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ANTIBIOTIC-RESISTANT BACTERIAL ISOLATES' DEVELOPMENT IN AL BAHA WATER SAMPLES: THE EFFECT OF BACTERIAL LOAD AND CHLORINATION

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ABSTRACT

*Chlorination is a widely used disinfection method in water treatment; however, its efficacy against antibiotic-resistant bacteria and its role in promoting resistance remain areas of concern. This study evaluates the relationship between bacterial load, chlorination, and the creation of bacterial isolates resistant to antibiotics in water samples from Al Baha. In all, 207 water samples were gathered, of which 53 exhibited bacterial growth. The samples underwent microbiological and physicochemical analysis to determine bacterial load, antibiotic resistance patterns, and biofilm formation. Free chlorine levels and pH were also measured. Antibiotic susceptibility was assessed applying the disk diffusion method of Kirby-Bauer, and biofilm formation was quantified using crystal violet staining. Multidrug-resistant bacteria, including *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Salmonella spp.*, were prevalent in the samples. *Acinetobacter baumannii* showed complete resistance to all tested antibiotics. Chlorination reduced bacterial counts but was ineffective in eradicating resistant strains, particularly in samples with lower chlorine concentrations (<0.2 ppm). Biofilm-forming bacteria demonstrated significant resistance, even in samples with higher chlorine levels (>0.25 ppm). The pH (6.5–8.4) had minimal influence on resistance patterns. The findings highlight the limitations of chlorination as a sole disinfection method, especially against biofilm-associated resistance. Supplementing chlorination with alternative methods such as UV irradiation is recommended. Regular monitoring of chlorine levels and bacterial profiles is necessary to mitigate the risks of antibiotic resistance as well as ensure water safety*

KEYWORDS: Chlorination, Water disinfection, Antibiotic resistance, Multidrug-resistant bacteria (MDR).

1. INTRODUCTION

Water being such an important element in human activities, turns out to be carrying fatal diseases within it (Organization 2019). It is a clear fact that water borne diseases which are due to various microorganisms are a threat not only to human lives but also the eco system (Nwabor, Nnamonu et al. 2016). Water borne diseases are not in doubt and these include; E.coli, Salmonella, cryptosporidium, and Giardia all of which cause gastrointestinal infections, diarrhea, among other health complications (Forde, Izurieta et al. 2019), (Bridle 2020).

In addition, new contaminants sources and antibiotic-resistant bacteria that are present in water have therefore posed new risks of transmitting infectious diseases and antimicrobial resistance, thus the need to have an integrated approach towards combating these challenges (Muñoz, Aransiola et al. 2024).

The control of microbiological risks in water supply and distribution is an essential aspect of health and environmental promotion (Olatunji, Olaboye and Otaye 2024). Important trends in technologies used in water treatment are filtration, chlorination, and other relevant disinfection processes to get rid of dangerous microorganisms, including bacteria, viruses, and parasites (Sarma 2020).

In addition, prevention of contaminating distribution systems through consistent inspection for possible contamination outlets, as well as quick identification of water quality issues is important for containing the spreading of waterborne diseases (Brunkard, Ailes et al. 2011). Commitment to high regulatory requirements in drinking water and introduction of new technologies that allow monitoring and controlling microbiological risks associated with water treatment and distribution are among the key factors responsible for the protection of microbiological safety when introducing safe and potable water supplies to communities in a given country (Organization 2022). Chlorination as a strategy has been known for many years to have major benefits in ensuring microbiological water security in treatment and distribution networks (Ngwenya; Ncube et al. 2012). Chlorination is very important in defense against waterborne infections because chlorination process is very effective in eradicating or neutralizing of dangerous bacteria (Adefisoye and Olaniran 2022). Inefficient application of chlorine for instance appropriate dose or treatment affects the diversity of water supplies in a negative manner. High level of chlorine usage is likely to interfere with aquatic systems and reduce functional microorganisms for the harmony in ecosystems (Douterelo, Dutilh et al. 2020).

However, excessive application of chlorination has also been linked to the appearance of antibiotic-resistant bacterial strains in aquatic facilities (Jia, Shi et al. 2015). Investigations discovered that chlorination can raise the choice and distribution of antibiotic-resistant bacteria, which enhances the rate of antibiotic resistance genes in aquatic microbial communityomes (Zhong, Zhou et al. 2022). This is no longer only a threat to human life but also contributes to aggravating one the antibiotic resistance that leads to the failure of medical therapies and public health measures (Cai, Sun et al. 2021).

However, chlorine and chlorinated compounds have numerous effects: On the one hand, it contributes to the antibiotic resistance of bacteria that are prevalent in water Distribution and storage networks; However, it also displays the arrangement and formation of biofilm-forming bacteria underlying water distribution and storage pipes (Zhang, Li et al. 2018).

Biofilms are multi-species microbial structures contained in a polymeric matrix (Flemming, van Hullebusch et al. 2023), representing gene depots for resistance to antibiotics, and ensuring the safety of bacterial life and proliferation. Chlorination efficacy in water structures is impacted both in terms of inhibiting bacterial growth and eliminating biofilm communities which had formed on the material's surface (Luo, Zhang et al. 2022). Therefore, biofilms are significant in the case of antibiotic resistance, and this microbial growth in chlorinated water systems (Zhang, Li et al. 2018).

It is, therefore, important to comprehend the relationships between chlorination, waterborne pathogens reduction, antibiotic resistance, as well as the development of biofilms to address these complicated challenges of achieving microbiological security of water sources. (Muhammad, Idris et al. 2020). When more knowledge on how chlorine affects microbial communities and the formation of antibiotic resistance is gained, specific methods to reduce the negative outcomes almost chlorine use along with satisfactory disinfection of water can be found (Zhu, Ghosh et al. 2021).

The determination of bacterial presence and water chlorination effectiveness in samples is important in the tracking of AMR and the evolution of antibiotic-resistant bacterial isolates (Li, Jay et al. 2019). The use of water in Al Baha is considered one of the major public health risks caused by antibiotic-resistant microorganisms in aqueous habitats. Thus, the purpose of this research is to assess connection between bacterial load, chlorination as well as antibiotic resistance in bacterial isolates from water

samples in Al Baha region. This knowledge is very crucial to facilitate successful application of measures in the water treatment process and reduction of environmental antibiotic resistance.

Therefore, the current study is to assess the bacteriological safety of water in Al-Baha city to the optimal extent. To achieve this objective, a sequential approach will be followed by sampling water from internal and external sources. The obtained samples will undergo severe physical and chemical parameter analysis and strong bacterial tests.

In order to give a comprehensive overview of microbial profile the microbial density shall be analyzed and the microbial diversity shall be analyzed using Vitek 2 compact and molecular analysis of bacterial 16s rRNA gene. In addition, special emphasis will be laid on the studies on the chlorine tolerance of microbial populations by employing chlorine resistance assays. In addition, the determination of development of biofilms, which is among the major elements of

microbial persistence and resistance, utilizing the method of microdilution as well as the crystal violet technique.

In addition, the antimicrobial susceptibility will also be based on the disk diffusion approach of Kirby-Bauer. This method is made comprehensive to provide crucial information on antibiotic-resistant bacterial isolates in water samples from Al-Baha region and shed light to the risks of waterborne antibiotic resistance. For these reasons, this study will focus on best and improved understanding of bacterial load, chlorination, and consequent formation of the antibiotic-resistant bacterial isolates, and to provide direction for the new activities related to the safety of water and public health.

2 MATERIALS AND METHODS

Materials:

The instruments and chemicals used for sample collection and analysis were as follows:

Sterile 250mL glass sample bottles

Ice box for holding the sample while in transit.

Thermometer, for water temperature checking.

Qualitative colorimeter DPD (N-diethyl phenylenediamine) - for the determination of free residual chlorine.

pH meter for check the pH and conductivity which is portable.

The following chemicals and instruments were used to conduct microbiological examination on water samples:

For the presumptive test, use double-strength MacConkey broth (Fluka).

For the confirmation test, use single-strength MacConkey broth

Eosin Methylene Blue (EMB) agar (Fluka) for the last step.

Kovac's reagent for detecting thermotolerant *E. coli*.

Sample collection and analysis

Gathering samples and performing physicochemical analysis This research was carried out between October as well as Desember 2024, 207 water samples in all were chosen at random from

different locations in Albaha city with colaberation with National water company . Only 53 sampls were conformed with positive bacterial growth. This company is resposibal for providing the fresh water (water dams and desalinated water) to homes in Albaha reigion. This water is bought form different locations, then kept in larg water tanks (40 in total), before distriputed to homes through undreground network. To represent the water quality , water samples from the tanks and crossing points of pipes were collected . Every sample was taken from the surface, shallow, and deep points of each source as well as put in sterile glass bottles with a capacity of 250 ml. Following that, the samples were sent right away to the lab for microbiological and physiochemical using a portable ice box for analysis (4°C).

Preliminary information about the water quality was obtained with the help of controlling the temperature and pH of the water at the collecting point. Besides these data other physico chemical analysis were also made in order to analysis the overall water quality. An electronic thermometer and the classical colorimetric method using N-diethyl-phenylenediamine (DPD) reagent were employed to measure the temperature and quantify the free residual chlorine concentration. Other physio chemical parameters including the pH and conductivity of water samples were also examined in a lab setting with a portable pH meter to ascertain a detailed analysis of the samples collected.

Microbiological Analysis of Water Samples

The aim is looking for diseases or the microbes that live with them. Mammals' intestines contain high concentrations of these marker bacteria, which makes them indicators of fecal contamination. Therefore, their existence in water would suggest that it is unfit for human consumption.

The bacterial load in water samples was quantified by enumerating total coliforms, fecal coliforms as well as *Escherichia coli*. The total as well

as fecal coliforms in water sample were determined utilizing standard 5-tube method of Most Probable Number (MPN).

The MPN test is based on the principle that coliform groups ferment lactose and form acid and gas. The MPN was determined in two steps: total coliform test, and specific fecal coliform.

Five tubes of double strength Macconkey broth (Fluka) were filled with 10 mL of the water sample for the presumptive test, which was then incubated for 24 hours at 37°C. The count for coliforms that included total coliform was positive when the tubes contained acid and/or gas.

A *Streptococcus faecalis* detection test was conducted. Cellulose nitrate membrane filters were used to filter 100 mL of samples. A sterile forcep was then used to put membrane filters, face up, onto KF Agar. After that, the agar was incubated for 48 hours at 35°C. The presence of *S. faecalis* was indicated by pink colonies that ranged in diameter from 0.5 to 2.0 mm. In order to perform an additional confirmation test, the colony was inoculated onto Kanamycin Esculin Azide Agar and incubated for 72 hours at 35°C. *S. faecalis* was identified using a catalyst test. Filtered cellulose nitrate membrane filters were applied to basic *Pseudomonas* agar in order to detect *P. aeruginosa*. For 40–44 hours, the medium was incubated at 42°C. To find the pigment believed to be *P. aeruginosa*, the agar was later examined under ultraviolet light.

Identification of isolated bacteria:

The API 20 system was used to identify and differentiate bacteria based on their biochemical properties. It is widely used in microbiology laboratories for rapid identification of Gram-negative as well as Gram-positive bacteria. The system contains a set of 20 different miniaturized biochemical tests, each of which detects a specific bacterial metabolic or enzymatic activity. **Sample Preparation:** The bacterial sample is grown on a suitable agar medium, typically overnight, and then a suspension of the bacteria is made in a sterile saline solution. This suspension is used to inoculate the API strip.

Due of their sensitivity to change during transit to the laboratory, water conductivity and pH are evaluated at the sample collecting location [6]. Then, using a thermometer and the conventional colorimetric DPD (N, N-diethyl-phenylenediamine) method, some physiochemical analyses of the water quality were carried out, including measurements of pH and free residual chlorine. In the lab, a portable pH meter (HI 9811-5 Portable pH/EC/TDS/°C Meters, USA) was used to assess the pH results.

Antibiotic susceptibility test

The Kirby-Bauer disk diffusion technique was used to determine the antimicrobial susceptibility test [26]. The McFarland scale (McFarland Densitometer, Grant-Bio) was set to 0.5 for overnight cultures of all isolated bacterial strains in nutritional broth, which corresponds to 1.5×10^8 CFU mL⁻¹. Then, using a cotton swab, 100 µl of the culture was equally dispersed throughout the Muller-Hinton agar plate's surface, and the plates were allowed to dry. The plates were then incubated at 37°C for 18 hours after the antimicrobial-containing disks (Sulfamethoxazole 30 µg, Chloramphenicol 30 µg, Nitrofurantoin 30 µg, Nalidixic acid 30 µg, and Tetracycline 30 µg) from Bioanalyse & HIMEDIA firm, India, were applied with sterile forceps [27]. The inhibitory zone widths around the disks were measured and compared to the 2013 CLSI recommendation, which is set forth by the Clinical and Laboratory Standards Institute [28].

Biofilm quantitation

As previously explained by Yuan et al. (2015) and Destiani et al. (2019), a 0.5 McFarland bacterial suspension was made independently from the overnight bacterial culture [15,30]. Next, 100 µl of bacterial suspension was added to 100 µl of Lysogeny broth (LB) (Thermofisher) in 96-microwell flat-bottom polystyrene plates (SPL Plastic Labware, Korea) to accomplish inoculation (1:1 ratio). For eighteen hours, the microwell plates were incubated at 37°C. Following the removal of planktonic cells, the plates underwent three rounds of washing in sterile distilled water. After staining the preserved biofilm with 200 µl of 0.1% (v/v) crystal violet, it was allowed to sit at room temperature for fifteen minutes. After three rounds of washing with sterile distilled water to get rid of extra crystal violet, the stained biofilm was allowed to air dry at room temperature. 200 µl of an 80:20 (v/v) ethanol:acetone mixture was used to dissolve the biofilm that had been dyed in each well. A microplate reader (Thermo Scientific, USA) was used to quantify the density of biofilm formation at optical density 595 (OD 595 nm).

3 RESULTS

The results of water sample analysis is presented in figure 1. Of special interest, results concern the bacterial isolates resistance profile as well as the water pollution variables pointed in the figure 1. Of equal importance is the fact that most of the bacterial isolates exhibited multidrug resistance especially *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Salmonella* spp. These bacteria exhibited resistance

to multiple antibiotics, including nalidixic acid, tetracycline, and chloramphenicol, suggesting their ability to survive even in chlorinated water environments. For instance, the presence of *Salmonella arizonae* singled out highly moderate to high resistance levels, hence questioning the current disinfection measures in place.

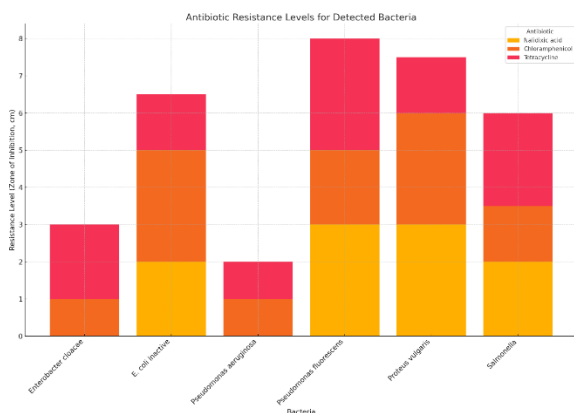


Figure 1: results of the analysis of water samples (N=53)

Some of the isolates for example *Acinetobacter baumannii* had zero susceptibility to all antibiotics analysed, this discussed the fact that some of the water samples had highly resistant strains. These findings are also consistent with results from other studies that demonstrated that while chlorination decreases the total bacterial counts, it increases the selection pressure prerequisite to the development of resistant strains.

Free chlorine concentrations were identified as significant causes of persistent resistant bacteria since higher concentrations in water indicated the absence of resistant bacteria. The corresponded chlorine concentrations in the samples, ranged from 0.07 – 0.29 ppm. More so, densities of resistant bacteria that emanated from the samples above eight log reductions were higher in samples with low chlorine level (<0.2 ppm), implying inadequate disinfection. Nevertheless, even in chlorinated samples with higher chlorine concentrations (> 0.25 ppm), *Pseudomonas aeruginosa* were not completely eradicated. This shows that chlorine alone cannot effectively disinfect water, particularly the bacteria that form biofilms on the inner surface of pipes etc. The pH value of the water samples ranged between 6.5 as well as 8.4 and is therefore within the limit permissible for drinking water.

4 DISCUSSION

This study found that several bacterial isolates, including *Pseudomonas aeruginosa*, *Proteus*

vulgaris, and *Salmonella* spp., exhibited multidrug resistance, a pattern consistent with global concerns about antimicrobial resistance (AMR) in aquatic environments.

Although chlorination suppressed bacterial densities, resistant types remained discernible, especially those responsible for biofilm development. Scholars support our conclusions by stating that chlorination creates selection pressure which leads to the persistence of viable but non-culturable (VBNC) states that harbor resistance genes, according to Yuan et al. (2015). Jiang et al., 2023 also noted that chlorination can potentially miss VBNC *E. coli* and *Enterococcus*, which chlorination should not be used as the sole method of bacterial sterilization.

P. aeruginosa, and *Proteus vulgaris* were isolated from samples having high chlorine concentration (>0.25 ppm) proving the role of biofilm. Some important principles for biofilm microorganisms include the biofilms' ability to act as a repository for antibiotic resistance genes due to disinfectant practices. This observation corresponds to the findings of Zhang et al. (2023) who noted that formation of biofilm by *Acinetobacter baumannii* under varying chlorine and UV disinfection stresses promoted multidrug resistance. More recently and in tandem with the above concept of biofilms, Adefisoye and Olaniran (2022) also made the point that biofilms reduce the effectiveness of chlorination by providing microenvironments that shield bacteria from oxidative stress.

With the case of free chlorine ranging between 0.07- 0.29 ppm during the analysis of the samples our results showed that water with low chlorine concentrations was likely to harbor resistant bacteria. Microbial cultures from water samples with less chlorine content (< 0.2 ppm) were characterised by higher numbers of bacteria counts, and resistance patterns. As noted by Najmuldeen and Razaq, (2023), regarding insufficient chlorination in public swimming pools raising the rate of antibiotic-resistant isolates. But even if that was accomplished, higher levels of chlorine failed to kill some of the more resistant strains, proving the need for multiple strategies for disinfection. In this context, Umar (2022) suggested the combination of UV irradiation and chlorination to improve microbial elimination and prevent bacterial resistance.

Another note is that samples contain highly resistant species like *Acinetobacter baumannii* and *Salmonella enterica* that pose big threats to public health. These species were not only immune to all the antibiotics under test they also have high ability to form biofilm. This conclusion accords with the

observations of Gholipour et al. (2024) who noted *Pseudomonas aeruginosa* and other chlorine-tolerant bacteria as pathogens in hospital water management systems and their contribution to hospital-acquired diseases.

The results also highlighted that changes in pH (6.5-8.4) did not affect bacterial resistance and that it is microbial resistance accompanied by biofilm formation and microbial adaptation that plays the crucial parts. This finding supports the work done by Zhang et al. (2018) where they observed that biofilms in water distribution systems are very stable to pH shocks within the range of 6 to 8 and retain their resistance profiles.

In conclusion, these findings imply important knowledge of bacterial load, chlorination and antibiotic resistance of water systems. This primary method of water disinfection, the chlorination process, is currently being challenged owing to the fact that it has failed to effectively tackle biofilm

associated resistance hence the need to pursue other multiple strategies of water management. Therefore, it is possible In order to lower the threats to antibiotic resistance in water systems and prevent adverse health impacts in a community that uses this water systematically with the help of following proper disinfection procedure in coordination with constant examination of water samples and raising aware of people through creating awareness about such existing problems.

It is recommended that Chlorination should be done alongside other complementary disinfection procedures including UV irradiation to make sure of better microbial removal and shell out less effort towards resistance building up. In addition, it has been noted that the determination of chlorine residual and the bacteria count has to be done occasionally so as to monitor the best chemical dose and the efficient bacterial efficacy to disinfect the water.

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