Quantitative detection of human enteric adenoviruses in river water by microfluidic digital polymerase chain reaction

Naohiro Kishida, Naohiro Noda, Eiji Haramoto, Mamoru Kawaharasaki, Michihiro Akiba and Yuji Sekiguchi

ABSTRACT

We describe an assay for simple and accurate quantification of human enteric adenoviruses (EAdVs) in water samples using a recently developed quantification method named microfluidic digital polymerase chain reaction (dPCR). The assay is based on automatic distribution of reaction mixture into a large number of nanolitre-volume reaction chambers and absolute copy number quantification from the number of chambers containing amplification products on the basis of Poisson statistics. This assay allows absolute quantification of target genes without the use of standard DNA. Concentrations of EAdVs in Japanese river water samples were successfully quantified by the developed dPCR assay. The EAdVs were detected in seven of the 10 samples (1 L each), and the concentration ranged from 420 to 2,700 copies/L. The quantified values closely resemble those by most probable number (MPN)-PCR and real-time PCR when standard DNA was validated by dPCR whereas they varied substantially when the standard was not validated. Accuracy and sensitivity of the dPCR was higher than those of real-time PCR and MPN-PCR. To our knowledge, this is the first study that has successfully quantified enteric viruses in river water using dPCR. This method will contribute to better understanding of existence of viruses in water.

Key words | absolute quantification, digital PCR, gastroenteritis, real-time PCR, waterborne infectious diseases

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INTRODUCTION

The accurate quantification of pathogenic microorganisms in water is crucial for estimating and controlling health risk of waterborne infectious diseases. Real-time polymerase chain reaction (PCR) is widely used for quantifying microorganisms in water (Rajal *et al.* 2007; Wyn-Jones *et al.* 2011; Kishida *et al.* 2012a). However, the quantified value by real-time PCR is unstable if the copy number of target genes in the reaction mixture is small, because the effect of annealing error at the beginning of the PCR cycle (PCR drift) on the amplification efficiency is high (Wagner *et al.* 1994). Although it is possible to increase copy number by concentrating microorganisms in water by filtering or centrifugation, excess concentration increases inhibitory substances for PCR, such as humic acids, fulvic acids and polysaccharides, which are typically co-extracted with target DNA from natural water samples (Hata *et al.* 2011). Due to the inhibitory effect, the concentration of microorganisms in water is often underestimated (Hamza *et al.* 2009; Hata *et al.* 2011). If copy number is small, and inhibitory effect is high, estimated quantified value can be less than one copy. This phenomenon sometimes occurred in our previous experiments (data not shown). In addition, accurate preparation of copy number of standard DNA is generally difficult, which increases quantification error.

In this study, we focused our attention on a recently reported novel molecular method for the quantification of nucleic acid sequences named microfluidic digital PCR (dPCR) (Dube *et al.* 2008; Spurgeon *et al.* 2008) as an improved method for the quantification of microorganisms in water. The microfluidic dPCR is a new technology that allows absolute quantification of target genes without the use of standard DNA. As shown in Figure 1, the reaction

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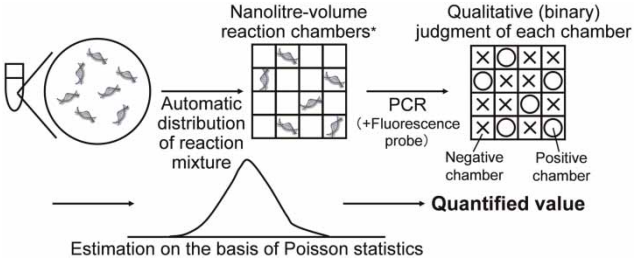

Figure 1 | Schematic presentation of microfluidic dPCR for estimating the target DNA without the use of standard DNA. (*The number of reaction chambers is much larger in an actual device.)

mixture for PCR is automatically distributed and partitioned into a large number of nanolitre-volume reaction chambers. After conducting PCR in the chambers, the amount of target DNA is estimated from the number of chambers containing amplification products on the basis of Poisson statistics, which can predict how input DNA is distributed into all small chambers (Dube *et al*. 2008). This method provides binary output because the PCR reaction in each nanolitre-volume chamber is either positive or negative, and the quantified copy number is independent of the PCR amplification efficiency (Hoshino & Inagaki 2012). Therefore, this method can accurately and simply quantify microorganisms even if inhibitory substances exist in the reaction mixture (Hoshino & Inagaki 2012). However, this method has rarely been applied to quantification of pathogenic microorganisms in water.

In the present study, we developed a microfluidic dPCR assay for accurate quantification of human enteric adenoviruses (EAdVs) as representative microorganisms, and applied the assay to the detection in water samples collected from a Japanese river. To investigate validity of the microfluidic dPCR, conventional real-time PCR assay and most probable number (MPN)-PCR assay were also performed, and the quantification data for the three techniques were compared.

MATERIALS AND METHODS

Collection of river water samples, virus concentration and DNA extraction

In total, 10 river water samples were collected from one sampling site in the Tone River in Japan, from October 2011 to March 2012. The Tone River has a total length of 322 km and a catchment area of 16,840 km^2, with approximately 800 tributaries. The sampling site was located on the right bank at the Tone Diversion Weir, where the surface water is utilized for the production of drinking water for the Tokyo Metropolitan Area. Over 2 million people live in the upper river basin of the sampling site, and there are many wastewater treatment plants and private septic tanks in the area. Enteric AdVs have been frequently detected at this sampling site in a previous study by us (Kishida *et al*. 2012b).

One litre of sampled river water was concentrated to approximately 0.6 mL using electronegative and ultrafiltration membranes as described previously (Haramoto *et al*. 2005). For detection of EAdVs, 200 μL of the concentrated sample was subjected to DNA extraction using a QIAamp DNA mini kit (Qiagen, Tokyo, Japan) to obtain 200 μL of the resulting DNA.

Quantification of EAdVs by dPCR, real-time PCR and MPN-PCR

The primer pairs and the TaqMan probe used for dPCR, real-time PCR and MPN-PCR were designed to amplify adenovirus stereotypes 40 and 41 (Ko *et al*. 2005). The dPCR was performed using the BioMark Real-time System and 12.765 Digital Array, which consisted of 12 panels containing 765 reaction chambers each (Fluidigm Corporation, San Francisco, USA). Aliquots of 1.15 μL of extracted DNA solution were mixed with 3.45 μL of reaction buffer containing 2.3 μL of TaqMan Gene Expression Master Mix (Life Technologies Japan, Tokyo, Japan), 900 nM of each primer, and 250 nM of TaqMan probe. The reaction mixtures were incubated at 50 °C for 2 min and 95 °C for 10 min followed by 45 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. After amplification, Digital PCR Analysis Software (Fluidigm Corporation) was used to count the number of positive chambers. Finally, the software statistically estimated the absolute copy number of target DNA in panels using Poisson statistics. The theory and equations used in this study have been described elsewhere (Dube *et al*. 2008; Bhat *et al*. 2009). Briefly, the copy number of template DNA (M) is given by

$$M = -C \times \ln\left(1 - \frac{H}{C}\right)$$

where C is the number of all chambers in a panel (=765) and H is the number of positive chambers (Hoshino & Inagaki 2012). Because the molecules in the reaction mix are distributed in small chambers, dPCR quantifies the number of molecules instead of the copy number of genes estimated by real-time PCR. For example, a genome fragment

possessing three copies of the target gene in a reaction chamber should be quantified as one by digital PCR, whereas real-time PCR quantifies it as three (Hoshino & Inagaki 2012).

Real-time PCR and MPN-PCR were performed using LightCycler 480 System II (Roche Diagnostics, Tokyo, Japan). For real-time PCR, aliquots of 1.15 μL of extracted DNA solution were mixed with 18.85 μL of reaction buffer containing 10 μL of 2 × LightCycler 480 Probe Master (Roche Diagnostics), 400 nM of each primer, and 200 nM of TaqMan probe. The reaction mixtures were incubated at 95 °C for 10 min followed by 50 cycles of 95 °C for 10 s and 55 °C for 30 s. When fluorescence intensity is over the threshold value within 45 cycles, the sample was considered to be positive. Tenfold serial dilutions of chemically synthesized oligo-DNAs of adenovirus serotype 40 (Accession No.: X16583; position: 614-723) were used to make a standard curve. The concentration of the standard was calculated from the optical density measurement. Validation of concentration of standard DNA was also performed using dPCR. Namely, standard DNA with the concentration of 500 copies/reaction calculated from the optical density measurement was quantified using dPCR.

For MPN-PCR, DNA sample was manually diluted as described previously (Katayama *et al.* 2008). The reaction mixture composition and incubation condition of MPN-PCR were the same of those of real-time PCR except for template volume. To compare detection sensitivity of each PCR, the total volume of template DNA solution before dilution for MPN-PCR was set as 1.15 μL. Each PCR test was performed in triplicate.

RESULTS AND DISCUSSION

Validation of concentration of standard DNA using dPCR

Figure 2 shows binary results of each reaction chamber of dPCR. Positive and negative chambers were clearly divided. Therefore, the number of positive chambers was successfully counted, and copy number was quantified on the basis of Poisson statistics. The quantified values for standard DNA tested in triplicate resemble each other (78 ± 10 copies/reaction). However, these values were much less than the input copy number (500 copies/reaction). This result indicates that the copy number of standard DNA prepared by the optical density measurement and dilution was not accurate.

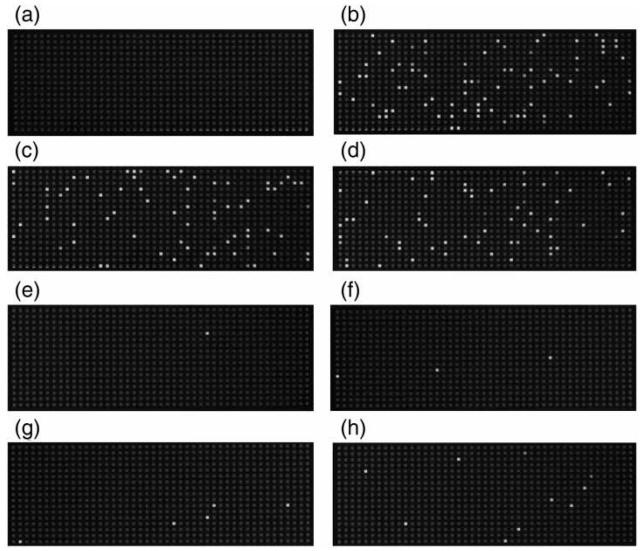

Figure 2 | Images of the digital PCR array after 45 cycles of PCR. Each panel contains 765 chambers, and white chambers are positive. (a) Negative control, (b)–(d) standard DNA ($n = 3$), (e)–(h) river water samples (examples).

Quantification of EAdV concentration in river water sample using dPCR

The EAdVs were successfully detected from river water samples using dPCR. As shown in Table 1, EAdVs were detected in seven of the 10 samples (70%). In this study, the water sampling was conducted only in the winter season when detection frequency of EAdVs is high. In fact, the detection efficiency is higher compared to a previous study conducted year-round which covered all seasons at the same sampling site (Kishida *et al.* 2012b).

Table 1 | Concentration of EAdVs in river water samples from the Tone River in Japan determined by microfluidic dPCR

	Concentration (copies/L) Test no. ($n = 3$)				
Sample no.	1	2	3	Mean	Standard deviation
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
4	420	420	420	420	0
5	1,000	1,500	1,500	1,300	290
6	460	920	1,400	920	460
7	1,300	2,500	4,000	2,700	1,500
8	440	880	1,800	1,000	670
9	440	1,300	1,300	1,000	510
10	1,600	1,600	2,000	1,700	230

Although the reason why detection efficiency is high in the winter season is not clear, a seasonal variation in the number of infected patients may be one of the reasons. According to limited clinical data reported to the National Institute of Infectious Diseases, Japan, the number of confirmed EAdVs (patients/surveillance site) was larger in the winter season in 2011/2012 compared with other seasons (National Institute of Infectious Diseases 2014). Hence, it is suggested that EAdVs were shed from humans and discharged into the water environment via wastewater treatment plants in this season, which may cause an increase in the concentration of EAdVs in river water. Another reason may be a seasonal variation in rainfall. Near the sampling site, the amount of rainfall was small in the winter season in 2011/2012 (Japan Meteorological Agency 2014), which decreases the water level of the river and the dilution effect by rainfall, and may cause an increase in the concentration of EAdVs in river water. Meanwhile, the first three samples (No. 1–3 in Table 1) were negative for EAdVs. This may be because the amount of rainfall near the sampling period was different. The amount of rainfall in the early winter season that the first three samples were obtained was larger than that in the late winter season when the other samples were obtained (Japan Meteorological Agency 2014). Dilution effect by rainfall would be higher in the early winter season, which decreased the concentration of EAdVs in river water.

Although river water samples were concentrated using electronegative and ultrafiltration membranes, the quantified copy number of each reaction was less than 10, as shown in Figure 2, because the EAdV concentration in river water sample is normally lower compared to those in sewage samples (Haramoto *et al.* 2007). Nevertheless, relatively stable quantified values were obtained. The mean concentration of each sample tested in triplicate ranged from 420 to 2,700 copies/L. This contaminant level is similar to that of the previous study conducted at another Japanese river (Haramoto *et al.* 2007).

Comparison of quantified data among dPCR, real-time PCR and MPN-PCR

Table 2 shows quantified data of EAdVs by dPCR, real-time PCR and MPN-PCR. Standard deviations of positive samples quantified by dPCR are lower than those by real-time PCR. This indicates that stable quantification data can be obtained using dPCR even when the target copy number in the reaction mixture is small. This is because dPCR provides binary output since the PCR reaction in

Table 2 | Concentration of EAdVs in river water samples using three different PCR methods (unit: copies/reaction; $n = 3$)

	dPCR		MPN-PCR		Real-time PCR			
					Adjustment[a]		Non-adjustment[b]	
Sample no.	Mean	S.D.[c]	Mean	S.D.[c]	Mean	S.D.[c]	Mean	S.D.[c]
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	1.0	0	1.9	1.6	0	0	0	0
5	2.7	0.58	4.0	3.4	3.7	3.0	23	19
6	2.0	1.0	0	0	1.2	0.42	7.7	2.7
7	6.3	3.5	6.2	2.7	3.7	3.9	24	25
8	2.3	1.5	4.7	2.7	2.2	2.0	14	13
9	2.3	1.2	2.1	1.8	1.5	0.66	9.7	4.2
10	4.3	0.58	3.7	0.94	1.7	1.2	11	7.6
Mean[d]	3.0	1.4	3.8	2.2	2.3	1.9	15	12
Detection frequency	70%		60%		60%		60%	

[a]Quantified values when the concentration of standard DNA was adjusted (validated) by dPCR.
[b]Quantified values when the concentration of standard DNA was not adjusted (validated) by dPCR.
[c]Standard deviation.
[d]Mean values of the positive samples.

each nanolitre-volume chamber is either positive or negative, and the quantified copy number is independent of the PCR amplification efficiency. In addition, the standard deviations quantified by dPCR are lower than those by MPN-PCR. This is because the handling error does not exist in the dPCR measurement. The reaction mixture is automatically distributed and partitioned into a large number of nanolitre-volume reaction chambers in dPCR whereas it is manually diluted and distributed in MPN-PCR.

The detection frequency of dPCR is a little higher than those of real-time PCR and MPN-PCR. This can be expected because amplification efficiency of dPCR is higher. Since the reaction chamber is very small, heat transfer of reaction mixture during PCR cycles would be efficiently completed. In a further study, the detection sensitivity could be thoroughly investigated by applying dPCR to quantification of microorganisms in a larger number of water samples.

The mean values of positive samples determined by real-time PCR are similar to those determined by dPCR and MPN-PCR when the standard DNA was adjusted (validated) by dPCR. However, they varied substantially when the standard was not adjusted as shown in Table 2. This strongly indicates that the standard DNA concentration calculated from the optical density measurement is not accurate. Generally, accurate preparation of standard DNA for real-time PCR is difficult and troublesome. Although the reaction time of dPCR and real-time PCR is almost the same, handling time considerably decreases in dPCR because accurate data can be obtained without the use of standard DNA.

CONCLUSIONS

Concentrations of EAdVs in Japanese river water samples were quantified by dPCR and other current detection techniques. The accuracy and sensitivity of the dPCR were higher than those of current detection techniques. Therefore, the dPCR can be applicable to quantify pathogenic microorganisms in aquatic environments in the cases where accuracy and sensitivity are more important than analytical costs. Accurate analytical data are strongly required when the data are used for regulation. In addition, dPCR measuring may be suitable for quantitative microbial risk assessment because accurate and sensitive data are required to increase the precision of the assessment.

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