Concentration and detection of SARS coronavirus in sewage from Xiao Tang Shan hospital and the 309th Hospital of the Chinese People's Liberation Army

X.W. Wang*, J. Li, T. Guo***, B. Zhen**, Q. Kong*, B. Yi****, Z. Li*, N. Song*, M. Jin*, W. Xiao**, X. zhu***, C. Gu*, J. Yin*, W. Wei**, W. Yao***, C. Liu*****, J. Li***, G. Ou*, M. Wang**, T. Fang******, G. Wang**, Y. Qiu***, H. Wu***, F. Chao* and J. Li***

*Institute of Hygiene and Environmental Medicine, Academy of Military Medical Sciences, 1 Da Li Road, Tianjin, 300050, P.R. China (E-mail: *junwenli@eyou.com*)

** Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, 172 Dong Da Jie street, Feng Tai, Beijing, 100072, P.R. China

***Xiao Tang Shang Hospital, Beijing, 102211, P.R. China

****309 Hospital of PLA of China, 17 Mushanhu Road, Beijing, 100091, P.R. China

*****Beijing Institute of Pharmacology & Toxicology, 100850, P.R. China

******Beijing Institute of Basic Medicine, 100850, P.R. China

Abstract A worldwide outbreak of severe acute respiratory syndrome (SARS) had been reported. Over 8439 SARS cases and 812 SARS-related deaths were reported to the World Health Organization from 32 countries around the world up to 5 July 2003. The mechanism of transmission of SARS-CoV has been limited only to close contacts with patients. Attention was focused on possible transmission by the sewage system because laboratory studies showed that patients excreted coronavirus RNA in their stools in Amoy Gardens in Hong Kong. To explore whether the stool of SARS patients or the sewage containing the stool of patients would transmit SARS-CoV or not, we used a style of electropositive filter media particle to concentrate the SARS-CoV from the sewage of two hospitals receiving SARS patients in Beijing, as well as cell culture, semi-nested RT–PCR and sequencing of genes to detect and identify the viruses from sewage. There was no live SARS-CoV detected in the sewage in these assays. The nucleic acid of SARS-CoV was found in the sewage before disinfection from both hospitals by PCR. After disinfection, SARS-CoV RNA could be detected from some samples from the 309th Hospital of the Chinese People's Liberation Army, but not from Xiao Tang Shan Hospital after disinfection. In this study, we found that the virus can survive for 14 days in sewage at 4°C, 2 days at 20°C, and its RNA can be detected for 8 days though the virus had been inactivated. In conclusion, this study demonstrates that the RNA of SARS-CoV could be detected from the concentrates of sewage of both hospitals receiving SARS patients before disinfection and occasionally after disinfection though there was no live SARS-CoV; thus much attention should be paid to the treatment of stools of patients and the sewage of hospitals receiving SARS patients.

Keywords Concentration; detection; hospital; SARS-Cov; sewage

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Introduction

Over 8439 SARS cases and 812 SARS-related deaths were reported to World Health Organization (WHO) from 32 countries around the world up to 5 July 2003. 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 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). From analysisi of a cluster of SARS cases in an apartment block in Hong Kong, sewage is believed to play a role through droplets containing coronavirus from the sewage system (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.

To confirm whether the sewage is a possible major transmission path of SARS-CoV or not, 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 of China, and cell culture and RT–PCR were used to detect and identify the viruses from sewage.

Materials and methods

Viruses and the culture methods

Bacteriophage f_2 (f_2), which was used as a model for the coronavirus that may be present in sewage, was prepared and detected according to the methods described by Womack *et al* (Wommack *et al.*, 1995). To identify viruses in the sewage system, we inoculated a variety of specimens (sewage before or after disinfection by chlorine) onto Vero E6. All cell cultures were grown in Eagle's growth medium (Difco Laboratories, Detroit, MI) containing 8% fetal bovine serum (FBS), 0.015 M DMEM buffer and antibiotics (50 μg [each] of kanamycin and gentamycin per millilitre) 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 inoculated 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 for 1 to 2 days of incubation. Culture was terminated 7 days after inoculation, and the culture was observed daily for a cytopathic effect. Any cultures exhibiting identifiable a cytopathic effect were subjected to several procedures to identify the cause of the effect (WHO, 2003c, d, e). If there is no cytopathic effect in the cell culture, the culture suspensate was harvested and added into additional flasks to isolate viruses. Then the cultures were used until three generations passed without cytopathic effect.

Sewage and disinfection

The sewage was collected at seven o'clock every morning from Xiao Tang Shan Hospital and the 309th Hospital of the Chinese PLA (People's Liberation Army), which were specially assigned to receive SARS patients in Beijing. 2500 ml before disinfection or 25,000–50,000 ml after disinfection by chlorine were collected. The sewage of these hospitals was not treated except for disinfection by chlorine.

Electropositive filter media particle

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

Detection of residual chlorine

The residual chlorine in sewage was determined by the N, N, dithyl-p-phenyldiamine (DPD) colorimetric method.

Concentration experiments

Evaluation of concentration efficiency of f_2 and SARS-CoV from seeded sewage by positive charged filter. 100 ml sewage water from the 309th Hospital or a residential quarter was placed in 500-ml capacity beaker. SARS-CoV and f_2 were then seeded to a final concentration of approximately 10^2-10^3 pfu or $TCID_{50}$ per ml. After mixed, the samples were taken for initial viruses assay. The concentration methods followed the directions reported by Li *et al.*, 1998.

Concentration of SARS-CoV from sewages. 2500 ml and 25,000 ml sewage from the hospitals before or after disinfection by chlorine was collected and added to 10 ml $Na_2S_2O_3$ (10% w/v) to neutralize the residual chlorine, and then added f_2 with 10^3-10^5 pfu. The concentration methods followed the directions reported by Li *et al.*, 1998.

RNA extraction

Virus RNA extracting kit (TRIzol Reagent) made by Invitrogen™ Life Technologies for extraction of exceedingly pure viral RNA was used in our experiment to extract virus RNA, and all operations were strictly implemented in accordance with the reagent instruction manual.

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′, and Cor-p-R1 (−) 5′-CAGGTAAGCG-TAAAACTCATC-3′. Cor-p-F2/Cor-p-R1 gave a 368 base pair (bp) product, and Cor-p-F3/Cor-p-R1 yielded 348 bp section.

Primer design for assay of enteroviruses nucleic acid

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

Detection of SARS-CoV by RT–PCR

2 μl of RNA solution was 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).

Detection of Enteroviruses by RT–PCR

The RT–PCR was similar to that for SARS-CoV.

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 millilitre. These were visualized with UV illumination and photographed. DNA marker (pUC19 DNA/MSP I Marker, Gibco/BRL) was included in each agarose gel electrophoresis run.

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 instructions. The sequences were compared with the genome of SARS-CoV in the GenBank and EMBL databases by using the FASTA program of the GCG.

Results

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

The recoveries of SARS-CoV varied from 0% to 21.4%; the average was 1.02% from small-volume sewage, while the recoveries of f_2 changed from 33.6% to 260.0%, and the average was 127.1% when the concentration of SARS-CoV and f_2 seeded sewage were about 10^3–10^4 $TCID_{50}$/100 ml and 10^2 pfu/ml, respectively. It is shown that the recoveries of SARS-CoV were much lower than that of f_2 on the same conditions (Table 1).

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.

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 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 semi-nested PCR in which the product of first amplification reaction was the template of the second PCR gave the positive amplification results (Figure 1). No residual chlorine was detected in any of the sewage samples (Table 2).

Concentration and detection of SARS-CoV from sewage after disinfection

The samples (25,000 ml or 50,000 ml) from the two hospitals were all negative by the infectivity methods. The SARS-CoV RNA was detected from the concentrates and inoculated cells of three samples (June 11, 13 and 15) from the 309th Hospital by semi-nested PCR, while the other samples were negative (Figure 2). The recoveries of f_2 seeded in sewage from the two hospitals ranged from 13.5% to 161.2%; the average was 61.2%

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 (%)	SARS-CoV (pfu/ml)	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	260.0
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	127.1

[a]100 ml sewage;
[b] sewage from the 309th Hospital;
[c] sewage from a residential quarter.

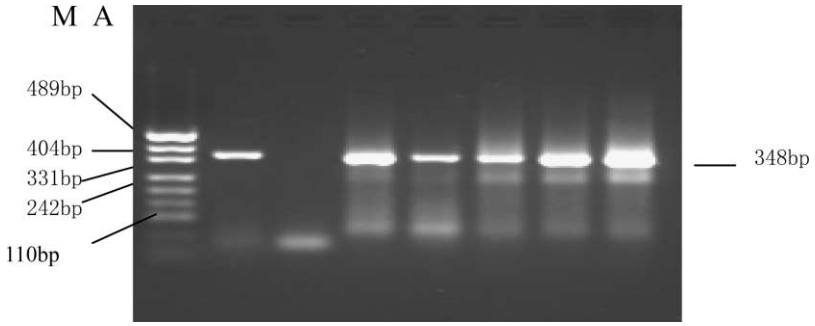

Figure 1 Amplification of SARS-Cov RNA from recoveries of sewage before disinfection from Xiao Tang Shan Hospital by semi-nested PCR. M: DNA marker (pUC19 DNA/MSP I Marker), A: positive control of SARS-CoV,348 bp, B: negative control, C–G: the samples of 11–15 June

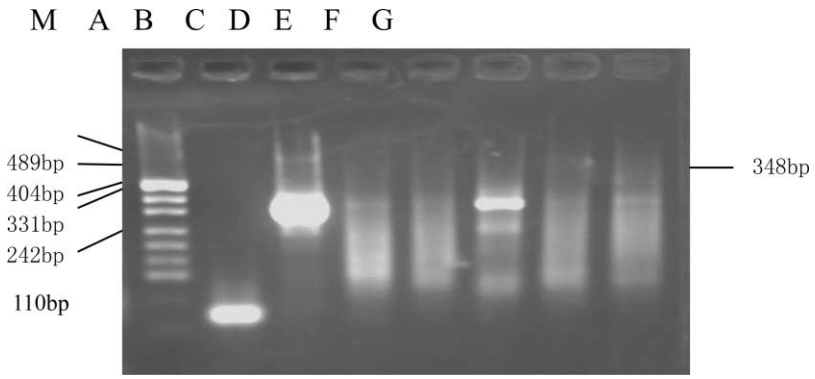

Figure 2 Amplification of SARS-Cov RNA from recoveries of sewage after disinfection from the 309th Hospital by semi-nested PCR. M: DNA marker (pUC19 DNA/MSP I Marker), A: negative control, B: positive control of SARS-CoV,348 bp, C-G: the samples of 11–15 June

and 85.5%. The total residual chlorine varied from 0 mg/L to 1.0 mg/L, and free residual chlorine from 0 mg/L to 0.5 mg/L in sewage from Xiao Tang Shan Hospital; while for the samples from the 309th Hospital, the total residual chlorine varied from 3.0 mg/L to 12.5 mg/L, and free residual chlorine from 1.5 mg/L to 5.0 mg/L. (Table 3).

Survival, infectivity and inactivation of SARS-CoV in sewage

100 ml sewage water from the 309th Hospital was placed in a 500 ml beaker. SARS-CoV was seeded to a final concentration of approximately 10^5 $TCID_{50}$ per ml, and then 2 ml of sewage was cultured everyday. The virus remained infectious more than 14 days at

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

Date	Patients in hosp.	Patients[#] with sympt.	f_2 input	f_2 recov.(%)	Cell cult *	concentrate + PCR[@]	inoculated cell + PCR[&]	residual chlor[+]
11 June	7	0	1.3×10^6	93.1	−	+	+	×
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.
[#] With any one of the symptoms of fever, malaise, cough, dyspnea, except chest radiography signs.
* Cell culture was maintained for 14 days to observe the cytopathic effect.
[@] PCR template was from the concentrates.
[&] Template from the cultured cells.

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

Date	Patients in hosp.	patients[#] with sympt	f_2 input	f_2 recov. (%)	Cell cult	concent + PCR	inoculated cell + PCR	residual chlor[+] total	free
11 June[a]	179	12	2.8×10^6	123.4	–	–	–	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]Volume of sewage was 50 000 ml;
[#] With any one of the symptoms of fever, malaise, cough, dyspnea, except chest radiography signs;
[+]Residual chlor (mg/L): total- total residual chlorine; free residual chlorine.

4°C in sewage, but only 2 days at 20°C. The viral RNA can be detected in all sewage samples at 4°C for 14 days, and at 20°C for 8 days (Table 4).

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 are: bankit579728 and bankit579738, respectively. Comparison of the nucleotide sequence of PCR products with data from GenBank revealed that the sequences of PCR product were close to these of SARS-COV genome, showing about 99% nucleotide homologue.

Discussion

Although SARS has been brought close to defeat by diligent and unrelenting application–isolation, contact tracing and follow-up, quarantine, and travel restriction (WHO, 2003a, g, h), epidemiologists are still trying to understand how and why the SARS-CoV has spread so readily throughout Asia and certain other regions of the world.

The mechanism of transmission of SARS-CoV has been limited only 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 a total of 321 individuals affected by SARS who were residents in Amoy Gardens. The attention was focused on possible transmission via the sewage system because the laboratory studies showed that patients with the disease excreted coronaviruses in their stools (WHO, 2003b, i; AGIFMP, 2003). However, except that positive PCR results had been obtained in some patients' stool, there were no reports on the detection of SARS-CoV or RNA from sewage samples containing patients stool.

We attempted to concentrate SARS-CoV or the RNA in sewage from Xiao Tang Shan Hospital and the 309th Hospital, which were assigned specially to receive SARS patients in Beijing of China by the electropositive particle adsorption method (Li *et al.*, 1998).

Table 4 SARS–CoV survival and infectious in sewage

Conditions and detection methods	seeded time (day) 0	1	2	3	4	5	6	8	14
4°C, culture	+	+	+	+	+	+	+	+	+
20°C, cultrue	+	+	+	–	–	–	–	–	–
20°C, RT–PCR	+	+	+	+	+	+	+	+	–

Since f_2 is a kind of bacterial virus with single positive-stranded RNA (similar to SARS-CoV), and testing f_2 was simple and relatively cheap (Olivieri *et al.*, 1975), f_2 was chosen as a model for SARS-CoV to evaluate the concentration efficiency. Because most enteroviruses also could grow on the Vero cell and yield cytopathic effects like the SARS-CoV, enteroviruses must first be excluded if there are viruses growing on the Vero cell culture or yield cytopathic effects.

There was no live SARS-CoV detected in the sewage in these assays. We believed that if the assays started early, i.e., the sewage samples were collected when the SARS patients were at an acute course, the alive virus should be isolated, since the SARS-CoV only remained infectious for 2 days in sewage based on this research. While the patients in these hospitals all exceed the acute course, most recovered from SARS. The following reasons may be responsible for the negative results: (1) the SARS-CoV was killed by the high concentration of disinfectants that were required to use after patient had a bowel movement; (2) the quantity of SARS-CoV was not enough to be detected by the current methods; (3) SARS-CoV may lose its infectivity to cells during the processes of concentration by some unknown factors.

The nucleic acid of SARS-CoV had been 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 on June 11, 13 and 15 from the 309th Hospital. It is interesting that the RNA of SARS-CoV could be detected in the samples from the 309th Hospital, but not from Xiao Tang Shan Hospital after disinfection. Where was the RNA from? It was found that two seriously ill patients had been moved to other hospitals in Beijing on June 2 and 9, respectively, before the experimental period. It was reported that the RNA of SARS-CoV could survive for five days or longer (AGIFMP, 2003). In this study, we found that the RNA can be detected in sewage for 8 days though the virus had been inactivated.

Conclusion

1. There was no live SARS-CoV in the sewage of both hospitals receiving SARS patients.
2. However, the RNA of SARS-CoV could be detected from the concentrates of sewage before disinfection and occasionally after disinfection.
3. SARS-Cov can survive for 14 days in sewage at 4°C, 2 days at 20°C, and its RNA can be detected for 8 days though the virus had been inactivated.
4. It provides strong evidence that SARS-CoV can be excreted through the stool/urine of patients into sewage system, thus the sewage system is a possible transmission way.30

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