



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

3

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26 **Short title:** Inactivation of EKC-related HAdV

27 **ABSTRACT**

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

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

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

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

43

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

45 **Introduction**

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

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

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

84 **Methods**

85 **Reagents**

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

91

92 **Cell culture**

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

97

98 **Virus**

99 HAdV-B3 and HAdV-D8 were provided from the Department of Medicine,
100 Ophthalmology, Fukuoka Dental College. HAdV-D37, -D53 and -D56 were obtained from
101 Kumamoto Institute of Public-Health and Environmental. HAdV-D54 was provided from the
102 Department of Ophthalmology, Hokkaido University. For virus propagation, A549 cells were
103 infected with each virus solution and incubated. After observation of cytopathic effects (CPE)
104 in the infected culture, cells and culture media were collected followed by 3 times of freeze
105 and thaw cycles. The supernatant including virus was collected by centrifuge at 3,000 rpm
106 for 10 min. All viral titers were determined by TCID₅₀.

107

108 **Examination of HAdV replication**

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

113

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

116 A 0.02%, 0.2%, and 2% (w/v) potassium peroxymonosulfate solution and 0.002%,
117 0.02%, and 0.2% (w/v) NaClO solution were prepared. HAdV solutions were incubated with
118 0.01%, 0.1%, and 1% (w/v) potassium peroxymonosulfate solution and 0.001%, 0.01%, and
119 0.1% (w/v) NaClO solution at final concentration (the effective chlorine concentration is 10
120 ppm, 100 ppm and 1,000 ppm, respectively) for 30 sec or 1 min. After incubation, 0.1% (w/v)
121 sodium thiosulfate at final concentration was added. The alcoholic chlorbenzarconium also
122 reacted with HAdV for 1 min, then diluted 260 times with DMEM. Next, the HAdV solutions
123 after treatment by three different disinfectants were used to infect A549 cells. After 6 days,
124 viral DNAs were isolated from culture cells and their medium. Real-time PCR was performed
125 using SYBR Premix Ex Taq (TOYOBO) and analysed on a StepOnePlus real-time PCR
126 system (Applied Biosystems). Primers for amplification of HAdV DNA by real-time PCR
127 were as follows. Forward primer: 5'-TTCCCCATGGC(A/T/C/G)CACAA(C/T)AC-3',
128 reverse primer: 5'-TGCC(T/G)(A/G)CTCAT(A/G)GGCTG(A/G)AAGTT-3' (product
129 length: 554 bp, product Tm: 88.0 °C). The real-time PCR condition included a first step at
130 98 °C, followed by 45 cycles of 10 sec at 98 °C, 10 sec at 50 °C and 45 sec at 68 °C. Copy
131 numbers were calculated using a standard curve method.

132

133 **Cell growth assay**

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

137

138 **Statistical analysis**

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

141 **Results**

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

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

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

170 **Discussion**

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

180 0.1% Hypochlorous acid NaClO is widely known as the most effective in mid-level
181 disinfectants¹⁴ despite drawbacks such as corrosive properties that limit its use with medical
182 devices and the irritating smell that is inappropriate to use in clinical settings.¹⁵ In contrast,
183 potassium peroxymonosulfate produce few effects on metals, especially non-reactive to
184 stainless steel.²⁰ Furthermore, potassium peroxymonosulfate is odourless, easy to prepare and
185 store. Our current study indicated that the use of potassium peroxymonosulfate is as effective
186 as NaClO against HAdV-D types, without the main drawbacks of the former. These results
187 support the use of potassium peroxymonosulfate for disinfection of medical devices such as
188 tonometer, slit lamp microscope and eyedrop bottle in healthcare facilities, in particular, at
189 eye clinics.

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

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

203 We demonstrated that 1% potassium peroxymonosulfate was more effective than
204 0.1% NaClO against HAdV-D54. HAdV-D54 was firstly reported and isolated from a
205 nosocomial outbreak in a Japanese hospital in 2000.²⁷ HAdV-D54 has been often isolated
206 since 2011, according to surveillance of EKC causative agents in Japan, and has unique
207 genome sequences encoded capsid protein,² however, the detailed virological features of
208 HAdV-D54 remain poorly understood. *Kaneko et al.* reported that HAdV-D54 is
209 characterized by cytopathic effects (CPE) harder to observe than other EKC-related types.²⁸
210 In relation to this report, our data showed that the replication rate of HAdV-D54 in A549
211 cells is the lowest among all HAdVs we tested (Figure 1e). In this regard, HAdV-D54 or its
212 interaction with host cells may have some factors behind the slow replication rate and CPE
213 due to its gene polymorphism or interaction with host factors. Despite these conditions, this
214 type is an important infectious EKC agent in Japan with severe cases leading to corneal
215 complications.^{2,28}

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

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224 (Tokyo, Japan).

225

226 **Declaration of conflicting interests**

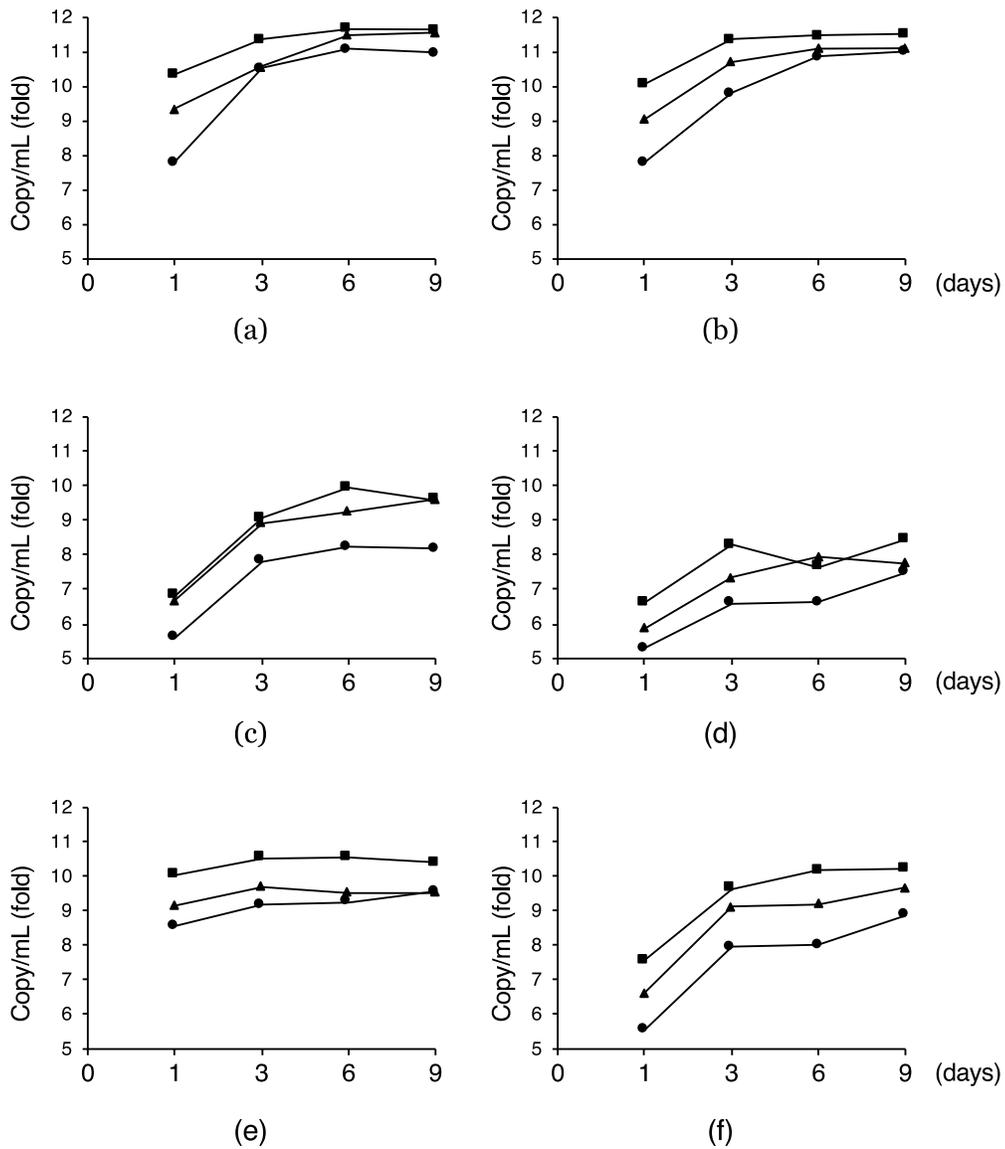
227 None.

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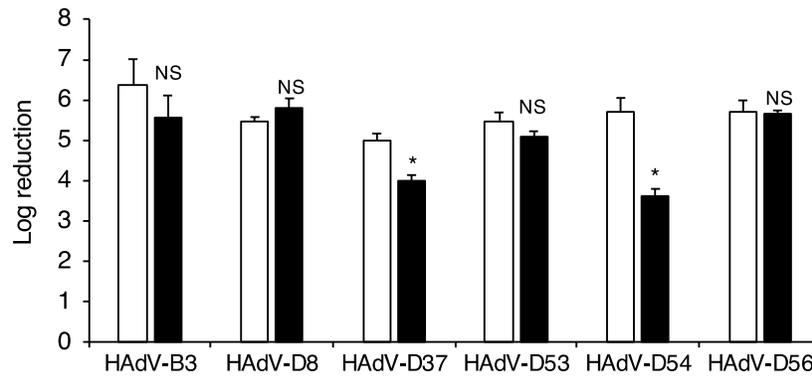
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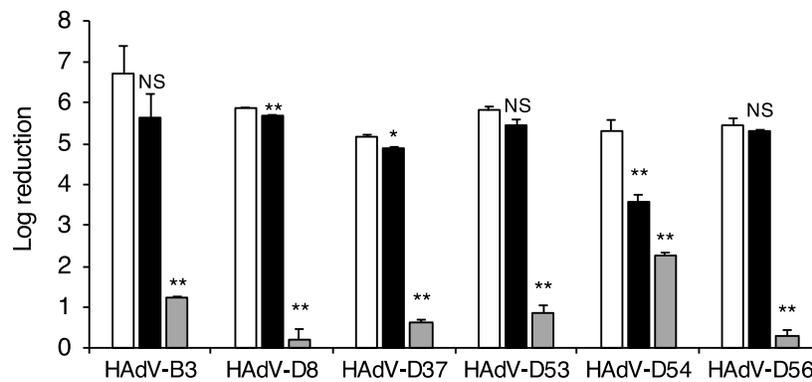


303

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.



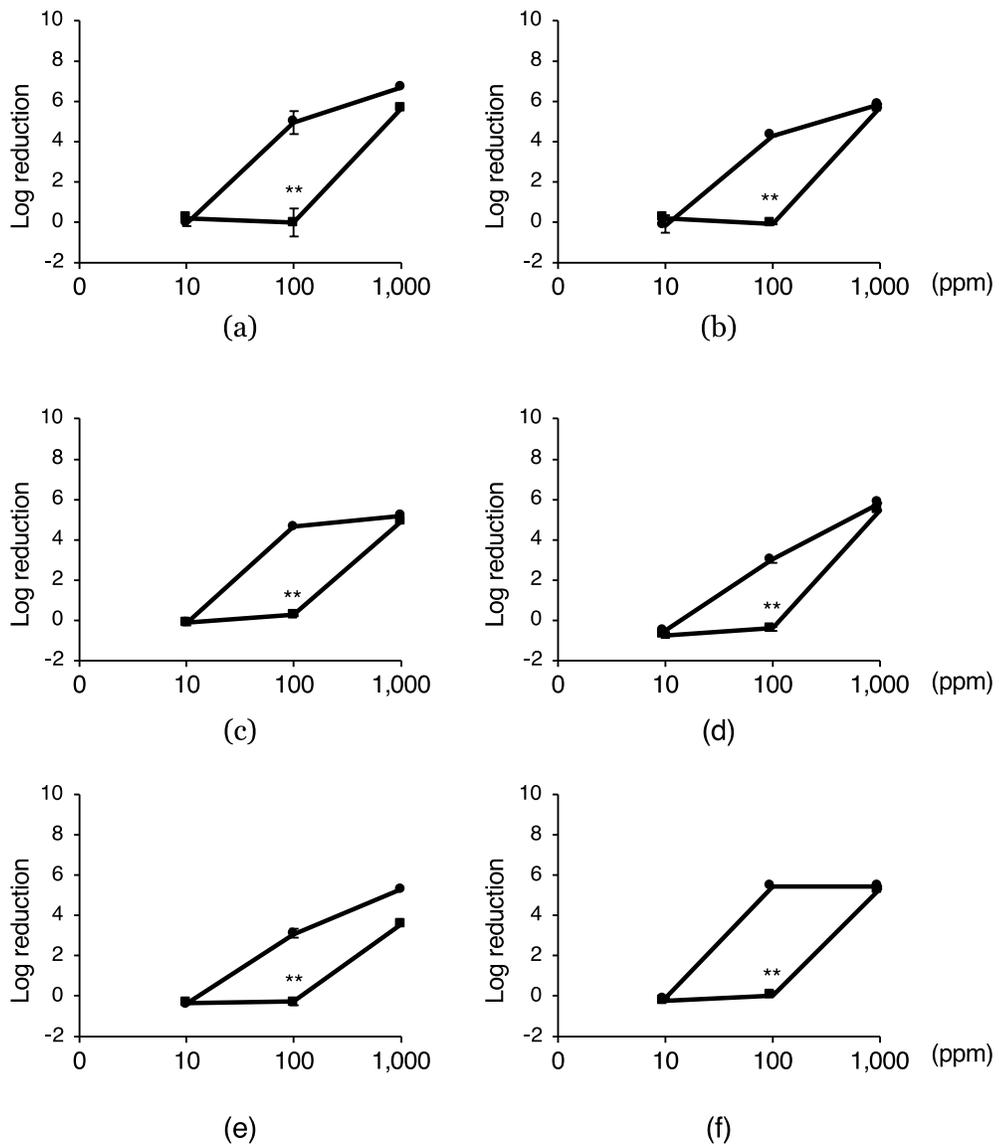
(a)



(b)

308

309 **Figure 2.** Effect of 1% potassium peroxymonosulfate (blank bar), 0.1% sodium hypochlorite
 310 (black bar) and alcoholic chlorbenzarconium (grey bar) on the inactivation of HAdV-D for
 311 30 sec (a) or 1min (b). ** $P < 0.01$ and * $P < 0.05$ vs potassium peroxymonosulfate. NS, not
 312 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).