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Evaluation the Impact of Chlorination on the Development of Chlorine and Antibiotic-Resistant Bacterial Isolates in Public Swimming Pools

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ABSTRACT

Water is necessary for human daily activities and acts as a vehicle for the transmission of several waterborne diseases. This study was designed to investigate the bacteriological safety of water in swimming pools in Sulaymaniyah city-Iraq. For this purpose, during summer time (August to September 2021), sixty-seven water samples were collected from eight indoor public swimming pools. The physicochemical parameters were measured and the bacteriological assessment was done using the Most Probable Number test. The diversity of microbial communities was then assessed using the Vitek 2 compact and the 16S rRNA gene. In addition, the chlorine resistance test and biofilm formation were quantified using the microdilution technique and crystal violet staining method, respectively. Moreover, the Kirby- Bauer disk diffusion technique was used to test antimicrobial susceptibility.

Variable results of the physicochemical analysis were recorded for each of the temperatures (25.81±1.16 to 28.69±1.03 °C), pH (7.02±0.24 to 7.46±0.31), and free chlorine (0.12±0.05 to 1.38±1.24 mg L^{-1}). MPN negative results revealed that non-lactose fermenting bacteria were detected in 80% of the water samples, including *Enterobacter cloacae* 34/59 (57.63%), and *Pseudomonas* spp. 10/59 (16.95%), and other important opportunistic pathogens 15/59 (25.42%). The contamination rate was strongly correlated with the sources of water used for the swimming pool and the free chlorine concentrations in the pools. Chlorine resistance test results revealed variations for all isolated bacteria ranging from 1.25 to 5 mg L^{-1}. Moreover, a negative linear correlation was found between chlorine concentrations, and both bacterial growth and biofilm formation. Most significantly, there was a strong positive correlation (r=0.90 to 0.98) between the degree of biofilm formation and bacterial growth. Moreover, different antibiogram profiles were recorded for the 15 antibiotics used in this study.

It can be concluded that most of the studied swimming pools are heavily contaminated by chlorine and antibiotic-resistant waterborne pathogens. Therefore, continuous monitoring and use of maximum permissible chlorine disinfectant are highly recommended.

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Keywords: Public swimming pools, Chlorine treatment, Biofilm formation, Chlorine resistant, Antibiotic-resistant, Waterborne bacterial diseases.

1. Introduction

Water is essential for life, it also acts as an excellent vehicle for the spreading of numerous infectious diseases caused by parasites, fungi, bacteria, and viruses[1, 2, 3]. Swimming promotes health and provides major physical activity[4]. However, microbial contamination of swimming pools from the environment and swimmers can result in the transmission of pathogens that cause infectious diseases such as acute gastrointestinal, cutaneous, and respiratory tract infections[5]. Additionally, the warm and moist environment of swimming pools provides an ideal growth condition for the growth of microorganisms and biofilm former pathogens[5]. The risk of potential pathogens in swimming pools is mainly associated with *Escherichia coli* O157:H7, *Shigella*, *Giardia*, *Cryptosporidium* spp., *Legionella* spp., *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Leptospira spp.*, *Salmonella sp.*, and *adenoviruses*[6,7]. As a result, disinfection is essential for keeping pool water quality and lowering the risks posed by pathogenic bacteria[8]. Chlorine, ozone, and ultraviolet radiation are the most suitable disinfectants for water treatment[9]. Chlorination is still the most applied disinfectant to ensure the microbiological safety of water and control waterborne diseases[10]. However, as a result of frequent use and variations in residual chlorine concentrations in the water of swimming pools across different countries and regions[11–14] many bacterial species have developed resistance to chlorine, which results in cross-resistance to commonly used antibiotics[15,16,17,18]. Several studies have reported that chlorination increases the antibiotic resistance genes (ARGs) in

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bacteria and promotes the transmission of antibiotic resistance bacteria (ARB) and (ARG) in the aquatic mediums that eventually pose a potential risk to human health[10,19]. Additionally, biofilms protect bacterial contaminants from the action of chlorine and act as a potential reservoir for waterborne infections[20].

In this perspective, identifying bacterial diversity and evaluating swimming pool water quality are considered important steps in protecting swimmers from acquiring waterborne infections. Based on the lack of proper scientific studies on the quality of the swimming pool water in Iraqi cities, especially in Sulaymaniyah city. This study was designed to assess the water quality of the selected public swimming pool of Sulaymaniyah city and to evaluate the occurrence of chlorine resistance and its impact on the development of multidrug resistance among isolated bacteria. Moreover, the isolated bacteria were also characterized for biofilm-forming ability.

2. Material and methods

2.1 Sample collection and physicochemical analysis

This study was conducted between August and September 2021, a total of 67 water samples were randomly collected from eight indoor swimming pools in Sulaymaniyah city. To represent the water quality of each pool on a daily basis, water samples were collected from the deep point, shallow point, and surface point of each pool and placed in 250 ml sterile glass bottles. The samples were then immediately transferred to the lab for microbiological analysis using a portable ice box (4°C). Water temperature, pH, and conductivity are measured on the site of sample collection since they are sensitive to change during transportation to the laboratory[6]. Therefore, on-site of water sample collection, some physiochemical analysis of water quality was performed, such as temperature and free residual chlorine using the thermometer and the standard colorimetric DPD (N, N-diethyl-phenylenediamine) method, respectively. While other physiochemical parameters, such as pH values, and conductivity, were analyzed in the laboratory using a portable pH meter (HI 9811-5 Portable pH/EC/TDS/°C Meters, USA).

2.2 Questionaries

A standard questionnaire was established to document the management information (opening hours, source of water they used, cleaning procedure of swimming pools, precaution for swimmers), and technical details of water treatment and recycling, concentration and frequency of daily injection of chlorine per swimming pool.

2.3 Microbiological analysis of swimming pool water samples

The microbiological water quality of the selected public swimming pools was evaluated through the detection of bacterial indicators such as total coliform and fecal coliform *Escherichia coli*. Among the most common techniques regularly used to evaluate microbiological water quality is membrane filtration (MF) and most probable number (MPN)[21].

The most probable number (MPN) test is used to estimate the probable number of the viable coliform groups present in the water sample[22]. In this study, total and fecal coliforms in the water sample were measured using a single set of 5-tube MPN as a standard counting method[23]. The principle of the MPN test depends on the lactose fermentation by the coliform group into acid and gas[24]. Then, the decision of MPN is based on three successive steps, including the presumptive test (total coliform), confirmation test (fecal coliform), and (thermotolerant *E. coli*). The presumptive test involved mixing 10 ml of water sample in five tubes of double-strength MacConkey broth (Fluka) and the tubes were incubated for 24 hours at 37 °C. The presence of acid and gas in any tube indicates positive detection of total coliform. The confirmation test was then carried out by transferring 0.1 ml from the first step's positive tube into a fresh single-strength MacConkey broth tube and incubating for 24 hours at 44°C. Finally, the positive growth was recognized as a positive fecal coliform. The test was finalized through inoculating on Eosin Methylene Blue (EMB) agar (Fluka) and peptone broth. The appearance of a green metallic sheen on EMB agar and the formation of a red ring after addition of Kovac's reagent on top of peptone broth indicate the presence thermotolerant *E. coli.*

2.4 Identification of isolated Gram-negative bacteria in water samples

This research was focused on the isolation and identification of each of the Gram-negative lactose fermenter and non-lactose fermenter bacteria in both MPN positive and negative results. To isolate of lactose fermenter in MPN positive and non-lactose fermenter in MPN negative water samples, 1 mL from each tube of MPN sets (5-tubes) was mixed in a sterile syringe and sub-cultured on MacConkey agar (Fluka) and incubated at 37°C for 24 hours. The bacterial isolates were identified by biochemical and molecular techniques using automated Vitek-2 (VITEK® 2: Healthcare, bioMérieux USA) and sequencing.

2.4.1 Molecular identification of bacterial isolates

Molecular identification was performed using colony PCR according to the protocol previously described by[25]. From the overnight culture of each bacterial isolate, 500 µl of broth culture was centrifuged at 10000 xg for 3 min, and the pellet was re-suspended in 300 µl of nuclease-free water. The mixture was then centrifuged at 10000 xg for 2 minutes after being heated at 95 °C for 10 minutes.

The PCR Mixture was prepared using 10µl of master mix (TransGene Biotech, China), 0.2 µl of each forward primer (27 Fp, AGAGTTTGATCCTGGCTCAG) and reverse primer (534 Rp, ATTACCGCGGCTGCTGG) (Macrogen company, South Korea), 4 µl of crude DNA and the volume was completed to 20 µl using nuclease-free water. The PCR program (Applied Biosystems, USA) set up on denaturing at 94 °C for 5 min, 30 cycles of 30 seconds at 94 °C (denaturing), 30 seconds at 55 °C (primer annealing), 30 seconds at 72 °C (primer extension), and a final extension step at 72 °C for 7 min using.

The PCR products were extracted from the gel after being separated using a 1% (w/v) agarose gel. Thermo Scientific's NANODROP1000 spectrophotometer was then used to measure the DNA concentration and adjusted to 20 ng µl-1. Then, according to the sequencing service provider's recommendations (Macrogen Company, South Korea), each premix should comprise 2 µl of (10 pmol µl-1) forward or reverse primers, and

15 µl of DNA template (20 ng µl-1). The sequencing data was then analyzed using the NCBI blast program, which is available at http://www.ncbi.nlm.nih.gov.

2.5 Antibiotic susceptibility test

The antimicrobial susceptibility test was determined using the Kirby-Bauer disk diffusion technique[26]. Overnight cultures of all isolated bacterial strains in nutrient broth were adjusted to 0.5 on the McFarland scale (McFarland Densitometer, Grant-Bio) corresponding to (1.5×10^8 CFU mL^{-1}). Then, Muller-Hinton agar plates were inoculated by spreading 100µl culture evenly across the surface of a Muller-Hinton agar plate using a cotton swab and left to dry. Then, the antimicrobial-containing disks (meropenem 10 µg, cefotaxime 30 µg, chloramphenicol 30 µg, ceftriaxone 10 µg, nalidixic acid 30 µg, tobramycin 10 µg, tetracycline 30 µg, erythromycin 15 µg, amikacin 30 µg, nitrofurantoin100 µg, ciprofloxacin 5 µg, levofloxacin 5 µg, gentamicin 10 µg, imipenem 10 µg, ceftazidime 30µg) (Bioanalyse &HIMEDIA company, India) were applied with sterile forceps, the plates were incubated at 37°C for 18 hours[27]. The diameters of the inhibitory zones surrounding the disks were measured and compared to the Clinical and Laboratory Standards Institute guidelines, CLSI-guideline 2013[28].

2.6 In vitro chlorine resistant test

The presence of chlorine-resistant bacteria was assessed using a microtiter plate test, slightly modified from the method described by Huang *et al.*, (2011)[29]. The stock chlorine solutions were prepared using sodium dichloroisocyanurate dihydrate ($C_3Cl_2N_3NaO_3 2H_2O$) in accordance with the manufacturer's instructions (Chanelle Medical Limited Co, Ireland). From the stock chlorine solution, six different chlorine concentrations were prepared in sterile nutrient broth medium 0, 0.07, 0.15, 0.3, 0.6, 1.25, 2.5, and 5.0 mg $L^{-}1$. According to the procedure which was as previously described by Destiani *et al.*, (2019) and Yuan *et al.*, (2015), 0.5 McFarland bacterial suspension was separately prepared from the overnight bacterial culture[15,30]. Then, inoculation was performed (1:100 ratio) by adding of 2 µl of bacterial suspension to each 200 µl of nutrient broth with different chlorine concentrations in 96-microwell flat-bottom polystyrene plates (SPL Plastic Labware, Korea). The microwell plates were incubated at 37°C for 18 hours and bacterial growth was measured at optical density 600 (OD 600nm) using a microplate reader (Thermo scientific, USA). Subsequently, the MIC (Minimal Inhibitory Concentration) was recorded as the lowest concentration of chlorine that had no turbidity, and MBC (Minimal Bactericidal Concentration) was determined using the viable counting method[31].

2.7 Biofilm formation assay

The chlorination biofilm assay was performed for 14 different bacterial genera to examine the impact of chlorine on the development of biofilm in the swimming pools. The quantitative crystal violet staining assay has been commonly suggested to quantify the level of biofilm formation as described previously[32,33]. The chlorination biofilm experiment was performed in a fresh nutrient broth medium mixed with different concentrations of chlorine (0, 0.07, 0.15, 0.3, 0.6, 1.25, 2.5, and 5.0 mg L^{-1}) and inoculated with (1:100 ratio) through the addition of 2 µl of bacterial suspension to each 200 µl of nutrient broth with different chlorine concentrations. The uninoculated nutrient broth was used as a negative control, and the inoculation of nutrient broth without chlorine was used as a positive control. Then, the 96-well flat-bottom polystyrene plate (SPL Plastic Labware, Korea) was incubated for 18 hours at 37°C. Planktonic cells were eliminated, and the plates were washed three times with sterilized distilled water (SDW). The preserved biofilm was stained by adding 200 µl of 0.1% (v/v) crystal violet and left at room temperature for 15 minutes. The stained biofilm was washed three times with SDW to remove excess crystal violet and left at room temperature for air-drying. Each well's-stained biofilm was dissolved in 200 µl of an 80:20 (v/v) ethanol: acetone mixture. The density of biofilm formation was measured at Optical density 595 (OD 595 nm) using a microplate reader (Thermo Scientific, USA).

2.8 Statistical Analysis

GraphPad Prism version 9 (GraphPad, California, USA) was used to analyze the data. Descriptive statistical analysis was used to show the results in graph charts and heatmap analysis. The correlation between the measured parameters was analyzed using the Pearson correlation coefficient, and $p<0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Characteristics of swimming pools

This study was carried out in the summer when people were mostly using swimming pools (Sp). Table-S1 displays the information about the eight swimming pools that were selected, including the daily average number of bathers, safety precautions to take before entering the pool, the water source, water exchange, and the process of disinfection. Each swimming pool is depending on one or two water sources, such as (well water, tanker water, and water distribution system). In addition, pool water exchange was taken place according to the filtration system (table-S1), and several precautionary actions were taken to prevent the spread of waterborne diseases and algal growth. Swimming pools were continuously injected with chlorine using either manual or automatic systems at various times (table-S1). In order to reduce the risk of viruses and bacteria in swimming pool water, WHO (2006) recommended that both filtration and adequate application of chlorine disinfectant are important to be used[6].

Due to their affordability and practicality, chlorine disinfectant is the most effective and commonly used disinfectant in municipal water supply networks[34]. Pool water quality from public and semi-public pools should be monitored to ensure the water safety of pool users and protect them from any negative health impacts caused by chemical and microbiological contaminants of water[1,35,36]. A previous study by Skibinski *et al.* (2016) confirmed that water re-circulation system and continuous chlorine injection are extremely required to mitigate microbial activity in swimming pools and to provide safety for pool users[37]

3.2 Physicochemical analysis and their correlation with bacterial content

During summer (August to September), the swimming pool water's pH, electrical conductivity, and temperature are more suitable for bacterial growth and survival. In the current study, the results of physicochemical analysis of water samples collected from eight public swimming pools showed that temperature ranged between 25.81±1.16 to 28.69±1.03 °C, pH ranged from 7.02±0.24 to 7.46±0.31, and water conductivity from 334±30 to 487±29 µS cm^{-1} (table-1). Moreover, the present study revealed great fluctuation in the mean values of free chlorine 0.12±0.05 to 1.38±1.24 mg L^{-1} (table 1).

According to WHO (2006), the degree of hypochlorous acid dissociation into H+ and OCl– (hypochlorite ion) depends on pH and temperature[38]. Results of the physicochemical analysis of the water, the temperature range of the water in agreement with the temperature ranges recorded in Egyptian swimming pools[39], and the pH values in agreement with the WHO acceptable limits (6.5-8.5)[23].

Table 1: Shows the number of bacterial isolates (n) and mean values of physicochemical parameters (Mean S.D) collected from eight public swimming pools in Sulaymaniyah city.

Swimming pools	Physicochemical parameters				No. of bacterial isolates	Percentage %
	Temperature (°C)	Conductivity µS cm^{-1}	pH values	Free chlorine ppm (mg L^{-1})		
SP1	26.00±0.55	462±67	7.23±0.17	0.17±0.09	11	18.64
SP2	25.81±1.16	487±29	7.14±0.25	0.45±0.47	4	6.80
SP3	26.89±0.87	388±23	7.16±0.23	0.18±0.13	8	13.55
SP4	27.33±0.86	334±30	7.02±0.24	0.12±0.05	9	15.25
SP5	27.64±1.00	491±24	7.23±0.15	1.38±1.24	6	10.16
SP6	28.25±1.25	367±26	7.46±0.31	0.23±0.10	10	16.94
SP7	28.69±1.03	469±29	7.26±0.22	0.28±0.16	6	10.16
SP8	27.95±1.19	448±36	7.20±0.18	0.42±0.25	5	8.50

Person correlation was used to determine the strength of the correlation between the number of bacteria isolates with physicochemical parameters of water samples, as well as, Correlation among physicochemical parameters themselves (water temperature, free chlorine, and pH). Statistical analysis results showed that bacterial isolates and pH values with weak positive (r=0.24) non-linear correlation, a moderate negative non-linear correlation (r= -0.45) with free chlorine, and no logical correlation was found between water temperature and bacterial isolates. According to this finding, Luo *et al.*, (2018) emphasized that increasing free chlorine greatly decreased the number of bacteria in the medium[40]. Moreover, a weak correlation (r= 0.09) was detected between pH values and free chlorine, and (r=0.11) between water temperature and free chlorine; a weak positive non-linear correlation was found between water temperature and the pH values with (r=0.44) (table 2). A weak correlation between free chlorine and both temperature and pH may indicate that these factors have little impact on the concentration of free chlorine in the studied swimming pools. Contrary to the obtained results, the degree of hypochlorous acid dissociation into H^+ and OCl^- (hypochlorite ion) depends on both pH and temperatures[38]. Therefore, it is important to note that even if there is a weak correlation, the relationship between these variables should not be dismissed completely.

Table 2: Person correlation between bacterial isolates and physiochemical parameters (water temperature, pH, and free chlorine), and person correlation between physiochemical parameters.

Statistical analysis	Bacterial isolates and Water temperature	Bacterial isolates and pH values	Bacterial isolates and Free chlorine
(r) Pearson correlation	-0.07	0.24	-0.45
p-values	0.86	0.56	0.26
Level of significant p<0.05	ns	ns	ns
Statistical analysis	**Water temperature and pH values**	**Water temperature and Free chlorine**	**pH values and Free chlorine**
(r) Pearson correlation	0.44	0.11	0.09
p-values	0.27	0.79	0.83
Level of significant p<0.05	ns	ns	ns

3.3 Bacteriological evaluation of water samples

Microbiological examinations were performed on pool water samples that were collected weekly over two months (August to September 2021). Water analysis mainly focuses on detecting fecal coliform as a microbial indicator for water contamination[24]. According to the findings of the microbiological analysis, all water samples had negative MPN

results, which suggests that no coliform group had been found in any of the tested swimming pools. This suggests that non-lactose fermenter bacteria dominate in swimming pools over the lactose fermenter coliform groups, which will ultimately increase the risk to swimmers that frequently visiting the swimming pools. In parallel to this finding, a previous study by Cabral (2010) confirmed that chlorine disinfectant rapidly kills the coliform group and leaves chlorine-resistant bacteria unaffected[24]. Moreover, Jin *et al.*, (2020) demonstrated that *Enterococcus faecalis* could survive and be high resistant to chlorine exposure compared to fully sensitive *Escherichia coli*[10].

Results of the bacteriological analysis showed variations in contamination of the water at Sulaymaniyah city's public swimming pools, including Sp1, Sp6, Sp4, and Sp3 with 11 (18.64%), 10 (16.94%), 9 (15.25%), and 8 (13.55%) isolates, respectively. However, the lowest bacterial isolation was found in Sp5, Sp7, Sp8, and Sp2 with 6 (10.16%), 6 (10.16%), 5 (8.50%), and 4 (6.80%) isolates, respectively (table 1). The contamination in the water of public swimming pools has been recognized globally such as (17.22%) of indoor pools in Yazd City in Iran[41], (32.9%) of indoor swimming pools in Northwestern Greece[42], (68%) of swimming pools around Kampala City in Uganda[43], and (18%) of studied pools in Biała Podlaska were above the permissible value[44].

3.4 The impact of water resources and free chlorine on bacterial load in swimming pools

The rate of water contamination might have resulted from the source of water that is used for the swimming pools, including mixed water from well water and water distribution system with 20/59 isolates (33.9%), mixed water from water tanker supply and water distribution system 19/59 (32.2%), well water 16/59 (27.12%), and water distribution system 4/59 (6.77%) isolates (figure 1).

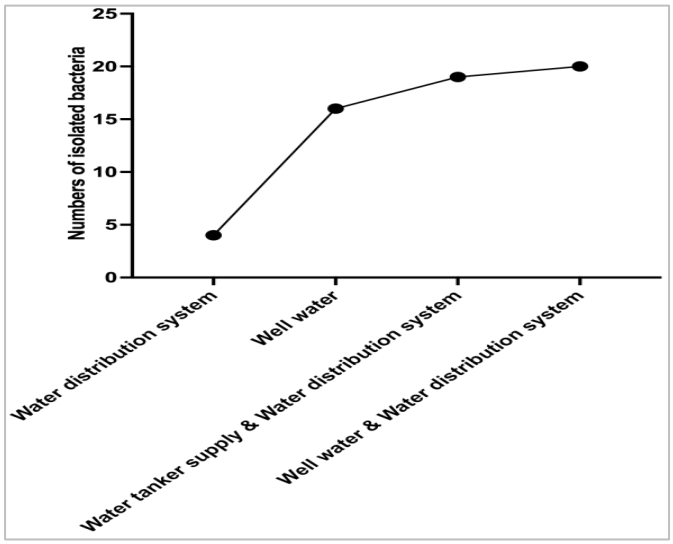

Figure 1: A water contamination according to the source of water supply to the swimming pools (Water distribution system) 4 isolates, (Well water) 16 isolates, (mixed water supply from a water tanker and water distribution system) 19 isolates, (mixed water supply from well water and water distribution system) 20 isolates.

The number of isolated bacteria in a swimming pool can be influenced by a variety of factors, including the concentration of chlorine in the water. The results of bacterial contamination showed that the number of isolated bacteria directly depends on the chlorine concentrations applied in the swimming pools. As it is obvious the maximum number of water samples with positive bacterial growth 31/67 (46.26%), was recorded at the lowest free chlorine concentration (≤0.2 mg L^{-1}) in swimming pools, followed by free chlorine concentrations (0.3-0.5 mg L^{-1}) and (>0.5 mg L^{-1}) with 13/67 (19.40%) and 4/67 (5.97%), respectively (figure 2). Czeczelewski (1994) stated that swimmers are in danger of infectious diseases when free chlorine concentration is insufficient to eliminate the bacteria in the pool[44], which is consistent with the results of a proportionally growing bacterial contamination in swimming pools with lower chlorine concentration. In addition, Luo et al. (2018) confirmed that lowering the probability of bacterial survival can be achieved by increasing free chlorine concentrations[40].

The high rates of water contamination by non-lactose fermenter

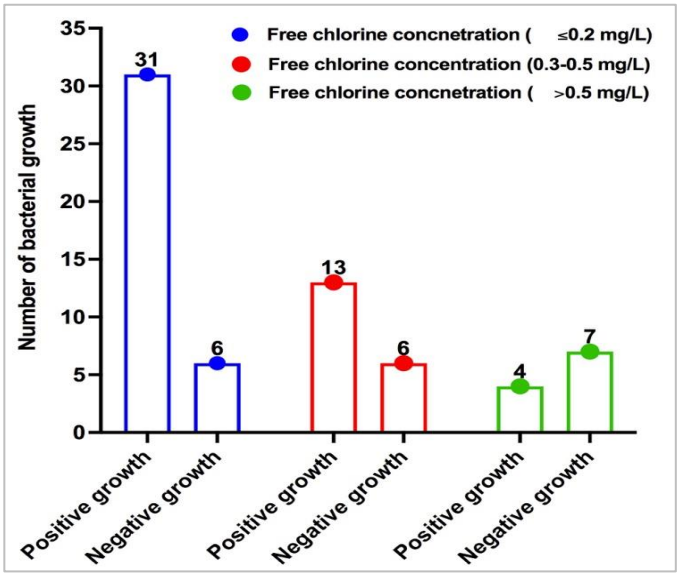

Figure 2: Represents the effect of different free chlorine concentrations (≤0.2, 0.3-0.5, and >0.5 mg L^{-1}) in swimming pool water samples on a number of negative and positive bacterial growth throughout the period of study over summer time (August to September).

bacteria might be attributed to several factors, including hygienic precautions, filtration system, source of water in swimming pools, contamination from bathers, inadequate pool disinfection, periodic pool cleaning, and poor water exchange in swimming pools. This explanation is supported by Bello *et al.*, (2012), who found that the presence of large numbers of coliform loads in pool water suggests either insufficient chlorination or insufficient protection of the source of untreated water [45]. A high level of organic matter might be other potential contributing factors related to pool contamination. The level of free chlorine in the water was believed to be directly impacted by organic substances found in swimmers' urine and sweat, temperature, and chemical components of the water[46].

In addition, a pool filled with water from the well, water tanker, and distribution water system might have a significant amount of contamination. The contaminants might be introduced through leakages in the pipes, and regrowth due to prolong storage of water, contaminated pumps, and sanitation systems[47]. Another study also confirmed that distribution system water could easily get contamination by fecal coliforms from the leakage and throughout water supply networks[25]. Moreover, Bartram *et al.*, (2014) described that unprotected springs, surface water, dug wells, and tanker trucks are considered not suitable and unimproved water for use[48].

3.5 Identification of chlorine resistance bacterial isolates

Results of the most probable number (MPN) of all water samples collected during the study showed negative growth of lactose fermenter bacteria. Therefore, the main objective of this investigation was to isolate and characterize non-lactose fermenter bacteria in MPN negative tests. Results of Vitek-2 compact indicated that all non-lactose fermenter Gram-negative divided into seven Gram-negative bacterial genera, including *Enterobacter cloacae* ssp. *cloacae* (11.86%) (7 isolates), *E. cloacae* ssp. *dissolvens* (22.03%) (13 isolates), *E. cloacae* ssp. *hormaechei* (23.72%) (14 isolates), *E. amnigenus* (1.7%) (single isolate), *Pseudomonas aeruginosa* (13.56) (8 isolates), *P. fluorescens* (1.7%) (single isolate), *P. stutzeri* (1.7%) (single isolate), *Raoultella ornithinolytica* (3.38%) (2 isolates), *Burkholderia cepacian* (6.77%)(4 isolates), *B. multivorans* (1.7%) (single isolate), *Stenotrophomonas maltophilia* (5.1 %) (3 isolates), *Acinetobacter sp*. (1.7%) (single isolate), *Acinnetobacter pittii* (3.38%) (2 isolates), *Atlantibacter hermannii* (1.7%) (single isolate) (figure 3). Molecular identification by PCR amplification was used for confirmation of identification. The sequence analysis of the amplified 16S rDNA revealed that *Enterobacter cloacae ssp. hormaechei* (with an accession number OQ421629), *E. cloacae ssp. cloacae* (accession number OQ401393), *E. cloacae ssp. dissolvens* (accession number OQ401398, *Pseudomonas aeruginosa* (accession number OQ401394), *P. fluorescens* (accession number OQ421630), *Stenotrophomonas maltophilia* (accession number OQ401399).

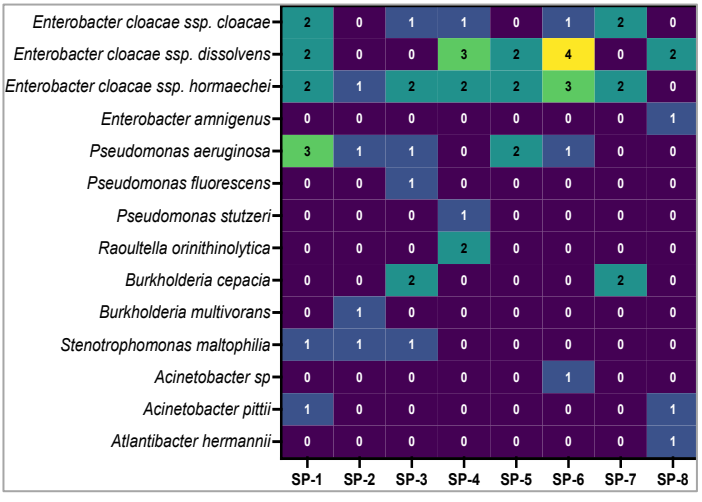

Figure 3: Heat map showing the relative abundances and distribution of the (n=7 genera) from eight swimming pools (SP).

Although, chlorine-resistant bacteria are a global issue. Therefore, this study provides insight to find the impact of different chlorine concentrations (0, 0.07, 0.15, 0.30, 0.60,.1.25, 2.5, 5 mg L^{-1}) on the growth and biofilm formation of all isolated bacteria. Results of chlorine resistance test (MIC and MBC) showed that all *E. cloacae* isolate resistant to more than 5 mg L^{-1} chlorine, followed by *P. aeruginosa, P. stutzeri, Raoultella ornithinolytica, Burkholderia cepacia, Acinetobacter pittii, Atlantibacter hermannii* resistance to 5 mg L^{-1} chlorine, *Enterobacter amnigenus, P. fluorescencea, and Stenotrophomonas maltophilia* resistance to 2.5 mg L^{-1} chlorine, and *Burkholderia multivorans* to 1.25 mg L^{-1} chlorine.

Results of correlation between different chlorine concentrations and growth of isolated bacteria exhibited negative linear correlation in *E. cloacae ssp. cloacae, E. cloacae ssp. dissolvens, E. cloacae ssp. hormaechei, E. amnigenus, P. aeruginosa, P. fluorescence, Raoultella ornithinolytica, Stenotrophomonas maltophilia, Acinetobacter sp., Acinetobacter pittii, Atlantibacter hermannii* ranging between (r =-0.74 to -0.82) (table 3) (figure 3) ($p<0.05$). However, a non-linear weak negative correlation was observed in *Pseudomonas stutzeri* (r=-0.17), *Burkholderia cepacia* (r=-0.17), and *Burkholderia multivorans* (r=-0.54) (table 3) (figure 4) ($p>0.05$).

On the other hand, a moderate negative linear correlation ranging between (r = -0.72 to r = -0.81) ($p<0.05$) (table 3) was found between different concentrations of chlorine and biofilm formation of *E. cloacae ssp. dissolvens, E. cloacae ssp. hormaechei, E. amnigenus, Burkholderia cepacian, Burkholderia multivorans, Stenotrophomonas maltophilia, Acinetobacter sp., Acinetobacter pittii, Atlantibacter pittii.* Moreover, weak to the moderate negative non-linear correlation between the effect of different chlorine concentrations and the growth was determined in each of *Enterobacter cloacae ssp. cloacae, Pseudomonas aeruginosa, Pseudomonas fluorescence, Pseudomonas stutzeri, Raoultella ornithinolytica,* and *Atlantibacter hermannii* (r = -0.13 to r = -0.68) (table 3) (figure 4) ($p>0.05$).

Most interestingly, a significant positive linear correlation found between the growth and biofilm formation *E. cloacae ssp. cloacae, E. cloacae ssp. dissolvens, E. cloacae ssp. hormaechei, E. amnigenus, Stenotrophomonas maltophilia, Acinetobacter sp., Acinetobacter pittii,* ranging from 0.90 to 0.98 (figure 4) (table 3) ($p<0.05$). However, other isolates *P. aeruginosa, P. fluorescence, P. stutzeri, Raoultella ornithinolytica, B. cepacia, B. multivorans,* and *A. hermannii* exhibited weak positive non-linear correlation (table 3) (figure 4). The overall results in quantitative biofilm analysis showed that the degree of biofilm formation was positively correlated with the ability of bacteria to grow at different chlorine concentrations. As contrary to growth and biofilm formation are both negatively correlated with chlorine levels.

The current study demonstrated the presence of free chlorine in variable concentrations, which was significantly lower than the permissible level in the whole water of swimming pools. According to previous study, the spread of pathogens that are susceptible to chlorine can be effectively stopped by using the adequate disinfectant concentration[49]. According to WHO

(2006), good filtration, and sufficient free chlorine level (1 mg/L) throughout the pool, are critically required for public and semi-public swimming pools[38]. Moreover, to control bacterial survival in the pools Yahya et al.(1990), confirmed that chlorine disinfectant needs to be supplied in high quantities and throughout all the times[50].

Table 3: Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of chlorine. Person correlation between different chlorine concentrations with bacterial growth and biofilm formation of isolated bacteria from all swimming pools. Nutrient broth (NB) mixed with different concentrations of chlorine (0, 0.07, 0.15, 0.30, 0.60,.1.25, 2.5, 5 mg L^{-1}) in 96 microwell plate was used and incubated at 37°C for 18 hrs. Biofilm formation was estimated by the crystal violet staining method. P<0.05 was used as a level of significance.

Bacterial stains	MIC & MBC of chlorine (mg L^{-1})	Chlorine and Growth	Chlorine and Biofilm	Growth and Biofilm
Enterobacter cloacae ssp. cloacae	>5	-0.79*	-0.68 ns	0.81*
Enterobacter cloacae ssp. dissolvens	>5	-0.77*	-0.79*	0.98***
Enterobacter cloacae ssp. hormaechei	>5	-0.80*	-0.80*	0.98***
Enterobacter amnigenus	2.5	-0.74*	-0.80*	0.92***
Pseudomonas aeruginosa	5	-0.75*	-0.52 ns	0.55^{ns}
Pseudomonas fluorescence	2.5	-0.81*	-0.61 ns	0.71^{ns}
Pseudomonas stutzeri	5	-0.17 ns	-0.16 ns	0.26^{ns}
Raoultella ornithinolytica	5	-0.80*	-0.63 ns	0.61^{ns}
Burkholderia cepacia	5	-0.17 ns	-0.72*	0.33^{ns}
Burkholderia multivorans	1.25	-0.54 ns	-0.75*	0.70^{ns}
Stenotrophomonas maltophilia	2.5	-0.77*	-0.81*	0.94***
Acinetobacter sp.	5	-0.82*	-0.80*	0.94***
Acinetobacter pittii	5	-0.79*	-0.78*	0.90***
Atlantibacter hermannii	5	-0.75*	-0.13 ns	0.09^{ns}

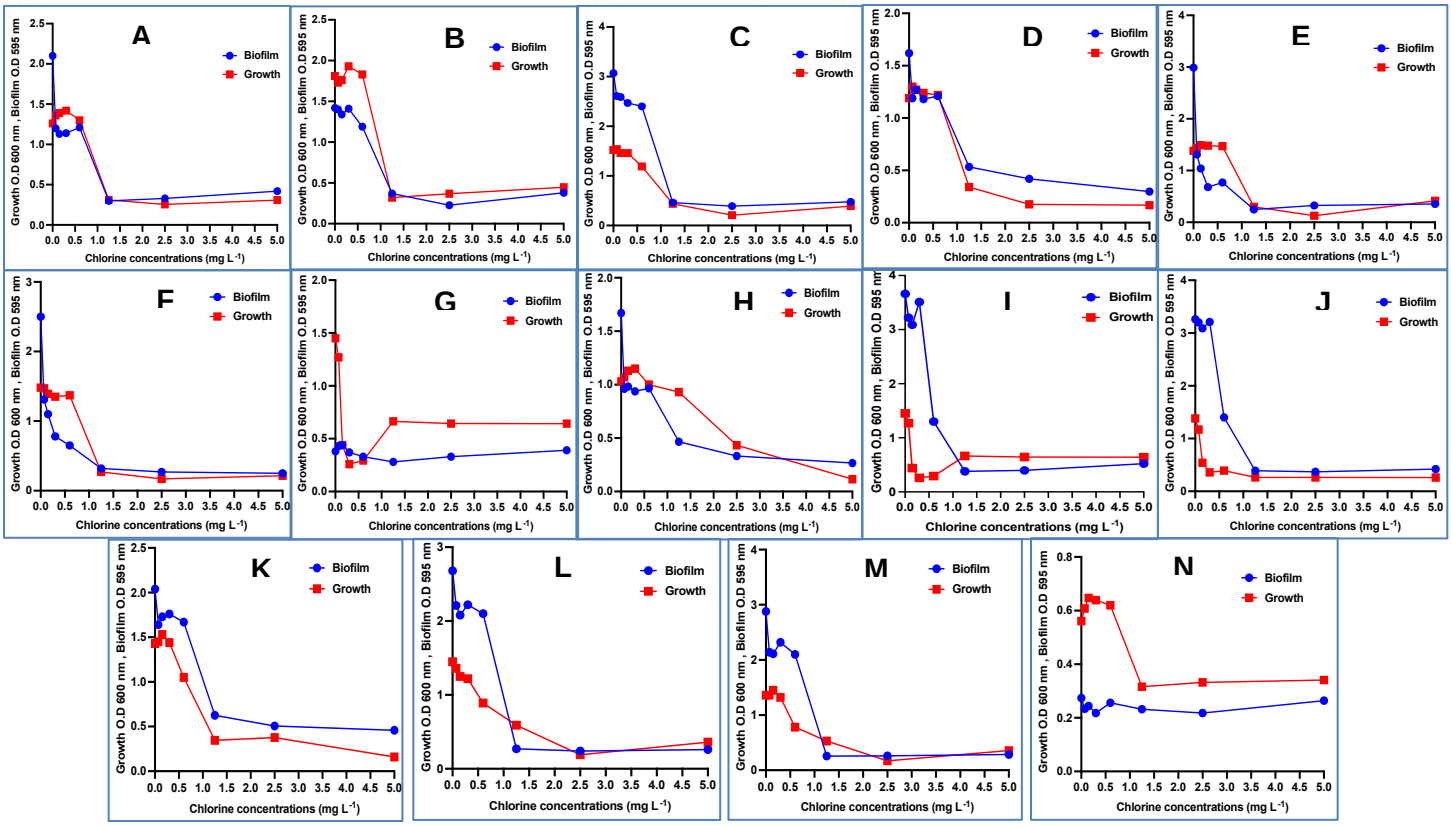

Figure 4: Evaluation the effect of the different chlorine concentrations (0, 0.07, 0.15, 0.30, 0.60,.1.25, 2.5, 5 mg L^{-1}) on growth and biofilm formation of **(A)** *Enterobacter cloacae ssp. cloacae*, **(B)** *Enterobacter cloacae ssp. dissolvens*, **(C)** *Enterobacter cloacae ssp. hormaechei*, **(D)** *Enterobacter amnigenus*, **(E)** *Pseudomonas aeruginosa*, **(F)** *Pseudomonas fluorescence*, **(G)** *Pseudomonas stutzeri*, **(H)** *Raoultella ornithinolytica*, **(I)** *Burkholderia cepacian*, **(J)** *Burkholderia multivorans*, **(K)** *Stenotrophomonas maltophilia*, **(L)** *Acinetobacter sp.*, **(M)** *Acinetobacter pittii*, **(N)** *Atlantibacter hermannii*. Nutrient broth (NB) mixed with different chlorine concentrations (0, 0.07, 0.15, 0.30, 0.60,.1.25, 2.5, 5 mg L^{-1}) in 96 microwell plate was used and incubated at 37°C for 18 hrs. biofilm formation was estimated by the crystal violet staining method. Data represents the mean of five replicates. (red line: represents the bacterial growth), (blue line: represent the biofilm formation).

Some of the Gram-negative bacteria isolated in this study belonged to the non-pathogenic, opportunistic, and common pathogens to humans. The high contamination level in pool water posed a potential risk to people[51]. In most countries, chlorination is the main water treatment method in swimming pools[10]. However, it is impossible to eliminate the threat posed by chlorine and antibiotic-resistant bacteria[47]. According to the previous study conducted in Sulaymaniyah city, the microbiological content of water samples did not reveal the existence of fecal coliform bacteria. However, non-lactose fermenter chlorine-resistant *Enterobacter cloacae* were found to be considerably determined[20].

Obtained results of this study showed that *Enterobacter cloacae* 57.5% (34/59) and *Pseudomonas aeruginosa* 13.56% (8/59) are the most commonly isolated bacteria from swimming pools (table 1). The absence of international bacterial indicators of water quality, such as total and fecal coliform *E. coli* might be due to the efficiency of chlorination to eliminate chlorine-sensitive fecal coliform and survival of chlorine-resistant *Enterobacter spp. and Citrobacter freundii*[24,52]. It has been documented that chlorine disinfectant failed to inactivate chlorine resistance *E. cloacae*[20], *Pseudomonas* sp.[51], and *Acinetobacter*[53] in water. According to several research, bacteria can survive in chlorinated water[20,54,55,56] . The prevalence of *Enterobacter cloacae* in chlorinated swimming water is consistent with the previous study by Najmuldeen(2021), who isolated (56%) chlorine-resistant *E. cloacae* from chlorinated water storage tanks[20]. Another study isolated *P. aeruginosa* from swimming pool water that causes otitis media in swimmers' ears, respiratory problems, and wound infections[54,55].

The usage of irregular quantities of chlorine-containing compounds in swimming pools may be directly related to the emergence of bacterial isolates with hyper-chlorine resistance in Sulaymaniyah public pools. Environmental isolates that have previously been exposed to chlorine may become resistant to disinfectants[15] . Furthermore, additional research showed that the excessive and frequent usage of sodium hypochlorite has led to the emergence of chlorine-resistant microorganisms (NaOCL)[16,56].

Additionally, the survival of waterborne opportunistic pathogens in treated water with disinfectants is linked to several mechanisms, including modification in the cell surface, attachment to surfaces or particles, exopolysaccharide barrier, biofilm formation, efflux pump activity, and spore formation[1,52,57,58], potential reduction in cell permeability[15], several genes that regulate oxidative stress, DNA repair, pore protein regulation, and cell wall repair have a protective role[47].

3.6 Bacterial Antibiotic Resistant tests

The most significant factor impacting the level of antibiotic resistance in treated water samples was revealed to be residual chlorine. Therefore, all isolated bacteria in this study were subjected to antibiotic sensitivity tests for 15 different antibiotics using the disc diffusion method recommended by CLSI guidelines (CLSI-2013).

The obtained results demonstrated that all bacterial isolates were resistant to cefotaxime *β*-lactam antibiotics of third-generation cephalosporins, amikacin, an aminoglycoside antibiotic, similarly resistant to other classes of antibiotics, including Nitrofurantoin, Erythromycin. Likewise, with the exception of a few isolates of *Enterobacter cloacae*, resistance to additional antibiotics with the variable modes of action such as minocycline second-generation of tetracycline, chloramphenicol, nalidixic acid was recorded (figure 5). With the exception of a few strains of *P. aeruginosa*, *Burkholderia cepacia*, and *Stenotrophomonas maltophilia*, levofloxacin, tobramycin, and ciprofloxacin were found to be the most efficient antibiotics against all identified bacteria (figure 5). Finally, a variable pattern of resistance and sensitivity was noticed to imipenem, meropenem, ceftazidime, ceftriaxone, and gentamicin antibiotics (figure 5).

Antibiogram analysis indicated that isolated bacteria belong to *Burkholderia cepacia*, *P. aeruginosa*, *Stenotrophomonas maltophilia*, *E. cloacae spp. cloacae*, *E. cloacae spp. dissolvens*, *and E. cloacae spp. hormeichae* are resistant to the most applied antibiotics. However, the most sensitive bacterial isolates to more than four antibiotics classes belong to *E. amnigenus*, *P. fluorescens*, *P. stutzeri*, *Raoultella ornithinolytica*, *Acinetobacter sp. Acinetobacter pittii*, *Atlantibacter hermannii*, and *Burkholderia multivorans.*

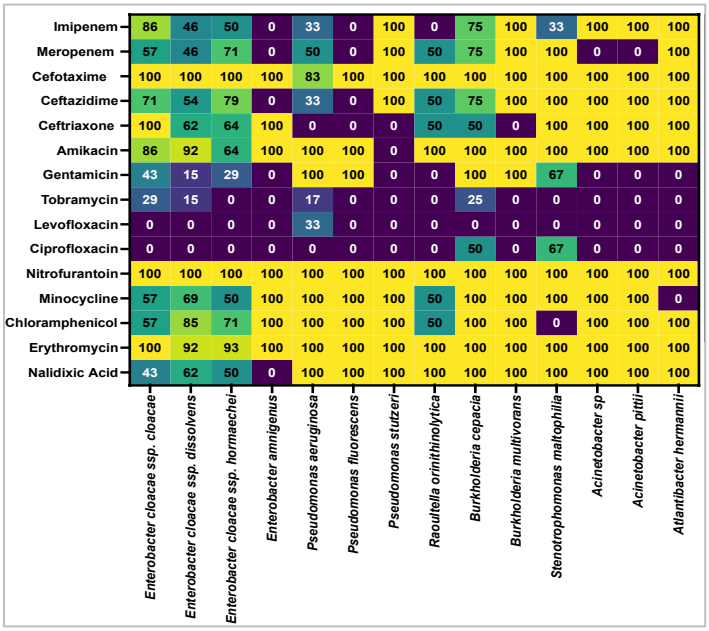

Figure 5: Heatmap pattern of antibiotic resistance profile of all isolated bacteria from eight swimming pools. Rows represent seven bacterial genera with different species and columns represent 15 different antibiotics with different modes of action, including inhibitors of cell wall synthesis, protein biosynthesis, and DNA replication[59].

There is yet no conclusive physiological link between the mode of antibiotic action and resistance to chlorine disinfectants. A recent study suggested that the aquatic environment could serve as an important reservoir of antibiotic-resistant genes (ARGs) that are horizontally transmitted to other closely related bacterial species[60]. It has been hypothesized that chlorination encourages the transformation of plasmids among bacteria in their natural environment, which promotes the development of antibiotic-resistant strains[31,61]. This concept has been proven by Jin *et al.*,

(2020), who stated that the chlorination process enhances the natural transformation of plasmid, which leads to the exchange of ARGs between bacterial genera and the emergence of new antibiotic-resistant bacteria (ARB)[10].

In agreement with this finding, other studies have verified that *E. cloacae* are susceptible to levofloxacin, ciprofloxacin, and resistant to nitrofurantoin and cefotaxime[62,63]. According to a previous study, *E. cloacae* has a high capacity to acquire genes encoding resistance to a variety of antibiotic classes, including *β*-lactamase inhibitors, aminoglycoside, tetracycline, and carbapenem[62,64]. Similarly, *B. cepacia* complex strains can survive for long durations in water, and disinfectant and exhibit resistant to a wide range of antimicrobial drugs, including polymyxin, aminoglycosides, carboxypenicillins, first and second-generation cephalosporins[65,66]. 96% of isolated *P. aeruginosa* from swimming pools have shown multidrug resistant[66].Furthermore, Govender *et al.*, (2020) noted that the presence of multidrug-resistant *Acinetobacter* spp., and *Stenotrophomonas maltophilia* in water increases the probability of community-acquired infections[67]. Maintaining safe pool water quality is crucial to prevent bathers' health problems. Regular water quality checks are necessary to avoid these undesirable effects related to the contamination of swimming pools. The findings of this study supported the existence of waterborne pathogens that may cause a risk for various bacterial illnesses.

Conclusion

The findings of this study supported the existence of slow or non-lactose fermenter Gram-negative waterborne pathogens in all selected public swimming pools that may cause a risk for various bacterial illnesses. The contamination is mainly attributed to water sources, contamination from swimmers, uneven operation of the recirculation-filtration systems, and inadequate chlorination. The failure to completely remove microbial contamination is directly related to low free chlorine levels in pool water. Additionally, optimum pH and temperature provide suitable conditions for bacterial growth and biofilm formation. Also, the presence of waterborne pathogens that are resistant to chlorine and antibiotics in swimming pool water suggests a possible risk to the general public's health in this locality.

Therefore, maintaining safe pool water quality is crucial to prevent bathers' health problems. Regular chemical and microbiological water quality checks are necessary to avoid these undesirable effects related to the contamination of swimming pools. Data from this study and subsequent comparable studies will give public health organizations and communities a foundation for better public swimming pool management to avoid waterborne diseases linked to swimming pools. Raising the bather’s knowledge and awareness of the risks is also fundamental to ensuring a safer environment in these swimming pools.

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Conflict of interests

The authors declare that there are no conflicts of interest.

Author contribution

Hastyar H. Najmuldeen: Developed the theory, planned the experiments, supervised the findings of this work, performed the statistical analysis and designed the figures, drafted and finalized the manuscript. Chawan Hazhar Razaq: Experimental design, sample collection, laboratory experiments, writing the drafted manuscript, and contributed to the interpretation of the results. The manuscript has been read and approved by the authors.

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