World Journal of Biology Pharmacy and Health Sciences
eISSN: 2582-5542
Cross Ref DOI: 10.30574/wjbphs
Journal homepage: https://wjbphs.com/

(RESEARCH ARTICLE)

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Multiple drug resistant enterobacteria from biofilms in some tap water supplies in a Nigerian university

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World Journal of Biology Pharmacy and Health Sciences, 2023, 16(01), 089–098

Publication history: Received on 06 August 2023; revised on 18 September 2023; accepted on 21 September 2023

Article DOI: https://doi.org/10.30574/wjbphs.2023.16.1.0387

Abstract

Tap outlets have been found to contain biofilms, which are a consortium of bacteria, fungi, viruses and protozoa. These microorganisms, especially Gram negative bacteria, could be pathogenic and highly resistant to antibiotics, leading to an increase in the prevalence of diseases worldwide. The university community depends on water from boreholes for consumption and domestic uses; as such, the need to identify the Gram negative bacteria is expedient. The main objective of this research is to determine the potability of the water released from the taps, and ultimately reduce the incidence of biofilm-related water infections. Water samples were collected from five boreholes and five tap outlets were swabbed with a sterile swab stick. Coliforms were estimated from the water samples using the Most Probable Number (MPN) technique. Gram negative bacteria were isolated and identified from the swabbing using standard bacteriological and biochemical tests. The antibiotic resistance patterns of the isolates were also determined, and the genes responsible for the multiple antibiotic resistance were identified using the DNA extraction and Polymerase Chain Reaction (PCR) methods. Bacterial counts of the samples ranged from 6.0×10^5cfu/mL to 1.4×10^6cfu/mL. MPN values of the tap water samples ranged from 4 to 1100^+cfu/100mL, which exceeded the WHO standards of water quality. A total of 31 bacteria were isolated, of which 8 (25.8%) were *Pseudomonas* sp, 7 (22.6%) were *Proteus* sp, 4 (12.9%) were *Klebsiella* sp, 4 (12.9%) were *Escherichia coli,* 3 (9.7%) were *Enterobacter* sp, 3 (9.7%) were *Citrobacter* sp and 2 (6.5%) were *Salmonella* sp. All the bacteria showed multiple drug resistance to different antibiotics used, especially the cephalosporins. The gene found to be responsible for the cephalosporin resistance was the TEM - 445. Tap swabbing and water samples were found to contain a high value of coliforms (4 – 1100cfu/100mL), showing heavy faecal pollution, many of which could be pathogenic microorganisms that renders the water unfit for human consumption. The presence of these multidrug resistant microorganisms in the tap outlets could pose a serious threat to public health.

Keywords: Water; Biofilms; Enterobacteria; Antibiotics; Resistance

1. Introduction

Water passed through distribution pipes is usually found to contain extremely diverse but poorly identified microbial flora and complex organic matter [1]. The transport of water through a distribution system especially in poor environments allows the growth and multiplication of microorganisms, making the eventual presence of biofilms in tap outlets difficult to control. This is because the proliferation of bacteria is followed by detachment of the bacteria, removal of the bacteria from the pipe-water interface (solid-liquid interface) through erosion, transport of the dislodged bacteria into the circulating water, and eventually into the tap outlet as they form biofilms [2].

Biofilms in distribution systems have been found to serve as reservoirs for pathogens such as *Helicobacter pylori* (which can cause ulcers and cancers), *Legionellae* sp (which can lead to legionellosis) and *Mycobacterium avium* (which can cause lung infections) [3]. Biofilms have also been implicated in the presence of human infections such as chronic and

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acute wounds, infectious kidney stones, bacterial endocarditis, cystic fibrosis airway infections, otitis media, acute, osteomyelitis, biliary tract infections, periodontitis, ophthalmic infections etc [4] [5].

Antibiotic resistance of the individual bacteria in the biofilm mode of growth contributes to the chronicity of infections such as those associated with medical devices [6]. According to then [7], most chronic nosocomial infections are found to be caused by the presence of biofilms in the biomaterial surfaces of medical implements and equipment such as catheters, prosthetic heart valves and orthopaedic devices. These infections share common characteristics even though the sites of infection and microbial pathogens differ, and are chronic because the bacteria in biofilms evade host defenses and may withstand antimicrobial chemotherapy [8]. Susceptibility tests with *in vitro* biofilm models consistently show the survival of bacterial biofilms after treatment with antibiotics at concentrations of a hundred or thousand times the minimum inhibitory concentration of the bacteria measured in a suspension culture [9]. Biofilm based infections are rarely resolved because of the complexity of the matrix formed and increased resistance to antibiotics and they usually persist until the colonized surface is surgically removed from the body. These infections compromise the quality of life but are rarely fatal in nature, and are often traced to species of bacteria that are ubiquitous in water, air, soil or skin, such as *Pseudomonas aeruginosa* and *Staphylococcus epidermidis.* These organisms can persist in the body by tenacious survival as opposed to aggressive virulence [10]. *In vivo*, antibiotics may suppress or inhibit to an extent symptoms of infections by killing free-floating bacteria shed from the attached population of the biofilm, but usually will fail to eradicate the bacterial cells still embedded in the biofilm matrix. When antimicrobial chemotherapy stops, the biofilm can act as a vehicle for recurrence of infections [11] [12].

This study therefore aims at isolating members of the family Enterobacteriaceae in biofilms found in various tap supplies in a Nigerian university community, and determining their antibiotic resistance patterns to different drugs.

2. Material and methods

2.1. Sample collection

Water samples were collected from 5 tap water points in labeled sterile bottles, stored and transported to the laboratory where analyses were carried out immediately [13]. The tap outlets were also swabbed using sterile swab sticks which were transported to the laboratory to be analysed. Samplings were before rainfall i.e. dry season (Oct- Dec 2018) and during rainfall i.e. wet season (Feb- April 2018).

The coliform density count of the water samples was taken using the multiple tube fermentation technique, also known as the most probable number technique (MPN). The results were compared with a statistical table, the Most Probable Number table [13]. In each of the water samples, 3 tubes containing double strength MacConkey broth and 6 tubes containing single strength MacConkey broth were prepared with inverted Durham tubes added to each tube to capture the production of gas.

2.2. Isolation of bacteria from samples

Isolation of bacteria was carried out by inoculating nutrient agar to aid the total viable bacterial count, and MacConkey agar to aid the selective bacterial count for Gram negative organisms. Routine serial dilutions of the water samples was carried out up to dilution factor 10^{-6}. The swab sticks containing biofilms from the tap outlets were used to swab sterile nutrient agar and MacConkey plates. The inoculated plates were incubated for 24 hours at 37^{o}C. After isolation, the bacteria were identified using standard routine biochemical tests (which included Gram's reaction, catalase, MR-VP, sugar fermentation).

2.3. Antibiotic sensitivity test

After identification of the organisms, antibiotic sensitivity test on the various bacteria were carried out using Mueller Hinton agar. The inoculum was prepared using the 0.5 McFarland standard. A suspension of the inoculum was prepared, and its absorbance was measured using a spectrophotometer at a wavelength of 625nm. The suspension was inoculated onto the agar surface uniformly using a sterile swab stick. A Gram negative antibiotic multi-disc containing 8 antibiotics was placed aseptically on the inoculated agar plate firmly to allow for proper diffusion of the antibiotics onto the agar. The plates were incubated for 24 hours at 37 ^{o}C, after which the zones of inhibition were measured and recorded in millimeter

2.4. Polymerase chain reaction (PCR)

The DNA of each organism was extracted by boiling and centrifugation, and the extracted DNA was stored in fresh Eppendorf tubes and stored at 4 ^{o}C.

The polymerase chain reaction method (PCR) was used for the enzymatic synthesis of specific DNA sequences by Taq and other thermo-resistant DNA polymerases. The method was performed in repeated cycle, so that the products of one cycle serve as the DNA template for the next cycle, doubling the number of target DNA copies in each cycle. Multiple sets of different microorganisms were detected in a single PCR reaction by amplifying (for 30-35 cycles) the corresponding loci simultaneously, and all necessary primers (a small piece of DNA of about base pair long) were combined in a single tube for detecting the presence of the main pathogens or the main subtypes within a given species.

3. Results

The mean bacterial counts of the water samples ranged from 6.0×105cfu/mL to 1.7×106cfu/mL during the first sampling while during the second sampling, the counts ranged from 6.0×105cfu/mL to 1.4×106cfu/mL. Figure 1 shows the bacterial counts of the organisms isolated from the water taps.

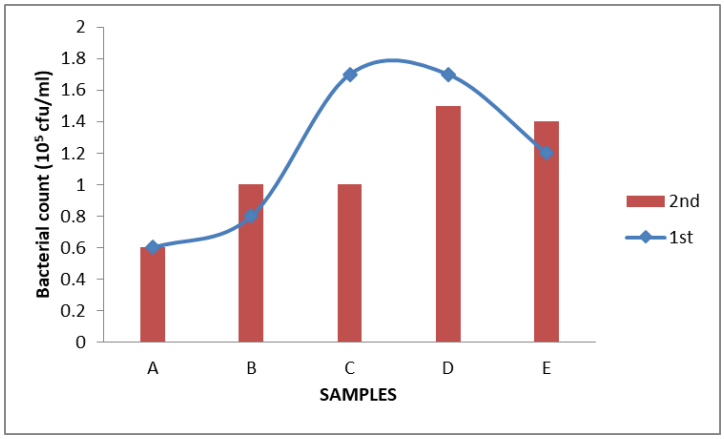


Figure 1 Bacterial counts of organisms isolated from water taps

A total of 31 Gram negative bacteria were isolated from different taps during the study, of which tap C had the highest distribution of 8 isolates (25.8%), followed by taps A and B with a distribution of 7 isolates each (22.6%), while tap E had the lowest distribution of 3 isolates (9.7%). Table 1 shows the distribution of the isolates obtained from the different taps.

Table 1 Distribution of isolates found in different taps

Probable Organism	A	B	C	D	E
Pseudomonas sp	1	1	2	2	2
Proteus sp	2	1	-	3	1
Escherichia coli	1	-	3	-	-
Klebsiella sp	1	1	1	1	-
Enterobacter sp	1	2	-	-	-
Citrobacter sp	-	1	2	-	-
Salmonella sp	1	1	-	-	-
TOTAL	7(22.6%)	7(22.6%)	8(25.8%)	6(19.3%)	3(9.7%)

Table 2 Most Probable Number (MPN) of coliform organisms in treated water sampled

				SAMPLING				
Quantity of H_2O per tube	**10ml**	**1ml**	**0.1ml**		**10ml**	**1ml**	**0.1ml**	
Number of tubes	3	3	3	Estimated number of coliforms/100ml	3	3	3	Estimated number of coliforms/100ml
Sample A	3	3	0	240	3	0	0	23
Sample B	2	1	1	20	3	0	0	23
Sample C	3	3	3	1100^{+}	3	3	3	1100^{+}
Sample D	3	2	0	93	1	0	0	4
Sample E	3	3	2	1100	3	3	1	460

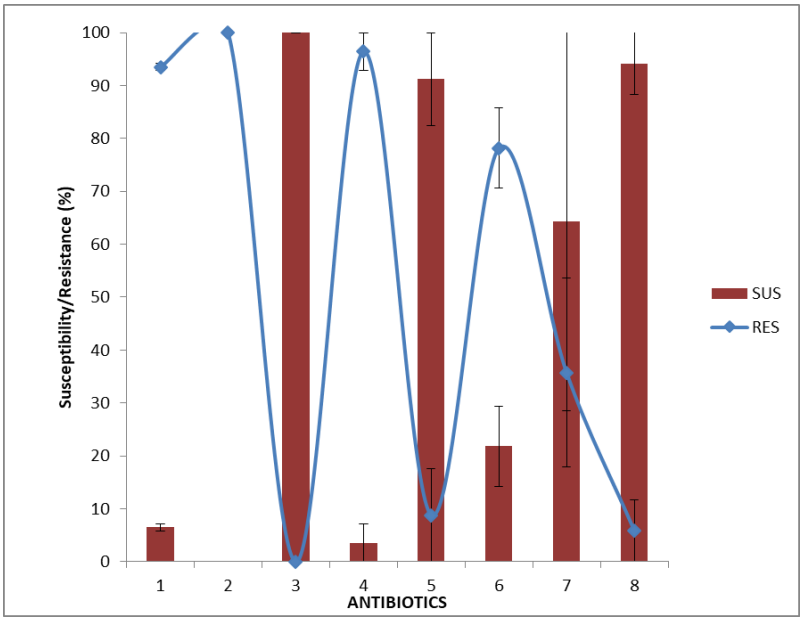
Table 3 Distribution of Bacteria Isolated from different tap outlets

Probable Organisms	**Identified Isolates**	**Percentage (%)**
Escherichia coli	4	12.9
Pseudomonas aeruginosa	8	25.8
Klebsiella pneumoniae	4	12.9
Enterobacter aerogenes	3	9.6
Proteus sp	7	22.5
Citrobacter sp	3	9.7
Salmonella sp	2	6.5
TOTAL	31	100

Table 2 shows the most probable number of coliforms present in the water samples obtained from the tap outlets. The coliform counts ranged from 4 to 1100 coliforms/100mL. In total, thirty one isolates were recorded, with seven genera identified during the study. Table 3 shows the distribution of all the isolates from the different taps sampled. Pseudomonas sp recorded the highest prevalence (25.8%), followed by *Proteus* sp (22.5%) while *Salmonella* sp. (6.5%) recorded the lowest prevalence.

Figure 2 shows the antibiotic sensitivity of the different tested bacterial isolates. All isolates (100%) were resistant to cefuroxime and susceptible to gentamicin. Of the 31 isolates, 29 (93%) organisms were resistant to ceftazidime, 30 (96%) were resistant to cefixime, 3 (9%) were resistant to ofloxacin, 24 (78%) were resistant to augmentin, 10 (35%) were resistant to nitrofurantoin, and 2 (6%) were resistant to ciprofloxacin. All isolates were found to be multi-antibiotic resistant (MAR), forming resistance to three or more antibiotics.

Figure 3 shows the agarose gel electrophoresis of PCR-amplified CTX-M, SHV and TEM genes from isolated organisms. Only 11 isolates (75%) show the amplification of TEM-445 gene while no isolate shows amplification for CTX-M and SHV genes. Table 4 shows the occurrence of amplified resistance genes in multiple antibiotic resistant isolates. Table 5 shows the distribution of each isolate for the positive resistant genes. *Proteus* sp and *Pseudomonas* sp showed the highest occurrence for the presence of the TEM genes, with 3 isolates each (20%). Escherichia coli had 2 positive isolates (13.3%), followed by *Citrobacter* sp, *Enterobacter* sp and *Klebsiella* sp, each having only 1 isolate with TEM-positive gene (6.7%).



Key:1 = Ceftazidime, 2 = Cefuroxime, 3 = Gentamicin, 4 = Cefixime, 5 = Ofloxacin, 6 = Augmentin, 7 = Nitrofurantoin, 8 = Ciprofloxacin, SUS = Susceptibility, RES = Resistance.

Figure 2 Cumulative susceptibility and resistance of isolates to antibiotics used

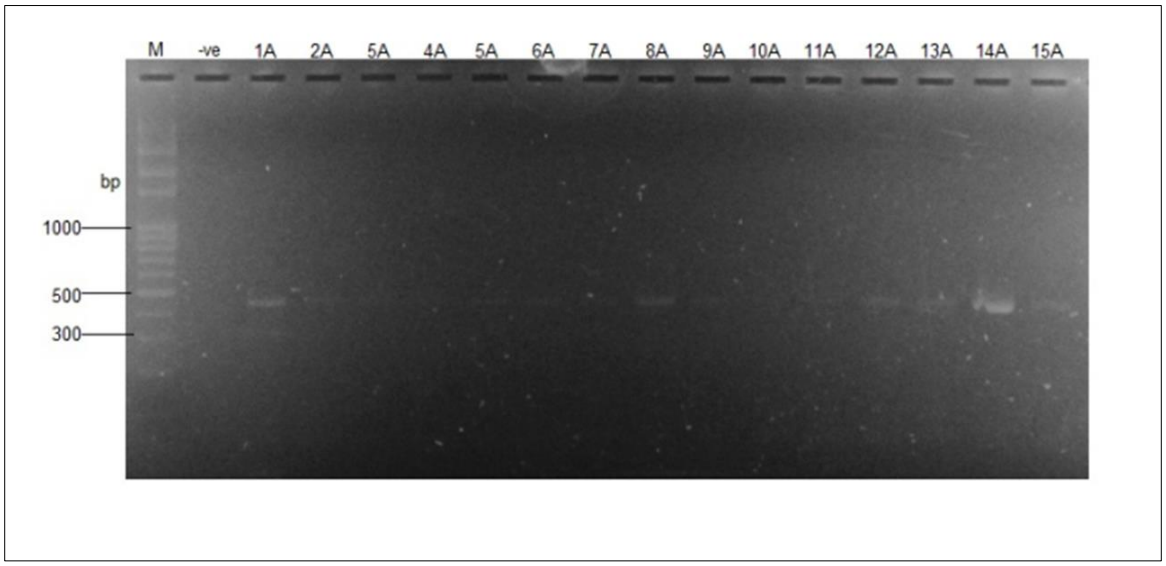
Table 4 Occurrence of amplified resistance genes in multi-antibiotic resistant isolates

			GENE		
S/N	**Bacteria**	**Lane**	***Bla*$_{TEM}$**	***Bla*$_{CTX-M}$**	***Bla*$_{SHV}$**
1	*Proteus* sp	3	+	-	-
2	*Escherichia coli*	4	+	-	-
3	*Salmonella* sp	5	-	-	-
4	*Klebsiella* sp	6	-	-	-
5	*Escherichia coli*	7	+	-	-
6	*Pseudomonas* sp	8	+	-	-
7	*Klebsiella* sp	9	+	-	-
8	*Pseudomonas* sp	10	+	-	-
9	*Proteus* sp	11	+	-	-
10	*Klebsiella* sp	12	-	-	-
11	*Pseudomonas* sp	13	-	-	-
12	*Proteus* sp	14	+	-	-
13	*Citrobacter* sp	15	+	-	-
14	*Pseudomonas* sp	16	+	-	-
15	*Enterobacter* sp	17	+	-	-

KEY: + = Positive to gene, - = Negative to gene

Table 5 Distribution of isolates with positive TEM gene

S/N	Probable Bacteria	TEM-+VE Isolates	Percentage (%)
1.	*Escherichia coli*	2	13.3
2	*Pseudomonas aeruginosa*	3	20
3	*Klebsiella pneumoniae*	1	6.7
4	*Enterobacter aerogenes*	1	6.7
5	*Proteus* sp	3	20
6	*Citrobacter* sp	1	6.7
7	*Salmonella* sp	-	-
	TOTAL	11	73.4



Lane 1: Marker, Lane 2: negative control, Lane 3: Proteus sp, Lane 4: Escherichia coli, Lane 5: Salmonella sp, Lane 6: Klebsiellasp, Lane 7: Escherichia coli, Lane 8: Pseudomonassp, Lane 9: Klebsiellasp, Lane 10: Pseudomonassp, Lane 11: Proteus sp, Lane 12: Klebsiellasp, Lane 13: Pseudomonassp, Lane 14: Proteus sp, Lane 15: Citrobactersp, Lane 16: Pseudomonassp, Lane 17: Enterobacter sp

Figure 3 Agarose gel electrophoresis of PCR-amplified CTX-M, SHV and TEM genes from cephalosporin resistant organisms

4. Discussion

Results of this work shows that Gram negative organisms, especially coliforms are present in biofilms found in taps, and this may reduce the potability of the treated water that comes out from the taps as well as posing a huge health risk to consumers. This corroborates the report of [14] who reported that biofilms contribute to coliform re-growth and detection of coliforms in biofilms found in water distribution systems is an indication of possible faecal contamination and could lead to potential risks from waterborne pathogens [15] also reported that poor personal hygiene of the water treatment plant workers and environmental hygiene contributes significantly to the level of contamination in treated water samples.

The most probable number (MPN) of the coliforms from the water samples collected from the taps ranged from 4 to 1100+cfu/100ml. The values were far above the [16] recommended safe limit for the potability of water; as such, the water is unfit for drinking [17]. Similar results were obtained by previous studies [18] [19]. According to [20], the presence of coliforms has been attributed to recontamination of tap water as a result of the absence of non-residual chlorine which could prevent the water from pollution. [21] and [22] have also suggested that the presence of coliforms, especially *Klebsiella* sp, *Escherichia coli*, *Enterobacter* sp, and *Citrobacter* sp in treated water is an indication of the

presence of other pathogens, and is a possible threat or an indication of microbiological water quality deterioration. It is therefore very important to identify potential sources of contamination of treated water which could be of public health concern.

The organisms isolated from the taps include *Pseudomonas* sp, *Proteus* sp, *Klebsiella* sp, *Escherichia coli*, *Enterobacter* sp, *Citrobacter* sp and *Salmonella* sp. This negates the work of [23] who did not find any organisms in the distribution line (tap water) after treatment, but found several organisms such as *Pseudomonas, Enterobacter, Escherichia coli* and *Klebsiella* in the original source of water (untreated water) in a rural water treatment plant in Louisiana. [24] reported that the microbial composition of potable water could reflect the microbial characteristics of the raw water source. Attachment of pathogenic bacteria to the surface in water distribution systems has been found to increase rapidly [25]. [26] is of the opinion that the presence of enteric bacteria of the genera *Escherichia, Salmonella* and *Klebsiella* in water, especially treated water and water distribution systems is a major threat to human health and are causative agents for many diseases.

All the organisms isolated proved to be multi-drug resistant, as they were mostly found to be resistant to ceftazidime, cefuroxime and cefixime which belong to a class of beta-lactam antibiotics known as cephalosporin, used to treat most bacterial infections [27]. Pseudomonas was found to be 100% resistant to the cephalosporins and augmentin, but highly susceptible to gentamicin and ofloxacin. Escherichia coli was 100% resistant to the cephalosporins but susceptible to gentamicin and nitrofurantoin. Klebsiellawas also 100% resistant to cephalosporins but was susceptible to gentamicin and ciprofloxacin. Enterobacter and Salmonella were susceptible to gentamicin, ofloxacin and ciprofloxacin, and 100% resistant to the cephalosporins. However, not all the isolates of Proteus and Citrobacter showed complete resistance to the cephalosporin antibiotics, but were susceptible to gentamicin, ofloxacin and ciprofloxacin. According to [28] who studied the diversity of bacteria isolated from samples of household drinking water, ciprofloxacin is one of the most active antibiotics used, and is proving to be more effective than the cephalosporins due to the plasmid-mediated genes, and indiscriminate use of the cephalosporins. In a similar study by [29], enterobacteria have been found to increasingly produce extended-spectrum beta lactamase, thereby increasing their resistance to the beta-lactam antibiotics such as the cephalosporins.

Among the antibiotics studied in this work, the third class cephalosporins such as cefuroxime, ceftazidime and cefizime were found to be the least active agents against all the organisms isolated, while gentamicin was the most active agent. [30] corroborated this finding in their study, indicating the increasing resistance of Gram negative bacteria to antibiotics, especially the third class cephalosporins. [31] and [32] suggested that the use of cefuroxime, ceftazidime and other cephalosporins should be used cautiously in treating infections to counter the related increase in resistance levels.

During the molecular analysis, the TEM genes were identified for all the organisms (11 isolates), and are found to be among those found responsible for the extended-spectrum beta-lactamase (ESBL) phenomenon [33]. The phenotype was found in all the organisms isolated, but they lacked the CTX-M and SHV genes which could be due to the presence of other ESBL encoding genes in the studied bacterial population. The polymerase chain reaction (PCR) detected only the presence of the TEM genes, in contrast to the presence of CTX-M genes in a study carried out by [27]. While the CTX-M genes are usually the most frequent ESBL-producing genes for Gram negative bacteria as reported in studies carried out by [29], it was not found in this study, and is corroborated by [34] who showed that among Enterobacteriaceae isolates, CTX-M genes were the least isolated. The emergence of the TEM gene could be due to selective pressure from the incorrect use of the cephalosporin class of antibiotics. The transfer of resistant genes by plasmids and extra chromosomal elements may also be responsible for the resistance of isolates who were previously susceptible to the beta-lactam antibiotics, according to [30].

The study showed that thirty isolates were resistant to three or more antibiotics, suggesting that these isolates are multi-resistant to different antibiotics of beta-lactam, macrolide and aminoglycoside origins.

Of all the isolates, *Pseudomonas* (25.8%) had the highest occurrence in the water taps, followed by *Proteus* (22.6%), *Klebsiella* and *Escherichia coli* (12.9% each), *Citrobacter* (9.7%), *Enterobacter* (9.7%), and least of all *Salmonella* (6.5%). Presence of isolates with multiple antibiotic resistant in water corroborates the work of [35] which suggests that some members of the Enterobacteria with multiple resistant genes can survive treatments such as disinfection and filtration. [36] suggests that an effective cleaning of the water pipes and taps will significantly reduce the incidence and occurrence of these bacteria.

In this study, it was discovered that the water taps are rarely cleaned, allowing for the growth and formation of biofilms containing coliforms and pathogenic organisms that could cause infections and water-borne diseases. The high prevalence of these multiple antibiotic resistant bacteria in the water taps used in this study is likely due to human activities and interactions including poor hygiene, or mutation by the plasmids of the bacteria due to the environment. This results in the positive selection of bacterial cells containing these plasmids, and can be transferred to daughter cells either by conjugation or transduction [37].

5. Conclusion

Multi antibiotic resistance of microorganisms has been found to increase steadily, and it is a concern to public health. The presence of multiple resistance genes from the bacteria isolated from water taps in this study makes the control of antibiotic resistance difficult, and this is a threat to public health. Therefore, more attention should be paid to water taps, as the continuous presence of these organisms is hazardous to health and could lead to an outbreak of waterborne infections. To reduce the incidence of the multiple antibiotic resistance bacteria, supportive efforts are required to reduce the rate of emerging diseases, as the organisms isolated in this study are known to be opportunistic pathogens. The prevalence of these microorganisms in the water environment could be due to the indiscriminate and inappropriate use of antibiotics; poor hygiene and sanitation practices. Given the increasing prevalence of these resistance genes, the use of antibiotics should be duly monitored to reduce indiscriminate use. Also, cleaning of the water taps should be carried out frequently to curb the increase of these organisms, and to prevent an outbreak of waterborne diseases.

Recommendation

A large population of the university community depends on the tap water supplies for consumption. The presence of multiple antibiotic resistant microorganisms which could be pathogenic in these water supplies however suggests that water treatment methods should be intensified and properly carried out. In addition, the following are recommended:

- A policy should be established in accordance with health regulations on microbiological safety of water pipes and boreholes.
- There should be increased awareness and monitoring on the use of antimicrobial products, especially antibiotics.
- The water should be subjected to filtration and chlorination to make the water good enough for use.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare no conflict of interest in this work.

Author's Contributions

Ayansina, A.D.V. is the major coordinator of the work. Popoola, O.T. and Aromolaran, O. did the practical work and supplied important literature.

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