Molecular characterization and antimicrobial susceptibility of bacterial isolates present in tap water of public toilets

Rajanbir Kaur[a], Drishtant Singh[b], Anup Kumar Kesavan[b] and Rajinder Kaur[a],*

[a]Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab 143005, India;
[b]Department of Molecular Biology and Biochemistry, Guru Nanak Dev University, Amritsar, Punjab 143005, India

*Corresponding author: Tel: +91 9814860975; E-mail: rajinder.botenv@gndu.ac.in

Background: The present study was carried out to investigate the tap water quality of public toilets in Amritsar, Punjab, India.

Methods: Water samples from the taps of the public toilets were collected in sterile containers and physico-chemical and bacteriological analysis was performed using standard methods. Also, genotypic and phenotypic characterization of the bacterial isolates was performed using different biochemical tests and 16S ribosomal RNA analysis. An antibiotic susceptibility test was performed using antibiotics based on their mode of action. A biofilm assay was performed to assess the adhesion potential of the isolates.

Results: A total of 25 bacterial isolates were identified from the water samples, including *Acinetobacter junii, Acinetobacter pittii, Acinetobacter haemolyticus, Bacillus pumilus, Bacillus megaterium, Bacillus marisflavi, Bacillus flexus, Bacillus oceanisediminis, Pseudomonas otitidis, Pseudomonas* sp. RR013, *Pseudomonas* sp. RR021, *Pseudomonas* sp. RR022, *Escherichia coli* and *Enterobacter cloacae*. The results of the antimicrobial susceptibility test revealed that the antibiotics cefodroxil, aztreonam, nitrofurantoin, cefepime, ceftazidime and amoxyclav were found to be mostly ineffective against various isolates. The biofilm assay revealed the weak, moderate and strong biofilm producers among them.

Conclusions: The tap water in the public toilets was microbially contaminated and needs to be monitored carefully. The antibiotic susceptibility profile showed that of 25 bacterial isolates, 5 were multidrug resistant. Bacterial isolates exhibited strong to weak adhesion potential in the biofilm assay.

Keywords: antimicrobial susceptibility test, bacteriological analysis, biofilm assay, public toilets, 16S rRNA analysis

Introduction

Water-borne diseases are a major water quality concern in economically developing countries such as India, where water supply and sanitation services do not keep pace with increasing population growth, urbanization and industrialization. According to a recent article published in the New York Times, half of India's population defaecates outdoors and it is one of the major reasons for stunted growth in children, as poor sanitation and hygiene contribute to chronic bacterial infections, making them unable to absorb nutrients from food. Many children have toilets at home, but they live in areas where other people lack toilets and get infections through flies and unsafe water sources.[1] Moreover, inadequate water availability; poor quality of the water supply; poorly maintained water and sewage pipelines; improper

disposal of human, animal and household wastes; and lack of awareness among people about good sanitation and personal hygiene are the main factors that are responsible for water pollution.[1,2]

The most common water-borne diseases are mainly caused by washing of hands with unclean water, and half of the malnutrition cases in India are due to repeated diarrhoea or intestinal infections due to poor water and sanitation conditions. According to the United Nations Children's Fund, the only way to prevent the prevalence of diarrhoea in developing countries is by constructing toilets and creating awareness among the general public about the importance of toilets.[3,4]

A lack of toilet facilities and open defecation in developing countries contributes to contamination of water resources. But

when toilets are not cleaned properly they also act as sources of infection, because opportunistic pathogens present in such environments are capable of causing diseases in aged, cancer or immunocompromised patients. Some opportunistic pathogens present in water distribution networks are *Pseudomonas, Klebsiella, Escherichia coli, Aeromonas, Legionella* and *Mycobacterium* spp.[5,6] Moreover, the presence of bacteria resistant to antibiotics, especially in contaminated environments, leads to high morbidity and mortality worldwide. According to the Infectious Diseases Society of America, antimicrobial resistance among bacteria poses a serious threat to human health.[7] Carbapenem resistance among Gram-negative bacteria, particularly those that belong to the family Enterobacteriaceae, is a global concern.[8]

Public toilets in schools, offices, factories, railway stations, restaurants, etc. are used to urinate and defaecate, wash hands, access mirrors, attend to menstrual hygiene needs and access dustbins for waste disposal. Improper sanitary conditions in public toilets lead to various diseases, especially urinary, reproductive and gastrointestinal tract infections.[9] Public toilets are frequently used by individuals with varying hygienic practices. Therefore it is necessary to explore and investigate the microbial diversity in public toilets, as they directly affect the health of users. Keeping all this in mind, the present study was designed to investigate the microbial diversity of the tap water of public toilets of Amritsar, susceptibility of bacterial isolates to different antibiotics and adherence ability of the microbes to the pipeline surfaces.

Materials and methods

Study area

This study was conducted in Amritsar, Punjab, India. Geographically it is located at 31.63°N latitude and 74.87°E longitude, having an average elevation of 234 m above sea level. The city had a population of 1 219 478 in 2016. The sampling sites of the present study are marked on the map in Figure 1.

Sample collection

A total of 20 water samples were collected from the taps of public toilets of Amritsar. The samples were collected in the months of August and September 2016 from the public toilets of different sites, including tourist spots, religious places, hospitals, educational institutions, government organizations, bus stands and the railway station. The water samples were collected in sterile screw-cap containers. To avoid contamination, disposable gloves were worn during sampling and the outer surface and mouth of the tap were sterilized using 70% ethanol before collecting the sample. The tap water was allowed to run for a few minutes in order to collect fresh water. For bacteriological analysis, samples were collected in sterilized containers, kept in an icebox during sampling and stored at 4°C in the laboratory. The samples were processed within 2 h from the time of collection for bacteriological analysis.

Water analysis

Physicochemical analysis

The physical and chemical properties of tap water samples were analysed using the standard methods as stated by the American Public Health Association.[10] The samples were analysed for seven physicochemical parameters, including pH, electrical conductivity (EC), total hardness, total dissolved solids (TDS), nitrate, phosphate and chloride content. The analytical methods used for testing of water quality were ensured through careful standardization, blank measurements and processing of samples in triplicate.

Bacteriological analysis of water samples

Isolation and enumeration. The water samples were diluted (10^{-4}–10^{-6}) and then plated on nutrient agar medium for enumeration. For enrichment of water samples, 1 mL of each water sample was inoculated in 5 mL of nutrient broth and incubated overnight at 37°C and 180 rpm. The isolated colonies were further streaked on various agar media (i.e. MacConkey agar, eosin methylene blue agar, mannitol salt agar) for selective differentiation of bacterial isolates. Gram staining was performed to identify whether the isolates were Gram positive or Gram negative and to determine the purity of the culture. The selected isolates were then transferred to Luria broth and grown overnight at 37°C on an orbital shaker at 180 rpm for further morphological and biochemical characterization. The colony-forming units (CFU) per millilitre were calculated using the formula: CFU/mL=(number of colonies×dilution factor)/volume of sample plated.

Bacterial identification. The primary identification of the bacterial isolates was performed on the basis of their culture characteristics on agar plates and also by microscopic observations using Gram staining. For Gram staining, each bacterial culture was spread over a sterile glass slide to form a uniform smear. After heat fixing, the smear was stained with crystal violet (1 min) followed by iodine (1 min), which acts as a mordant, followed by 70% ethanol (30 s) for decolourization and counterstained by safranin (1 min). Gram-positive bacteria appear purple, while Gram-negative bacteria appear pink after staining. The biochemical and morphological characterization was performed according to Bergey's Manual of Systematic Bacteriology.[11] The biochemical characterization was performed using biochemical tests such as IMViC (indole, methyl red, Voges–Proskauer, citrate), motility, triple sugar iron, oxidation/fermentation, catalase, oxidase and urease production, and sugar fermentation and gas formation (glucose, sucrose, fructose, mannitol, lactose).

For molecular characterization, the bacterial samples were grown on nutrient agar plates and a single colony was inoculated in the nutrient broth for DNA isolation. The cultures were grown overnight and centrifuged at 5000 rpm for 5 min at room temperature and the cell pellet was washed with Tris buffer (1 M Tris-hydrochloride [HCl], 0.1M ethylenediaminetetraacetic acid [EDTA] and 0.1 M sodium chloride [NaCl]). The pellet was suspended in Tris buffer and treated with lysozyme and RNase at 37°C. The suspension was further treated with sodium dodecyl sulphate at 65°C for 30 min and then by proteinase K at 65°C for 2 h. The mixture was mixed with NaCl and the supernatant was collected after centrifugation. An equal amount of alcohol was added to the supernatant to precipitate DNA. The DNA was suspended in Tris–EDTA buffer after washing with 70% alcohol. The 16S ribosomal RNA (rRNA) sequence of the bacterial samples was amplified using primers previously described by Lane.[12] The primers used were 27F (5′-CAGGCCTAACACATGCAAGTC-3′) and
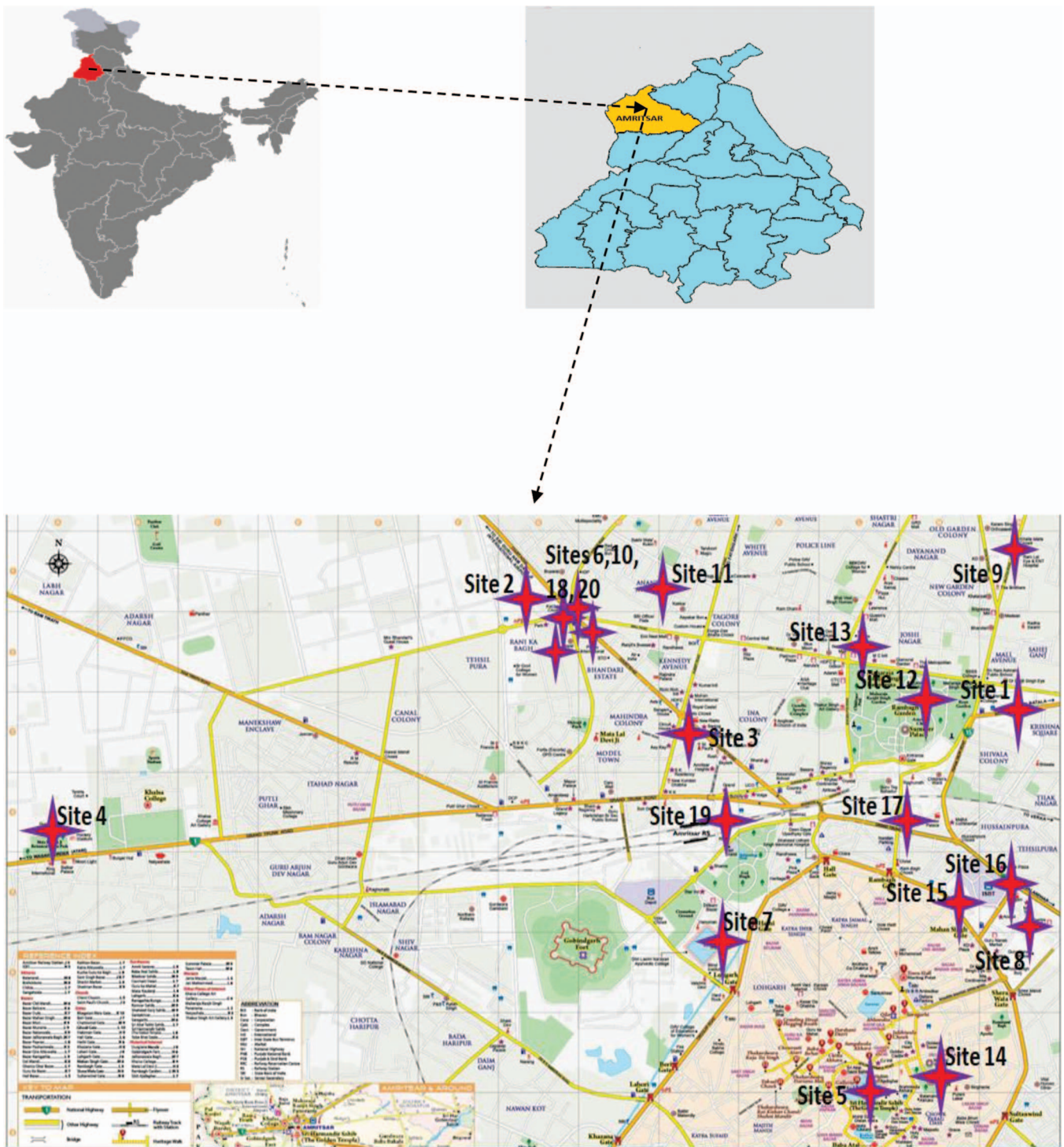
Figure 1. Map showing sampling sites in Amritsar, Punjab, India. Source: http://punjabtourism.gov.in/Downloads/TravelMap/travelMap_1.pdf.

1492R (5′-GGGCGGWGTGTACAAGGC-3′). The 16S rRNA gene was amplified using PCR in a 20 μl reaction mixture. The reaction mixture included 2 μl of Taq buffer (10X), 0.6 μl each of the forward and reverse primers (10 μM), 2 μl of deoxyribonucleotide triphosphates (10 mM), 3 μl of DNA, 0.2 μl of Taq polymerase (5 U/μl) and 11.6 μl of double-distilled water. The PCR was performed in a thermocycler (Agilent Technologies, Santa Clara, CA, USA). An initial denaturation step was performed at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 1 min, and a final step of extension was performed at 72°C for 10 min. The PCR-amplified product was purified using a PCR or Gel Extraction Kit (IBI Sci-

entific, Dubuque, IA, USA) following the manufacturer's instructions. The purified PCR products of 16S rRNA were sequenced using the same primers provided by the DNA sequencing services of BioServe Biotechnologies (Hyderabad, India). The sequences obtained were used for a gene similarity search against the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) algorithm. The 16S rRNA sequences of all the isolates were submitted in the NCBI GenBank using BankIt (www.ncbi.nlm.nih.gov/Banklt/).

Enumeration of coliforms. The most probable number (MPN) method was used to detect the presence of total and faecal coliforms in the water samples.[10] The test was performed sequentially in three stages: presumptive, confirmed and complete. The test series consisted of three groups, each having three tubes of the MacConkey Broth Purple medium. The tubes containing double-strength MacConkey Broth Purple were inoculated with 10 mL of the water samples while tubes containing single-strength medium were inoculated with 1 mL and 0.1 mL of the water samples. The tubes were kept in a biochemical oxygen demand (BOD) incubator for 24–48 h. Gas production along with a colour change of the medium in any of the tubes was the presumptive evidence of the presence of coliform bacteria. The positive broth tubes were further analysed by inoculating lactose broth containing inverted Durham tubes to confirm the presence of coliforms by the turbidity of the medium plus gas production. Tubes showing positive results were further streaked on eosin methylene blue agar. Colonies having a dark centre with a green metallic sheen are characteristic of *E. coli,* a major indicator of faecal contamination of water.

Antibiotic sensitivity test. This test was performed using the disc diffusion method with some modifications.[13] Discs of 24 different antibiotics (ceftriaxone [30 μg], cefuroxime [30 μg], levofloxacin [5 μg], co-trimoxazole [25 μg], carbenicillin [100 μg], ceftazidime [30 μg], aztreonam [30 μg], cefadroxil [30 μg], imipenem [10 μg], ampicillin [10 μg], nitrofurantoin [300 μg], piperacillin [100 μg], meropenem [10 μg], amikacin [30 μg], ciprofloxacin [5 μg], norfloxacin [10 μg], tobramycin [10 μg], amoxyclav [30 μg], fosfomycin [200 μg], gentamicin [10 μg], nalidixic acid [30 μg], polymyxin B [300 units], cefepime [30 μg] and cefpirome [30 μg]; HiMedia Laboratories, Mumbai, India) were used. The cultures were initially grown in Luria–Bertani broth medium and diluted with fresh medium to attain a cell density equivalent to 0.5 McFarland standards. The agar plates were prepared with Luria–Bertani agar medium and 0.1 mL of inoculum of the diluted bacterial culture was spread on each agar plate. Using sterile forceps, antibiotic discs were placed on the surface of the agar plates. A maximum of six discs were placed in a Petri plate (9 cm diameter). The discs were lightly pressed to ensure complete contact of the discs with the agar surface. The plates were incubated for 24–48 h in a BOD incubator at 37°C. The antibacterial activity of the given antibiotic was evaluated by measuring the clear zone or zone of inhibition around the discs.

Biofilm assay. The biofilm assay was performed according to the protocol given by O'Toole,[14] with a few modifications. The culture was grown overnight in the Luria broth and diluted in a ratio of 1:100 in fresh medium. From this dilution, 100 μl was placed in a sterile 96-well microtitre plate. For quantitative purposes, we used triplicate wells for each culture. The microtitre plate was covered and incubated for 16–24 h at 37°C. After incubation, the cells were dumped by flipping the plate and gently shaking to remove the liquid from the wells. The wells were washed twice with phosphate-buffered saline (1×), thus removing the planktonic cells and media components if attached to the wells. The wells were stained using 125 μl of 0.1% crystal violet. After 10–15 min the stain was removed by flipping the plate and gently shaking. The microtitre plate was washed with autoclaved distilled water until all the excess stain had been removed. The plate was shaken and blotted vigorously on a stack of tissue paper and was then dried at room temperature overnight. For quantification, 33% glacial acetic acid was added into each well to solubilize the dye. The plate was incubated for 10–15 min at room temperature. Optical density (OD) was measured at 595 nm using a microtitre plate reader (Synergy HT, BioTek, Winooski, VT, USA) with 33% acetic acid as the blank. The bacterial adhesion and biofilm mass were represented as the OD at 595 nm. Bacteria-producing biofilms were classified on the basis of the cut-off OD (ODc) value as non-adherent or non-biofilm producer (OD<ODc), weakly adherent or weak biofilm producer (ODc<OD≤2×ODc), moderately adherent or moderate biofilm producer (2×ODc<OD≤4×ODc) or strongly adherent or strong biofilm producer (OD>4×ODc).

Results

Physicochemical analysis

The results of the physicochemical analysis of the water samples are given in Table 1 and compared with WHO and International Organization for Standardization (ISO) standards. In the present study, the pH of the samples ranged from 7.6 to 8.3 (i.e. slightly alkaline in nature). The water sample of site 8 was found to be highly alkaline, with a pH of 8.3. The EC of all the tap water samples recorded ranged from 423.6 to 1485 μS/cm. The minimum EC observed was at site 2, i.e. 423.6 μS/cm, and the maximum was at site 20, i.e. 1485 μS/cm. The recorded TDS of the samples ranged between 221.3 and 470.3 mg/l. Sites 16 and 20 possessed the minimum and maximum amounts of TDS, i.e. 221.3 and 470.3 mg/l, respectively. The pH is an important parameter that tells us about the nature of water, whether it is acidic or basic, whereas the electrical conductivity and TDS directly indicate the presence of ions in water. The total hardness of the samples ranged between 174 and 380 mg/l. Site 1 possessed the minimum water hardness and site 19 the maximum. Total hardness reflects the amount of calcium and magnesium present as carbonates and bicarbonates in the water. The phosphate level of the tap water samples ranged from 0.9 to 3.5 mg/l, with a maximum at site 20 and a minimum at site 11. The presence of nitrate in several samples was found to be negligible or in trace amounts. The maximum level of nitrate was observed at site 7, i.e. 14.29 mg/l. The presence of phosphates and nitrates gives us indication about the contamination of water with organic material. The chloride level in the samples ranged from 12.78 to 83.78 mg/l. The maximum level of chloride was present at site 14 and the minimum at sites 16 and 17. A higher value of chloride in tap water indicates contamination of water with human excreta, which contains high amounts of chloride.

Table 1. Different water quality parameters at different sites in Amritsar

Parameters and sites	pH	Electrical conductivity (µS/cm)	Total hardness (mg/l)	TDS (mg/l)	Chloride (mg/l)	Nitrate (mg/l)	Phosphate (mg/l)
WHO (maximum allowable limit)	6.5–8.5	–	60–180	300–600	250	50	–
ISO (desirable limit)	6.5–8.5	–	200–600	500	250	45	–
Site 1	8.2	730.7	174±6	361.1	49.7±1.42	1.65±1.09	1.20±0.03
Site 2	8.0	423.6	224±12	226.6	17.04±2.84	–	1.12±0.08
Site 3	7.9	867.1	340±24	433.8	42.6±2.84	0.75±0.04	1.15±0.12
Site 4	7.8	491.7	210±14	245.9	14.2±2.84	–	1.16±0.07
Site 5	8.0	427.7	220±32	264.2	21.29±1.41	–	1.13±0.12
Site 6	7.6	1168.0	210±6	371.0	24.14±4.26	0.36±6	1.29±0.07
Site 7	7.7	837.3	306±6	419.4	49.7±4.26	14.29±0.01	1.40±0.28
Site 8	8.3	457.5	202±2	229.0	18.46±1.42	0.52±0.13	1.37±0.10
Site 9	8.2	532.1	246±26	266.8	21.3±1.42	–	1.21±0.16
Site 10	7.8	1259.0	270±10	397.2	26.98±1.42	0.34±0.01	1.31±0.11
Site 11	8.0	771.3	322±18	386.0	45.44±2.84	–	0.92±0.13
Site 12	7.6	1172.0	232±4	372.2	26.98±1.42	0.61±0.02	1.65±0.14
Site 13	7.8	751.1	374±26	383.9	59.64±2.84	–	1.16±0.02
Site 14	8.0	959.6	272±40	480.7	83.78±1.42	–	1.19±0.13
Site 15	7.9	492.6	320±12	351.1	48.28±2.84	3.13±0.16	1.19±0.15
Site 16	8.0	441.2	218±34	221.3	12.78±4.26	–	1.08±0.06
Site 17	8.1	463.7	222±14	236.4	12.78±1.42	0.50±0.01	1.16±0.05
Site 18	7.7	1094.0	242±2	348.2	24.14±4.26	–	1.73±0.21
Site 19	7.7	826.0	380±4	416.6	62.48±2.84	–	1.26±0.07
Site 20	7.7	1485.0	278±6	470.3	35.5±1.42	2.59±0.21	3.50±0.56

Results are presented in mean±standard deviation.
—: not detected.

Bacteriological analysis

The microbial analysis of tap water from the public toilets was performed according to the standard methods and guidelines provided by the WHO.[15] The results are summarized in Table 2. The standard plate count (SPC), which indicates the total microbial number in water samples, ranged from 5.77 to 6.96 log(cfu/mL) in different localities, indicating that the water sources were highly contaminated.

A total of 25 bacterial isolates were found in the samples of different localities. The 16S rRNA PCR amplification of different bacterial isolates gave approximately 1400 bp amplicons. The alignment of partial 16S rRNA sequences of different bacteria against the NCBI database suggested that they belong to 14 different bacterial species, as shown in Table 2. The accession numbers of the 16S rRNA sequences of different bacteria are listed in Table 2. Of the 25 isolates, 3 were of *Acinetobacter* (*A. junii, A. haemolyticus* and *A. pittii*), 5 were of *Bacillus* (*B. pumilus, B. flexux, B. megaterium, B. marisflavi* and *B. oceanosediminis*), 4 were of *Pseudomonas* (*P. otitidis, P.* sp. RR013, *P.* sp. RR021 and *P.* sp. RR022), as well as *E. coli* and *Enterobacter cloacae* (Table 2).

Although the occurrence and distribution of microbial species varied greatly between different localities in the city, *A. junii* and *B. pumilus* were most frequently recovered from the water samples.

We isolated two crucial coliform bacteria that belong to the family Enterobacteriaceae, i.e. *E. coli* and *Enterobacter cloacae*, from the water samples collected at sites 9 and 15 (Table 2). The results of the MPN method were interpreted with McCrady's probability tables from the number of tubes showing positive results with acid and gas formation. The presence of total coliform was calculated as the MPN index/100 mL of water sample, ranging from <1 to 93, as shown in Table 2. Faecal coliform, i.e. *E. coli*, was isolated from site 15, which is an indicator organism for the faecal contamination of water (Table 2).

Antimicrobial assay

The results of the antimicrobial sensitivity test are given in Table 3. For *Acinetobacter* sp., the isolates *A. junii* RR008 and RR011 were found to be resistant to ampicillin and nitrofurantoin, whereas isolates RR024 and RR009 were resistant to ceftazidime and nalidixic acid. The *A. pittii* RR009 isolate was found to be resistant to more than three antibiotics, i.e. aztreonam, cefadroxil, cefepime and nitrofurantoin. The *A. haemolyticus* RR019 and RR020 isolates showed resistance to nitrofurantoin and cefadroxil.

In the case of *Pseudomonas* sp., the *P. otitidis* RR006 isolate was found to be resistant to cefadroxil and amoxyclav.

**Table 2.** Prevalence of bacterial species in the tap water of public toilets

Sampling site	Isolation sites	Bacteria isolated	Standard plate count (log [CFU/mL])	Total coliform count (MPN index/100 mL)	Faecal coliform (E. coli)	Biofilm formation	Accession number
1	Celebration Mall	Bacillus pumilus RR005	6.96±0.02	<1	–	+++	MF426260
2	District Court	Pseudomonas otitidis RR006	6.76±0.06	<1	–	+	MF426243
3	General Post Office	Acinetobacter pittii RR009	6.92±0.01	<1	–	++	MF426244
4	Administrative block (GND University)	Bacillus marisflavi RR014	5.77±0.07	<1	–	+++	MF426247
5	Religious place 1	Bacillus flexus RR010	6.96± 0.03	<1	–	+	MF426245
6	Bhai Dharam Singh Satellite Hospital	Bacillus flexus RR023	6.50±0.07	<1	–	+	MF426355
7	Religious place 2	Bacillus megaterium RR004	6.87±0.02	<1	–	++	MF426242
8	Mall of Amritsar	Acinetobacter junii RR008	6.94±0.04	<1	–	+++	MF426262
9	Sri Guru Nanak Dev Hospital	Enterobacter cloacae RR026	6.95±0.03	21	–	+++	MF426257
10	Public toilet (Ranjit Avenue)	Acinetobacter junii RR016	6.09±0.10	<1	–	+++	MF426265
11	Customs Office	Bacillus megaterium RR015, Pseudomonas sp. RR021	6.74±0.11	<1	–	++, ++	MF426248, MF426253
12	Rose Garden (Ranjit Avenue)	Acinetobacter haemolyticus RR019	5.85±0.08	<1	–	+++	MF426251
13	Nehru Shopping Complex (Lawrence Road)	Bacillus pumilus RR002	6.67±0.12	<1	–	+++	MF426258
14	Jallianwala Bagh	Acinetobacter junii RR0024, Bacillus pumilus RR003	6.68±0.06	<1	–	+++, +++	MF426266, MF426259
15	Bus stand	Pseudomonas otitidis RR017, Escherichia coli RR025	6.96±0.02	93	+	+, ++	MF426249, MF426256
16	Dental College	Pseudomonas sp. RR013	6.93±0.01	<1	–	+++	MF426246
17	Civil Hospital	Pseudomonas sp. RR022, Acinetobacter junii RR012	6.95±0.03	<1	–	+++, +++	MF426254, MF426264
18	Religious place 3	Acinetobacter haemolyticus RR020	6.33±0.06	<1	–	++	MF426252
19	Railway station	Acinetobacter junii RR011, Bacillus oceanisediminis RR018	6.87±0.02	<1	–	+++, ++	MF426263, MF426250
20	Beant Park (Ranjit Avenue)	Acinetobacter junii RR007	6.86±0.04	<1	–	+++	MF426261

+: weak; ++: moderate; +++: strong biofilm producers.

Table 3. Antibiotic susceptibility of different bacterial isolates to various antibiotics

Serial No.	Antibiotics	Zone of inhibition (mm)																								
		RR007	RR008	RR009	RR011	RR012	RR016	RR019	RR020	RR024	RR006	RR013	RR017	RR021	RR022	RR002	RR003	RR004	RR005	RR010	RR014	RR015	RR018	RR023	RR025	RR026
1	CTR (30 µg)	4.03	6.90	7.53	6.80	6.20	10.4	4.46	3.40	6.5	6.9	7.8	6.8	8.4	6.9	–	6.90	6.90	4.36	5.80	9.86	3.36	4.40	5.50	6.90	4.40
2	CXM (30 µg)	4.4	5.40	4.40	6.96	5.0	4.50	4.90	3.0	6.96	–	8.5	–	4.5	2.8	–	0.96	6.93	1.86	2.90	6.40	4.36	1.86	3.53	2.90	1.80
3	LE (5 µg)	6.4	9.90	4.50	9.46	6.86	4.80	4.50	6.46	6.93	10.36	6.03	9.96	11.96	6.90	4.46	11.53	5.36	9.83	4.36	7.86	5.50	6.90	0.06	4.36	8.86
4	COT (25 µg)	7.4	7.0	6.90	6.86	6.46	5.50	2.50	4.53	6.66	4.46	6.86	8.90	10.36	6.90	–	13.40	5.46	12.86	6.50	8.90	5.86	6.90	4.40	6.80	7.0
5	CB (100 µg)	4.36	7.53	5.86	9.53	7.56	0.11	5.46	5.50	6.36	9.40	2.90	10.40	9.36	4.86	11.36	11.36	5.86	5.40	6.80	6.20	6.83	4.40	6.90	6.53	4.40
6	CAZ (30 µg)	4.46	6.90	4.50	5.40	4.43	5.80	1.86	3.96	–	5.36	5.46	6.46	5.36	1.80	–	–	5.36	–	4.86	4.80	4.36	5.86	1.86	4.86	3.86
7	AT (30 µg)	3.3	7.43	–	6.86	5.0	4.30	4.46	4.53	4.63	5.90	–	4.96	6.90	6.90	–	–	–	–	3.50	–	2.80	5.80	5.36	3.80	4.96
8	CFR (30 µg)	1.96	4.40	–	6.96	3.03	1.96	–	–	0.46	–	9.46	11.03	2.0	2.0	4.90	9.33	9.40	11.40	0.86	6.90	1.86	3.90	–	1.53	–
9	IPM (10 µg)	7.83	10.9	11.9	14.4	7.76	4.53	9.53	9.80	13.5	10.96	11.83	9.40	11.86	7.46	9.40	16.90	11.36	12.86	11.36	7.0	9.40	6.96	8.40	4.83	4.40
10	AMP (10 µg)	1.96	–	9.53	6.50	1.76	2.0	2.96	1.90	3.9	2.66	2.86	4.40	2.53	2.53	–	7.43	1.86	4.83	1.96	11.86	1.93	3.80	3.46	2.80	2.0
11	NIT (300 µg)	1.96	6.83	–	–	2.76	5.50	–	5.50	0.46	3.50	5.46	4.56	5.40	4.30	2.86	3.76	3.40	3.86	1.90	4.40	–	–	4.50	3.40	3.46
12	PI (100 µg)	4.43	8.0	4.46	9.46	6.33	4.46	3.50	4.53	6.63	4.50	3.90	6.53	6.90	6.90	1.80	7.80	1.86	3.93	4.46	12.5	4.50	4.53	3.50	5.46	2.86
13	MRP (10 µg)	6.9	11.9	6.80	10.4	7.56	6.9	4.83	7.83	6.73	13.50	6.90	13.36	9.46	4.90	4.53	11.76	6.80	10.40	4.50±	9.0	7.40	8.06	8.90	6.50	8.90
14	AK (30 µg)	1.96	9.40	9.03	8.03	6.06	4.50	0.96	1.83	6.63	11.40	7.46	7.40	6.36	6.86	6.40	7.80	6.90	7.36	2.90	7.96	6.90	2.03	1.86	3.0	4.36
15	CIP (5 µg)	6.8	11.8	6.80	10.8	7.60	7.03	6.03	5.40	7.0	7.0	7.83	6.93	10.96	6.9	4.50	11.63	4.86	10.86	5.46	6.86	4.53	7.50	8.86	6.40	8.80
16	NX (10 µg)	5.3	9.36	4.83	9.53	6.60	5.40	2.40	5.50	5.96	9.36	6.83	8.96	9.40	6.9	4.46	10.46	6.53	8.40	5.0	5.03	4.60	7.03	7.83	5.80	7.80
17	TOB (10 µg)	2.90	14.3	3.40	9.50	4.36	4.46	0.46	1.86	6.90	9.96	5.86	9.86	4.50	4.40	4.36	9.46	6.6	7.83	2.36	4.96	6.80	6.40	0.40	2.86	1.96
18	AMC (30 µg)	1.96	6.53	1.96	8.70	2.76	1.90	2.40	1.90	4.86	–	1.96	8.83	–	2.90	–	8.80	1.83	5.36	1.86	11.8	4.86	5.86	2.83	2.46	–
19	FO (200 µg)	9.43	7.0	5.03	6.40	7.53	4.43	4.46	4.46	6.46	6.80	–	–	5.03	6.76	0.11	4.36	4.90	11.83	8.36	4.50	5.40	4.90	6.86	11.96	3.36
20	GEN (10 µg)	4.46	9.46	9.53	9.50	7.96	4.36	5.46	5.46	8.03	11.96	6.83	7.80	3.86	3.86	4.36	10.36	6.40	9.40	2.40	5.40	6.90	6.86	6.60	2.36	4.46
21	NA (30 µg)	4.56	2.50	4.40	0.11	3.76	4.53	3.03	5.46	–	0.40	2.96	2.0	8.0	–	1.86	3.83	1.86	4.46	3.36	4.36	2.83	4.50	4.36	1.96	2.86
22	PB (300 units)	1.86	3.96	4.0	3.90	3.36	1.90	1.96	1.96	3.50	2.46	1.90	2.46	–	0.36	0.36	0.96	2.0	1.86	0.86	3.03	4.40	1.90	2.50	1.90	1.90
23	CPM (30 µg)	1.80	4.46	–	4.40	2.50	4.50	1.53	1.86	2.46	7.83	0.46	4.46	9.40	5.53	–	–	0.86	–	4.36	0.11	4.50	1.93	3.50	4.46	4.50
24	CFP (30 µg)	3.46	5.36	0.56	5.53	3.63	3.46	1.96	1.96	4.40	2.46	5.90	4.50	3.86	5.50	–	–	0.46	–	4.36	6.86	4.53	4.53	3.36	5.36	1.86

Results are expressed as the mean of three replicates. The antibiotics used are ceftriaxone (CTR), cefuroxime (CXM), levofloxacin (LE), co-trimoxazole (COT), carbenicillin (CB), ceftazidime (CAZ), aztreonam (AT), cefadroxil (CFR), imipenem (IPM), ampicillin (AMP), nitrofurantoin (NIT), piperacillin (PI), meropenem (MRP), amikacin (AK), ciprofloxacin (CIP), norfloxacin (NX), tobramycin (TOB), amoxyclav (AMC), fosfomycin (FO), gentamicin (GEN), nalidixic acid (NA), polymyxin B (PB), cefepime (CPM) and cefpirome (CFP). -: bacterium is resistant to antibiotic at a specified concentration.
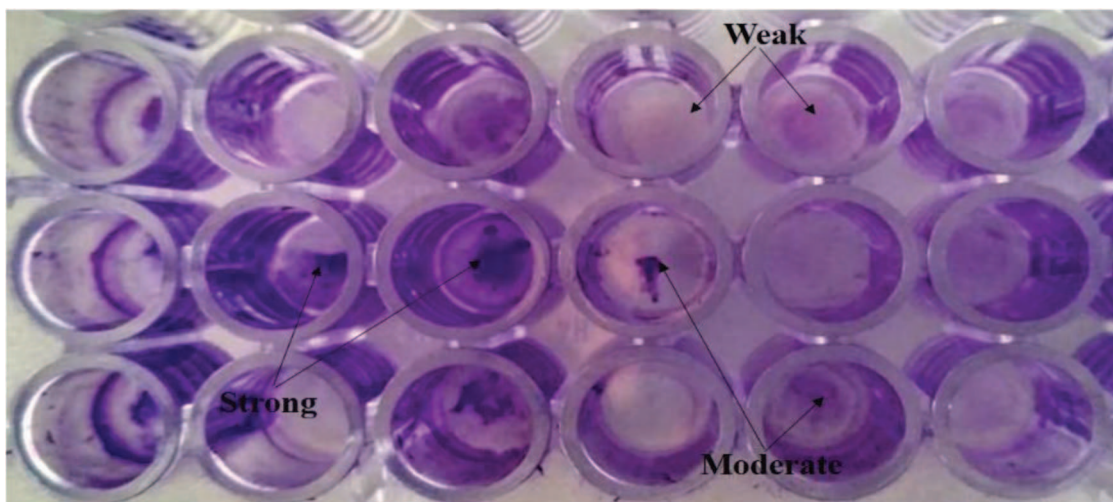
Figure 2. A 96-well microtitre plate with adherent bacterial films stained with crystal violet stain.

Furthermore, isolates RR017 and RR013 were resistant to cefuroxime, aztreonam and fosfomycin. In addition, isolate RR021 was found to be resistant to four antibiotics, i.e. cefadroxil, ampicillin, amoxyclav and polymyxin B. Similarly, isolate RR022 showed resistance to nalidixic acid. In the case of *Bacillus* sp., the *B. pumilus* RR002 isolate was resistant to several antibiotics, including ceftriaxone, cefuroxime, co-trimoxazole, carbenicillin, ceftazidime, aztreonam, ampicillin, amoxyclav, cefepime and cefpirome. Isolates RR003 and RR005 showed resistance to ceftazidime, aztreonam, cefepime and cefpirome. Isolates *B. megaterium* RR004 and RR015 were resistant to aztreonam and nitrofurantoin. *B. oceanisediminis* showed resistance to nitrofurantoin. In addition, isolate *B. flexus* RR010 showed resistance to cefadroxil, whereas the *B. marisflavi* RR014 isolate was resistant to aztreonam. Fosfomycin and ceftriaxone are more effective against the *E. coli* RR025 isolate. Similarly, the *E. cloacae* RR026 isolate is most sensitive to meropenem and ciprofloxacin and resistant to cefadroxil and amoxyclav.

The antibiotic susceptibility profile showed that of 25 bacterial isolates, 5 were multidrug resistant. Antibiotics cefodroxil, aztreonam, nitrofurantoin, cefepime, ceftazidime and amoxyclav were found to be mostly ineffective against various isolates.

Biofilm assay

Among the isolated bacteria in the water samples, *P. otitidis, B. flexus* and *A. pittii* were classified as weak; *E. coli, B. megaterium, B. oceanisediminis* and *Pseudomonas* sp. RR022 were classified as moderate and *A. junii, B. pumilus, Pseudomonas* sp. RR013 and RR021, *B. marisflavi, A. haemolyticus* and *E. cloacae* were classified as strong biofilm producers (Figure 2).

Discussion

The chemical and microbial contamination of water in the water distribution system is a global health concern. Therefore proper monitoring and management of water distribution systems has recently generated substantial interest among researchers. In the present study, most of the physicochemical parameters were found within the desirable limits of ISO (2004) and WHO guidelines (2011). In all the samples, the pH was within the permissible limits. pH is one of the most important parameters in determining water quality, as it relates to the acidity or alkalinity of the water. According to the US Environmental Protection Agency, a pH <6.5 imparts a bitter and metallic taste and enhances the rate of corrosion. It also enhances the leaching of metallic ions such as iron, manganese, lead, copper and zinc from the pipes and plumbing fixtures, whereas a pH >8.5 imparts a soda-like taste and reduces the effectiveness of chlorination. The study of drinking water conducted by Karthick et al.[16] in Kerala, India revealed pH values between 5.9 and 9.0, which is slightly higher than the permissible limits of the WHO. In another study on drinking water by Rout and Sharma[17] in Haryana, India, the pH was within permissible limits (i.e. 6.5–8.5). Yasin et al.[18] in Ethiopia and Okodoma et al.[19] in Nigeria observed the pH of potable water to be within the range of the WHO guidelines.

Similarly, the presence of dissolved solids and minerals contributes to high TDS and total hardness. Enhanced levels of TDS contribute to hard water, corrode water pipes and impart a metallic taste, whereas low TDS levels in water provide a flat taste. Sometimes contamination of water with agriculture and urban runoff and industrial wastes also leads to high TDS level. The most common ions that contribute to TDS are Ca^{2+}, Na^+, K^+, Mg^{2+}, Cl^-, SO_4^{2-}, HCO_3^- and CO_3^{2-}.[20] Studies conducted in Bangladesh and Nigeria by Adhikary and Hossain[21] and Oluyemi et al.[22] showed the TDS of the local water sources was above the permissible limit, while a study by Mohsin et al.[23] in Pakistan revealed a mean TDS value of drinking water of 438.50 mg/l, which is within the WHO limits (i.e. 300–600 mg/l).

The high level of total hardness is mainly due to high levels of calcium and magnesium salts of carbonates and bicarbonates, which can lead to the scaling of boilers. Consumption of hard water for a long time also causes health problems.[24,25] The study conducted by Srivastava et al.[26] in Allahabad, India showed the level of total hardness of drinking water was slightly lower than the prescribed limits of the ISO (i.e. 200–600 mg/l) but were

within the WHO limits (i.e. 60–180 mg/l). In a study by Shah et al.[27] in Gwalior, India the hardness of drinking water in the rainy season was found to be 464 mg/l.

Phosphates at low concentrations are harmless, but at higher concentrations they can produce ill effects such as nausea, stomach cramps, drowsiness and kidney damage. A high concentration of phosphate initiates algal growth in water sources.[28] Similarly, the presence of nitrate in the samples was found to be negligible or in trace amounts. The presence of nitrate in water indicates contamination with fertilizers and sewage. Excess nitrate in drinking water can be harmful for small children, as it causes methaemoglobinaemia.[29] In the present study, the nitrate levels at all the sampling sites were found to be within the permissible limits of the WHO and ISO. In Maharashtra, India, Srivastava et al.[30] observed the presence of nitrate in the drinking water supply that was within the permissible limit as stated by the WHO (i.e. 50 mg/l). Kamboj et al.[31] also observed nitrate levels of 54 mg/l in the municipal supplied water, which is higher than the permissible limit.

The present study revealed severe bacterial contamination in water samples at all the sites tested, making the water unsuitable for human use. The tap water samples of various public toilets showed different bacterial diversity (Table 2). The SPC of bacteria showed the level of the general bacterial count in water samples. The higher the SPC of water, the higher the amount of organic and dissolved salts in it. The study revealed contamination of water with coliforms. The coliforms, mainly referred to as total coliforms, belong to the family Enterobacteriaceae, which are Gram negative, rod-shaped, non-spore-forming and capable of growing at 37°C. The presence of coliforms showed faecal contamination of the water, which poses major health risks.[32] A study conducted in Nepal showed the presence of heterotrophic bacteria in both tap and bottled water samples. The tap water samples were found to be positive for the presence of total coliforms as compared with the bottled water.[33] A study by Deji-Agboola et al.[34] in Nigeria revealed the presence of coliforms in the potable water. The isolated coliforms included *E. coli, Klebsiella oxytoca, K. pneumonia* and *Enterobacter aerogenes*, and the presumptive total coliform count ranged from <3 to 1100 MPN/100 mL, which is very high compared with the permissible limits of the WHO guidelines.

The 14 bacterial species isolated from the public toilets of different localities have some clinical importance, as they may not be pathogenic in nature but can act as opportunistic pathogens. The bacterial isolates belonging to the family Moraxellaceae were *A. junii, A. haemolyticus* and *A. pittii*. In a similar study, Narciso-da-Rocha et al.[35] isolated different species of *Acinetobacter* from tap water. Three *Acinetobacter* species isolated in the present study are involved in several health-related issues. *Acinetobacter* is a well-known nosocomial pathogen that causes infections in hospitalized patients. *A. junii* is considered to be a human pathogen but is rarely reported as causing infections in humans. It has been reported to be associated with septicaemia in neonates and paediatric oncology patients,[36–39] and with meningitis,[40] refractory peritonitis,[41] ocular infection[42] and bloodstream infections in patients with acute lymphoblastic leukemia.[43] Wang et al.[44] reported that *A. pittii* causes hospital-acquired pneumonia and Wisplinghoff et al.[45] stated that it causes nosocomial bloodstream infections

in hospitalized patients. Similarly, Grotiuz et al.[46] found that *A. haemolyticus* causes bloody diarrhoea and is known to produce Shiga toxins.

The various *Bacillus* species isolated from the water samples belonged to the family Bacillaceae, including *B. pumilus, B. megaterium, B. flexus, B. marisflavi* and *B. oceanisediminis.* They occur in extreme conditions of high pH, temperature and salt concentrations. *B. pumilus* causes severe sepsis in infants, although it is rarely associated with clinical infections.[47] Similarly, Guo et al.[48] reported *B. megaterium* causes brain abscess in adult patients. In rare cases, *B. flexus* causes infections in burn patients.[49]

There are many studies that have shown the presence of different *Pseudomonas* spp. in drinking water.[50–52] In this study, *P. otitidis*, belonging to the family Pseudomonadaceae, was reported to cause otic infections in patients.[53] The members belonging to the family Enterobacteriaceae, including *E. coli* and *E. cloacae*, are known to be potential pathogens found in soil and water. In some reported cases, *E. coli* is known to cause bloody diarrhoea in humans[54] and is a common pathogen of urinary tract infections.[55–57] Although *E. cloacae* is not a primary human pathogen, it has been reported to cause nosocomial infections.[58] In addition, it has been reported to cause urinary tract infections in patients on dialysis.[59]

This study also provides evidence of the prevalence of antibiotic-resistant bacteria in tap water samples of different localities, similar to previous studies.[60,61] Antibiotic resistance among bacterial species is spreading at an alarming rate and the lack of effective antibacterial drugs against them is a major concern. Our findings revealed that most of the bacterial isolates were resistant to cefodroxil (7), aztreonam (7), nitrofurantoin (5), cefepime (4), ceftazidime (4) and amoxyclav (4). Antibiotic resistance in bacteria is mainly because of the overuse and abuse of antibiotics by humans against various bacterial diseases and infections, and the vertical and horizontal transfer of antibiotic resistance genes from one bacterium to another of the same or a different genus through the process of conjugation, transformation and transduction.[62,63] Incomplete antibiotic courses and little knowledge about multidrug-resistant bacteria further worsen the condition. The study conducted by Khan et al.[63] showed a correlation between antibiotic and disinfectant resistance profiles in bacteria isolated from tap water. A study by Bergeron et al.[64] revealed the presence of antibiotic-resistant bacteria in raw and treated drinking water.[65]

In the current study, all bacterial species showed the ability to form biofilms. In biofilm assays, bacterial adsorption to the surface is mainly because of van der Waals forces, which contribute to weak adsorption, or cellular appendages or extracellular polymers excreted by the cell, which contribute to strong adsorption.[66] Bacteria use extracellular polymers to form a strong, permanent attachment, which can hold the bacteria to surfaces even in the presence of large shear forces. Bacterial cells coat their surfaces with glycocalyx or extracellular polymers, allowing them to strongly adhere to surfaces in flowing water systems.[67]

Leakage in sewage pipelines or unhygienic groundwater may cross-contaminate potable water pipes, leading to the spread of pathogenic bacteria and depletion of chlorine residuals. Furthermore, the addition of animal manure to agricultural land is considered a major source of pathogenic microorganisms in the surface and groundwater systems.[68]

In addition, microorganisms present in the air of toilets may make their way into taps and hence into the water distribution system. Toilet flushing also contributes microorganisms in the toilet environment and may contaminate water sources and form biofilms.[69] Pit latrines, predominantly used in developing countries, contaminate soil and groundwater, produce bad odours and are breeding places for mosquitoes. The results of this study have demonstrated poor water quality in terms of bacterial contamination, which may pose serious health threats to users. However, further studies are required to establish the sources of contamination of the water of public toilets and the health concerns caused by this contamination.

Conclusions

The study revealed microbial contamination of the tap water of public toilets. Most of the physicochemical parameters were found to be within the permissible limits of WHO and ISO standards, but microbial contamination makes water unfit for human use. Most of the bacteria isolated from the water samples can cause diseases in human beings. The presence of *E. coli* in water samples indicates faecal contamination, which needs to be monitored carefully. The antibiotic susceptibility profile showed that of 25 bacterial isolates, 5 were multidrug resistant. The antibiotics aztreonam, cefdroxil, nitrofurantoin, ceftazidime, amoxyclav and cefepime were ineffective against them. Such bacteria may pose serious threats to patients with weak immune systems. Bacterial isolates exhibited strong to weak adhesion potential in biofilm assays. This problem can be reduced by the use of antibacterial materials in toilets, such as antibacterial-coated tiles, toilet seat covers and taps. Maintenance of proper sanitary conditions in toilets, cleaning of dustbins, proper air ventilation, disinfection of floors and chlorination of water can decrease the chances of microbial contamination. In addition, touch-free flushing, taps and door-opening devices should be recommended to avoid pathogen transmission through contact. Materials used in the construction of water distribution systems should be carefully selected to prevent biofilm formation by microbes.

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