Bidet toilet seats with warm-water tanks: residual chlorine, microbial community, and structural analyses

Toru Iyo, Keiko Asakura, Makiko Nakano, Mutsuko Yamada and Kazuyuki Omae

ABSTRACT

Despite the reported health-related advantages of the use of warm water in bidets, there are health-related disadvantages associated with the use of these toilet seats, and the bacterial research is sparse. We conducted a survey on the hygienic conditions of 127 warm-water bidet toilet seats in restrooms on a university campus. The spray water from the toilet seats had less residual chlorine than their tap water sources. However, the total viable microbial count was below the water-quality standard for tap water. In addition, the heat of the toilet seats' warm-water tanks caused heterotrophic bacteria in the source tap water to proliferate inside the nozzle pipes and the warm-water tanks. *Escherichia coli* was detected on the spray nozzles of about 5% of the toilet seats, indicating that the self-cleaning mechanism of the spray nozzles was largely functioning properly. However, *Pseudomonas aeruginosa* was detected on about 2% of the toilet seats. *P. aeruginosa* was found to remain for long durations in biofilms that formed inside warm-water tanks. Infection-prevention measures aimed at *P. aeruginosa* should receive full consideration when managing warm-water bidet toilet seats in hospitals in order to prevent opportunistic infections in intensive care units, hematology wards, and other hospital locations.

Key words | bidet toilet, microbial community, *Pseudomonas aeruginosa*, residual chlorine

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LIST OF ABBREVIATIONS

CFU	colony forming units
HPC	heterotrophic plate count
MPN	most probable number
PCR-DGGE	polymerase chain reaction-denaturing gradient gel electrophoresis

INTRODUCTION

Warm-water bidet toilet seats are equipped with a device that sprays warm water (spray water) on the external genitalia and anus after urination and defecation. A toilet seat equipped with bidet functions first appeared on the market in the United States in 1964 (Altman 2007). This was followed by the development of functional electric warm-water bidet toilet seats in Japan, of which at least 30 million units have been sold nationwide to date (Hasegawa 2012). A consumer behavior survey conducted by the Japanese Cabinet Office showed that 74.0% of households had warm-water bidet toilet seats in March 2013, with 102.9 units per 100 households (effectively one per household) (Cabinet Office Government of Japan 2013). Broadly, there are two types of warm-water bidet toilet seats: tank types and on-demand types. In the tank type, the spray water is warmed to a suitable temperature in a tank, whereas in the on-demand type the water is warmed as needed inside a tube. Tank-type products

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are both cheaper and more common than on-demand types in Japan. In the United States, consumers have generally resisted the use of warm-water bidet toilet seats, with only around 200,000 units sold in the past 45 years (Altman 2007).

The use of warm-water bidet toilet seats has been reported to promote defecation in people with spinal injuries (Uchikawa *et al.* 2007) and improve toilet usage in elderly nursing home residents (Cohen-Mansfield & Biddison 2005). However, there are also potential health-related disadvantages to the use of these toilet seats. It has been proposed that the habitual use of such toilet seats by women can change the vaginal flora, increase vaginal secretions, and lead to bacterial vaginosis. Although this increase in vaginal secretions is usually mild, bacterial vaginosis in pregnant women can lead to intrauterine infections and chorioamnionitis, which can cause premature delivery (Goldenberg *et al.* 2000; Morris *et al.* 2001). Ogino *et al.* (2010) reported that the regular use of warm-water bidet toilet seats causes the condition of the intervaginal flora to deteriorate. Asakura *et al.* (2013) found that regular use of bidet toilet seats increased the risk of early delivery and bacterial vaginosis in pregnant women.

While hygienic evaluations of the warm water sprayed from bidet toilet seat nozzles are important to consider from the perspective of public health, there has been almost no bacterial research conducted on these toilet seats to date. The water sprayed from bidet toilet seats is disinfected by the residual chlorine in tap water, which is the source of the bidet spray water. However, we hypothesized that the heat of the warm-water tank could eliminate the residual chlorine, thus negating the effect of this important built-in factor for disinfection. Therefore, we surveyed the state of residual chlorine and microbial indicators in the spray water of warm-water tanks of bidet toilet seats at the campus of Kitasato University, Kanagwa, Japan. We also evaluated the disinfection status and microbial hygiene of the spray water.

MATERIALS AND METHODS

Warm-water bidet toilet seats

To evaluate the hygiene status of bidet toilet seats with warm-water tanks (hereafter, warm-water bidet toilet seats), a survey of their microbial communities was conducted at the Sagamihara campus of Kitasato University in 2012 and 2013. Of the total 127 seats analyzed, there were 43 toilet seats for men's use, 71 for women's use, and 13 for barrier-free use. The campus contains both university and hospital buildings. Of the toilet seats studied, 86 were in a research building (33 men's, 45 women's, eight barrier-free) and 41 were in an outpatient hospital building (10 men's, 26 women's, 10 barrier-free). Between about 2 to 10 years had passed since these toilet seats had been installed, and all were functioning properly.

Survey methods

Spray water

Residual chlorine and microorganism indicators in the spray water from the warm-water bidet toilet seats were surveyed twice. For the first survey, spray water was collected directly as it came out of the nozzle, and tap water was used as a control. In the second survey, the surface of the nozzle was first disinfected with 70% ethanol and then rinsed with sterile purified water so that the sample could be collected without any contamination from the nozzle surface. About 200 mL of spray water from the nozzle was collected in sterilized bottles (containing 30 mg sodium thiosulfate) for microorganism testing. About 50 mL of spray water was collected in sterilized bottles for residual chlorine testing. The samples were kept at 4 °C in refrigerated storage until analysis. Measurements were performed quickly.

Tap water

Tap water for control specimens was collected from faucets in the restrooms that were surveyed. To eliminate as much contamination as possible on the faucet, the tap water was allowed to run for about 1 min before samples (~200 mL) were collected for microorganism testing in sterilized bottles (containing 30 mg sodium thiosulfate). Next, about 50 mL of tap water was collected for residual chlorine testing in sterilized bottles. The samples were kept at 4 °C in refrigerated storage until analysis. Measurements were performed quickly.

Residual chlorine

For spray water and tap water samples, residual chlorine levels (mg/L) were measured using a DR2010 multi-item rapid water-quality measuring instrument (Hach Co.; Tillamook, OR, USA) with the *N,N*-Diethyl-*p*-phenylenediamine (DPD) method (USEPA-approved Hach Method 8167).

Total viable count

Samples of 1 mL, diluted as necessary with sterilized physiological saline solution (0.85%), were cultured in CompactDry® TC (Nissui Pharmaceutical Co.; Tokyo, Japan), an adjusted culture medium, at 35 °C for 48 h. After culturing, the number of red colonies was counted to calculate the total viable count (colony-forming units [CFU]/mL). Note that CompactDry® TC is an AOAC Performance Tested Method (Kodaka *et al.* 2005).

Heterotrophic plate count (HPC)

Samples of 1.0 mL or 0.1 mL, diluted as necessary with sterilized physiological saline solution (0.85% NaCl), were cultured in a pour culture plate (Bartram *et al.* 2003; World Health Organization 2011; Health Canada 2012) using R2A agar culture medium (Eiken Chemical Co.; Tokyo, Japan) at 20 °C for 7 days to calculate the HPC (CFU/mL).

Fecal indicator bacteria

Coliform bacteria, Escherichia coli

Using the United States Environmental Protection Agency (USEPA)-approved Defined Substrate Technologies Colilert® and Quanti-Tray® systems (IDEXX Laboratories Inc.; Westbrook, ME, USA), samples were cultured at 35 ± 0.5 °C for 24 h. Yellow wells were considered positive for coliform bacteria, and wells that emitted fluorescence at 365 nm were considered to be *E. coli*-positive. The number of positive large wells and positive small wells were combined to calculate the most probable number (MPN) per 100 mL.

Enterococci

Using the USEPA-approved Defined Substrate Technologies Enterolert® and Quanti-Tray® systems (IDEXX Laboratories Inc.; Westbrook, ME, USA), samples were cultured at 41 ± 0.5 °C for 24 h. Wells that emitted fluorescence at 365 nm were considered enterococcus-positive. The number of positive large wells and positive small wells were combined to calculate the MPN/100 mL.

Pseudomonas aeruginosa

A smear test (smear amount 0.3–0.5 mL) was performed using nalidixic acid cetrimide agar (Eiken Chemical Co.; Tokyo, Japan). After culturing at 37 °C for 24 h, the number of yellow-green to blue colonies was counted (CFU/mL). Using the Defined Substrate Technologies Pseudalert® and Quanti-Tray® systems (IDEXX Laboratories Inc.; Westbrook, ME, USA), samples were cultured at 38 ± 0.5 °C for 24 h. Wells that emitted fluorescence at 365 nm were considered *P. aeruginosa*-positive. The number of positive large wells and positive small wells were combined to calculate the MPN/100 mL.

Infectious microorganisms

For determination of infectious microorganisms, *Salmonella*, *Vibrio parahaemolyticus*, *Bacillus cereus*, and *Staphylococcus aureus* were cultured using the Compact Dry SL, Compact Dry VP, Compact Dry X-BC, and Compact Dry X-SA kits, respectively, according to the culture temperatures and times recommended by the manufacturer.

Microbial community structure analysis

Samples of spray water were collected from warm-water bidet toilet seats on the first floors of the research and outpatient buildings (three research buildings and two outpatient buildings), which were considered to receive the most frequent use. For comparison, spray water from a warm-water bidet toilet seat in an individual residence (one of the authors' homes) in the same city was also collected. Spray water (100 mL) was passed through a 0.22 μm microfilter. The filter containing the microorganisms from the spray water

was placed in a 50 mL microtube. DNA from the microorganisms in the filters was extracted and purified with a WaterMaster™ DNA Purification kit (Epicentre; Madison, WI, USA). Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis of the V3 region of the 16S rRNA gene was performed according to previously reported methods (Hoefel *et al.* 2005; Xue *et al.* 2012).

In brief, using the extracted solution as the template, PCR was performed with 0.2 mM dNTP, 1.0 μM each primer (27f:5′- AGA GTT TGA TYM TGG CTC AG-3′; 1492r: 5′-TAC GGY TAC CTT GTT ACG ACT-3′), 1.5 mM $MgCl_2$, 1 × PCR Gold Buffer, 5% dimethyl sulfoxide (DMSO), and 0.05 U/μL AmpliTaq Gold DNA Polymerase at 95 °C for 9 min, 94 °C for 30 s, 50 °C for 60 s, 72 °C for 2 min (40 cycles), with a final extension at 72 °C for 10 min to obtain PCR products. Using these PCR products as the template, PCR was performed with 0.2 mM dNTP, 1.0 μM each primer (357fGC: 5′- CGC CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′; 518r: 5′- ATT ACC GCG GCT GCT GG-3′), 1.5 mM $MgCl_2$, 1 × PCR Gold Buffer, 5% DMSO, and 0.05 U/μL AmpliTaq Gold DNA Polymerase at 95 °C for 9 min, 94 °C for 30 s, 55 °C for 60 s, 72 °C for 2 min (for 40 cycles), with a final extension at 72 °C for 10 min. Formamide (40%) and 7 M urea was defined as the 100% denaturant concentration. DGGE was performed for 5 h at 60 °C and 130 V on an 8% acrylamide gel with a 40–70% denaturant concentration. Then, staining was performed for 30 min with SYBR Green I. DGGE bands were confirmed with an ultraviolet transilluminator, and specific bands were cut out for repeat PCR (primers 357fGC, 518r), followed by repeated PCR-DGGE analysis to confirm that these were indeed single bands, and a repeat of the cutting-out manipulation. Using the DNA eluted from the cutout bands as the template, PCR (primers 357f, 518r) was performed again. Then, the primers were removed using NucleoSpin Extract II (Takara Bio Inc.; Tokyo, Japan) and sequence analysis was performed with an Applied Biosystems 3730xl sequencer (Applied Biosystems; Foster City, CA, USA).

Statistical analysis

The geometric means (GM) and geometric standard deviations were calculated for total viable count, HPC, and residual chlorine. When there were non-detected data, the detection limit was assigned for use in calculation. Statistical comparisons of total viable count, HPC, and residual chlorine in spray water and tap water were performed using Mann-Whitney U tests and a non-parametric Kruskal-Wallis test. Chi-squared tests and exact probability tests were used for cross-tabulations between microorganism detection rates and each factor. Correlations between total viable count and residual chlorine, and between HPC and residual chlorine, were analyzed with the Spearman's rank correlation test and Pearson's correlation test, respectively. Statistical analysis was performed with the Statistical Package for the Social Sciences ver.22 (IBM; Armonk, NY, USA). The significance level was set at less than 5%.

RESULTS

Residual chlorine

The GM of residual chlorine in the spray water of warm-water bidet toilet seats and in tap water was 0.04 mg/L and 0.24 mg/L, respectively, which represented a significant difference ($P < 0.01$). This result demonstrated that there is clearly less residual chlorine in spray water than in tap water (Table 1). Moreover, when these data were log-normalized, it became clear that artificial factors (e.g., heating) caused the residual chlorine to disappear in the spray water. However, no significant difference in the residual chlorine concentration of spray water was observed between the male and female restrooms. Residual chlorine levels were higher in the spray water from toilet seats in the outpatient building than from those in the research building ($P < 0.01$; Figure 1). Frequent inflow of tap water into a toilet's warm-water tank is needed to maintain the chlorine concentration in spray water; that is, the residual chlorine concentration cannot be maintained without frequent use. As such, we surmised that the toilet seats in the outpatient building were used more often than those in the research building.

Total viable count and HPC in spray water

The total viable count and HPC of spray water from warm-water bidet toilet seats and control tap water are shown in

Table 1 | Residual chlorine in spray water and tap water

		Spray water			Tap water		
		n	GM	(GSD)	*n*	AM	(SD)
Residual chlorine (mg/L)	Total	127	0.04	(2.63)	103	0.23	(0.12)
	Male	43	0.05	(2.63)	40	0.23	(0.10)
	Female	71	0.04	(2.59)	50	0.24	(0.12)
	Barrier-Free	13	0.04	(2.91)	13	0.17	(0.12)

GM: geometric mean, GSD: geometric standard deviation.
AM: arithmetic mean, SD: standard deviation.

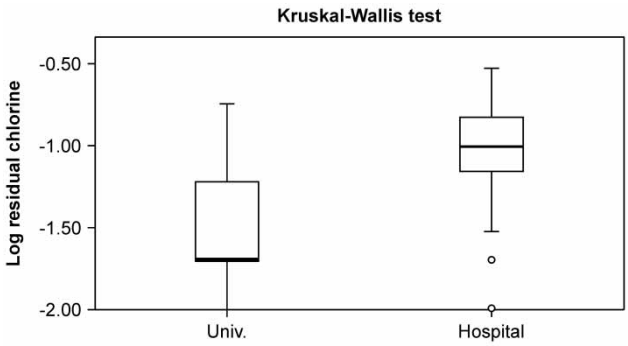

Figure 1 | Difference in residual chlorine levels between buildings. Chi-square value: 40.468, Total number: 127.

Table 2. The total viable count and HPC were both significantly higher in spray water than in tap water, and the difference was particularly apparent for HPC, with an increase of around 2–3 log10 ($P < 0.01$). Detection rates were also higher in spray water than in tap water ($P < 0.01$). Significant differences in total viable count were not observed between male and female restrooms (Table 2) or between the research and outpatient buildings (data not shown).

A significant difference in HPC was observed between the male and female restrooms ($P < 0.01$) (Figure 2). Examining the data by building, a significant difference was observed between the male and female restrooms in the research building, but not in the outpatient building (data not shown). In addition, HPC was clearly higher in the outpatient building's male restrooms than in the male restrooms in the research building ($P < 0.01$; Figure 3).

Relationship of residual chlorine with total viable count and HPC

The correlations of residual chlorine with total viable count and HPC are shown in Figures 4 and 5, respectively. The threshold of detection for total viable count (≤100 CFU/mL) was around a residual chlorine level of 0.1 mg/mL, while the threshold of detection for HPC (≤1,000 CFU/mL) was around a residual chlorine level of 0.2 mg/mL.

Residual chlorine levels were significantly negatively correlated with both total viable count and HPC. The Spearman's rank correlation coefficient for residual chlorine and

Table 2 | Total viable count (TVC) and heterotrophic plate count (HPC) of tap water and spray water from warm-water bidet toilet seats

		Spray water					Tap water				
		n	GM	(GSD)	Positive number	Detection ratio (%)	*n*	GM	(GSD)	Positive number	Detection ratio (%)
TVC (CFU/mL)	Total	127	5.7	(9.2)	71	55.9	103	1.2	(2.2)	18	17.5
	Male	43	4.8	(8.9)	23	53.5	40	1.3	(2.9)	7	17.5
	Female	71	6.4	(9.7)	42	59.2	50	1.1	(1.7)	9	18.0
	Barrier-Free	13	5.2	(11.7)	6	46.2	13	1.2	(1.7)	2	15.4
HPC (CFU/mL)	Total	127	18,000	(12.1)	127	100	103	22	(13.3)	103	100
	Male	43	7,300	(7.0)	43	100	40	15	(12.0)	40	100
	Female	71	33,200	(13.0)	71	100	50	28	(13.7)	50	100
	Barrier-Free	13	12,800	(13.6)	13	100	13	32	(17.8)	13	100

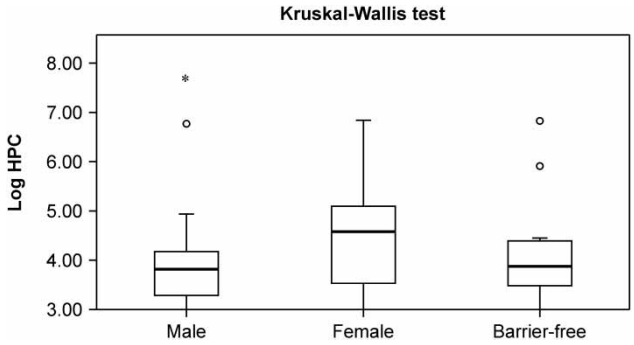

Figure 2 | Differences in heterotrophic plate count (HPC) by gender. Chi-square value: 10.544, Total number: 127.

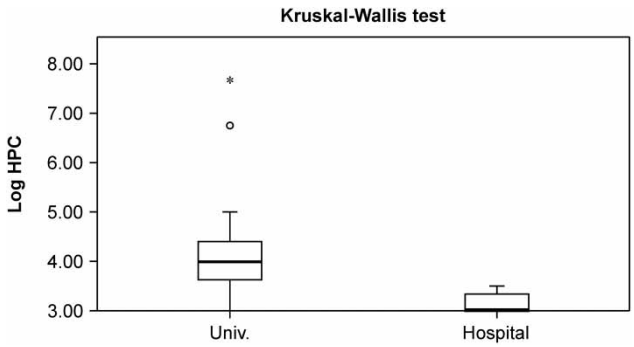

Figure 3 | Differences in heterotrophic plate count (HPC) by building (male restrooms only). Chi-square value: 16.667, Total number: 43.

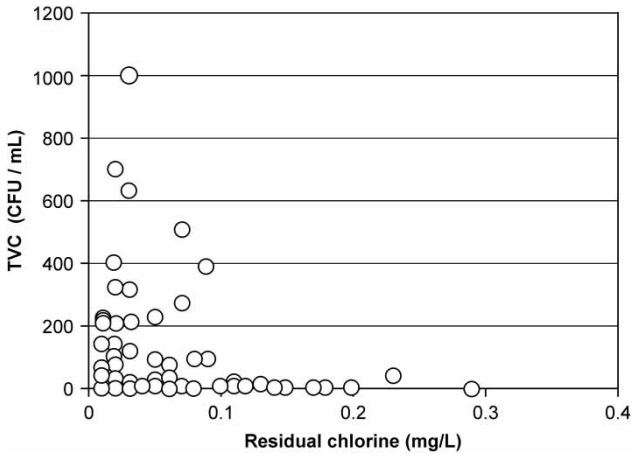

Figure 4 | Relationship between residual chlorine and total viable count (TVC).

total viable count was $R = -0.395$ ($R^2 = 0.156$; $P < 0.01$), and that for residual chlorine and HPC was $R = -0.451$ ($R^2 = 0.203$; $P < 0.01$). Although the correlation coefficients between HPC and residual chlorine did not show a strong relationship, the coefficients of determination (R^2) suggest

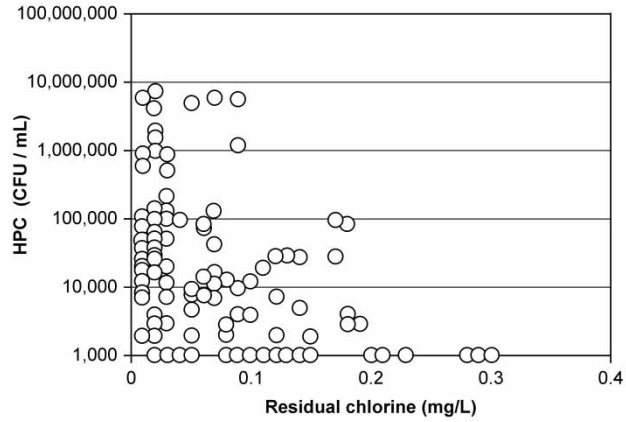

Figure 5 | Relationship between residual chlorine and heterotrophic plate count (HPC).

that a factor other than residual chlorine affects the microorganism count.

Effect of nozzle cleaning on the survey results

Figure 6 shows a comparison of the total viable count and HPC in the first and second surveys, in which the water

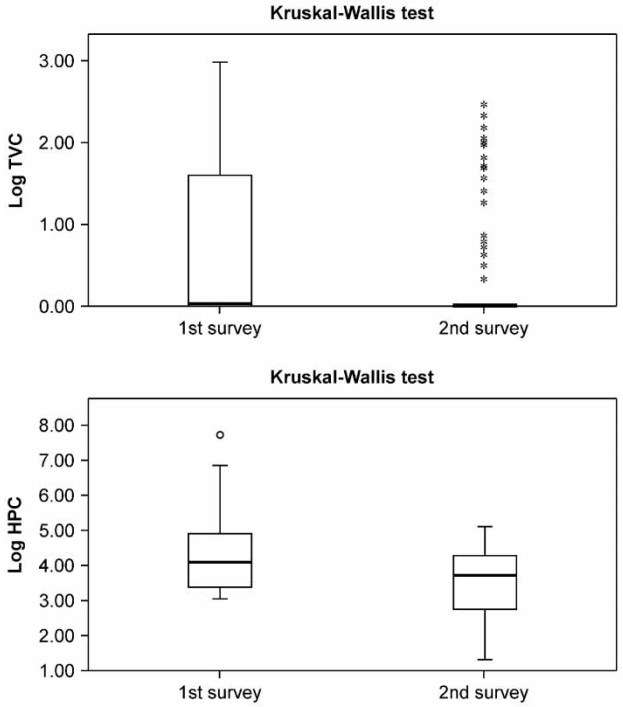

Figure 6 | Differences in total viable count (TVC) (Chi-square value: 23.300, Total number: 254) and heterotrophic plate count (HPC) (Chi-square value: 23.189, Total number: 254) between the first and second surveys.

was collected directly or following disinfection of the nozzle, respectively. These results indicate that the total viable count and HPC decreased when spray water was collected after cleaning the nozzle ($P < 0.01$).

Detection of fecal indicator bacteria and *P. aeruginosa*

Surveys of fecal indicator bacteria and *P. aeruginosa* in the spray water were conducted from early September to early October (for the first survey) and from late October to late November (for the second survey). The detection rates of fecal indicator bacteria and *P. aeruginosa* were nearly identical in both surveys (Table 3). Coliform bacteria were detected in the spray water collected from seven toilet seats (5.5%), *E. coli* was detected in the water from three toilet seats (2.4%), enterococci were detected in the water from four toilet seats (3.1%), and either *E. coli* or enterococci were detected in the water from six toilet seats (4.7%). When tap water was analyzed as a negative control, no fecal indicator bacteria were detected, as expected.

A Chi-squared test of the fecal indicator bacteria detection rates in male and female restrooms showed a tendency for coliform bacteria to be detected more often in spray water from toilet seats in male restrooms ($P < 0.05$) than in female restrooms. Most fecal indicator bacteria were detected at a concentration of 1 MPN/mL or less (Table 4), and fecal indicator bacteria (coliform bacteria, *E. coli*, enterococci) appeared in only one toilet seat in both surveys (Table 4, No. 6). This indicates that feces stuck to the nozzle that discharges spray water is able to mix with the spray water when it comes out, although in very small amounts, leading to the detection of fecal indicator bacteria in spray water.

In the first and second surveys, *P. aeruginosa* was specifically detected in water from the same male and female toilet seats. Of the 127 warm-water bidet toilet seats, *P. aeruginosa* was detected in the spray water of only two seats (Table 4) at concentrations of 5 CFU/mL (male restroom) and 10 CFU/mL (female restroom).

Infectious microorganisms

Neither *Salmonella*, *V. parahaemolyticus*, *B. cereus*, nor *S. aureus* was detected in the spray water from the warm-water bidet toilet seats or in tap water.

Microbial community structure

Figure 7 shows the microbial community structure in spray water collected from five warm-water bidet toilet seats on the university campus (spray waters 1–5, lanes 1–5) and from one toilet in a residence in the same city, which was evaluated as a control (spray water 6, lane 6).

Table 3 | Detection of fecal indicator bacteria and *P. aeruginosa*

			Coliforms		*E. coli*		*Enterococcus*		*E. coli* or *enterococcus*		*P. aeruginosa*	
Survey		*n*	Positive number	Detection ratio (%)	Positive number	Detection ratio (%)	Positive number	Detection ratio (%)	Positive number	Detection ratio (%)	Positive number	Detection ratio (%)
1st	Total	127	7	5.5	3	2.4	4	3.1	6	4.7	2	1.6
	Male	43	6	14.0	3	7.0	2	4.7	4	3.1	1	2.3
	Female	71	1	1.4	0	0.0	2	2.8	2	1.6	1	1.4
	Barrier-Free	13	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
2nd	Total	127	6	4.7	6	4.7	3	2.4	6	4.7	2	1.6
	Male	43	5	11.6	5	11.6	1	2.3	5	3.9	1	2.3
	Female	71	1	1.4	1	1.4	2	2.8	1	0.8	1	1.4
	Barrier-Free	13	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
1st + 2nd	Total	254	13	5.1	9	3.5	7	2.8	15	5.9	4	1.6
	Male	86	11	12.8	8	9.3	3	3.5	11	4.3	2	2.3
	Female	142	2	1.4	1	0.7	4	2.8	4	1.6	2	1.4
	Barrier-Free	26	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0

Table 4 | Detection concentration of fecal indicator bacteria and *P. aeruginosa*

	Coliforms		*E. coli*		*Enterococcus*		*P. aeruginosa*	
No.	1st Survey MPN/100mL	2nd Survey MPN/100mL	1st Survey MPN/100mL	2nd Survey MPN/100mL	1st Survey MPN/100mL	2nd Survey MPN/100mL	1st Survey CFU/mL	2nd Survey MPN/100mL
1							10	345
2	52				288			
3		52		52				
4	10						5	624
5					10			
6	20	10	10	10				
7	109		85					
8		135		135		10		
9					31			
10		160		121		6,488		
11	41							
12	175							
13	2,613		2,481		52			
14		10		10		10		
15		408		408				

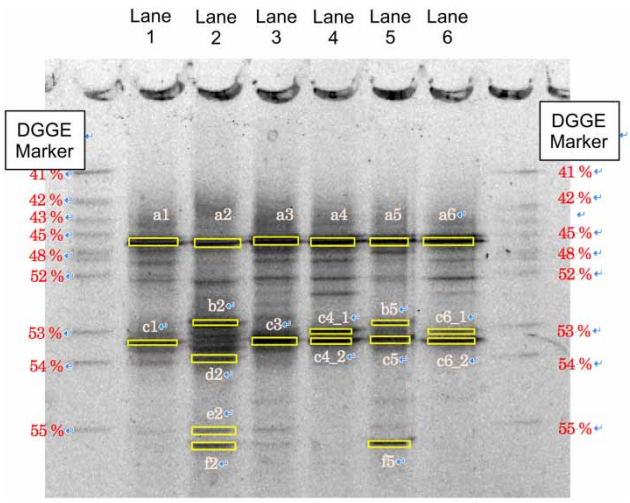

Figure 7 | Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) products of the analysis of the spray water from bidet toilet seats with warm-water tanks. Lanes 1–3: spray waters from the research buildings, Lanes 4–5: spray waters from the outpatient buildings, and Lane 6: spray water from the individual residence.

Spray waters 1–5 were collected from the research building and spray water 6 was collected from a location about 6 km from the university campus. However, the tap water supplying all of the toilets was from the same water source and the same water-purification plant. The band patterns in Figure 7 show two systems (band a, bands b and c) in almost the same location in spray waters 1–6; these are thought to represent bacteria from the shared water source. Note that band groups are evident below bands a1 to a6 in Figure 7, but these bands could not be confirmed or cut out. Sequence analysis was performed on the cutout bands shown in Figure 7, and the sequences that were obtained were identified by a Basic Local Alignment Search Tool (BLAST) search of the DNA Data Bank of Japan (Table 5).

The bacterial groups that the spray water samples had in common were *Arthrobacter*, a type of actinomycete; *Novosphingobium*, a soil bacterium; *Sphingomonas*; and *Sphingopyxi*. Phylogenetic analysis with the neighbor-joining method revealed that *Novosphingobium* (bands b2, b5) and *Sphingomonas* (bands c1, c3–c6) have similar DNA sequences (Figure 8).

PCR-DGGE isolation and sequence analysis and identification indicated that the environmental microorganisms found in trace amounts in tap water create biofilms and proliferate inside the warm-water tanks of

Table 5 | Identification of denaturing gradient gel electrophoresis bands

Band	Accession number	Most closely related organisms	Partial sequence identities	Gap
a1, a2	NR 024783.1	*Arthrobacter russicus* strain A1-3 1	186/188 (99%)	2/188 (1%)
a4	NR 024783.1	*Arthrobacter russicus* strain A1-3	151/152 (99%)	1/152 (1%)
a5	NR 024783.1	*Arthrobacter russicus* strain A1-3	184/188 (98%)	2/188 (1%)
a6	NR 024783.1	*Arthrobacter russicus* strain A1-3	185/188 (98%)	2/188 (1%)
b2, b5	NR 044320.1	*Sphingomonadaceae bacterium* E4A9 strain	169/169 (100%)	
	NR 040827.1	*Novosphingobium subterraneum* strain IFO 16086	169/169 (100%)	
	NR 025838.1	*Novosphingobium capsulatum* strain GIFU 11526	169/169 (100%)	
c1, c3, c4_2, c5, c6_2	NR 044187.1	*Sphingomonas insulae* strain DS-28	169/169 (100%)	
	NR 043612.1	*Sphingomonas dokdonensis* strain DS-4	169/169 (100%)	
	NR 024631.1	*Sphingopyxis chilensis* strain S37	169/169 (100%)	
d2	NR 042358.1	*Mesorhizobium thiogangeticum* strain	169/169 (100%)	
	NR 028901.1	*Ochrobactrum grignonense* strain OgA9a	169/169 (100%)	
e2	NR 044095.1	*Filomicrobium insigne* strain SLG5B-19	165/169 (98%)	0/169 (0%)
f2, f5	NR 036877.1	*Craurococcus roseus* strain NS130	168/169 (99%)	0/169 (0%)

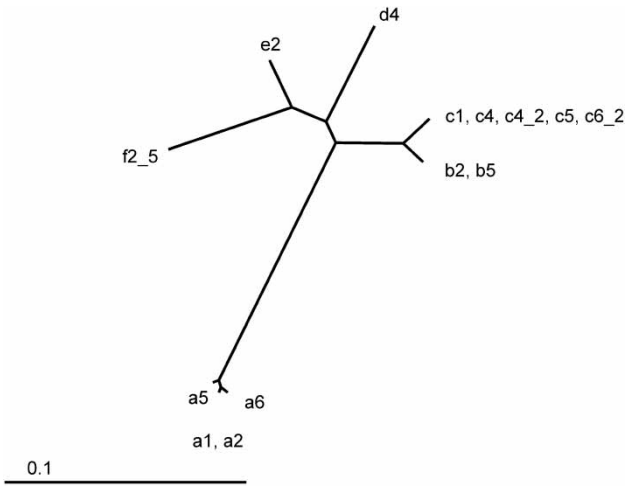

Figure 8 | Phylogenetic tree of microorganisms obtained from spray water. Symbols of taxa refer to the PCR-DGGE bands shown in Figure 7 and Table 5. Scale bar represents 0.1 substitutions per nucleotide site (Neighbor-Joining method).

bidet toilet seats, and are then discharged into the spray water.

DISCUSSION

The ability to maintain and regulate residual chlorine is an important built-in factor for maintaining the hygiene and disinfection of spray water. In this study, we surveyed 127 warm-water bidet toilet seats and found that the concentration of chlorine decreased due to heating and remaining stagnant for long periods. Since there was no obvious change in the toilet seats surveyed over time, the observed decrease in residual chlorine was not due to deterioration of the toilet seats themselves.

The effectiveness of disinfection depends on the CT value, which is the product of residual chlorine concentration and contact time (Zamyadi *et al.* 2012, 2013). When water is sprayed from a bidet toilet seat's nozzle, the tap water flows in to replace it, thereby increasing the amount of residual chlorine in the warm-water tank. However, if a toilet seat is not used from nighttime to the next morning, the water in the tank will stay warm without any new influx of tap water, causing the residual chlorine levels in the tank to decrease. Therefore, a warm-water bidet toilet seat's frequency of use and the interval between uses will affect the amount of residual chlorine in the warm-water tank as well as the contact time, thereby changing the CT value.

When heterotrophic bacteria are cultured at low temperatures for long periods using culture media with relatively low concentrations of organic nutrients, the number of bacteria that form colonies on the culture medium can be used as an indicator of the bacterial count and biofilm growth inside pipes, which represent an aquatic environment of low nutrition (Gagnon *et al.* 2005; Ndiongue *et al.* 2005; Berry *et al.* 2006; Abdul *et al.* 2012). HPC in the

spray water showed a marked increasing trend, rising to levels 100 to 1,000 times higher than those found in tap water. This suggests that microorganisms repopulate and biofilms form at higher rates in the warm-water tanks and internal tubing of bidet toilet seats than in tap water pipes due to heating, and because of remaining stagnant for long periods.

Furthermore, the nozzles of warm-water bidet toilet seats can become contaminated with feces because they are used to wash the area around the anus after defecation. When feces contaminate the nozzle or the region around the hole from which the spray water exits, fecal indicator bacteria can be detected in the spray water. Fecal indicator bacteria were detected at a higher rate in the spray water of warm-water bidet toilet seats in male restrooms, which suggests that toilet seats in male restrooms are used more frequently than those in female restrooms. Although there are non-fecal types of coliform bacteria (Caplenas & Kanarek 1984; Leclercq *et al.* 2002), all of the bacteria detected in the present survey are considered to have originated from feces. As both heterotrophic bacteria and viable bacteria exist in tap water, these could repopulate inside the warm-water tanks and then be detected in spray water. However, it is difficult to imagine that fecal indicator bacteria, which require high nutrient conditions, could have repopulated in the low-nutrient environment of a warm-water tank (Qi *et al.* 2008). It follows that the fecal indicator bacteria detected likely came from the users' feces, and that the fecal indicator bacteria on nozzle surfaces and spray holes could mix with spray water. Warm-water bidet toilet seats are equipped with a mechanism that cleans the nozzle before and after use. Considering the relatively low frequency of fecal indicator bacteria detection, it appears that this cleaning function was generally working properly. However, we believe that further improvements should be made to the nozzle cleaning functions.

Trace amounts of *P. aeruginos*a were detected in the spray water of warm-water bidet toilet seats. *P. aeruginosa* is a gram-negative aerobic bacillus that exists in soil, freshwater, and seawater. It is also a member of the enterobacterial community and is sometimes detected when sewage mixes with environmental water (van der Kooij *et al.* 1982). *P. aeruginosa* secretes a biopolymer that enables it to form biofilms and shows strong resistance to chlorine (Silva *et al.* 2008; Baghal *et al.* 2013). The warm-water tanks in the bidets also promote biofilm formation and long-term survival of *P. aeruginosa* on the inner walls of these tanks. As such, it is difficult to achieve complete deactivation of *P. aeruginosa* with the concentration of residual chlorine found in tap water, and there are reports that this bacterium can remain inside biofilms on pipes for long periods (Mena & Gerba 2009). Silver ions have been shown to be effective in deactivating *P. aeruginosa* (Silvestry-Rodriguez *et al.* 2007), indicating that coating warm-water tanks with silver ions could be a potential option for the effective elimination of this bacterium.

P. aeruginosa is not pathogenic in immunocompetent, healthy individuals; therefore, there is no need to worry about the risk of infection from this organism from warm-water bidet toilet seats in regular households. However, it can cause opportunistic infections in individuals with reduced immunity (Reuter *et al.* 2002; Aumeran *et al.* 2007; Trautmann *et al.* 2008). *P. aeruginosa* can easily become resistant to antibiotics (Bert *et al.* 1998; Ferroni *et al.* 1998; Durojaiye *et al.* 2011). The ability of *P. aeruginosa* infections to spread via tap water has become a particular problem in intensive-care units (Bert *et al.* 1998; Trautmann *et al.* 2001, 2005; Rogues *et al.* 2007; Silva *et al.* 2008; Fujitani *et al.* 2011). Therefore, when warm-water bidet toilet seats are placed in hospital wards, sufficient attention needs to be paid to the possibility of *P. aeruginosa* contamination and propagation, which should include regular testing for *P. aeruginosa*.

P. aeruginosa has been detected in groundwater at 1 to 2,300 CFU per 100 mL (Allen & Geldreich 1975). Moreover, a *P. aeruginosa* detection rate of 7.3% (32 of 440 tests were positive) in tap water was reported in Hungary (Szita *et al.* 2007). It is unclear what water-treatment methods were used on these samples or the local characteristics of these areas, making it difficult to directly apply these data to Japan. Nonetheless, this report does indicate that *P. aeruginosa* can exist in groundwater, and if water-treatment plants are not 100% efficient, the possibility of extremely low concentrations of *P. aeruginosa* in tap water cannot be ruled out. Further detailed studies should focus on *P. aeruginosa* detected in spray water from warm-water bidet toilet seats, including its source, behavior inside warm-water tanks, ability to survive for long periods, and multidrug resistance.

As total viable count is a widely used indicator of hygiene and disinfection, our results indicate that the disinfection effect was clearly lower in spray water than in tap water (Codony *et al.* 2005). Although residual chlorine decreased and total viable count and HPC increased in the spray water, there were no marked increases in the amount or detection frequency of fecal indicator bacteria and *P. aeruginosa*. Therefore, hygienic safety was being maintained overall, as the concentrations of total viable bacteria, fecal indicator bacteria, and *P. aeruginosa* were low.

PCR-DGGE isolation and sequence analysis and identification also indicated that environmental microorganisms found in trace amounts in tap water create biofilms and proliferate inside the warm-water tanks of bidet toilet seats, and are discharged in the spray water. No clear pathogenic microorganisms were detected in the microbial community structure analysis with PCR-DGGE. The common taxa isolated from the spray water across the survey were *Arthrobacter, Sphingomonas,* and *Novosphingobium. Arthrobacter* species were reported to have been isolated from air inside the Russian space station Mir. *Sphingomonas* and *Novosphingobium* are soil bacterial groups that were classified separately from *Pseudomonas* about 20 years ago. Although these are gram-negative bacteria, instead of possessing the lipopolysaccharides specific to gram-negative bacteria, they are characterized by the sphingoglycolipids that are seen in eukaryotic cells (Godoy 2003; Li *et al.* 2004; Yoon *et al.* 2006, 2008). Other bacterial species that are known to be inherent to spray water include the sulfur-oxidizing bacterium *Mesorhizobium thiogangeticum*, the soil bacteria *Ochrobactrum grignonense* and *Filomicrobium insigne*, and the acetobacter *Craurococcus roseus* (Saitoh *et al.* 1998; Bathe *et al.* 2006; Wu *et al.* 2009).

In summary, our survey of 127 warm-water bidet toilet seats installed in restrooms in a university research building and an outpatient building showed that when the residual chlorine concentration decreased owing to heating or remaining stagnant for long periods in warm-water tanks, there was an increase in HPC in the tanks and growth of biofilms on nozzle surfaces, inside nozzle tubing, and inside the tanks. Total viable bacteria, fecal indicator bacteria, and *P. aeruginosa* were detected in the spray water of warm-water bidet toilet seats at low frequencies and concentrations, indicating that hygienic safety is being maintained overall. However, the existence of *P. aeruginosa* in spray water, even at low levels, should receive full consideration when these toilets are used in hospital locations such as intensive care units and hematology wards to prevent opportunistic infections in immunocompromised individuals. To improve the hygienic safety of the spray water from warm-water bidet toilet seats in hospital locations, self-cleaning mechanisms of the spray nozzle should be improved and structural changes should be implemented to prevent the growth of biofilms inside both the warm-water tanks and pipes.

DISCLOSURE

The authors declare no conflicts of interest. Supported by the Japan Toilet Seat Appliances Association.

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