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Occurrence and Diversity of Biofilm Producing Multidrug Resistant *Escherichia coli* in Dug Wells Used for Domestic Purposes in Ile-Ife, Southwest Nigeria

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ABSTRACT: Antibiotic resistance in *Escherichia coli* has emerged as a serious and growing threat to public and environmental health and domestic wells have been fingered as a potential reservoir of resistant *E. coli* due to faecal contamination, and are ideal for microbial biofilm formation. Therefore, this study was carried out to investigate biofilm production among 60 multidrug-resistant (MDR) *E. coli* recovered from domestic wells in Ile-Ife, Nigeria. All biofilm positive isolates were screened for seven associated (*papC, iroN, biofilm, fimH, cna, fbna* and *bcsa*) genes by multiplex polymerase chain reaction (PCR). The diversity of the isolates was investigated using (GTG)5-PCR, and a dendrogram was drawn with interactive-tree-of-life software. Overall, 45 of 60 (75%) isolates produced biofilms, comprising strong (n=15; 25%), moderate (n=16; 26.7%), and weak (n=14; 23.3%) producers. Moreover, 35 (77.8%) isolates had at least one biofilm gene, 30 (66.7%) had multiple genes, and 5 (11.1%) had one. The biofilm gene (*biofilm, fimH, bcsa, iroN*) combination was identified in 10 (28.7%) of the 60 isolates. The isolates (n=14; 40%) with moderate biofilm production had the highest number of genes dominated by the biofilm gene (*biofilm*, *fimH, bcsa*) combination, while isolates (n=8; 22.9%) with weak production had the least number of genes and comprised mainly the *fimH* and *cna* gene combination. The biofilm formers showed 32 distinct fingerprints and were grouped into nine clades, with clade 6 having the most strains (31.6%). The occurrence of MDR biofilm-producing organisms in domestic wells constitutes a public health concern. There is a need to develop strategies to curb faecal contamination of wells to prevent outbreaks of untreatable diseases.

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The poor access to safe, clean water in Nigeria is a major contributing factor to high morbidity and mortality rates particularly among children under five. The majority of the population especially people in rural and suburban areas, have had to resort to alternative sources of drinking water such as dug wells which are vulnerable to pollution because of their small depths and proximity to human activity (Odetoyin *et al*., 2022a; Shehu and Nazim, 2022). *E. coli* occupies a pivotal role in water microbiology, both as a marker of faeces-related contamination and as a potential pathogen in drinking water. Their occurrence in drinking water sources constitutes a problem to public health due to their ability to cause a range of diseases that humans or livestock can acquire if such water is ingested (Oyedeji *et al*., 2010; Adejuwon *et al*., 2011). The eradication of *E. coli* and other organisms from drinking water sources may be impeded by their ability to form biofilm which can protect them from disinfection, thereby affecting quality and constituting to public health threat (Agbabiaka *et al*., 2021). Biofilm is a group of microorganisms that stick together in a polymeric matrix, which protects from environmental and chemical stressors. Bacterial cells in biofilms are more resistant to antimicrobials than their non-biofilm producing counterparts (Chen *et al*., 2018). This decreased susceptibility may be due to inadequate

penetration of antibiotics into the polysaccharide matrix, the random existence of resistant cells and the presence of non-growing cells or cells that initiated stress responses in response to adverse chemicals inside the biofilm matrix (Stewart, 2002). These defense mechanisms work in tandem with those responsible for traditional antibiotic resistance in bacterial genomes or extrachromosomal elements, increasing biofilm resistance to antimicrobial drugs. *E. coli* possesses a variety of virulence factors, including P fimbriae, adhesins, enterotoxins and type-1 fimbriae among others, which enable it to adapt to a variety of settings, contribute to the production of biofilms, and cause an array of diseases. Virulence traits are often encoded on genetic elements and can be transferred to other strains to create a novel combination of virulence factors (Kaper *et al*., 2004). Biofilms are important to public health due to their role in some infectious illnesses. A greater understanding of biofilm dynamics could result in innovative and efficient biofilm management strategies, hence enhancing environmental control. There are several techniques for evaluating biofilm development in bacteria, including Congo red agar, the tube method, and microtitre plates (MTP) test (Dhaka *et al*., 2016; Katongole *et al*., 2020). The most trustworthy approach for determining biofilm development is the subject of debate among experts. However, the MTP biofilm development test has been recognized as an efficient technique (Dhaka *et al*., 2016). Additionally, molecular DNA-based methods like PCR have recently been employed to enhance our understanding of the molecular pathways underlying biofilm development (Tewawong *et al*., 2020). Several high-resolution genetic fingerprinting methods have been employed to reveal species and subspecies diversity in different settings. Among the fingerprinting techniques is (GTG)5 PCR, which provides a simple approach and discriminating power equivalent to pulse field gel electrophoresis (Mohapatra *et al*., 2008). This method groups bacteria into clades/clusters to indicate species diversity, which can be used to determine the origins of contaminating bacteria in aquatic environments. This is essential for developing measures to prevent environmental disease transmission. Studies on the prevalence and diversity of biofilm-forming bacteria in clinical and environmental settings have been described in Nigeria. In addition, previous reports in Ile-Ife have revealed the presence of pathogenic and multidrug -resistant *E. coli* in domestic wells which have great implications for public health (Odetoyin *et al*., 2022a, b). However, there is a dearth of information on the presence and diversity of biofilm forming *E. coli* in domestic wells (Olowe *et al*., 2019; Uzuegbunam *et al*., 2021). The ability of these multi-drug resistant *E. coli* to produce biofilm may hamper environmental control strategies, as biofilm producing strains have been shown to be resistant to antimicrobial agents including disinfectants. Therefore, this study was undertaken to detect biofilm producing multidrug-resistant *E. coli* in dug wells, their associated genes and the diversity of the isolates across the study environment, with a view to providing information to generate ideas for efficient biofilm management strategies in drinking water sources.

MATERIALS AND METHODS

Source of isolates: The multidrug-resistant *E. coli* isolates (n=60) were recovered from 143 dug wells at different locations in six wards in Ile-Ife, Nigeria as previously described (Figure 1). The isolates were resistant to different antibiotics ranging from 3 to 7 with MAR indices ranging from 0.3 to 0.6 km (Odetoyin *et al*., 2022a, b).

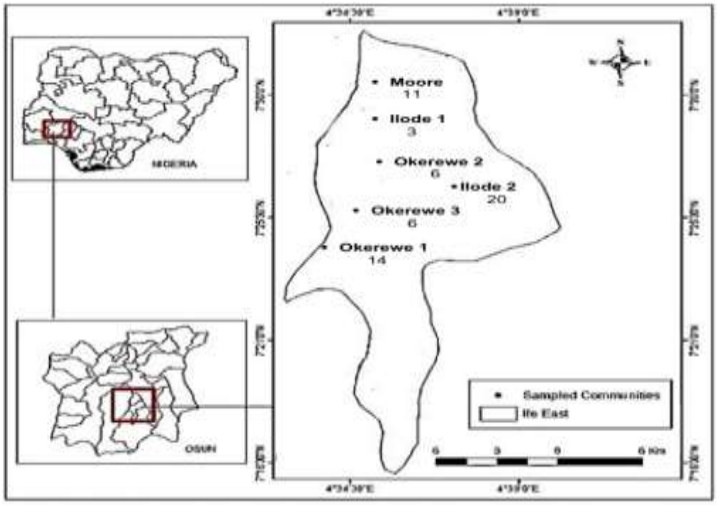

Fig 1: Geo-location of multidrug resistant *Escherichia coli* isolates

Quantitative Detection of Biofilm Formation: All the isolates were screened for biofilm formation using the microtitre plate technique (Merritt *et al*., 2006). They were cultured in 2% sucrose-supplemented brain heart infusion broth at 37ºC for 18-24 hours. The culture was diluted 1:100 with fresh medium, and 200µL of the resulting suspension was dispensed to a sterile 96-well microtitre plate and incubated at 37ºC overnight. Thereafter, the suspension was decanted and the wells were rinsed four times with 200µL of phosphate buffer saline (pH 7.2), eliminating any unfixed microbial cells. The biofilms created by adhering cells in a plate were fixed for 30 min with 2% sodium acetate and stained for the same amount of time with 250µL of 0.1% crystal violet solution in water. The excess stain was removed and the wells were washed with deionized water and the plate was air-dried for 30 min at ambient temperature. A positive result was indicated by the appearance of a layer of stained materials adhering to the wells' interior walls. The quantity of biofilm produced was determined by destaining the wells from the previous test with 250µL of ethanol-acetic acid (95:5 v/v), transferring 100µL from each well to a new microtitre plate, and measuring the optical density (OD) of the solution with a microtitre plate reader at a wavelength of 630 nm. The medium that was not inoculated was utilized as a control for the determination of the OD of the negative control. The cut-off value (ODc) was calculated by taking the average OD value of the negative control and multiplying it by three times the standard deviation of the negative control (Abdulrahim *et al*., 2019).

Adherence classification: The ability of the strains to produce biofilm was divided into four groups based on their OD. These groups are: non-adherent (OD < ODc), weakly adherent (ODc < OD < 2XODc), moderately adherent (2XODc < OD < 4XODc), and strongly adherent (4XODc < OD) (Abdulrahim *et al*., 2019).

Extraction of Isolates DNA: Isolates' DNA was extracted as previously described (Dashti *et al*., 2009). Briefly, three colonies of an overnight culture of the test isolates were suspended in 50µL of sterile distilled water and boiled for 10 minutes. Thereafter, the boilate was centrifuged at 10,000 rpm for 10 min. The recovered DNA suspension served as a template for PCR.

Molecular Detection of Biofilm genes: The primers used for biofilm (*papC, iroN, biofilm, fimH, cna, fbna, bcsa*) genes detection are described in Table 1. The reaction mixture contained 3.4 µL of DNA free water, 5 µL of the 2X PCR Master Mix (Biolabs, England), 0.2 µL of each primer pair, and 1.2 µL of bacterial DNA. The amplification of the genes was done in a Thermal Cycler and the conditions are as follows: for *fbna*, *papC* and biofilm, initial denaturation at 94°C for 4mins, 30 cycles of 94°C for 30s, 45°C for 1 min, 72°C for 2 mins, final extension of 72°C for 7 mins, for *fimH*, *cna*, *bcsa*, initial denaturation at 94°C for 4mins, 30 cycles of 94°C for 30s, 48°C for 1 min, 72°C for 2mins, final extension of 72°C for 7 mins, for *iroN*, initial denaturation at 94°C for 4mins, 30 cycles of 94°C for 30s, 56°C for 1 min, 72°C for 2mins, final extension of 72°C for 7 mins. Gel electrophoresis (1.5% agarose gel electrophoresis with 0.5µg/mL ethidium bromide) was performed on the PCR products. The migration was conducted for 45 minutes at a scale of 100 V/cm. The products were viewed under ultraviolet light.

Table 1: Primer sequences of the biofilm genes investigated

Gene	Function	Primers (5'→3')	Size of product (bp)	References
papC	Outer membrane usher protein *papC*	F: TGATATCACGCAGTCAGTAGC R: CCGGCCATATTCACATAA	501	(Olowe *et al*., 2019)
iroN	Salmochelin siderophore system gene	F: AATCCGGCAAAGAGACGAACCGCCT R: GTTCGGGCAACCCCTGCTTTGACTTT	533	(Subedi *et al*., 2018)
biofilm	Biofilm regulator	F: GATTCAATTTTGGCGATTCCTGC R: TAATGAAGTCATTCAGACTCATCC	225	(Dougnon *et al*., 2021)
fimH	mannose-binding adherence	F: TACTGCTGATGGGCTGGTC R: GCCGGAGAGGTAATACCCC	640	(Dougnon *et al*., 2021)
cna	collagen-binding protein	F: AAAGCGTTGCCTAGTGGAGA R: AAAGCGTTGCCTAGTGGAGA	192	(Arciola *et al*., 2005)
fbna	Fibronectin binding proteins	F; CATAAATTGGGAGCAGCATCA R; ATCAGCAGCTGAATTCCCATT	128	(Kasela *et al*., 2021)
bcsa	catalytic subunit of cellulose synthase	F: GCTTCTCGGCGCTAATGTTG R: GAGGTATAGCCACGACGGTG	826	(Olowe *et al*., 2019)

Determination of relatedness and diversity of isolates: Forty-five biofilm-forming isolates were subtyped by (GTG)5-PCR. Amplification was done with a 25 µL reaction mix that contained 2X Master mix (12.5 µL), 10 µM of the primer (5'GTGGTGGTGGTGGTG3'), bacterial DNA (2.4 µL) and nuclease free water (9.5µL). The amplification conditions included a five-minute initial denaturation at 95°C, followed by 35 cycles of 60 secs at 95°C, 60 secs at 40°C, and 8 minutes at 68°C for extension; followed by a final extension at 68°C for 8 minutes. Each amplicon (10 µl) was electrophoresed in 1X TAE on 1.5% agarose gel. Gels with 5µL of 0.5µg/mL of ethidium bromide were viewed with a UVitec transilluminator (Odetoyin *et al*., 2022a). In a data matrix generated using GelJ software, the presence of a band was classified as 1, while the lack of a band was entered as 0 (Heras et al., 2015). The Jaccard similarity was used to assess the degree of similarity between the strains. The Unweighted Pair-Group Method (UPGMA) was used in the interactive tree of life online programme to create the dendrogram based on the matrix's averaged similarity (Letunic and Bork, 2021).

Data Analysis: Data analysis was done with the aid of LibreOffice Calc software (vs.7.3.4.2). Data were presented as frequencies and percentages, and represented with tables and bar charts. (GTG)5 gel fingerprints were analysed with GELJ software and a dendrogram was drawn with the aid of an interactive tree of life online software.

RESULTS AND DISCUSSION

The MTP result is shown in Figure 2. The biofilm producers are classified as strong, moderate and weak. According to the classification, 75% (n=45) of all the tested isolates (n=60) produced biofilm, ranging from 66.7% of the tested isolates in Ilode1 and Okerewe 2 to 100% in Okerewe 3.

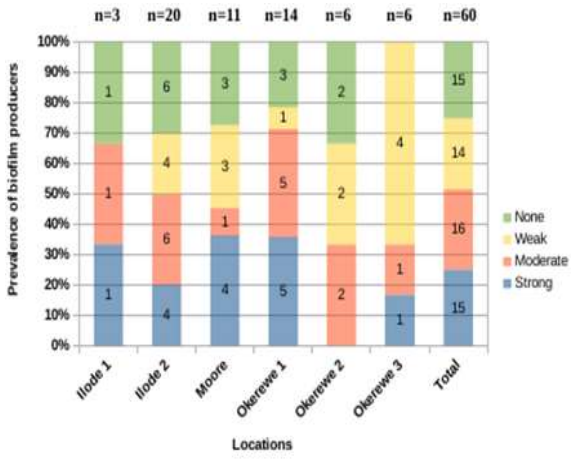

Fig 2: Prevalence of different biofilm formers in the locations

This result is consistent with the findings of other researchers who discovered a significant number of MDR isolates that produce biofilm in various environments (Olowe *et al*., 2019; Bandyopadhyay *et al*., 2021). It is possible that biofilm had a role in the development of multi drug-resistance in the isolates, given that the majority of the isolates in this study are biofilm formers (Chen *et al*., 2018). This occurrence threatens public health as a result of the inability of antibiotic therapy to eliminate biofilms due to their inherent antibiotic resistance and the emergence of mutational antibiotic resistance (Abd *et al*., 2020).

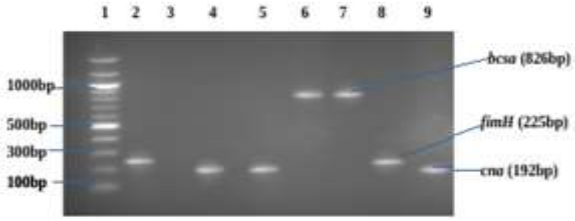

Fig. 3: Amplification of *fimH*, *cna* and *bcsa* genes

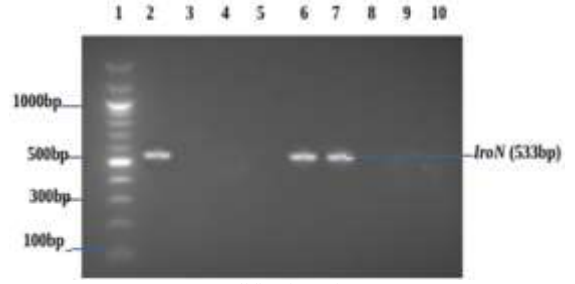

Fig 4: Amplification of *iroN* genes

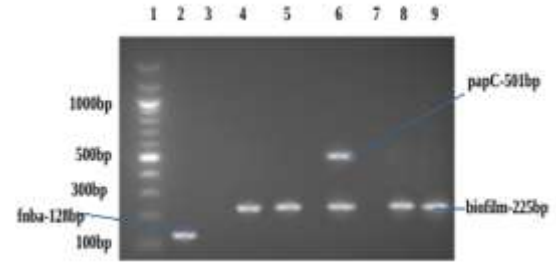

Fig 5: Amplification of *papC*, *biofilm*, *fnba*

Of all the assessed isolates, 25% (n=15) were strong biofilm producers, ranging from 0% in Okerewe 2 to 36.4% in Moore. Sixteen isolates did not produce biofilm ranging from 0% in Okerewe 3 to 21.4% in Okerewe 1. The observation of strong biofilm-positive *E. coli* in this study is similar to previous investigations by Effiok *et al*. (2019) and Agbabiaka *et al*. (2021) in Nigeria, but in contrast with the reports of other investigators that detected weaker biofilm producers in drinking water sources. This discrepancy might be attributed to a variety of factors such as isolation sources, method of detection, and geographical location.

All the sought genes were detected in the isolates (Figures 3-5 and Table 2). Of the 45 biofilm producers, 35 (77.8%) had at least one biofilm associated gene, 30 (66.7%) had multiple genes, and five (11.1%) had one. The most prevalent biofilm gene was *fimH* (n=29), followed by *bcsa* (n=23) and *biofilm* (n=22). There was a preponderance of the *biofilm*, *fimH*, *bcsa*, *iroN* gene combination (n=10; 28.7%), followed by *biofilm*, *fimH*, *bcsa* (n=5; 14.3%) and *fimH*, *bcsa* (n=4; 11.4%) combination.

Table 2: Distribution of biofilm associated genes

Biofilm Genes	Locations (No) n=45	Weak n=14	Moderate n=16	Strong n=15	Total n=45
One gene	Okerewe 2 (1), Okerewe 3 (2), Ilode 2 (1), Moore (1)	**4**	**0**	**1**	**5**
fbna	Okerewe 3 (1)	1	0	0	1
biofilm	Ilode 2 (1), Moore (1)	1	0	1	2
iroN	Okerewe 2 (1)	1	0	0	1
papC	Okerewe 3 (1)	1	0	0	1
Two genes	Okerewe 1 (4), Okerewe 2 (1), Moore (1), Ilode 2 (4)	**2**	**8**	**0**	**10**
fimH, cna	Okerewe 1(1), Moore (1), Ilode 2 (1)	2	1	0	3
biofilm, iroN	Ilode 2 (1)	0	1	0	1
biofilm, papC	Ilode 2 (1)	0	1	0	1
fimH, bcsa	Okerewe1 (3), Okerewe 2(1)	0	4	0	4
biofilm, fimH	Ilode 2 (1)	0	1	0	1
Three and more genes	Moore (4), Okerewe 1 (6), Ilode 1 (2), Ilode 2 (7), Okerewe 3 (1)	**2**	**6**	**12**	**20**
fimH, bcsa, cna	Moore (2), Okerewe1 (1)	1	2	0	3
biofilm, fimH, bcsa	Ilode 1 (2), Ilode 2 (2), Moore (1)	1	3	1	5
biofilm, fimH, bcsa, cna	Okerewe 3 (1), Okerewe1 (5), Moore (1), Ilode 2 (3)	0	1	9	10
biofilm, fimH, bcsa, iroN	Ilode 2 (1)	0	0	1	1
biofilm, fimH, cna	Ilode 2 (1)	0	0	1	1
Total	Ilode 1 (2), Ilode 2 (14), Moore (8), Okerewe 1 (11), Okerewe 2 (4), Okerewe 3 (6)	**8**	**14**	**13**	**35**

The detection of biofilm genes in the positive isolates suggests their correlation with biofilm formation which has been outlined by previous investigators (Olowe *et al*., 2019; Al-Marri *et al*., 2021). In this study, the most prevalent biofilm associated gene in the isolates was *fimH*. According to previous reports, *fimH* was discovered more often in uropathogenic *E. coli* (UPEC) causing urinary tract infections (UTI), indicating its significance in the pathogenesis of UTI (Zamani and Salehzadeh, 2018). Also, *fimH* has been identified in a variety of environmental isolates (Abd *et al*., 2020). Therefore, its occurrence in *E. coli* from drinking water sources is of public health concern due to its frequent association with uropathogenic *E. coli* (UPEC), which suggests that some of the isolates may be UPEC (Zamani and Salehzadeh, 2018). Second to *fimH* is *bcsa*, a cellulose synthase-encoding factor, which has been frequently reported as a virulence factor with a role in the formation of biofilm by *Salmonella* spp and *E. coli*. Apart from protecting *E. coli* with resistance to environmental insults, such as desiccation and bleach, the gene also protects against predation (DePas *et al*., 2014). The *cna* (collagen adhesin) gene is usually linked with clinical *S. aureus* isolates, but it has also been discovered in food and environmental isolates (Bougnom *et al*., 2019). The gene has been identified in *S. aureus* as encoding an adhesin with a known affinity for collagen. Interestingly, this adhesion gene was detected in 17 *E. coli* isolates, more frequently in strong biofilm producers, which suggests their role in biofilm formation by *E. coli*. The identification of a gene previously described only in *S. aureus* in *E. coli* is not surprising given that biofilm formation offers a platform for horizontal gene transfer across bacteria, facilitating the spread of drug resistance markers and other virulence factors (Abd *et al*., 2020). Isolates with moderate biofilm production had the highest number of genes detected (n=14; 40%) dominated by *biofilm*, *fimH, bcsa* gene combination, while isolates with weak biofilm production had the least (n=8; 22.9%) dominated by *fimH*, *cna* gene combination. According to locations, Ilode 2 had the highest no of isolates with biofilm genes (n=12) while Ilode 1 and Okerewe 2 had the least no (n=2) respectively. Isolates with biofilm, *fimH*, *bcsa*, *cna* gene combination were seen at Okerewe 3 (n=1), Okerewe1 (n=5), Moore (n=1) and Ilode 2 (n=3). None was seen at Ilode 1, Ilode 2 and Moore wards. All the two isolates (100%) from Ilode 1 carried biofilm, *fimH*, *bcsa* gene. The occurrence of multiple genes in biofilm formers has also been reported by previous investigators (Olowe *et al*., 2019; Divyashree *et al*., 2022). This study indicates that biofilm-forming isolates with moderate to strong capacity possessed a greater number of multiple genes than weak biofilm-producing isolates. In addition, the strong biofilm producers carried much more biofilm forming genes than the weak and moderate producers. The dominant gene combination was often found in producers of strong biofilm. This is the first time such a gene combination will be discovered in waterborne *E. coli* isolates. This indicates that the significant ability of the isolates to form biofilm may be attributable to the co expression of these genes. Figure 6 depicts a typical image of a (GTG)5 PCR fingerprint. The banding patterns of the isolates ranged from one

to eleven bands. The molecular weight of the bands ranged from 250 to 1500 bp. (GTG)5 typed 38 isolates, whereas seven isolates did not yield any bands and were therefore untypeable. Figure 7 shows the fingerprints dendrogram. All the isolates except 105c, 81, 129b clustered together. However, nine clades of strains were observed. Clade 6 had the highest number of strains (12/38; 31.6%), while clades 2, 7 and 9 had the least number (1/38; 2.6%). Two isolates each in clade 1 (23c-Moore and 101b-Okerewe 2) and clade 3 (102b-Okerewe 3 and 76-Ilode 2) from different locations are identical.

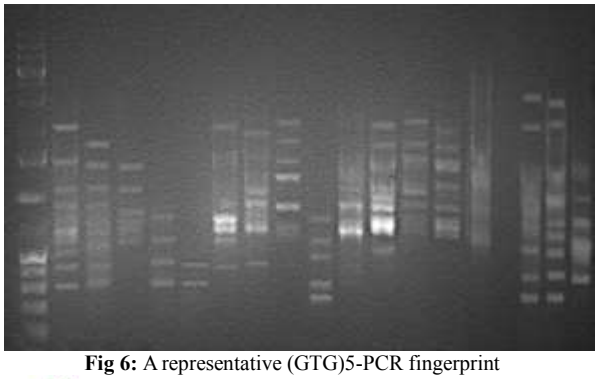

Fig 6: A representative (GTG)5-PCR fingerprint

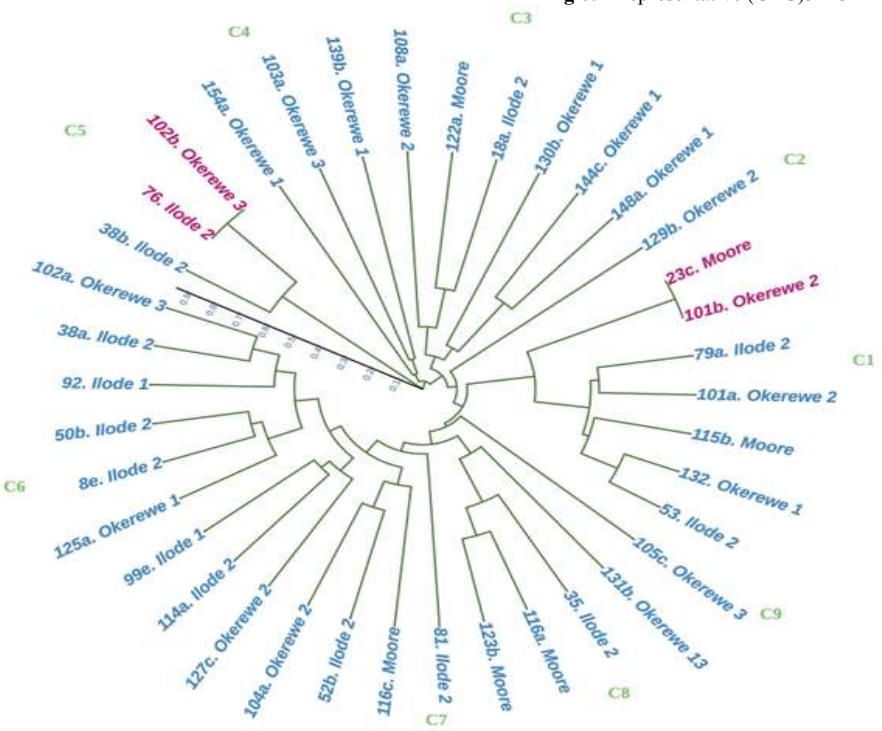

Fig 7: Dendrogram of biofilm producing isolates showing nine clades

The (GTG)5 result revealed complicated fingerprinting patterns for the isolates. Furthermore, with the exception of three isolates, the remaining isolates clustered together, into nine clades. In general, a wide range of profiles across and among isolates retrieved from various sources was found. *E. coli* strains have a higher level of genetic variation due to their highly adaptable character, short generation time intervals, and ease of mobile genetic components acquisition under selection pressure. The vast variety among the strains recovered from various sources mainly rules out the transmission of isolates between/within locations. Similarly, several independent investigations have revealed the presence of various biofilm producing *E. coli* populations in a variety of hosts and settings (Olowe *et al*., 2019; Souza *et al*., 2019). Clade 6 had the most strains (12/38, or 31.6 %), whereas clades 2, 7, and 9 had the fewest (1/38, or 2.6 %). Two isolates from separate places in clade 1 (23c-Moore and 101b-Okerewe 2) and clade 3 (102b-Okerewe 3 and 76-Ilode 2) are identical. This suggests that these isolates originated from a single source, from which they were either maintained or dispersed.

Conclusion: These findings indicate that dug wells in the study environment harboured genetically diverse, multidrug resistant biofilm producing *E. coli*. The capacity of these pathogens to form biofilms may increase their persistence and spread in the environment, thus increasing the risk of contamination or infection. There is a need to develop strategies to curb the development of biofilm in drinking water sources so as to prevent outbreaks of untreatable diseases.

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