Concentration and detection of SARS coronavirus in sewage from Xiao Tang Shan Hospital and the 309th Hospital

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Abstract

The transmission of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) is associated with close contact to SARS patients and droplet secretions of those patients. The finding of positive RT-PCR results from stools of SARS patients suggests that stools of SARS patients or sewage containing stools of patients could transmit SARS-CoV. We used a novel style of electropositive filter media particle to concentrate the SARS-CoV from the sewage of two hospitals receiving SARS patients in Beijing. We also used cell culture, RT-PCR and gene sequencing to detect and identify the viruses from sewage. No infectious SARS-CoV contamination was found in any of the samples collected, but the nucleic acid of SARS-CoV could be detected in the sewage from the two hospitals before disinfection. While the RNA was only detected in three samples from the 309th Hospital, the others were negative after disinfection. These findings provide strong evidence that SARS-CoV can be excreted through the stool/urine of patients into sewage system, thus making the sewage system a possible route of transmission.
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1. Introduction

Through 5 July 2003 over 8439 severe acute respiratory syndrome (SARS) cases and 812 SARS-related deaths were reported to the World Health Organization (WHO) from 32 countries. Most of these cases occurred after exposure to SARS patients in household or health care settings (Tsang et al., 2003; CDC, 2003; WHO, 2003a). A novel coronavirus from SARS patients was isolated and identified (Rota et al., 2003; Holmes, 2003; Fouchier et al., 2003; Ksiazek et al., 2003; Marra et al., 2003; Qin et al., 2003; Enserink and Vogel, 2003).

Investigations of the global outbreak of SARS have shown that the major mode of transmission of the SARS virus is through close personal contact, in particular, exposure to droplets of respiratory secretions from an infected person (Tsang et al., 2003; Lee et al., 2003; WHO, 2003b; Cyranoski and Abbott, 2003; Poutanen et al., 2003; Donnelly et al., 2003). Sewage is thought to have played a role in spreading the infection among a cluster of SARS cases in an apartment block in Hong Kong (WHO, 2003b;

Cyranoski and Abbott, 2003). However, there is no direct evidence to prove that the coronavirus exists in the sewage system and is contagious.

In order to confirm whether sewage is a possible major route of transmission of SARS-CoV, a novel style of electropositive filter media particle (Li et al., 1998) was used to concentrate the SARS-CoV from the sewage of hospitals receiving SARS patients in Beijing, China. Cell culture and RT-PCR were utilized to detect and identify the viruses from sewage.

2. Materials and methods

2.1. Viruses and the culture methods

Bacteriophage f_2 (f_2) was used as a model for the coronavirus that may be present in sewage. The f_2 was prepared and detected according to the methods described by Wommack et al. (1995). To identify viruses in sewage, a variety of specimens (sewage before or after disinfection by chlorine) were inoculated onto Vero E6. All cell cultures were grown in Eagle's growth medium (Difco Laboratories, Detroit, MI, USA) containing 8% fetal bovine serum (FBS), 0.015 M DMEM buffer and antibiotics (50 μg [each] of kanamycin and gentamycin per milliliter), and maintained in the same medium with 1.5% FBS. For virus propagation and isolation, cell cultures in 75-cm^2 flasks were drained of medium, inoculated with 2 ml of sample, and incubated for 2 h at 37 °C with periodic rocking for viral adsorption. Because of the toxicity of most sewage, all cell cultures were inoculated in the presence of growth medium. Medium was replaced after 1–2 days of incubation. The culture was observed daily for cytopathic effect and terminated 7 days after inoculation. Any cultures exhibiting an identifiable cytopathic effect were subjected to several procedures to identify the cause of the effect (WHO, 2003c,d,e). If there was no cytopathic effect in the cell culture, the culture suspensate was harvested and transferred into additional flasks to isolate viruses. The cultures were used for three generations without any observable cytopathic effect.

2.2. Sewage and disinfection

Hospital sewage was collected daily at 7:00 a.m. from Xiao Tang Shan Hospital and the 309th Hospital of PLA, specially assigned to receive SARS patients in Beijing. Domestic sewage was collected from a housing estate in Fengtai district of Beijing City. Disinfection of the sewage is by chlorine, however, a 2500 ml sample was collected prior to disinfection and a 25,000–50,000 ml sample was collected after disinfection.

2.3. Electropositive filter media particle

The positively charged filter media particle was prepared as previously described by Li et al. (1998). In summary, 57 ml of a 4 mol/l Na_2CO_3 solution was slowly added to 1260 ml of 0.025 mol/l $AlCl_3$ to form a precipitate and then adjusted to pH 7.2; 120 g of silica gel was added to 88 ml of $Al(OH)_3$ precipitate, mixed well, and then the silica gel was dried at 60 °C for 36 h or longer.

2.4. Detection of residual chlorine

The residual chlorine and chlorine dioxide concentrations were measured by N,N-diethyl-p-phenylenediamine colorimetric method (DPD method). For chlorine dioxide analysis, glycine was added to mask interferences (American Public Health Association, 1980).

2.5. Concentration experiments

2.5.1. Evaluation of concentration efficiency of f_2 and SARS-CoV from seeded sewage by positively charged filter

One hundred milliliters of hospital sewage or domestic sewage was placed in 500-ml beaker. The sewage was then seeded with SARS-CoV and f_2 to a final concentration of approximately 10^2–10^3 pfu or $TCID_{50}$ per milliliter. After mixing, samples were taken for an initial virus assay. The concentration methods followed the procedures reported by Li et al. (1998).

2.5.2. Concentration of SARS-CoV from sewage

Ten milliliters of $Na_2S_2O_3$ (10%, w/v) were added to the disinfected sewage collected from the hospitals to neutralize the residual chlorine. The sewage was then seeded with f_2 to a final concentration of approximately 10^3–10^5 pfu. The concentration methods were followed the directions reported by Li et al. (1998).

2.6. RNA extraction

A virus RNA extracting kit (TRIzol Reagent) made by Invitrogen^{TM} Life Technologies for extraction of exceedingly pure viral RNA was utilized in our experiment to extract virus RNA. All operations were strictly implemented in accordance with the reagent instruction manual.

2.7. Primer design for assay of SARS-CoV nucleic acid

Three sets of primers from WHO Network Laboratories (WHO, 2003f) were used to detect the SARS-CoV RNA:

- Cor-p-F2 (+) 5′-CTAACATGCTTAGGATAATGG-3′;
- Cor-p-F3 (+) 5′-GCCTCTCTTGTTCTTGCTCGC-3′;
- Cor-p-R1 (−) 5′-CAGGTAAGCGTAAAACTCATC-3′.

Cor-p-F2/Cor-p-R1 gave a 368 bp product and Cor-p-F3/Cor-p-R1 yielded 348 bp section.

2.8. Primer design for assay of enteroviruses nucleic acid

In order to distinguish SARS-CoV and enteroviruses, which also cause a cytopathic effect in the Vero cell, a pair of consensus primers of enteroviruses from the 5′ non-coding region was utilized because of their presence in many enteroviruses serotypes. The information from the primers was as follows: E1 5′-ATTGTCACCATAAGCAGCCA-3′, E2 5′-CCAGCACTTCTGTTTCCCCGG-3′, product size was 440 bp (Li et al., 2002).

2.9. Detection of SARS-CoV and enteroviruses by RT-PCR

Two microliters of a viral RNA solution were analyzed with RT-PCR assay. The KaTaRa one step RNA PCR kit (KaTaRa Biotechnology, Dalian) was used for the reaction. Positive and negative RT-PCR controls were included in each run, and all operations were strictly implemented in accordance with the kit instruction manual (OSRPK, 2003).

2.10. Detection of the PCR product

PCR products were analyzed by electrophoresis with 1.5% (w/v) agarose gels containing 0.5 μg of ethidium bromide per milliliter. These were visualized with UV illumination and photographed. A DNA marker (pUC19 DNA/MSP I Marker, Gibco/BRL) was included in each agarose gel electrophoresis run.

2.11. Nucleotide sequence analysis

The PCR products from four different samples were purified with the QIAquick PCR purification kit (QIANEN, INC.) and sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit with Ampli*Taq* DNA polymerase FS (Perkin-Elmer, Applied Biosystem) following the product instructions. The sequences were compared with the genome of SARS-CoV in the GenBank and EMBL databases by using the FASTA program.

3. Results

3.1. Recoveries of f_2 and SARS-CoV from 100 ml of seeded sewage

When the concentrations of SARS-CoV and the f_2 seeded sewage were about 10^3–10^4 $TCID_{50}$/100 ml and 10^2 pfu/ml, respectively, the recoveries of SARS-CoV varied from 0 to 21.4%, with an average of 1.02% from small-volume sewage, and the recovery of f_2 ranged from 33.6 to 260.0%, with an average of 127.1%. The recovery of SARS-CoV was much lower than that of f_2 under the same conditions (Table 1).

3.2. Detection limit of SARS-CoV by RT-PCR

The minimum amount of the viral RNA detected by RT-PCR was equivalent to 10^3 $TCID_{50}$ and 10 $TCID_{50}$ by semi-nested RT-PCR.

3.3. Concentration and detection of SARS-CoV from sewage before disinfection

The recoveries of f_2 from 2500 ml sewage from Xiao Tang Shan Hospital and 309th Hospital of PLA varied from 55.4 to 188.1%, the average was 79.2 and 85.8%, respectively. All sewage samples tested for the presence of infectious SARS-CoV in cell culture were negative. The RNA of SARS-CoV could be found in the concentrates of sewage from the two hospitals by semi-nested PCR, and was also detected in the inoculated cells of the sewage concentrates from the 309th Hospital but not from Xiao Tang Shan Hospital. However, the RNA copies of SARS-CoV in the samples were too low to be detected by the first amplification reaction. The positive amplification results occurred when the product of the first amplification reaction was used as the template of the second PCR (Fig. 1). No residual chlorine was detected in any of the sewage samples (Table 2).

3.4. Concentration and detection of SARS-CoV from sewage after disinfection

The samples (25,000 or 50,000 ml) from the two hospitals were all negative by the infectivity methods. The

Table 1
Recoveries of f_2 and SARS-CoV from the seeded sewages[a]

Sewage samples	Viruses input		Recovered viruses		Recoveries	
	SARS-CoV ($TCID_{50}$/100 ml)	f_2 (pfu/ml)	SARS-CoV ($TCID_{50}$/100 ml)	f_2 (pfu/ml)	SARS-CoV (%)	f_2 (%)
1[b]	$10^{2.67}$	157	$10^{2.0}$	125	21.4	79.6
2[c]	$10^{3.2}$	146	0	381	0	100.0[d]
3[c]	$10^{3.0}$	128	$10^{1.0}$	43	1.0	33.6
Mean	$10^{3.58}$	144	$10^{1.6}$	183	1.02	100.0

[a] One hundred milliliters sewage.
[b] Sewage from the 309th Hospital.
[c] Sewage from a housing estate.
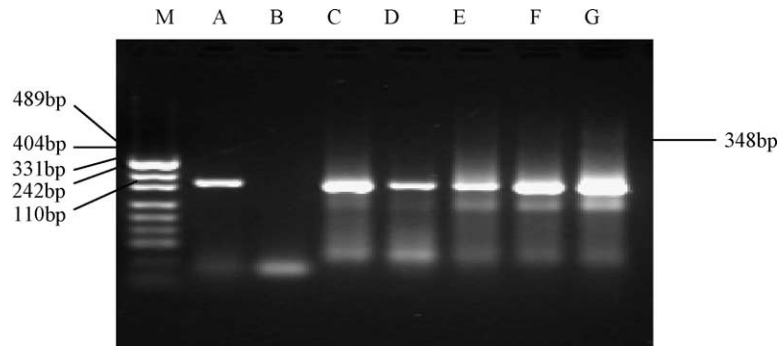[d] The recoveries were more than 100% in fact.

Fig. 1. Amplification of SARS-CoV RNA samples recovered from sewage before disinfetion from Xiao Tang Shan Hospital by semi-nested PCR. (M) DNA marker (pUC19 DNA/MSP I Marker); (A) positive control of SARS-CoV, 348 bp; (B) negative control; (C–G) the samples of 11–15 June.

Table 2
Concentration and detection of SARS-CoV from 2500-ml sewage in the 309th Hospital of PLA[a]

Date	Patients in hospital	Patients[b] with symptoms	f_2 input	f_2 recovered (%)	Cell culture[c]	Concentrate + PCR[d]	Inoculated cell + PCR[e]	Residual chlorine[a]
11 June	7	0	1.3×10^6	93.1	−[f]	+[g]	+	−
12 June	6	0	2.1×10^6	87.5	−	+	+	−
13 June	3	0	1.3×10^6	96.9	−	+	+	−
14 June	2	0	1.3×10^6	70.0	−	+	+	−
15 June	2	0	3.0×10^5	62.2	−	+	+	−

[a] Glass column diameter: 19 mm, bed height: 14 cm, eluate volume: 500 ml.
[b] With any one of the symptoms of fever, malaise, cough, dyspnea, except chest radiography signs.
[c] Cell culture was maintained for 14 days to observe the cytopathic effect.
[d] PCR template was from the concentrates.
[e] Template from the cultured cells.
[f] Negative.
[g] Positive.

SARS-CoV RNA was detected from the concentrates and inoculated cells of three samples (11, 13 and 15 June 2004) from the 309th Hospital by semi-nested PCR, while the other samples were negative (Fig. 2). The recovery of f_2 seeded in sewage from Xiao Tang Shan Hospital and 309th Hospital of PLA ranged from 13.5 to 161.2%. The average recovery rates were 61.2 and 85.5%, respectively. The total residual chlorine varied from 0 to 1.0 mg/l, and free residual chlorine from 0 to 0.5 mg/l in sewage from Xiao Tang Shan Hospital; while for the samples from the 309th Hospital, the total resid-

ual chlorine varied from 3.0 to 12.5 mg/l, and free residual chlorine from 1.5 to 5.0 mg/l. (Table 3).

3.5. Result of nucleotide sequence analysis

The PCR products from the sewage samples of the two hospitals were sequenced, and submitted to GenBank. The accession numbers were: bankit579728 and bankit579738. Comparison of the nucleotide sequence of PCR products with data from GenBank revealed that the sequences of PCR
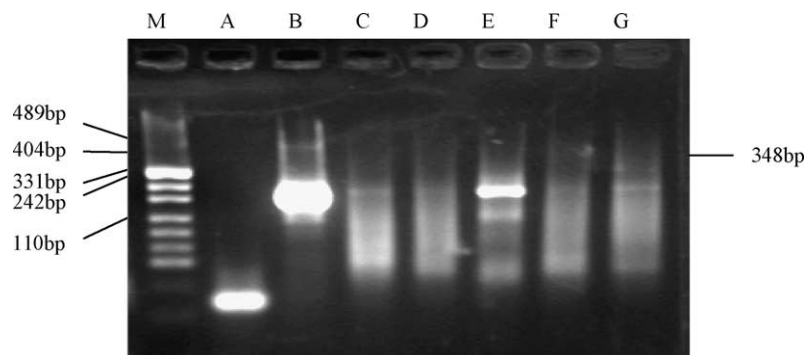

Fig. 2. Amplification of SARS-CoV RNA from recoveries of sewage after disinfetion from the 309th Hospital by semi-nested PCR. (M) DNA marker (pUC19 DNA/MSP I Marker); (A) negative control; (B) positive control of SARS-CoV, 348 bp; (C–G) the samples of 11–15 June.

Table 3
Concentration and detection of SARS-CoV from 25,000- or 50,000-ml sewage in Xiao Tang Shan Hospital

Date	Patients in hospital	Patients[b] with symptoms	f_2 input	f_2 recovered (%)	Cell culture	Concentrate + PCR	Inoculated cell + PCR	Residual chlorine[a]	
								Total	Free
11 June[c]	179	12	2.8×10^6	100.0	–	–	–	1.0	0.5
12 June	145	8	3.5×10^6	69.5	–	–	–	1.0	0.5
13 June	112	6	1.4×10^6	57.1	–	–	–	1.0	0.5
14 June	89	4	3.5×10^6	51.2	–	–	–	1.0	0.2
15 June	88	3	3.4×10^7	13.5	–	–	–	0.0	0.0

[a] Residual chlorine (mg/l): total, total residual chlorine; free, free residual chlorine.
[b] With any one of the symptoms of fever, malaise, cough, dyspnea except chest radiography signs.
[c] Volume of sewage was 50,000 ml.

product were close to these of SARS-CoV genome, showing about 99% nucleotide homologue.

4. Discussion

Control of the spread of SARS is possible through isolation of infected patients, contact tracing and follow-up, quarantine, and travel restriction (WHO, 2003a,h,g). However, epidemiologists are still trying to understand how and why the SARS-CoV has spread so readily throughout Asia and certain other regions. The primary mechanism of SARS-CoV transmission has been limited to close contacts with patients (Tsang et al., 2003; Lee et al., 2003; WHO, 2003b; Cyranoski and Abbott, 2003; Poutanen et al., 2003; Donnelly et al., 2003; Seto et al., 2003). On 15 April 2003, health authorities reported that 321 individuals affected by SARS were residents in Amoy Gardens of Hong Kong. Attention was focused on possible transmission via the sewage system because laboratory studies indicated that the Amoy Gardens patients excreted coronaviruses in their stools (WHO, 2003b,i; AGIFMP, 2003). However, except for the positive PCR results found in some patients' stools, there were no reports of the detection of SARS-CoV or RNA from sewage samples containing patients' stools.

Using the electropositive particle adsorption method (Li et al., 1998) we attempted to concentrate SARS-CoV or the RNA in sewage from Xiao Tang Shan Hospital and the 309th Hospital. These hospitals were specially assigned to receive SARS patients in Beijing. Since f_2 is a kind of bacterial virus with a single-positive-stranded RNA (similar to SARS-CoV), and testing f_2 was simple and relatively inexpensive (Olivieri et al., 1975), f_2 was chosen as a model for SARS-CoV to evaluate the concentration efficiency. Because most enteroviruses can also grow on the Vero cell, and yield a cytopathic effect similar to that of the SARS-CoV, enteroviruses must be first excluded from all the viruses that can grow on the Vero cell culture or yield a cytopathic effect.

There was no infectious SARS-CoV detected in the sewage in these assays. SARS-CoV only remains infectious for two days in sewage. We expected that if the assays were initiated with sewage samples collected when patients were in the early, acute phase of SARS the infectious virus could be isolated. The patients in these hospitals were all beyond the acute phase, and most had recovered from SARS. The following reasons may be responsible for the negative results: (1) the SARS-CoV was inactivated by the high concentration of disinfectants, which were used after a patient had a bowel movement; (2) the quantity of SARS-CoV was too low to be detected by the current methods; (3) SARS-CoV may become non-infectious by unknown factors during the concentration process.

The nucleic acid of SARS-CoV was found in the sewage before disinfection from both hospitals by semi-nested PCR. After disinfection, SARS-CoV RNA could only be detected from the samples collected on 11, 13 and 15 June 2004, from the 309th Hospital. It is interesting that, after disinfection, the RNA of SARS-CoV could be detected in the samples from the 309th Hospital but not from Xiao Tang Shan Hospital. It is possible that the RNA originated from two serious patients who had been moved to other hospitals in Beijing on 2 and 9 June, respectively, before the experimental period. It has been reported that the RNA of SARS-CoV can survive for five days or longer (AGIFMP, 2003). In this study, the RNA could be detected in sewage for 8 days, though the virus itself was inactive.

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