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Suitability of pepper mild mottle virus as a human enteric virus surrogate for assessing the efficacy of thermal or free-chlorine disinfection processes by using infectivity assays and enhanced viability PCR

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Abstract

Evaluating the efficacy of disinfection processes to inactivate human enteric viruses is important for the prevention and control of waterborne diseases caused by exposure to those viruses via drinking water. Here, we evaluated the inactivation of two representative human enteric viruses (adenovirus type 40 [AdV] and coxsackievirus B5 [CV]) by thermal or free-chlorine disinfection. In addition, we compared the infectivity reduction ratio of a plant virus (pepper mild mottle virus [PMMoV], a

21 recently proposed novel surrogate for human enteric viruses for the assessment of virus removal by
22 coagulation–rapid sand filtration and membrane filtration) with that of the two human enteric
23 viruses to assess the suitability of PMMoV as a human enteric virus surrogate for use in thermal and
24 free-chlorine disinfection processes. Finally, we examined whether conventional or enhanced
25 viability polymerase chain reaction (PCR) analysis using propidium monoazide (PMA) or improved
26 PMA (PMAxx) with or without an enhancer could be used as alternatives to infectivity assays (i.e.,
27 plaque-forming unit method for AdV and CV; local lesion count assay for PMMoV) for evaluating
28 virus inactivation by disinfection processes. We found that PMMoV was more resistant to heat
29 treatment than AdV and CV, suggesting that PMMoV is a potential surrogate for these two enteric
30 viruses with regard to thermal disinfection processes. However, PMMoV was much more resistant
31 to chlorine treatment compared with AdV and CV (which is chlorine-resistant) (CT value for 4-$\log_{10}$
32 inactivation: PMMoV, 84.5 mg-Cl_2·min/L; CV, 1.15–1.19 mg-Cl_2·min/L), suggesting that PMMoV
33 is not useful as a surrogate for these enteric viruses with regard to free-chlorine disinfection
34 processes. For thermal disinfection, the magnitude of the signal reduction observed with
35 PMAxx-Enhancer-PCR was comparable with the magnitude of reduction in infectivity, indicating
36 that PMAxx-Enhancer-PCR is a potential alternative to infectivity assay. However, for free-chlorine
37 disinfection, the magnitude of the signal reduction observed with PMAxx-Enhancer-PCR was
38 smaller than the magnitude of the reduction in infectivity, indicating that PMAxx-Enhancer-PCR
39 underestimated the efficacy of virus inactivation (i.e., overestimated the infectious virus
40 concentration) by chlorine treatment. Nevertheless, among the PCR approaches examined in the

present study (PCR alone, PMA-PCR or PMAxx-PCR either with or without enhancer), PMAxx-Enhancer-PCR provided the most accurate assessment of the efficacy of virus inactivation by thermal or free chlorine disinfection processes.

Keywords: Chlorine treatment, Heat treatment, Infectivity assay, Pepper mild mottle virus, Propidium monoazide, Quantitative real-time PCR

1. Introduction

Viruses associated with waterborne transmission are mainly those that can infect the gastrointestinal tract of humans or animals, which are known as enteric viruses (WHO, 2011). More than 140 enteric virus serotypes are known to infect humans, and the main health effect associated with human enteric virus infection is gastrointestinal illness (Health Canada, 2017). In addition to gastrointestinal disease, human enteric viruses can cause serious acute illnesses, such as meningitis, poliomyelitis, and hepatitis (Health Canada, 2017). Human enteric viruses are excreted in very high numbers in the feces of infected persons, even from those who are asymptomatic, and can persist in water environments for prolonged periods (WHO, 2011). In fact, drinking water sources often become contaminated with those viruses through a variety of routes, such as wastewater treatment plant effluent and disposal of sanitary sewage or sludge on land (Bosch, 2007; Health Canada,

61 2017). Measures to reduce the risk of infection by human enteric viruses focus not only on the
62 prevention of water source contamination but also on the detection of contamination and subsequent
63 disinfection of contaminated water (WHO, 2011). Thus, methods for the accurate validation of
64 disinfection processes are an essential part of these measures.

65 Historically, cell culture is the most widely used method for the detection and quantification of
66 infectious viruses in water. Consequently, because this approach detects only infectious viruses, it is
67 an important means of obtaining data regarding the potential risk of infection from contaminated
68 water (Health Canada, 2017). However, cell culture is labor intensive, time consuming, and
69 nonspecific for several enteric viruses. In addition, some enteric viruses cannot be cultured by using
70 current cell culture technology; a universal cell line that can be used to culture and detect all human
71 enteric viruses is unavailable at present; and some viruses do not produce a detectable
72 cytopathogenic effect, which is necessary for the visual detection of infectivity (Bosch, 2007;
73 Health Canada, 2017).

74 Recently, molecular methods targeting viral DNA or RNA, such as polymerase chain reaction
75 (PCR) and quantitative real-time PCR, have become a common means of detecting and quantifying
76 viruses in drinking water sources (Varughese *et al.,* 2018; Miura *et al.,* 2019). These methods have
77 also been used to evaluate the efficacy of drinking water treatment processes (Albinana-Gimenez *et*
78 *al.*, 2006, 2009). Compared with common cell-culture-based infectivity assays (e.g., plaque-forming
79 unit [PFU] assay, median tissue culture infectious dose [$TCID_{50}$] assay), PCR-based methods are
80 fast, highly sensitive, highly specific, and are able to detect and quantify any virus for which the

81 nucleotide sequence is known, including nonculturable and difficult-to-culture viruses (Bosch,
82 2007). However, PCR potentially overestimates the amount of infectious viruses because this
83 method detects the viral DNA/RNA of both infectious and inactivated viruses, unless the target part
84 of the DNA/RNA is damaged or lost (Parshionikar *et al.*, 2010; Leifels *et al.*, 2015; Fuster *et al.*,
85 2016; Prevost *et al.*, 2016; Lee *et al.*, 2018). This means that if the effectiveness of a disinfection
86 process is evaluated by a PCR-based method, the results likely lead to underestimation of the
87 efficacy of the disinfection process. Therefore, assessment methods that accurately discriminate
88 infectious from inactivated viruses are highly desired.

89 One advanced approach that has been used to distinguish between infectious and inactivated
90 viruses is viability PCR using the photoreactive intercalating dye propidium monoazide (PMA)
91 (Parshionikar *et al.*, 2010; Karim *et al.*, 2015; Leifels *et al.*, 2015; Fuster *et al.*, 2016; Randazzo *et*
92 *al.*, 2016; Lee *et al.*, 2018). In this approach, a sample is treated with PMA and then exposed to
93 intense visible light, which converts the azide group of the dye into a highly reactive nitrene radical
94 that then reacts with viral DNA or RNA and inhibits its subsequent amplification by PCR (Fittipaldi
95 *et al.*, 2012; Randazzo *et al.*, 2016). Because the dye cannot enter viruses with intact capsids, only
96 inactivated viruses with damaged capsids are targeted; thus, the DNA or RNA of inactivated viruses
97 is not amplified during PCR.

98 Several research groups have examined the suitability of PMA-PCR for evaluation of the
99 effectiveness of disinfection processes (Parshionikar *et al.*, 2010; Karim *et al.*, 2015; Lee *et al.*,
100 2018). However, these studies have shown that the ability of PMA-PCR to discriminate between

101 infectious and inactivated viruses varies depending on the target virus and disinfection process. For
102 example, Karim *et al.* (2015) reported that PMA-PCR was able to selectively differentiate between
103 infectious and inactivated poliovirus in water samples exposed to heat and chlorine treatment but
104 could not differentiate when poliovirus was inactivated by ultraviolet irradiation or when human
105 norovirus was treated with any disinfection process. Therefore, the suitability of PMA-PCR for the
106 assessment of disinfection processes with regard to virus inactivation remains under debate.
107 Recently, PMAxx, a newly improved version of PMA, and PMA Enhancer for Gram Negative
108 Bacteria, a product originally designed to improve PMA- or PMAxx-mediated discrimination
109 between live and dead gram-negative bacteria, have successfully been used for the assessment of
110 virus infectivity in the field of food safety (Randazzo *et al.*, 2016, 2018). Randazzo *et al.* (2016)
111 reported that the addition of PMA Enhancer to PMAxx-PCR (PMAxx-Enhancer-PCR) was more
112 effective than PMA-PCR or PMAxx-PCR at distinguishing between infectious and heat-inactivated
113 human norovirus, for which a cell culture-based infectivity assay is not routinely available (Oka *et*
114 *al.*, 2018). Thus, PMAxx-Enhancer-PCR may be a suitable approach for evaluation of the efficacy
115 of disinfection processes. However, Kim and Ko (2012) have reported that the bacteriophage MS2
116 was inactivated after exposure to PMA at high concentration, even without any disinfection process,
117 meaning that PMA itself, and by extension PMAxx, may cause virus inactivation. Therefore,
118 comparison of data obtained by using PMAxx-Enhancer-PCR with that obtained by infectivity
119 assay is necessary to determine whether PMAxx-Enhancer-PCR is a suitable alternative to
120 infectivity assay for evaluating the efficacy of disinfection processes to inactivate viruses.

121 A plant virus, pepper mild mottle virus (PMMoV; genus *Tobamovirus*, family *Virgaviridae*,
122 single-stranded RNA virus), which infects bell, hot, and ornamental peppers, has been proposed as a
123 novel surrogate for human enteric viruses for the assessment of the efficacy of physical and
124 physicochemical water treatment processes (Kitajima *et al.*, 2018; Symonds *et al.*, 2018). The
125 removal ratios of PMMoV, as evaluated by PCR, have been shown to be similar to or smaller than
126 those of human enteric viruses in laboratory-scale coagulation–rapid sand filtration or membrane
127 filtration processes (Shirasaki *et al.*, 2017a; Kato *et al.*, 2018; Shirasaki *et al.*, 2018). Also, virus
128 removal efficiencies in actual drinking water treatment plants have successfully been determined by
129 targeting indigenous PMMoV due to its high concentration in drinking water sources (Kato *et al.*,
130 2018; Canh *et al.*, 2019). Therefore, if the inactivation efficiencies of PMMoV and human enteric
131 viruses are comparable, PMMoV could be a useful surrogate for evaluating the efficacy of
132 disinfection processes for the inactivation of human enteric viruses. However, the efficacies of
133 disinfection processes against PMMoV have not yet been investigated, possibly because of the
134 difficulty of infectivity assays. Thus, it remains unclear in what contexts PMMoV is a suitable
135 surrogate for human enteric viruses.

136 Here, we examined whether PMMoV is a suitable surrogate for two human enteric viruses (one
137 adenovirus [AdV; genus *Mastadenovirus*, family *Adenoviridae*, double-stranded DNA virus] and
138 one coxsackievirus [CV; genus *Enterovirus*, family *Picornaviridae*, single-stranded RNA virus]) for
139 evaluation of the effectiveness of thermal or free-chlorine disinfection processes. Thermal and
140 free-chlorine disinfection processes were targeted because thermal disinfection is a point-of-use

141 technology (WHO, 2011) used in situations when other control measures have failed or cannot be
142 implemented, such as during emergencies or disasters, leading to inadequate sanitation, hygiene,
143 and protection of water sources. In addition, free-chlorine disinfection is currently widely used in
144 actual drinking water treatment plants to ensure drinking water safety. We then used the three
145 viruses to examine the ability of conventional and enhanced viability PCR approaches to distinguish
146 between infectious and inactivated viruses and to determine whether these approaches can be used
147 as alternatives to infectivity assays for validation of the two target disinfection processes.
148
149
150 **2. Materials and methods**
151 2.1. PBS and chlorine
152 Chlorine demand-free phosphate-buffered saline (PBS) (0.01 M, pH 7) was prepared by dissolving
153 Na_2HPO_4 and NaH_2PO_4 in Milli-Q water (Milli-Q Advantage, Millipore Corp., Billerica, MA,
154 USA). In the chlorine treatment experiments, sodium hypochlorite (Fujifilm Wako Pure Chemical
155 Corporation, Osaka, Japan) was used immediately after dilution with Milli-Q water.
156
157 2.2. Human enteric viruses and PMMoV
158 For our study, we chose two types of human enteric viruses—AdV as a representative DNA virus
159 and CV as a representative RNA virus—because the efficacy of disinfection processes for virus
160 inactivation can vary by type of viral genetic material (Health Canada, 2017). AdV type 40 Dugan

161 strain (ATCC VR-931) and CV B5 Faulkner strain (ATCC VR-185) were specifically chosen
162 because they are highly resistant to ultraviolet disinfection (Nwachuku *et al.*, 2005) and chlorine
163 disinfection (Cromeans *et al.*, 2010), respectively. The two strains were obtained from American
164 Type Culture Collection (ATCC, Manassas, VA, USA) and propagated in human lung carcinoma
165 epithelial cells (A549 cells) and buffalo green monkey kidney epithelial cells (BGM cells),
166 respectively. The details of the propagation and purification of AdV and CV are described in our
167 previous reports (Shirasaki *et al.*, 2016, 2017b). The concentrations of AdV and CV in the purified
168 solutions were approximately $10^{5–6}$ and 10^{7} PFU/mL, respectively, as evaluated by means of plaque
169 assay (Shirasaki *et al.*, 2016, 2017b).
170 PMMoV pepIwateHachiman1 strain (MAFF 104099) was obtained from the National Institute of
171 Agrobiological Sciences Genebank (Tsukuba, Japan) and propagated by using a plant host,
172 *Nicotiana benthamiana*. The details of the propagation of PMMoV are described in our previous
173 report (Shirasaki *et al.*, 2018). The concentration of PMMoV in the stock solution was
174 approximately 10^{7} lesions/mL, as evaluated by using a local lesion count assay with *Nicotiana*
175 *tabacum* cv. *Xanthi-nc* (see section 2.7).
176
177 2.3. Naked DNA and RNA
178 The ability of PMA-PCR, PMA-Enhancer-PCR, PMAxx-PCR, and PMAxx-Enhancer-PCR to
179 discriminate between infectious and inactivated viruses was evaluated by using naked DNA and
180 RNA. The details of the preparation of the naked DNA and RNA samples are provided in

181 Supplementary Information.

182

183 2.4. Heat treatment experiments

184 Heat treatment experiments were conducted with 1 mL of virus-spiked PBS in a sterile microtube
185 and a thermoblock (ND-S01, Nissinrika Corp., Tokyo, Japan). Purified solution of AdV or CV, or
186 stock solution of PMMoV, was added to PBS at an initial infectious virus concentration of $10^{2–3}$
187 PFU/mL for AdV, $10^{3–4}$ PFU/mL for CV, or $10^{4–5}$ lesions/mL for PMMoV. Because the purified
188 solution or stock solution of virus was diluted by their addition to the PBS, virus addition
189 contributed less than 0.2 mg/L of unintentional carry-over of dissolved organic carbon. The
190 virus-spiked PBS (N_0) was incubated in the thermoblock at 45, 50, 55, 60, 70, 80, or 90 °C for 5
191 min, and then immediately cooled in a refrigerator set at 4 °C for 10 min to stop the disinfection
192 process. After cooling, the virus concentration (N_h) in the samples was determined by infectivity
193 assay (i.e., plaque assays for AdV and CV; local lesion count assay for PMMoV).

194

195 2.5. Chlorine treatment experiments

196 Chlorine treatment experiments were conducted with 300 mL of virus-spiked PBS in a prepared
197 Erlenmeyer flask at 20 °C. The glass Erlenmeyer flask with stopper and magnetic stirrer were
198 soaked overnight in a solution containing 50 mg-Cl_2/L of free chlorine to eliminate the consumption
199 of chlorine by those items during the experiment. The soaked Erlenmeyer flask and magnetic stirrer
200 were rinsed carefully with Milli-Q water and then baked at 210 °C for 30 min prior to use. The

201 virus-spiked PBS (N_0) was continuously mixed with the prepared magnetic stirrer at 400 rpm during
202 the experiments. Sodium hypochlorite was added to the spiked PBS to obtain an initial free-chlorine
203 concentration of 0.1 or 0.5 mg-Cl_2/L, and then left for a contact time of 10 min for AdV, 10 min (0.5
204 mg-Cl_2/L) or 50 min (0.1 mg-Cl_2/L) for CV, and 1440 min for PMMoV. Periodically, a 10-mL
205 sample was taken from the flask, immediately added to a sterile tube containing sodium thiosulfate
206 (final concentration, 5 mg/L) to quench any residual free chlorine and stop the disinfection process,
207 and used for quantification of virus concentration (N_c). An additional 10 mL of sample was
208 collected at each time point for quantification of the residual free-chlorine concentration by using
209 the *N*,*N'*-diethyl-*p*-phenylenediamine method and a DR900 Multiparameter Portable Colorimeter
210 (Hach Company, Loveland, Co., USA).
211
212 2.6. Quantification of infectious human enteric virus by plaque assay
213 Infectious AdV type 40 or CV B5 was quantified by using a plaque assay. The details of the plaque
214 assay are described in our previous reports (Shirasaki *et al.*, 2016, 2017b).
215
216 2.7. Quantification of infectious PMMoV by local lesion count assay
217 Infectious PMMoV was quantified by local lesion count assay based on the fact that PMMoV
218 infection results in local lesions on *Nicotiana tabacum* cv. *Xanthi-nc*, as reported by Colson *et al.*
219 (2010). Seeds and seedlings of *Nicotiana tabacum* cv. *Xanthi-nc* were grown in 200-mL pods filled
220 with gardening soil (Kumiai gardening soil; Hokusan Co., Kitahiroshima, Japan) and cultured in a

221 growth chamber at 25 °C under a long-day photoperiod (16-h light, 8-h dark) for approximately 1
222 month. Milli-Q water was added to the pods every 1 or 2 days during cultivation. After cultivation,
223 three leaves of each plant were covered with 600-mesh carborundum, and then 100 μL of sample or
224 100 μL of sample 10-fold serially diluted with PBS was rubbed into each leaf with a gloved finger
225 for virus inoculation. After inoculation, the plants were incubated at 20 °C for 5 min and then the
226 leaves were washed with Milli-Q water to remove the inoculum and carborundum. After washing,
227 the plants were incubated in the growth chamber at 25 °C under the long-day photoperiod for 4–5
228 days. At the end of incubation, the number of lesions on each leaf was counted and the average
229 lesion count of one or two plants (i.e., 3–6 leaves) for each sample was considered as the infectious
230 PMMoV concentration for that sample. The quantification limit of the local lesion count assay was
231 1 lesion/600 μL (i.e., 10 lesions/6 mL) when six leaves were prepared.
232
233 2.8. Quantification of viral DNA and RNA, and of artificially synthesized plasmid DNA, by
234 real-time PCR or real-time reverse-transcription PCR
235 Viral DNA of AdV and artificially synthesized plasmid DNA were quantified by real-time PCR, and
236 viral RNA of CV and PMMoV were quantified by real-time reverse-transcription PCR (real-time
237 RT-PCR). The quantitative real-time PCR and the quantitative real-time RT-PCR methods were
238 executed following the MIQE guidelines (Bustin *et al.*, 2009). The details of the quantitative
239 real-time PCR and quantitative real-time RT-PCR methods are provided in Supplementary
240 Information.

241

242 2.9. Intercalating dye treatment

243 To investigate the effects of different intercalating dyes and the use of an intercalating dye enhancer
244 on the ability of viability PCR to discriminate between infectious and inactivated viruses, PMA,
245 PMAxx, and PMA enhancer were used in the present study. PMA or PMAxx solution (20 mM in
246 H_2O; Biotium, Inc., Fremont, CA, USA) was added to 200 μL of sample to achieve a final
247 concentration of 50, 100, 200, 500, or 1000 μM. In the cases of PMA-Enhancer-PCR and
248 PMAxx-Enhancer-PCR, PMA Enhancer for Gram Negative Bacteria (5× solution; Biotium, Inc.)
249 was added to the sample containing PMA or PMAxx to achieve a final concentration of 1×. The
250 sample was then mixed and incubated in the dark at 20 °C for 10 min with mild shaking to allow
251 the PMA or PMAxx to penetrate the viral capsid, after which the sample was exposed to intense
252 visible light (60 W, 465–475 nm, blue light) for 15 min by using a PMA-Lite LED Photolysis
253 Device (Biotium, Inc.) to photoactivate the PMA or PMAxx. After treatment, viral DNA or RNA
254 was extracted and purified (naked DNA or RNA was also purified) with a QIAamp MinElute Virus
255 Spin Kit (Qiagen, Tokyo, Japan) and then quantified by using real-time (RT-)PCR.

256

257 2.10. Kinetic modeling and CT value calculation for chlorine treatment experiments

258 The efficiency factor Hom (EFH) model (Haas and Joffe, 1994), which is widely applied to
259 calculate CT values (free-chlorine concentration [C] multiplied by contact time [T], i.e., the
260 integration of the residual free-chlorine concentration until a given contact time) for non-linear

261 virus inactivation curves for chlorine treatments (Cromeans *et al.*, 2010; Kahler *et al.*, 2010), was
262 used for the CT value calculations. Details of the EFH model and CT value calculation are provided
263 in Supplementary Information.
264
265
266 **3. Results and discussion**
267 3.1. Virus inactivation by heat treatment
268 First, we determined infectivity reduction ratios ($\log_{10}[N_0/N_h]$) for AdV, CV, and PMMoV after heat
269 treatment (pH 7 and 45–90 °C for 5 min) by means of infectivity assay (i.e., plaque assay for AdV
270 and CV; local lesion count assay for PMMoV) (Figure 1). For AdV and PMMoV, no or limited
271 inactivation ($\leq$0.1-$\log_{10}$) was observed at $\leq$55 ºC. For CV, limited inactivation (0.3-$\log_{10}$) was
272 observed at 45 ºC. However, inactivation of all three viruses markedly increased with increasing
273 temperature. At 60 ºC, moderate inactivation was observed for AdV (2.4-$\log_{10}$) and PMMoV
274 (1.1-$\log_{10}$), and CV was completely inactivated (>3.6-$\log_{10}$; i.e., virus concentration was below the
275 limit of quantification). At 80 ºC, AdV was also completely inactivated (>3.4-$\log_{10}$). In contrast,
276 PMMoV was not completely inactivated even after treatment at 90 ºC. These results indicate that
277 PMMoV is more resistant to heat treatment compared with AdV and CV.
278 Hewitt *et al.* (2009) evaluated the efficiency of heat treatment for the inactivation of two other
279 viruses: hepatitis A virus IB, a human enteric virus, and murine norovirus type 1, a surrogate of
280 human caliciviruses, and reported that these viruses were completely inactivated ($\geq$3.5-$\log_{10}$) after

281 treatment at 72 ºC for 5 min. Taken together, these data show that PMMoV is more resistant to heat
282 treatment than the representative human enteric viruses. This suggests that PMMoV probably has a
283 more robust capsid structure compared with that of the representative human enteric viruses,
284 because the dominant mechanism that leads to virus inactivation by heat treatment is capsid damage
285 (Wigginton *et al.*, 2012; Torrey *et al.*, 2019). Because PMMoV has a rod-shaped capsid, which is
286 different from the round capsid of human enteric viruses (Kitajima *et al.*, 2018), the high resistance
287 of PMMoV may partly be attributed to this difference in capsid morphology. Thus, PMMoV
288 appears to be a potential surrogate for human enteric viruses for the assessment of the efficacy of
289 disinfection by heat treatment.
290
291 3.2. Virus inactivation by chlorine treatment
292 Figure 2 shows the infectivity reduction ratios ($\log_{10}[N_0/N_c]$) for AdV, CV, and PMMoV after
293 chlorine treatment (pH 7 and 20 ºC), as evaluated by infectivity assay. In addition, Table 1 shows
294 the CT values required for 2-, 3-, and 4-$\log_{10}$ inactivation calculated from the experimental data by
295 using the EFH model. AdV was completely inactivated (>3.4-$\log_{10}$ inactivation) within the first 5 s
296 of the disinfection process (initial free-chlorine concentration, 0.5 mg-Cl_2/L; Figure 2a). Although
297 the EFH model could not be applied to AdV because of the rapid inactivation of the virus, a CT
298 value of 0.04 mg-Cl_2·min/L was experimentally observed to provide at least 3-$\log_{10}$ inactivation
299 (Table 1). These results are consistent with those of Cromeans *et al.* (2010), who also reported that
300 AdV type 40 was readily inactivated by chlorine treatment (initial free-chlorine concentration, 0.2

301 mg-Cl_2/L, pH 7–8, 5 ºC), with at least 3-log_{10} inactivation within the first 5 s of the process (CT
302 value calculated by using the EFH model for 4-log_{10} inactivation of AdV type 40: <0.04
303 mg-Cl_2·min/L).

304 CV required a longer contact time for inactivation compared with AdV (Figure 2b,c). When the
305 initial free-chlorine concentration was 0.1 or 0.5 mg-Cl_2/L, a contact time of 15–20 min or 2–3 min,
306 respectively, was required to achieve 4-log_{10} inactivation. These results indicate that CV is more
307 resistant than AdV to chlorine treatment. Other researchers have also reported that CV B5 is more
308 resistant to chlorine disinfection than other serotypes of CV (i.e., CV B3 and CV B4) and other
309 viruses (e.g., AdV type 2, type 40, and type 41; poliovirus type 1, type 2, and type 3; and echovirus
310 type 1 and type 11 [Payment *et al.*, 1985; Cromeans *et al.*, 2010; Kahler *et al.*, 2010]). The
311 calculated CT values for 2-, 3-, and 4-log_{10} inactivation of CV B5 in the present study were
312 comparable between the two initial free-chlorine concentrations and were in the range of 0.71–0.73,
313 0.95–0.97, and 1.15–1.19 mg-Cl_2·min/L, respectively (Table 1). Kahler *et al.* (2010) conducted
314 chlorine treatment with an initial free-chlorine concentration of 0.2 mg-Cl_2/L in three types of
315 natural water at pH 7 and 15 ºC and reported CT values calculated by using the EFH model for
316 3-log_{10} inactivation of CV B5 in the range of 1.0–2.0 mg-Cl_2·min/L. Our results roughly agree with
317 those of Kahler *et al.* (2010), although the efficacy of virus inactivation by chlorination is
318 dependent on the water type and temperature, and these factors differed between our study and
319 theirs (PBS vs. natural water, 20 ºC vs. 15 ºC).

320 PMMoV required an even longer contact time for inactivation compared with AdV and CV

(Figure 2d). At a contact time of 30 min (initial free-chlorine concentration, 0.5 mg-Cl_2/L), only <1-log_{10} inactivation was observed. PMMoV did not achieve >4-log_{10} inactivation until a contact time of 240 min. These results indicate that PMMoV has very high resistance to chlorine treatment compared with AdV and CV. As with heat treatment, this high resistance can probably be attributed to PMMoV's robust capsid structure compared with that of the two representative human enteric viruses. The calculated CT values for 2-, 3-, and 4-log_{10} inactivation of PMMoV were 51.2, 71.4, and 84.5 mg-Cl_2·min/L, respectively (Table 1).

Other research groups have found that the resistance of CV B5 to chlorine treatment varies depending on the strain, reporting that some environmental isolates of CV B5 are more resistant than the commercially available laboratory strain used in the present study (Payment *et al.*, 1985; Meister *et al.*, 2018). Although Meister *et al.* (2018) reported a CT value for 4-log_{10} inactivation of an environmental isolate of CV B5 of 11.2 mg-Cl_2·min/L, this CT value is still well below the CT value needed for 4-log_{10} inactivation of PMMoV. Because the resistance of PMMoV to chlorine treatment is much higher than that of the representative human enteric viruses, if the virus inactivation efficacy of chlorine treatment is evaluated by using PMMoV as a surrogate, it could lead to marked underestimation of the effectiveness of the disinfection process. Thus, PMMoV does not appear to be useful as a surrogate for human enteric viruses for assessment of the efficacy of disinfection by chlorine treatment. Taken together, our present heat- and chlorine-treatment results suggest that the high resistance of PMMoV to temperature and chlorine treatment may be one of the reasons for the virus's widespread presence in drinking water sources that receive sewage

341 discharges (Kitajima *et al.*, 2018; Symonds *et al.*, 2018).

342

343 3.3. Effect of different intercalating dye treatments on naked viral DNA and RNA

344 To confirm the ability of viability PCR to discriminate between infectious and inactivated viruses,
345 we evaluated the inhibition of PCR amplification of naked viral DNA or RNA by two intercalating
346 dyes and an intercalating dye enhancer (Figure 3). In the absence of an intercalating dye, incubation
347 and subsequent photoactivation did not affect the PCR quantification of viral DNA extracted from
348 AdV or viral RNA extracted from CV or PMMoV, as determined by comparing the PCR signal
349 obtained by using PCR alone and PCR without intercalating dye but with incubation and
350 subsequent photoactivation (data not shown). In contrast, treatment with an intercalating dye (PMA
351 or PMAxx) reduced the PCR signal for each of the target viruses compared with PCR alone (Figure
352 3); for example, the signal reduction by PMA and PMAxx compared with PCR alone for AdV was
353 approximately 1-$\log_{10}$ and 2-$\log_{10}$, respectively. The signal reductions obtained with PMAxx
354 treatment without the enhancer were larger than those obtained with PMA treatment without the
355 enhancer for AdV and CV, regardless of intercalating dye concentration. When the intercalating dye
356 enhancer was used, it improved the performance of the intercalating dyes, with the combination of
357 PMAxx and Enhancer showing the greatest signal reduction ($\geq$3-$\log_{10}$) for AdV and CV compared
358 with the other three treatments. These findings are consistent with those of Randazzo *et al.* (2016,
359 2018), although the virus type and condition differed between our study and theirs (AdV and CV vs.
360 human norovirus and hepatitis A virus, extracted viral DNA/RNA vs. heat-inactivated virus).

For PMMoV, approximately 2-$\log_{10}$ signal reductions were obtained for all of the treatments (Figure 3c). In addition, the signal reduction performance was not improved even when the initial concentration of the RNA extracted from PMMoV was reduced from $10^{9–10}$ to $10^{7–8}$ copies/mL, which is the concentration corresponding to that of the RNA extracted from CV (data not shown). When plasmid DNA containing the PCR target sequence of PMMoV was treated with 200 μM of intercalating dye, only a slight improvement of the signal reduction by PMAxx-Enhancer treatment was observed compared with PMAxx treatment alone. In addition, the signal reduction obtained with PMAxx-Enhancer treatment remained approximately 2-$\log_{10}$ regardless of the initial concentration of the plasmid DNA (Figure S1c), which is comparable with the data obtained using RNA extracted from PMMoV. In contrast, ≥3-$\log_{10}$ signal reductions by PMAxx-Enhancer treatment were observed for plasmid DNAs containing the PCR target sequence of AdV or CV (Figure S1a,b); these findings are comparable to the data obtained with viral DNA and RNA extracted from these viruses. These results indicate that the effectiveness of the intercalating dye treatment depends on the virus type and, more specifically, on the nucleotide sequence targeted by the PCR analysis; these differences may be more important than whether the genetic material is DNA or RNA or the size of the nucleotide sequence, because the signal reduction performance of PMAxx-Enhancer treatment was comparable between the viral DNA extracted from AdV and the viral RNA extracted from CV, as described above, and no marked difference in the size of the viral RNA and the PCR target between CV and PMMoV was observed (see Supplementary Information).

Of the different viability PCR approaches examined, we found that PMAxx-Enhancer-PCR

provided the greatest PCR signal reduction for AdV and CV, and comparable signal reductions were observed for all the treatments with PMMoV. In addition, no large differences in the signal reduction performances were observed for PMAxx-Enhancer treatment within the PMAxx concentration range of 50 to 500 μM. Based on these results, in the following inactivation experiments we used PMAxx-PCR and PMAxx-Enhancer-PCR with 200 μM of PMAxx.

3.4. Application of PMAxx-PCR and PMAxx-Enhancer-PCR to heat-inactivated virus

Next, we evaluated whether PMAxx-PCR and PMAxx-Enhancer-PCR could distinguish between infectious and heat-inactivated virus. Because PMAxx and PMAxx-Enhancer treatments may themselves have virucidal activity, we first evaluated the effect of PMAxx-Enhancer treatment on virus infectivity (Figure S2). Limited inactivation ($\leq$0.4-$\log_{10}$) of AdV, CV, or PMMoV, as evaluated by infectivity assay, was observed when infectious AdV, CV, or PMMoV were exposed to the combination of PMAxx (final concentration, 200 μM) and PMA Enhancer for 10 min with subsequent photo-activation for 15 min, indicating that virus inactivation by PMAxx treatment and PMAxx-Enhancer treatment was negligible for AdV, CV, and PMMoV.

Figure 4 shows the performance of PCR alone, PMAxx-PCR, and PMAxx-Enhancer-PCR to discriminate between infectious and heat-inactivated viruses. For AdV, although a reduction in infectivity was observed at ≥60 ºC (see section 3.1), PCR alone showed almost no reduction of the PCR signal at any of the temperatures tested (Figure 4a). Similarly, Leifels *et al.* (2015) reported a >4-$\log_{10}$ inactivation of AdV type 5 after heat treatment at 65 ºC for 10 min, as evaluated by $TCID_{50}$

401 assay, but no reduction of the PCR signal. Thus, these results indicate that PCR alone is unable to
402 discriminate between infectious and heat-inactivated AdV. In contrast, PMAxx-PCR and
403 PMAxx-Enhancer-PCR showed signal reductions consistent with the observed reduction in
404 infectivity at ≥60 ºC: 2.2–2.7-$\log_{10}$ and 2.4–3.7-$\log_{10}$ reductions were observed for PMAxx-PCR
405 and PMAxx-Enhancer-PCR, respectively. Similar trends were observed for CV and PMMoV
406 (Figure 4b,c). These results indicate that PMAxx enters heat-inactivated viruses and binds to the
407 viral DNA or RNA, and that heat treatment inactivates these viruses by damaging the viral capsid
408 (i.e., altering the viral capsid proteins and compromising capsid integrity) rather than by degrading
409 the viral genetic material; therefore, these data are consistent with the current understanding of the
410 viral component affected by this disinfection process (Wigginton *et al.*, 2012; Torrey *et al.*, 2019).
411 The signal reductions observed for PMAxx-Enhancer-PCR tended to be larger than those
412 observed for PMAxx-PCR, indicating that the enhancer increased the penetration performance of
413 the intercalating dye; in addition, the magnitude of the signal reduction was comparable with the
414 magnitude of the reduction in infectivity, regardless of the virus type (Figure 4). These results
415 indicate that PMAxx-Enhancer-PCR is more able than PMAxx-PCR to discriminate between
416 infectious and heat-inactivated viruses. Thus, PMAxx-Enhancer-PCR is a potential alternative to
417 the infectivity assays used in the present study for evaluating virus inactivation by heat treatment.
418
419 3.5. Application of PMAxx-PCR and PMAxx-Enhancer-PCR to chlorine-inactivated virus
420 Figure 5 shows the performance of PCR alone, PMAxx-PCR, and PMAxx-Enhancer-PCR to

421 discriminate between infectious and chlorine-inactivated viruses. For AdV, although >3.4-$\log_{10}$
422 inactivation was observed immediately after the start of the disinfection process (within a contact
423 time of 5 s at 0.5 mg-Cl_2/L; see section 3.2), PCR alone showed a signal reduction of only
424 <0.3-$\log_{10}$ for all of the contact times tested (5 s–10 min; Figure 5a). Leifels *et al.* (2015) reported
425 >4-$\log_{10}$ inactivation of AdV type 5, as evaluated by PFU assay, after chlorine treatment (initial
426 free-chlorine concentration, 2 mg-Cl_2/L; contact time, 1 min), and only a 0.3-$\log_{10}$ reduction of the
427 PCR signal; these previous findings are consistent with our present results. Even when
428 PMAxx-PCR and PMAxx-Enhancer-PCR were used, almost no signal reductions were observed
429 within a contact time of 1 min. Similar trends were observed for CV (initial free-chlorine
430 concentration, 0.1 or 0.5 mg-Cl_2/L; signal reduction <0.9-$\log_{10}$ within a contact time of 10 min or 2
431 min, respectively; Figure 5b,c) and PMMoV (signal reduction <1.1-$\log_{10}$ within a contact time of
432 360 min; Figure 5d). Together, these results indicate that PMAxx-PCR and PMAxx-Enhancer-PCR
433 were unable to discriminate between infectious and chlorine-inactivated viruses at low CT values
434 (e.g., during the initial stages of chlorine treatment). Because PMAxx was unable to penetrate the
435 capsid of chlorine-inactivated viruses, virus inactivation by chlorine treatment at low CT values
436 probably is mainly due to subtle alterations of the viral capsid.

437 Furthermore, unlike PCR alone, which showed <0.3-$\log_{10}$ signal reduction for AdV at all of the
438 contact times tested, PMAxx-PCR and PMAxx-Enhancer-PCR showed signal reductions at a
439 contact of 2 min or greater, and the magnitudes of the signal reductions were increased with
440 increasing contact time (Figure 5a). These results indicate that chlorine treatment at a high enough

441 CT value to inactivate AdV caused damage to the viral capsid such that PMAxx could penetrate into
442 the virus but not damage the viral DNA, and that damage to the capsid was increased with
443 increasing contact time (i.e., increasing CT value). Similarly, PMAxx-PCR and
444 PMAxx-Enhancer-PCR showed signal reductions for CV from a contact time of 15 min when the
445 initial free-chlorine concentration was 0.1 mg-Cl_2/L and a contact time of 3 min when the initial
446 free-chlorine concentration was 0.5 mg-Cl_2/L (Figure 5b,c), and for PMMoV from a contact time of
447 720 min (Figure 5d). In addition, the magnitudes of the signal reductions increased with increasing
448 contact time, as was found for AdV. Moreover, even when PCR alone was used, signal reductions
449 were observed for CV and PMMoV, unlike in the case of AdV, and the magnitudes of the signal
450 reductions were also increased with increasing contact time. These results indicate that chlorine
451 treatment at a high CT value results in inactivation of CV and PMMoV not only by damaging the
452 viral capsid but also by damaging the RNA, including the part targeted by PCR, and both types of
453 damage increased with increasing contact time. Thus, the degree of damage of the genetic material
454 by chlorine treatment was different between the representative DNA virus (i.e., AdV) and the two
455 representative RNA viruses (i.e., CV and PMMoV), suggesting that the resistance of viral DNA
456 against chlorine treatment was higher than that of viral RNA; these results are consistent with the
457 observations of Leifels *et al.* (2015).

458 The magnitudes of the signal reductions observed with PMAxx-PCR and PMAxx-Enhancer-PCR
459 were larger than those observed with PCR alone for chlorine-inactivated AdV, CV, and PMMoV. In
460 addition, the magnitudes of the signal reductions observed with PMAxx-Enhancer-PCR tended to

461 be comparable or somewhat larger than those observed with PMAxx-PCR (Figure 5). These results
462 suggest that PMAxx-Enhancer-PCR performs better than PCR alone and probably better than
463 PMAxx-PCR for discrimination between infectious and chlorine-inactivated virus. However, the
464 magnitudes of the signal reductions observed with PMAxx-Enhancer-PCR were still smaller than
465 those of the reduction in infectivity in most cases of chlorine treatment (Figure 5). This indicates
466 that infectious and chlorine-inactivated viruses cannot be fully discriminated even when
467 PMAxx-Enhancer-PCR is used, and that even PMAxx-Enhancer-PCR underestimates the efficacy
468 of virus inactivation by chlorine treatment (i.e., it overestimates infectious virus concentration after
469 chlorine treatment). Although the specific mechanisms that lead to virus inactivation by chlorine
470 treatment is still under discussion, it is generally considered to be a result of not only the loss of
471 capsid integrity but also the loss of virus-specific functions, such as host attachment, genome
472 internalization, and genome replication (Wigginton *et al.*, 2012; Torrey *et al.*, 2019). Because the
473 performance of PMAxx-Enhancer-PCR relies on the integrity of the viral capsid, completely
474 distinguishing between infectious and chlorine-inactivated viruses will be difficult. To optimize the
475 performance of PMAxx-Enhancer-PCR, we also examined the effects of incubation time and light
476 exposure time on performance, which are two important factors that are known to affect the
477 performance of PMA-PCR (Fittipaldi *et al.*, 2012), Unfortunately, we found that increasing
478 incubation time did not provide any additional improvement on the performance of
479 PMAxx-Enhancer-PCR, and increasing the light exposure time produced a non-negligible increase
480 in virucidal activity (data not shown). Thus, although PMAxx-Enhancer-PCR results in

481 overestimation of infectious virus concentration (i.e., the magnitude of the signal reduction
482 observed with PMAxx-Enhancer-PCR is not comparable with the magnitude of the reduction in
483 infectivity), particularly after chlorine treatment, this method still provides a more accurate
484 assessment of the efficacy of virus inactivation by disinfection processes compared with PCR alone,
485 conventional PMA-PCR with or without enhancer, and PMAxx-PCR. Nevertheless, further
486 investigation is needed to improve the ability of viability PCR, including PMAxx-Enhancer-PCR,
487 to provide accurate validation of disinfection processes for virus inactivation.
488
489
490 **4. Conclusions**
491 (1) PMMoV showed higher resistance to heat treatment than AdV and CV. Thus, PMMoV appears
492 to be a potential surrogate for human enteric viruses for the assessment of the efficacy of heat
493 treatment to inactivate viruses.
494 (2) PMMoV showed higher resistance to chlorine treatment than AdV and CV, but the resistance of
495 PMMoV was markedly higher than that of CV, which is a chlorine-resistant virus. Thus,
496 PMMoV does not appear to be useful as a surrogate for human enteric viruses for the
497 assessment of the efficacy of chlorine treatment to inactivate viruses.
498 (3) Because the magnitudes of the signal reductions observed with PMAxx-Enhancer-PCR were
499 comparable to the magnitudes of the reductions in infectivity in the case of heat treatment,
500 PMAxx-Enhancer-PCR appears to be a suitable alternative to infectivity assay for evaluating

the inactivation of viruses by heat treatment.

(4) The magnitudes of the signal reductions observed with PMAxx-Enhancer-PCR were smaller than the magnitudes of reductions in infectivity in most cases of chlorine treatment. Thus, PMAxx-Enhancer-PCR likely underestimates the efficacy of virus inactivation (i.e., overestimates the infectious virus concentration) by chlorine treatment.

(5) Although PMAxx-Enhancer-PCR likely overestimates infectious virus concentration, particularly after chlorine treatment, this method still provides a more accurate assessment of the efficacy of virus inactivation by the two disinfection processes examined compared with PCR alone, conventional PMA-PCR with or without enhancer, and PMAxx-PCR.

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Table 1. CT value ranges observed from the chlorine treatment experiments and CT values calculated by using the efficiency factor Hom (EFH) model for 2-, 3-, and 4-log_{10} inactivation.

Viruses	Initial free chlorine concentration (mg-Cl_2/L)	CT value [a] (mg-Cl_2 · min/L)					
		2-log_{10} inactivation		3-log_{10} inactivation		4-log_{10} inactivation	
		Observed	EFH model	Observed	EFH model	Observed	EFH model
Adenovirus	0.5	<0.04	ND [b]	<0.04	ND	ND	ND
Coxsackievirus	0.1	>0.41 to <0.76	0.71	>0.76 to <1.09	0.95	>1.09	1.19
	0.5	>0.48 to <0.94	0.73	>0.94 to <1.40	0.97	>0.94 to <1.40	1.15
Pepper mild mottle virus	0.5	>21.4 to <57.1	51.2	>57.1 to <74.8	71.4	>74.8 to <92.1	84.5

[a] CT value, free-chlorine concentration [C] multiplied by contact time [T], i.e., the integration of the residual free-chlorine concentration until a given contact time.

[b] ND, not determined.

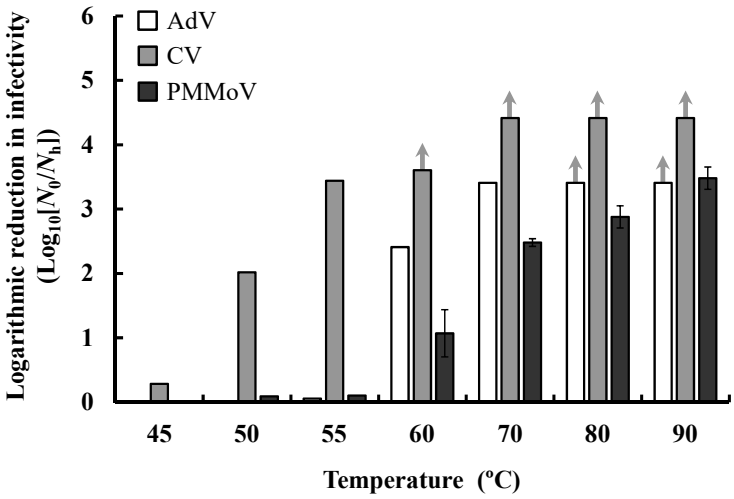
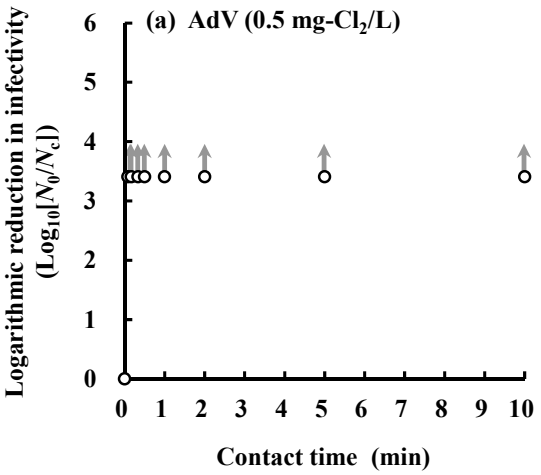
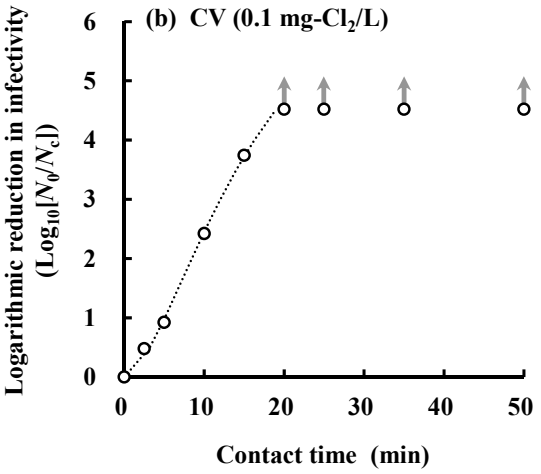
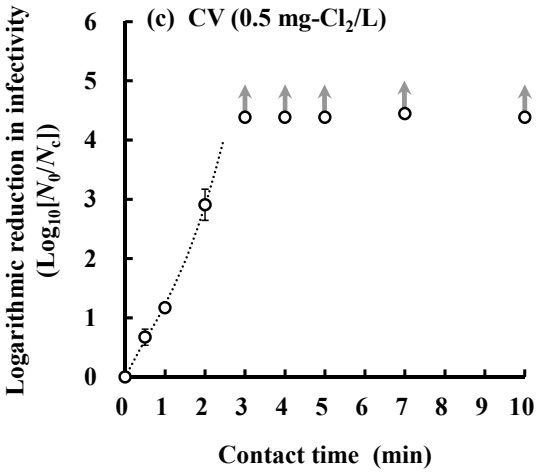


Figure 1. Inactivation of adenovirus type 40 (AdV), coxsackievirus B5 (CV), or pepper mild mottle virus (PMMoV) by heat treatment for 5 min, as evaluated by infectivity assay. Values were determined from a single experiment or are the means of duplicate experiments; error bars indicate standard deviations. Arrows indicate that the infectious virus concentrations were below the limit of quantification of the infectivity assay.


(a) AdV (0.5 mg-Cl2/L)
Logarithmic reduction in infectivity (Log10[N0/Nc])
Contact time (min)


(b) CV (0.1 mg-Cl2/L)
Logarithmic reduction in infectivity (Log10[N0/Nc])
Contact time (min)


(c) CV (0.5 mg-Cl2/L)
Logarithmic reduction in infectivity (Log10[N0/Nc])
Contact time (min)

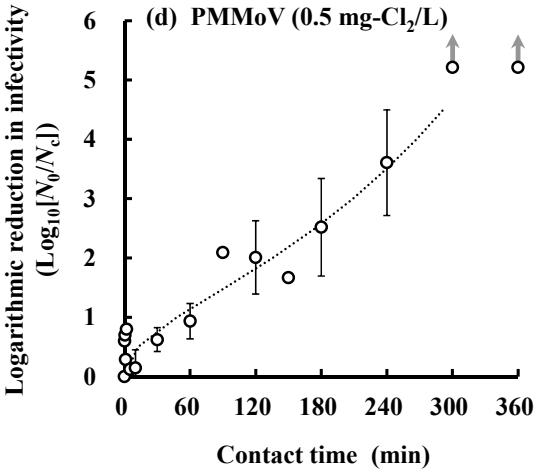

(d) PMMoV (0.5 mg-Cl2/L)
Logarithmic reduction in infectivity (Log10[N0/Nc])
Contact time (min)

Figure 2. Inactivation of adenovirus type 40 (AdV) (a), coxsackievirus B5 (CV) (b,c), or pepper mild mottle virus (PMMoV) (d) by chlorine treatment, as evaluated by infectivity assay. Values are means and error bars indicate standard deviations (n = 1–3). Dotted lines indicate the fit of the efficiency factor Hom model to the observed virus inactivation curves. Arrows indicate that the infectious virus concentrations were below the limit of quantification of the infectivity assay.

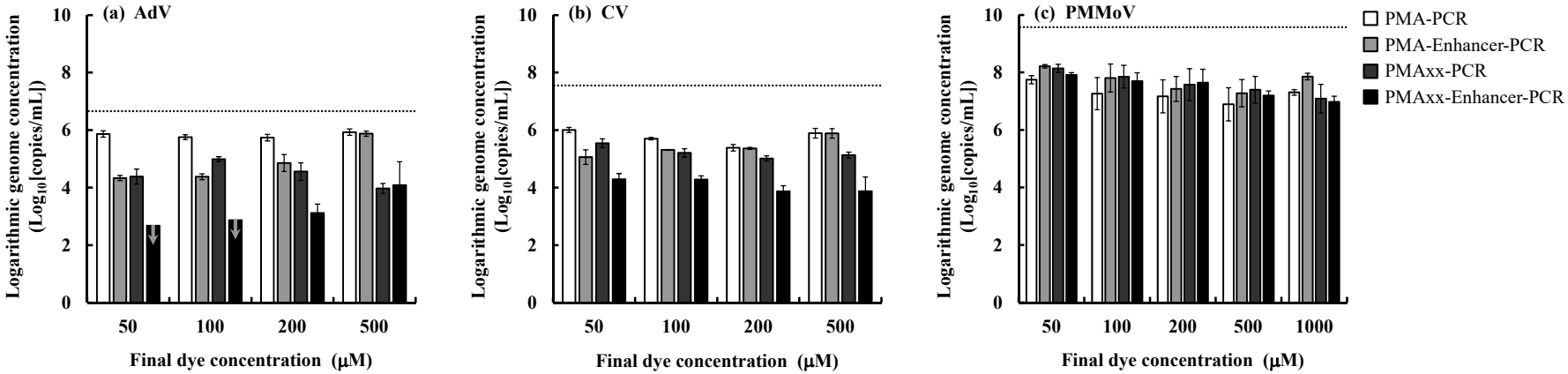


Figure 3. Effects of type and concentration of intercalating dye, and the effect of using an intercalating dye enhancer, on PCR amplification of viral DNA extracted from adenovirus type 40 (AdV) (a), or viral RNA extracted from coxsackievirus B5 (CV) (b) or pepper mild mottle virus (PMMoV) (c). Values are means and error bars indicate standard deviations (n = 3–6). Dotted lines indicate the concentration of extracted viral DNA/RNA determined by PCR alone. Arrows indicate that the extracted viral DNA/RNA concentrations were below the limit of quantification of the PCR.

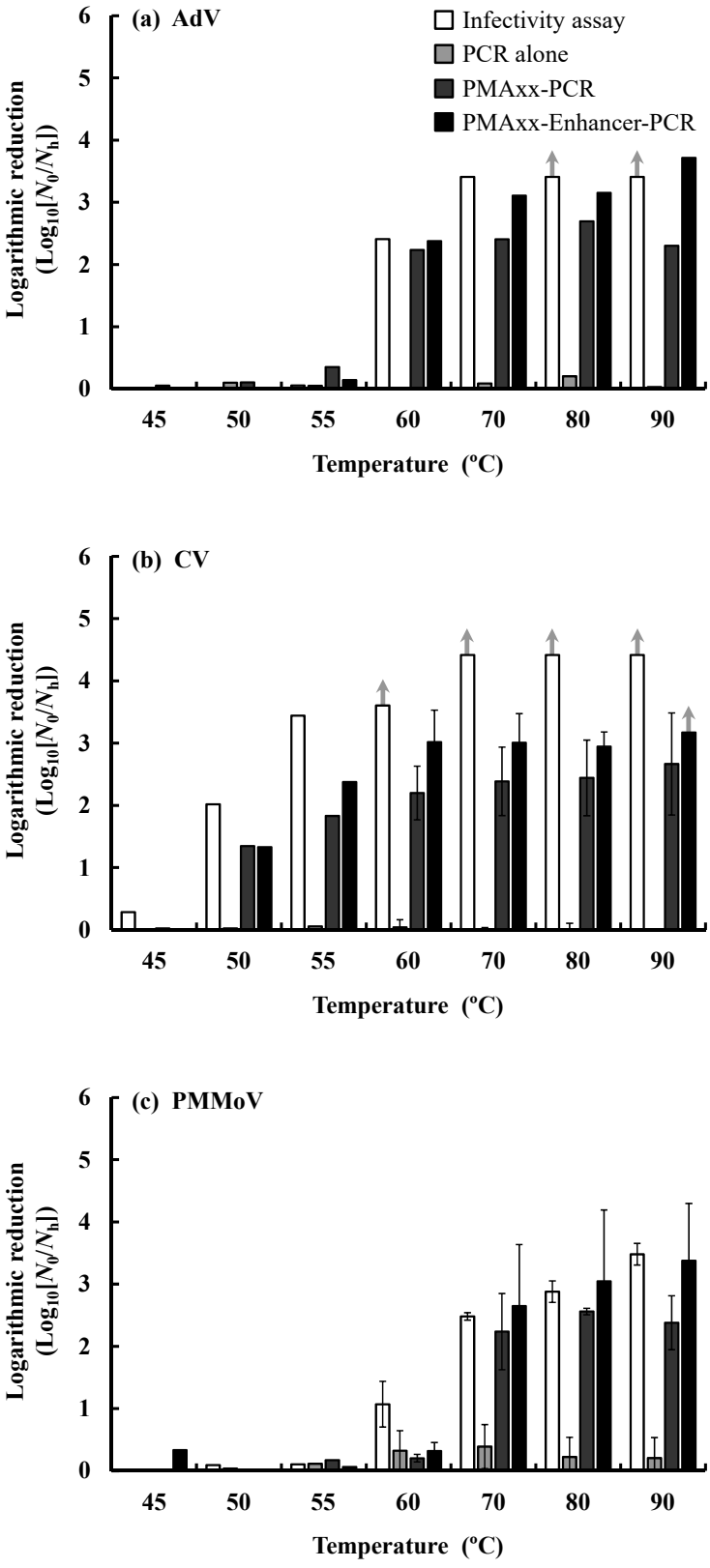
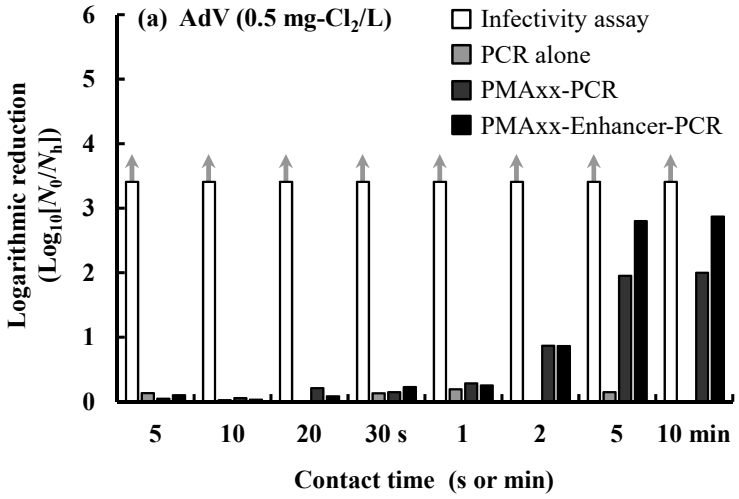
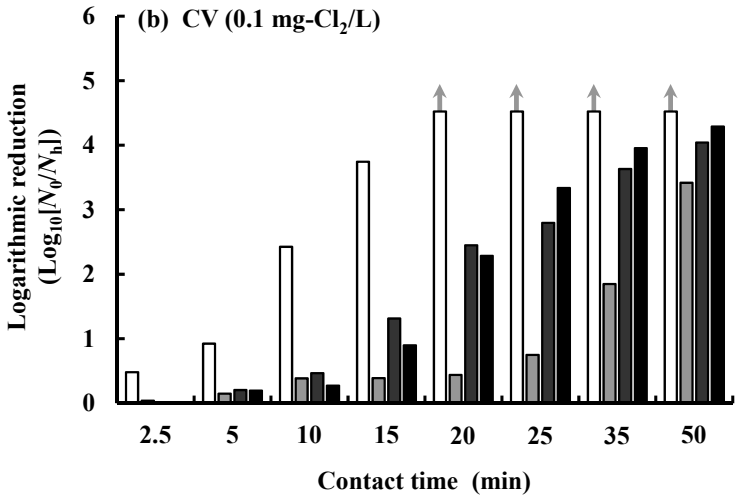
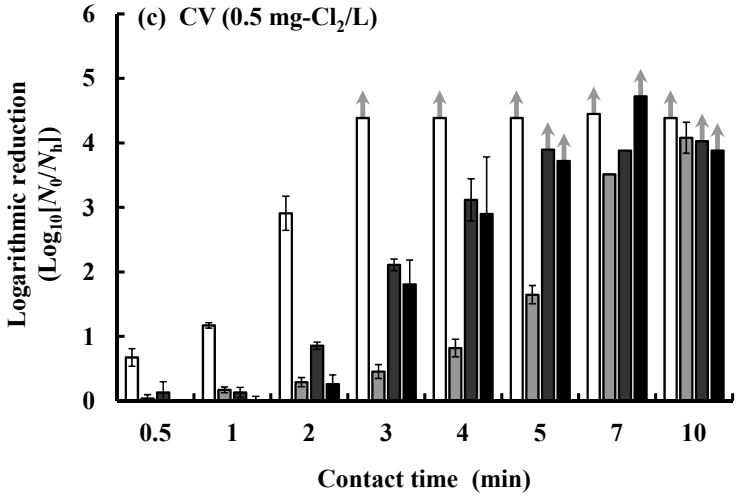

(a) AdV
Infectivity assay
PCR alone
PMAxx-PCR
PMAxx-Enhancer-PCR
Logarithmic reduction
(Log10[N0/Nh])
0
1
2
3
4
5
6
45
50
55
60
70
80
90
Temperature (ºC)
(b) CV
Logarithmic reduction
(Log10[N0/Nh])
Temperature (ºC)
(c) PMMoV
Logarithmic reduction
(Log10[N0/Nh])
Temperature (ºC)

Figure 4. Comparison of the ability of PCR alone, PMAxx-PCR, or PMAxx-Enhancer-PCR to assess the efficacy of inactivation of adenovirus type 40 (AdV) (a), coxsackievirus B5 (CV) (b), or pepper mild mottle virus (PMMoV) (c) by heat treatment, as evaluated by infectivity assay. Values were determined from a single experiment or are the means of duplicate experiments; error bars indicate standard deviations. Arrows indicate that the virus concentrations were below the limit of quantification of the infectivity assay or PCR.


(a) AdV (0.5 mg-Cl2/L)
Infectivity assay
PCR alone
PMAxx-PCR
PMAxx-Enhancer-PCR
Logarithmic reduction (Log10[N0/Nt])
0
1
2
3
4
5
6
5
10
20
30 s
1
2
5
10 min
Contact time (s or min)


(b) CV (0.1 mg-Cl2/L)
Logarithmic reduction (Log10[N0/Nt])
0
1
2
3
4
5
6
2.5
5
10
15
20
25
35
50
Contact time (min)


(c) CV (0.5 mg-Cl2/L)
Logarithmic reduction (Log10[N0/Nt])
0
1
2
3
4
5
6
0.5
1
2
3
4
5
7
10
Contact time (min)

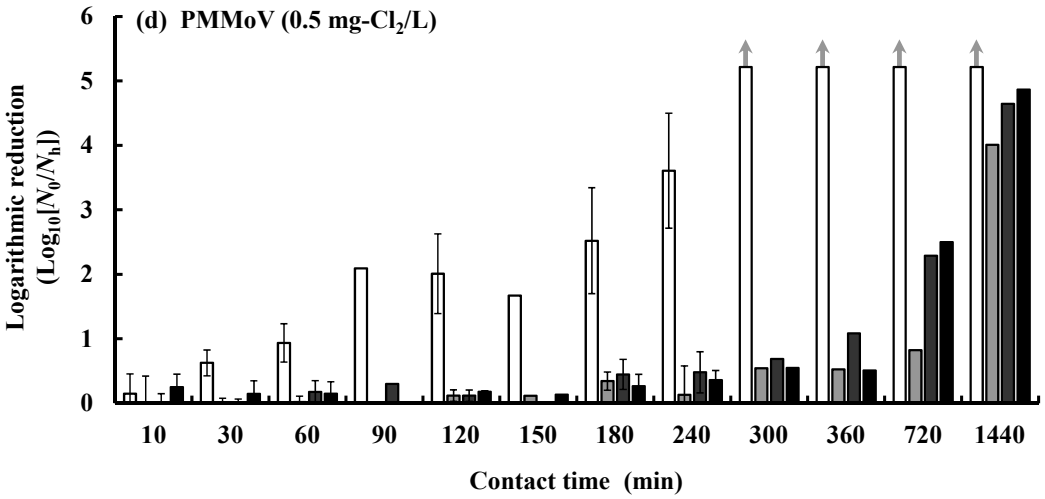


Figure 5. Comparison of the ability of PCR alone, PMAxx-PCR, or PMAxx-Enhancer-PCR to assess the efficacy of inactivation of adenovirus type 40 (AdV) (a), coxsackievirus B5 (CV) (b,c) or pepper mild mottle virus (PMMoV) (d) by chlorine treatment, as evaluated by infectivity assay. Values are means and error bars indicate standard deviations (n = 1–3). Arrows indicate that the virus concentrations were below the limit of quantification of the infectivity assay or PCR.