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Fate of Liberated Viral RNA in Wastewater Determined by PCR

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The use of a PCR method to investigate the fate of liberated Qβ coliphage RNA in wastewater suggests that a positive PCR result indicates the recent presence of potentially viable virus in the sample and therefore increases the public health significance of the result.

PCR as an alternative method for detecting enteric viruses in environmental samples has been well documented as having advantages over cell culture in reducing the time of detection and having higher detection sensitivity (3, 4, 6). Since the target of the PCR assay is viral nucleic acid, the stability of released viral nucleic acid from lysed virus in wastewater would provide substantial information for the positive detection of virus by PCR in wastewater. That is, the positive PCR products should be amplified from the RNA of intact viruses or from liberated RNA of lysed viruses. We have previously reported a reverse transcriptase-PCR (RT-PCR) method for the detection of Qβ coliphage from night soil wastewater samples that uses the RT-PCR developed for FRNA coliphage (group III) primers (5). The RT-PCR method just mentioned was used in the present study to determine the fate of liberated Qβ coliphage RNA in wastewater samples, including raw domestic wastewater and activated sludge (AS). The experiments were conducted with raw domestic wastewater and AS with Qβ RNA as the model virus.

The raw domestic wastewater samples (collected from Mikawashima Wastewater Treatment Plant, Tokyo, Japan) were divided for autoclaving and filtering with a 0.45-μm-pore-size membrane filter. Four aliquots of 4.5-ml samples (autoclaved Milli Q water, autoclaved raw wastewater, 0.45-μm-pore-size membrane filtrate of raw wastewater, and untreated raw wastewater) in glass tubes were seeded with 0.5 ml of Qβ RNA stock to achieve an initial concentration of 9×10^2 or 3.6×10^4 PFU/reaction, depending on the experiment. Raw wastewater samples without addition of Qβ RNA were kept in a control tube. All of the samples were incubated in the dark at 20°C. The samples were removed periodically and diluted decimally with autoclaved Milli Q water and then assayed by RT-PCR. Two series of experiments were conducted.

FRNA coliphages have been found in abundance in domestic wastewater at concentrations of more than 10^2 PFU/ml (1, 2). To test whether the amplified RT-PCR products were from the Qβ RNA inoculated into the samples, all of the wastewater samples tested were assayed by RT-PCR without heat extraction. In all cases, no positive band of Qβ RNA was found in the raw wastewater samples without Qβ RNA inoculation. The results obtained from the experiment in series 1 with the initial concentration of inoculated Qβ RNA of 9.0×10^2 PFU/reaction indicated that the presence of the inoculated Qβ RNA in raw wastewater was not found within 4 h after inoculation, whereas Qβ RNA in autoclaved wastewater and autoclaved Milli Q water was seen up to 3 and 7 days, respectively.

To investigate whether inoculated Qβ RNA is still found 4 h after inoculation in raw wastewater, the experiments in series 2 with the initial concentration of Qβ RNA of 3.6×10^4 PFU/reaction were performed by periodically removing samples at 30 min, 1 h, and 3 h after inoculation. The results showed that the seeded Qβ RNA in raw domestic wastewater and filtered wastewater was not found after 30 and 60 min of incubation, respectively. However, seeded Qβ RNA in autoclaved wastewater and autoclaved Milli Q water was detected up to ≥88 days (Table 1). In all cases, the inoculated Qβ RNA in autoclaved samples existed longer than that in nonautoclaved samples. These results implied that liberated Qβ RNA in wastewater could disappear soon after being released from the Qβ capsids into wastewater, and the activities of microorganisms existing in wastewater seem to be involved in the disappearance of the Qβ RNA inoculated in the sample. The observation is adhered to in the study of Tsai et al. (7), who reported that poliovirus genomic RNA was not stable in nonsterilized seawater and viral RNA could be detected by the RT-PCR in filter-sterilized seawater over a longer period.

Experiments similar to those with raw domestic wastewater were done to determine the fate of the Qβ RNA in the AS sample. The Qβ RNA was inoculated in autoclaved, filtered, and unfiltered AS samples at an initial concentration of 1.7×10^4 PFU/reaction. The results showed that the seeded Qβ RNA in unfiltered AS was not detected after 1 h of incubation (Table 2), while Qβ RNA in autoclaved AS and filtered AS was still detected for at least 2 and 5 days of incubation, respectively. No positive band of Qβ RNA was found in the unfiltered AS samples without Qβ RNA inoculation that were kept as controls.

Because the disappearance of the Qβ RNA inoculated in wastewater could result from many factors, including degradation of RNA itself and inhibitors in the sample that might be present during incubation because of the activities of existing microorganisms, the potential of a false-negative result caused by the effect of these inhibitors was investigated by the reseeding of Qβ RNA in the samples which gave a negative PCR result. Before reseeding to achieve an initial concentration of 1.7×10^4 PFU/reaction, the AS samples were divided for filtration with a 0.2-μm-pore-size membrane. After being thoroughly mixed, samples were collected for RT-PCR assay. The remaining samples were kept in the dark at 20°C and then were collected again after 1 h of incubation. All seeded AS samples were used directly in the RT-PCR assay without heat extraction. The results showed positive bands of Qβ RNA in all

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TABLE 1. Fate of Qβ RNA in wastewater detected by PCR[a]

Incubation period	Detection of Qβ RNA dilution by PCR[b]									
	Autoclaved Milli Q water + Qβ RNA		Raw sewage		Raw sewage + Qβ RNA		Autoclaved sewage + Qβ RNA		Filtered sewage + Qβ RNA	
	10^0	10^{-1}	10^0	10^{-1}	10^0	10^{-1}	10^0	10^{-1}	10^0	10^{-1}
0	+	+	−	−	−	+	+	+	+	+
30 min	+	+	−	−	−	+	+	+	+	+
1 h	+	+	−	−	−	−	+	+	−	+
3 h	+	+	−	−	−	−	+	+	−	−
6 h	+	+	−	−	−	−	+	+	−	−
1 day	+	+	−	−	−	−	−	+	−	−
4 day	+	+	−	−	−	−	+	+	−	−
10 day	+	+	−	−	−	−	+	+	−	−
12 day	+	+	−	−	−	−	+	+	−	−
18 day	+	+	−	−	−	−	+	+	−	−

[a] Results are from experiment series 2 with an initial Qβ RNA concentration of 3.6×10^4 PFU/reaction.
[b] +, positive; −, negative.

TABLE 2. Fate of Qβ RNA in AS detected by PCR[a]

Incubation period	Detection of Qβ RNA dilution by PCR[b]							
	Raw AS		Raw AS + Qβ RNA		Autoclaved AS + Qβ RNA		Filtered AS + Qβ RNA	
	10^0	10^{-1}	10^0	10^{-1}	10^0	10^{-1}	10^0	10^{-1}
0	−	−	−	+	−	+	+	+
30 min	−	−	−	+	−	+	+	+
1 h	−	−	−	+	−	+	+	+
3 h	−	−	−	−	−	+	+	+
1 day	−	−	−	−	−	+	+	+
2 days	−	−	−	−	−	+	+	+
5 days	−	−	−	−	−	−	+	−
8 days	−	−	−	−	−	−	−	−
20 days	−	−	−	−	−	−	−	−

[a] Results are from experiment series 1 with an initial concentration of Qβ RNA of 1.7×10^4 PFU/reaction.
[b] +, positive; −, negative.

seeded samples at the initial time. However, after 1 h of incubation, the bands of Qβ RNA in unfiltered samples entirely disappeared, while those in filtered samples were still obviously seen (data not shown). These results could confirm that disappearance of the liberated Qβ RNA from the sample should be due to the degradation of RNA rather than the effect of inhibitors contained in samples.

The results of this study demonstrate that the liberated viral RNA in wastewater could disappear in few minutes after being released from the Qβ capsids into wastewater. The detection of viral RNA, particularly single-stranded RNA, in wastewater could reflect the existence of intact virus in sample and therefore increases the public health significance of a positive PCR result. This finding is paramount for the scientist or utility director who requires rapid results. While a positive PCR result still cannot definitely determine the presence of viable virus, the data provided here suggest that a positive PCR result indicates the recent presence of potentially viable virus.

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