timepoint and MOI of HAdVs for measurement of viral titers, we first conducted time-course analyses of viral propagation in A549 cells. This cell line is known to provide an advantage of viral growth since it lacks expression of cGAS and STING, which are reported to be innate sensors for HAdVs.[18] As shown in Figure 1, HAdV-B3, HAdV-D8, HAdV-D37, HAdV-D53, HAdV-D54, and HAdV-D56, which are known to cause EKC, were successfully replicated in A549 cells in a dose-dependent manner. The levels of DNA copy number became saturated at day 6 after infection with any of the tested HAdVs. Therefore, we decided to assess viral titers at day 6 after infection with each type of HAdV-D.

To evaluate the effects of potassium peroxymonosulfate on the viability of EKC-related HAdV-D types, we tested five HAdV-D types: 8, 37, 53, 54, and 56, together with HAdV-B3 as a control, which does not directly cause EKC. We also analysed the effects of NaClO and alcoholic chlorbenzarconium for comparison. First we checked cytotoxic effect of potassium peroxymonosulfate, and confirmed that it has no effect on A549 cell growth in our experimental condition (data not shown). Thus we used 1% potassium peroxymonosulfate which is recommended concentration for using for clinical setting and compare with NaClO. As shown in Figure 2, the viral replication of all the types of HAdV-D tested were significantly suppressed by more than 4, which was comparable or more effective than NaClO in HAdV-D8, -D37 and -D54. Next, we examined the antiviral effect of several concentration disinfectants. Surprisingly, the 100 ppm (0.1%w/v) potassium peroxymonosulfate still had sufficient antiviral effect (Figure 3). In contrast low concentration of NaClO couldn't reduce infectivity of HAdV-D as previously reported.[14] These data indicated that potassium peroxymonosulfate sufficiently reduced the infectivity of EKC-related HAdV types.

Additionally, we also checked whether alcoholic chlorbenzarconium, which is commonly used for hand wash in hospital, could inactivate EKC-caused HAdV-D. For 1 min reaction, treatment with alcoholic chlorbenzarconium, did not affect the viabilities of all tested types of HAdV (Figure 2b).

Discussion

EKC is typically caused by HAdV-D8, -D19a, -D37, and in recent years new types of EKC-related HAdV, HAdV-D53, -D54 and -D56, have emerged widely in Japan.[2,19] Since these types are highly infectious and often spread in hospitals, control of such nosocomial infections is recognized as an important issue. This study demonstrated that potassium peroxymonosulfate is a promising disinfectant for the prevention of EKC nosocomial infections in ophthalmological clinics. We also showed that comparable to 0.1% NaClO, 1% potassium peroxymonosulfate sufficiently reduced the yield of infectious virions of all tested HAdV types, supporting the usefulness of potassium peroxymonosulfate against EKC-related HAdV-D types.

0.1% Hypochlorous acid NaClO is widely known as the most effective in mid-level disinfectants[14] despite drawbacks such as corrosive properties that limit its use with medical devices and the irritating smell that is inappropriate to use in clinical settings.[15] In contrast, potassium peroxymonosulfate produce few effects on metals, especially non-reactive to stainless steel.[20] Furthermore, potassium peroxymonosulfate is odourless, easy to prepare and store. Our current study indicated that the use of potassium peroxymonosulfate is as effective as NaClO against HAdV-D types, without the main drawbacks of the former. These results support the use of potassium peroxymonosulfate for disinfection of medical devices such as tonometer, slit lamp microscope and eyedrop bottle in healthcare facilities, in particular, at eye clinics.

It is noteworthy that potassium peroxymonosulfate has been shown to have antimicrobial activity against different types of pathogenic agents other than adenovirus, including hepatitis B and C viruses, poliovirus, enterovirus, feline calicivirus, several strains of different bacteria, spores, and fungi.[16,21-25] In the case of adenovirus, it was reported that potassium peroxymonosulfate inactivated HAdV-C5 and -C6,[17] which are known as respiratory pathogens. The chlorine-based disinfectants seem to disrupt capsid proteins of HAdV. Among various types of HAdV, there is a large difference in their amino acid composition especially in capsid proteins. Recent long term surveillance suggested that not only different type but also a genetic variation is associated with the epidemics of adenoviral keratoconjunctivitis.[26] The information was limited regarding the availability of potassium

peroxymonosulfate as a disinfectant against EKD-related types of HAdV. Our results extended such results to effective inactivation of HAdV-B3, -D8, -D37, -D53, -D54 and -D56.

We demonstrated that 1% potassium peroxymonosulfate was more effective than 0.1% NaClO against HAdV-D54. HAdV-D54 was firstly reported and isolated from a nosocomial outbreak in a Japanese hospital in 2000.[27] HAdV-D54 has been often isolated since 2011, according to surveillance of EKC causative agents in Japan, and has unique genome sequences encoded capsid protein,[2] however, the detailed virological features of HAdV-D54 remain poorly understood. *Kaneko et al.* reported that HAdV-D54 is characterized by cytopathic effects (CPE) harder to observe than other EKC-related types.[28] In relation to this report, our data showed that the replication rate of HAdV-D54 in A549 cells is the lowest among all HAdVs we tested (Figure 1e). In this regard, HAdV-D54 or its interaction with host cells may have some factors behind the slow replication rate and CPE due to its gene polymorphism or interaction with host factors. Despite these conditions, this type is an important infectious EKC agent in Japan with severe cases leading to corneal complications.[2,28]

In conclusion, we showed that 1% potassium peroxymonosulfate has a strong disinfectant activity against EKC-related types of HAdV. Considering that negligible odor and corrosive effect, this study suggests that potassium peroxymonosulfate is a superior disinfectant in as a prevention measure against nosocomial infections and accidental spreading of infectious agents.

Acknowledgements

We thank R. Hinokuma and S. Hashimoto for HAdV, and H. Watanabe for helpful advice. This work was supported by financial support from KYORIN Medical Supply Co., Ltd (Tokyo, Japan).

Declaration of conflicting interests

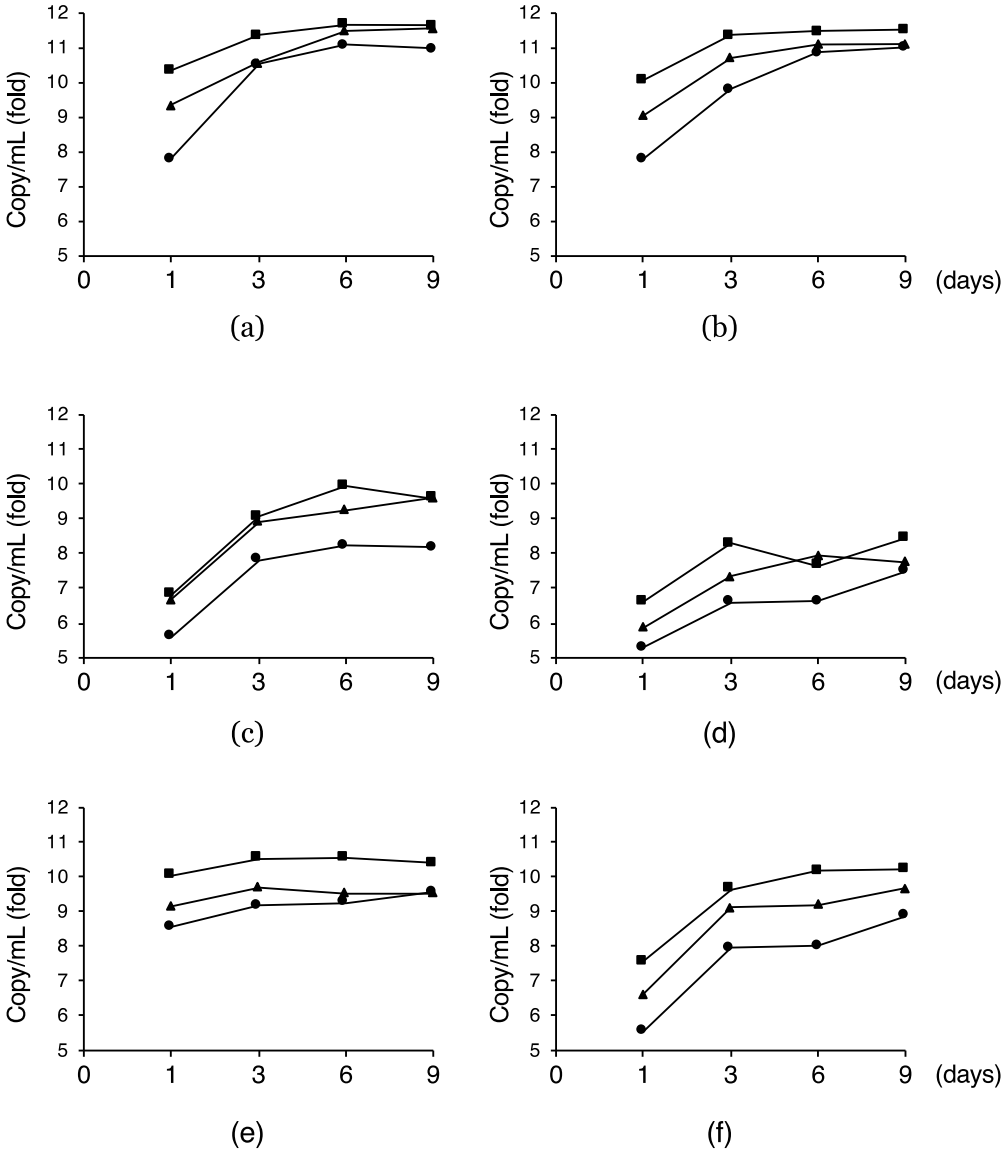
None.

References

1. Hall K, Blair Zajdel ME, Blair GE. Unity and diversity in the human adenoviruses: exploiting alternative entry pathways for gene therapy. *Biochem J* 2010; 431: 321–336.
2. Gonzalez G, Yawata N, Aoki K, et al. Challenges in management of epidemic keratoconjunctivitis with emerging recombinant human adenoviruses. *J Clin Virol* 2019; 112: 1–9.
3. Wutzler P, Sauerbrei A. Virucidal efficacy of a combination of 0.2% peracetic acid and 80% (v/v) ethanol (PAA-ethanol) as a potential hand disinfectant. *J Hosp Infect* 2000; 46: 304–308.
4. Uzuner H, Karadenizli A, Er DK, et al. Investigation of the efficacy of alcohol-based solutions on adenovirus serotypes 8, 19 and 37, common causes of epidemic keratoconjunctivitis, after an adenovirus outbreak in hospital. *J Hosp Infect* 2018; 100: e30–e36.
5. Nauheim RC, Romanowski EG, Araullo-Cruz T, et al. Prolonged recoverability of desiccated adenovirus type 19 from various surfaces. *Ophthalmology* 1990; 97: 1450–3.
6. Gordon YJ, Gordon RY, Romanowski E, et al. Prolonged recovery of desiccated adenoviral serotypes 5, 8, and 19 from plastic and metal surfaces in vitro. *Ophthalmology* 1993; 100: 1835–9; discussion 1839-40.
7. Uchio E, Ishiko H, Aoki K, et al. Adenovirus detected by polymerase chain reaction in multidose eyedrop bottles used by patients with adenoviral keratoconjunctivitis. *Am J Ophthalmol* 2002; 134: 618–9.
8. Ghebremedhin B. Human adenovirus: Viral pathogen with increasing importance. *Eur J Microbiol Immunol* 2014; 4: 26–33.
9. Hamada N, Gotoh K, Hara K, et al. Nosocomial outbreak of epidemic keratoconjunctivitis accompanying environmental contamination with adenoviruses. *J Hosp Infect* 2008; 68: 262–268.
10. Tabbara KF, Omar N, Hammouda E, et al. Molecular epidemiology of adenoviral keratoconjunctivitis in Saudi Arabia. *Mol Vis* 2010; 16: 2132–6.

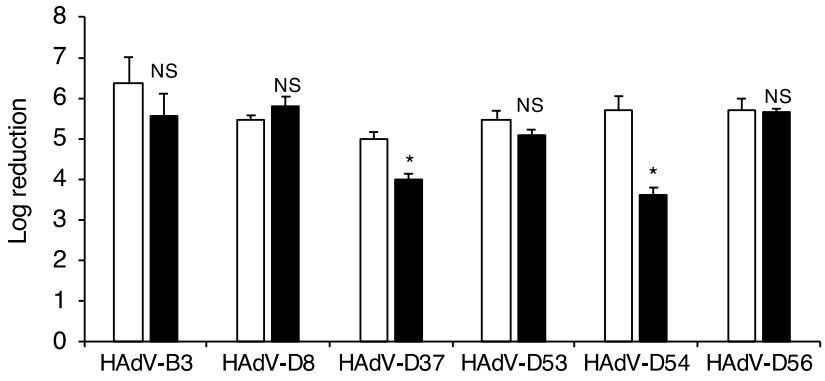
11. Warren D, Nelson KE, Farrar JA, et al. A large outbreak of epidemic keratoconjunctivitis: problems in controlling nosocomial spread. *J Infect Dis* 1989; 160: 938–43.
12. Takeuchi R, Nomura Y, Kojima M, et al. A nosocomial outbreak of epidemic keratoconjunctivitis due to adenovirus type 37. *Microbiol Immunol* 1990; 34: 749–54.
13. Alai N. Enhancing best practices in ophthalmology for prevention of nosocomial epidemic keratoconjunctivitis infections. *Curr Med Res Opin* 2016; 32: 1757–1758.
14. Akanuma M. Evaluation of Disinfectant against Adenovirus by Real-time Polymerase Chain Reaction. *J japanese opthalmological Soc* 2007; 111: 384–390.
15. Fukuzaki S. Mechanisms of Actions of Sodium Hypochlorite in Cleaning and Disinfection Processes. *Biocontrol Sci* 2006; 11: 147–157.
16. Eleraky NZ, Potgieter LND, Kennedy MA. Virucidal Efficacy of Four New Disinfectants. *J Am Anim Hosp Assoc* 2002; 38: 231–234.
17. McCormick L, Maheshwari G. Inactivation of adenovirus types 5 and 6 by Virkon® S. *Antiviral Res* 2004; 64: 27–33.
18. Lam E, Falck-Pedersen E. Unabated Adenovirus Replication following Activation of the cGAS/STING-Dependent Antiviral Response in Human Cells. *J Virol* 2014; 88: 14426–14439.
19. Ishiko H, Aoki K. Spread of Epidemic Keratoconjunctivitis Due to a Novel Serotype of Human Adenovirus in Japan. *J Clin Microbiol* 2009; 47: 2678–2679.
20. Okaue A, Ozawa T, Ogura Y, et al. Evaluation of Corrosiveness to Various Surface Materials of Complex Type Chlorine-Based Disinfectant Cleaner. *Japanese J Infect Prev Control* 2015; 30: 325–330.
21. Gasparini R, Pozzi T, Magnelli R, et al. Evaluation of in vitro efficacy of the disinfectant Virkon. *Eur J Epidemiol* 1995; 11: 193–7.
22. Hernndez A, Martró E, Matas L, et al. Assessment of in-vitro efficacy of 1% Virkon® against bacteria, fungi, viruses and spores by means of AFNOR guidelines. *J Hosp Infect* 2000; 46: 203–209.

23. Chan YF, Abu Bakar S. Virucidal activity of Virkon S on human enterovirus. *Med J Malaysia* 2005; 60: 246–8.
24. Roongruangchai K, Kummalue T, Sookkua T, et al. Comparison of Pouzolzia indica methanolic extract and Virkon against cysts of Acanthamoeba spp. *Southeast Asian J Trop Med Public Health* 2010; 41: 776–84.
25. Ogura Y, Ozawa T, Nojima Y, et al. Antimicrobial Efficacy of Complex-Type Chlorine-Based Disinfectant Cleaner Against Several Pathogenic Microorganisms. *Japanese J Infect Prev Control* 2015; 30: 391–398.
26. Ariga T, Shimada Y, Shiratori K, et al. Five New Genome Types of Adenovirus Type 37 Caused Epidemic Keratoconjunctivitis in Sapporo, Japan, for More Than 10 Years. *J Clin Microbiol* 2005; 43: 726–732.
27. Ishiko H, Shimada Y, Konno T, et al. Novel Human Adenovirus Causing Nosocomial Epidemic Keratoconjunctivitis. *J Clin Microbiol* 2008; 46: 2002–2008.
28. Kaneko H, Suzutani T, Aoki K, et al. Epidemiological and virological features of epidemic keratoconjunctivitis due to new human adenovirus type 54 in Japan. *Br J Ophthalmol* 2011; 95: 32–36.

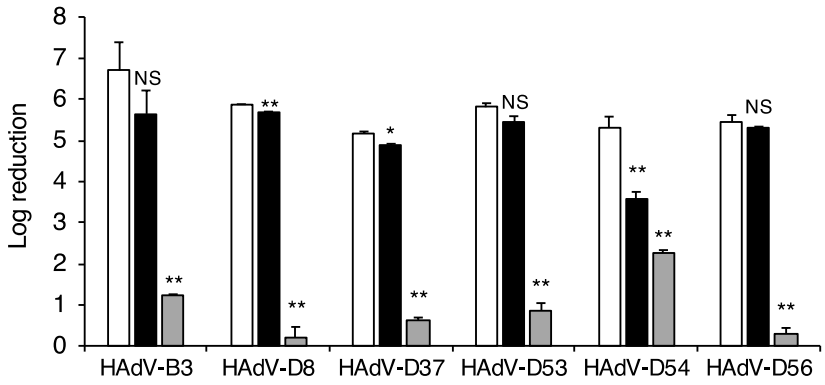


303

304 **Figure 1.** Time course analyses of HAdV DNA copy number at the indicated times by real-
305 time PCR (1, 3, 6 and 9 days) after HAdV-B3 (a), HAdV-D8 (b), HAdV-D37 (c), HAdV-
306 D53 (d), HAdV-D54 (e) and HAdV-D56 (f) infection (multiplicity of infection = 0.1; ●, 1;
307 ▲, 10; ■) in A549 cells.

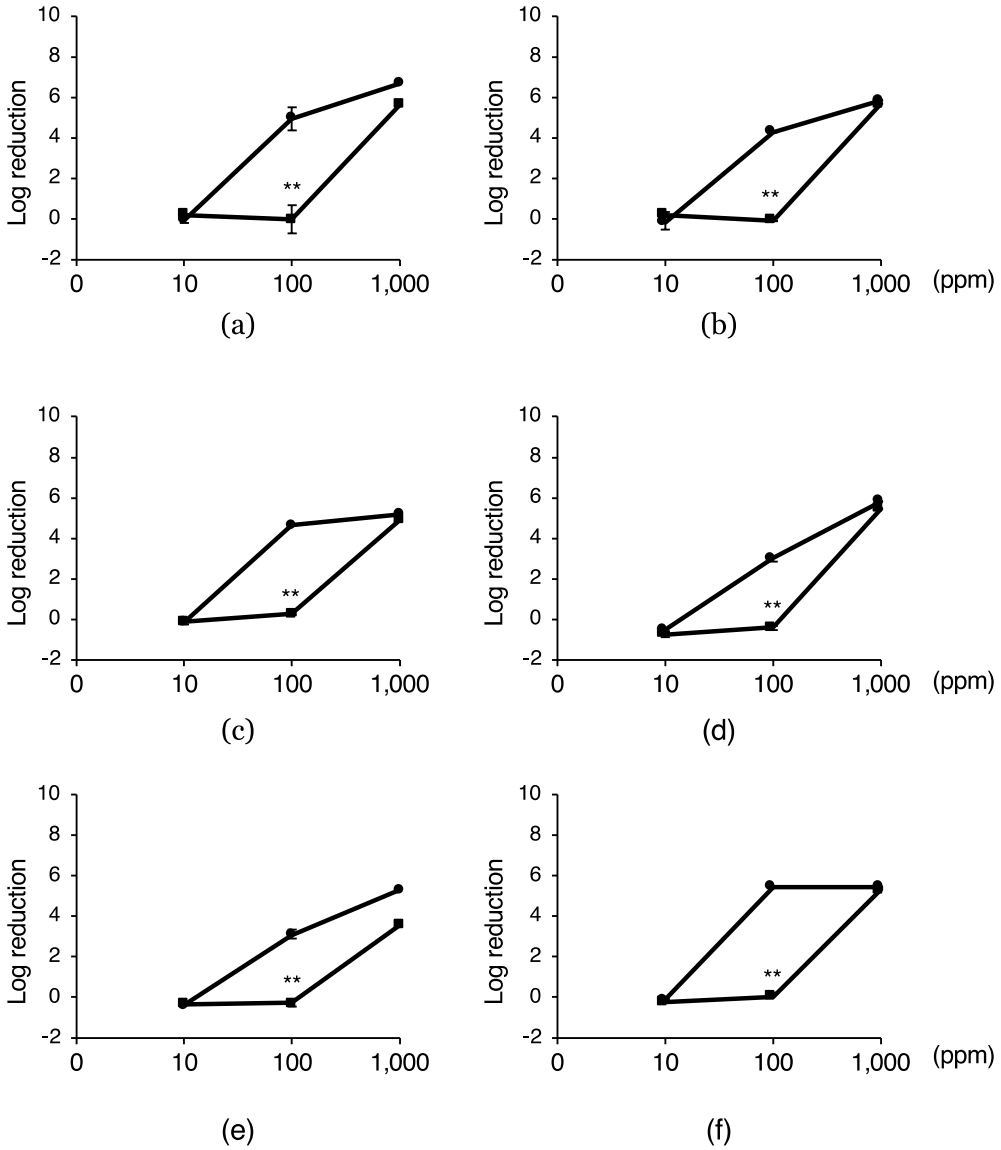


(a)



(b)

Figure 2. Effect of 1% potassium peroxymonosulfate (blank bar), 0.1% sodium hypochlorite (black bar) and alcoholic chlorbenzarconium (grey bar) on the inactivation of HAdV-D for 30 sec (a) or 1min (b). ** $P < 0.01$ and * $P < 0.05$ vs potassium peroxymonosulfate. NS, not significant. Data are presented as mean and standard deviation (n=3).



313

314 **Figure 3.** Effect of various concentration potassium peroxymonosulfate (circle) and sodium
315 hypochlorite (square) on the inactivation of HAdV-B3 (a), HAdV-D8 (b), HAdV-D37 (c),
316 HAdV-D53 (d), HAdV-D54 (e) and HAdV-D56 (f) for 1min. ** $P < 0.01$ vs potassium
317 peroxymonosulfate. Data are presented as mean and standard deviation (n=3).