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Detection of antibiotic resistant bacteria and sterol concentration in hand dug wells cited near pit latrine in Southwestern Nigeria

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Provision of safe water and improvement in sanitation has led to a reduction in occurrence of diseases, especially water borne diseases but citing of pit latrine near these wells can be of health concern. Assessment of microbiological quality, feacal sterol concentration and antibiotic resistant pattern of isolated bacteria from hand dug well in Oko, Nigeria were carried out during dry and rainy seasons using standard methods. A total of thirty-one and twenty-nine organisms were isolated during rainy and dry seasons, respectively. The total heterotrophic count of the water samples for dry and rainy seasons ranged from 1.14 to 5.53×10^5 Cfu/100 mL and 0.54 to 7.06×10^5 Cfu/100 mL respectively, while total enterobacteriaceae count ranged between 1.18 to 4.62×10^5 Cfu/100 mL and 4.58 to 14.1×10^5 Cfu/100 mL during dry and rainy season, respectively. All the isolates showed multiple antibiotics resistant (MAR) to the eleven antibiotics used in this study. U.V spectrophotometric analysis revealed the concentrations of coprostanol to be within the range of 1.654 to 2.676 abs which is an indication of contamination from human feacal sources. There was a significant relationship between the resistant pattern of both Cephalosporin and Penicillin classes of antibiotics, a justification from heavy pollution and possession of multidrug (commonly used antibiotics) resistant organisms of the studied well water samples, these calls for a major concern of public health workers.

Key words: Water, antibiotic, resistant, Enterobacteriaceae, sterol.

INTRODUCTION

Rural towns and villages in Africa are confronted with huge challenges and multiple issues that adversely affect public health. One of the major challenges is the ability of both rural and urban inhabitants to access clean water supply (WHO, 2006). Not only is there poor access to portable drinking water, even when water is available in

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these small towns, there are risks of contamination due to several factors. When wells are dug and water sanitation facilities are developed, they are not properly maintained due to limited financial resources (Jasmin and Malikarjuna, 2014; WHO/UNICEF, 2008). Both pit latrines and hand dug well are necessities in rural areas of low-income countries like Nigeria. Leachate from pit latrines to nearby wells may cause human and ecological health hazards associated with microbiological and chemical contamination of groundwater (Nwachukwu and Otokunefor, 2006). In rural areas, well water are not properly planned before drilling and are often times located near unlined septic tanks or pit latrines which are majorly not properly covered (Tukur and Amadi, 2014).

The quality of water is majorly affected by microbial pollution and human health can be affected by many pathogen-contaminated water resources from agricultural produce, body contact and drinking of this water (Azzam et al., 2017). Water borne diseases include many gastrointestinal disorders as well as urinary tract infections and associated skin diseases, though people are not affected the same way but immunocompromised people are more affected (Pillai and Rambo, 2014).

The use of antibiotics is not only limited to clinical uses presently; large amount of it is used in Agriculture, Food industries, and Aquaculture (Van Boeckel et al., 2015). The environmental spread of unused antibiotics and their incomplete metabolism in the environment has elicited a bacterial adaptation response to develop antibiotic resistance and genes (Purohit et al., 2017). Antibiotics resistance in wastewater, surface water and drinking water has been documented (Purohit et al., 2017). The problem of antibiotic resistant bacteria is a global phenomenon in drinking water, rivers, lakes, groundwater and waste water (Mulamattathil et al., 2014). Effects of water pollution are much on human health when it is further complicated with the spread of antibiotic resistant bacteria (Novo et al., 2013). This problem of antibiotic resistant is a major problem because infections or diseases caused by these bacteria might result in high cost of treatment and increased mortality (Lupan et al., 2017).

In the past, leachate of faecal contaminant in ground water, marine water, fresh water and portable water supplies has been determined traditionally by quantification of faecal coliform bacteria and by the determination of some inorganic compounds, such as ammonia and nitrogen (Tonny et al., 2004). However, faecal sterol such as coprostanol remains the most prominent human biomarkers indicator of faecal pollution (Bachtiar, 2002). These compound markers derivatives are coprostanol (5β-cholestan-3β-ol), which contains 40 to 60% of the aggregate sterols in human.

Therefore, this study was carried out to determine the concentrations of feacal sterols in the water samples as an indication of feacal contamination and prevalence of antibiotic resistant bacteria in the studied wells.

MATERIALS AND EXPERIMENTAL METHODS

Description of the study area

Oko is a densely populated rural residential/village area in Oyo State, Nigeria. The justification for selecting the study area was based on the high usage of pit latrine in the community. The elevation of the ground above sea level is above 400 m. The topography of the area is of gentle low land in the south, rising to the plateau by about 40 m. The town has an equatorial climate of dry and rainy seasons and relatively high humidity. The dry season begins from December to March while the rainy season starts from April and ends in October. Average daily temperature ranges from between 25°C (77.0°F) to 35° (95.0°F) almost throughout the year (Figure 1).

Sample collection

A total of 11 well were randomly chosen for this study, water samples were taken from the shallow hand-dug wells in the area following standard sampling procedures. Water samples were collected during the peaks of dry and rainy seasons. Water samples were collected using standard methods, water was collected into the sampling bottles, the caps were carefully replaced and the sample was transported in ice box to the laboratory for immediate analysis (Adejuwon et al., 2011). Table 1 shows the GPS coordination of the studied wells.

Bacteriological analysis

Samples were examined after collection according to standard methods for examination of water samples (APHA, 2012). Estimation of Total Heterotrophic Bacteria (THB) and Total Enterobacteriaceae Count (TEC) was determined with a little modification to conventional plate count techniques as described by Larry and James (2001). Nutrient Agar and Eosine Methylene Blue Agar were used for THB and TEC cultivation, respectively. The agar plates were incubated at 37°C for 24 to 48 h to enumerate the aerobe facultative bacteria and the faecal coliform. After incubation, the colonies that grew on the medium were counted and expressed as colony forming units (Cfu/100 ml) of the samples using previous methods (Guo et al., 2013; Hussain et al., 2013). Individual pure colonies were determined by morphological and biochemical techniques according to the methods described by Leonard et al. (2016). Microbial identification was performed using the keys provided in the Bergey's Manual of Determinative Bacteriology (Bergey et al., 1994).

Antimicrobial sensitivity test for the isolated microorganisms

Mueller-Hinton Agar procured from MICROMASTER, Maharashta, India and Antibiotics disks from Oxoid Company (UK) were used for this test. Diffusion technique as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2014) was used to test the sensitivity of organisms to the following antibiotics impregnated disks: Cefixime (5 µg); Cluverate/Augumentin (30 µg); Ceftazidine (30µg); Cefuroxime (30 µg); Ciprofloxacin (5 µg); Ofloxacin (5 µg); Nitrofurantin (300 µg); Gentamicin (10 µg); Erythromycin (15 µg); Novobiocin (5 µg) and Penicillin (10 µg). Results after 18 to 24 h of incubation were interpreted according to clinical breakpoints from CLSI (2014). Resistance values were recorded either as susceptible (S), intermediate (I), or resistant (R) (Jennifer, 2001).

Extraction of extractable organic matter (EOM)

Water samples (250 ml) was treated with 25 ml of dichloromethane

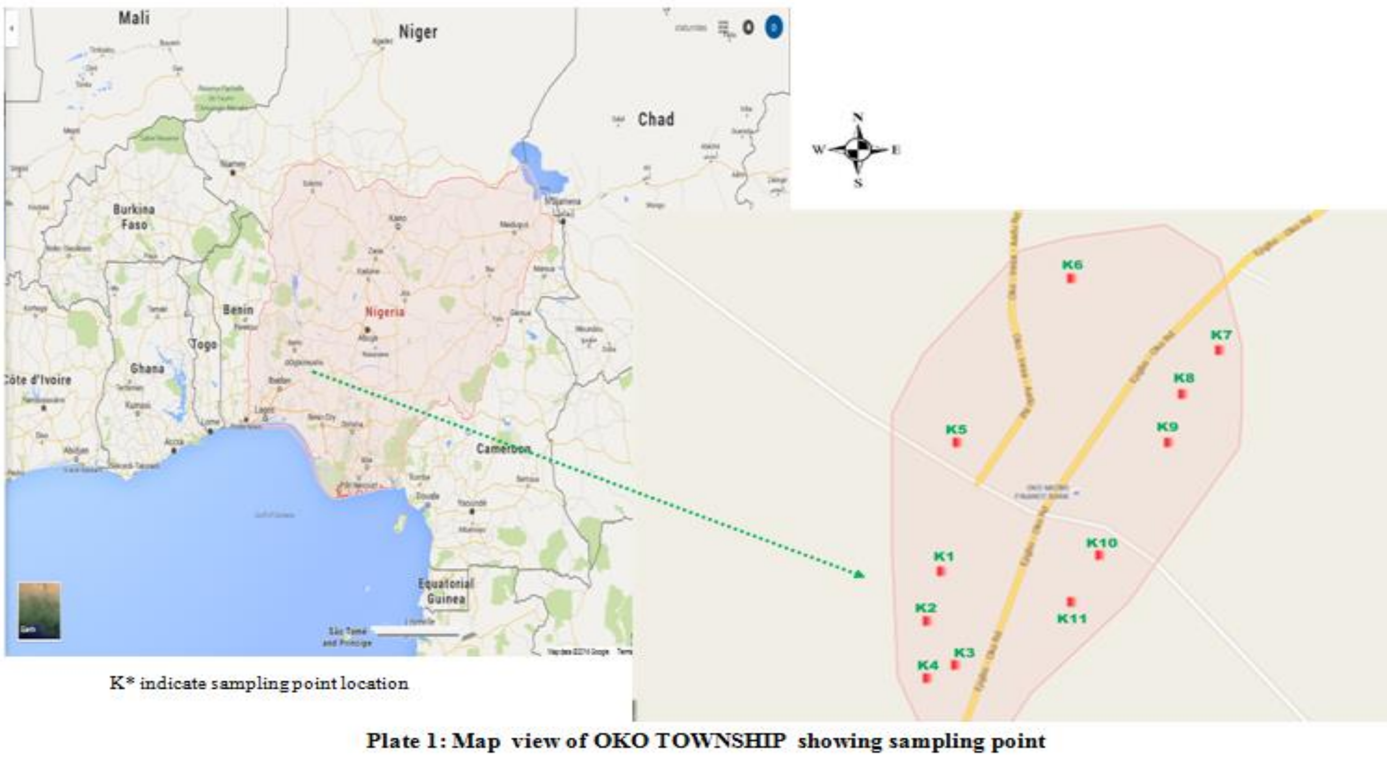

Figure 1. Map view of OKO Township showing sampling location.

Table 1. GPS coordination of sampling sites.

Code	Co-ordinates of well (GPS) location	Elevation (m)	Distance of well from pit latrine (m)	Co-ordinates of pit latrine (GPS) location
K1	7° 57' 7'' North 4° 20' 25'' East	389.50	30	7° 57' 7'' North 4° 20' 24'' East
K2	7° 57' 9'' North 4° 20' 25'' East	393.57	9	7° 57' 8'' North 4° 20' 24'' East
K3	7° 56' 58'' North 4° 20' 19'' East	377.50	10	7° 56' 57'' North 4° 20' 19'' East
K4	7° 56' 55'' North 4° 20' 17'' East	376.00	18	7° 56' 54'' North 4° 20' 17'' East
K5	7° 57' 21'' North 4° 20' 28'' East	399.00	20	7° 57' 21'' North 4° 20' 17'' East
K6	7° 57' 33'' North 4° 20' 43'' East	410.00	8	7° 57' 32'' North 4° 20' 43'' East
K7	7° 57' 27'' North 4° 20' 44'' East	410.00	11	7° 57' 26'' North 4° 20' 43'' East
K8	7° 57' 24'' North 4° 20' 44'' East	391.00	16	7° 57' 25'' North 4° 20' 44'' East
K9	7° 57' 27'' North 4° 20' 48'' East	394.50	12	7° 57' 26'' North 4° 20' 48'' East
K10	7° 57' 18'' North 4° 20' 37'' East	388.00	30	7° 57' 18'' North 4° 20' 36'' East
K11	7° 57' 18'' North 4° 20' 36'' East	390.00	11	7° 57' 18'' North 4° 20' 37'' East

Figure 2. The diagrams of wells and pits in close proximity in the studied area.

followed by rigorous shaking in separating funnel, the separating funnel was left to settle down on a retort stand for 4 or 5 h and the organic layer was separated from the inorganic content (Radwan et al., 2009). Final evaporation of the organic layer was carried out under a vacuum. Sterile foil paper was used to seal the conical flask containing the EOM to prevent interference of other organic matter. The dried extract was then dissolved in 25 ml of n-hexane and package for chromatographic analyses.

Chromatographic techniques (Fractionation) and spectrophotometry analysis

Modified method of Radwan et al. (2009) was used to separate the sterol from EOM through a column chromatographic techniques, the silica gel and alumina used were first activated and packed at ratio 3:6 inches. The dichloromethinated Extractable Organic Matter ware subjected to column chromatography on silica gel (at the top) and alumina (bottom). The column was eluted with (i) n-hexane (25 ml), (ii) 25 ml mixture of Dichloromethane and n-hexane (3:2) and (iii) 25 ml of methanol. The first fraction which is the saturated compound contained the aliphatic hydrocarbons, the second fraction which is the aromatic extract contained the polycyclic aromatic quantified using a UV– visible spectrophotometer.

Statistical analysis

The Statistical Package for Social Scientist (SPSS) 16.0 model was used in undertaking the statistical analysis. The t-test analysis of mean was used to establish the significant differences that exist between the microbial quality of well water between the dry and rainy season at P<0.05. Pearson correlation was used to determine the relationship between concentration of faecal sterol, distance of well from pit latrine and growth of Enterobacteriaceae.

RESULTS AND DISCUSSION

Table 1 shows the distance of the well water to the pit latrines, only 13.3% of the studied wells conformed to the standard set by WHO. According to the WHO standard of 2007, 30 m is the approved standard distance in citing a well in close proximity to a pit latrine.

Figure 2a to c showed examples of three sampling sites used in this study; the arrows are pointing to the wells and the pit latrines. The pictures showed that some of these latrines are in close proximity to the wells.

The mean Total Heterotrophic Bacteria Count and Total Enterobacteriaceae Count of the samples were shown in Table 2. K5 had the highest Total heterotrophic count of 8.58×10^5 Cfu/100 mL, while the least count of 1.14×10^5 Cfu/100mL was recorded for K8 during the dry season. The highest count of 11.6×10^5 Cfu/100 mL and least count of 0.54×10^5 Cfu/100 mL was observed in K9 and K10 respectively during the rainy season. The statistical analysis showed that there is no significant difference for all the values obtained during the two seasons. During the dry season, the highest total enterobacteriaceae count was observed in K3 to be 4.62×10^5 Cfu/100mL, followed by K4 (2.61×10^5 Cfu/100 mL) and K8 (2.37×10^5 Cfu/100 mL), while K5 had the least count of 1.18×10^5 Cfu/100 mL. The highest enterobacteriaceae count was found in K7, with count of 14.1×10^5 Cfu/100 mL followed by K6 and K5 with counts of 13.6×10^5 Cfu/100 mL and 13.3×10^5 Cfu/100 mL respectively, while the lowest count of 4.58×10^5 Cfu/100mL was found in K1 (Table 2).

A significant difference was noted to have accompanied the changes in season ($p > 0.05$) when results obtained during dry season was compared with that of rainy season for the Enterobacteriaceae count. Generally, highest counts for heterotrophic bacteria and total Enterobacteriaceae were observed during rainy season. Result obtained from Total Enterobacteriaceae count revealed that all wells were heavily contaminated

Table 2. Mean of total heterotrophic bacteria and total Enterobacteriaceae counts.

Sample	Growth of THB Cfu/100 ml		Growth of TEC Cfu/100 ml	
	Dry season (Cfu/100ml×10^5)	Rainy Season (Cfu/100ml×10^5)	Dry season (Cfu/100ml×10^5)	Rainy season (Cfu 100/ml×10^5)
K1	3.30[f]	4.20[d]	2.16[g]	4.58[a]
K2	3.91[h]	6.06[g]	1.73[e]	9.09[c]
K3	5.53[j]	5.55[f]	4.62[k]	12.30[g]
K4	3.83[g]	6.07[g]	2.61[j]	12.42[h]
K5	8.58[k]	7.06[h]	1.18[a]	13.30[i]
K6	4.74[i]	7.06[h]	1.67[d]	13.61[j]
K7	2.28[d]	4.31[e]	1.61[c]	14.10[k]
K8	1.14[a]	3.19[c]	2.37[i]	9.60[e]
K9	1.63[b]	11.61[i]	1.82[f]	9.93[f]
K10	2.13[c]	0.54[a]	2.21[h]	7.03[b]
K11	2.38[e]	2.69[b]	1.42[b]	9.11[d]

K* = Sampling point; Values are calculated in colony forming unit per 100 mL.

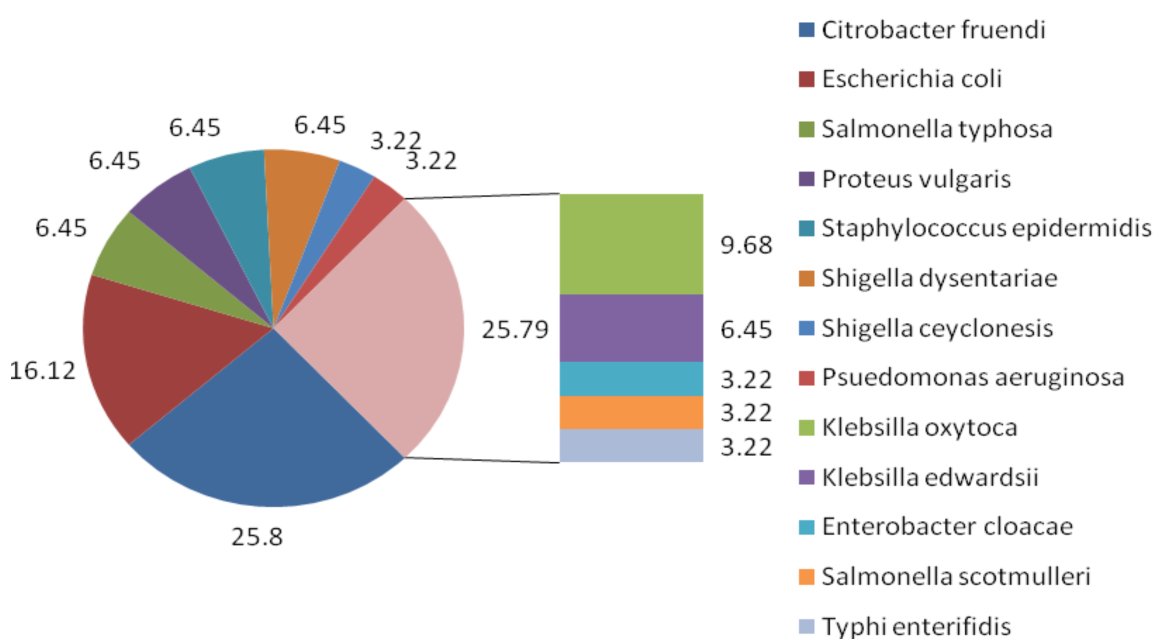

Figure 3. Frequency of occurrence of the identified microorganisms during rainy season (Percentage).

and did not meet up with the WHO (2007) standard that stated that coliforms or feacal coliforms should not be detectable in any 100 ml of drinking water. High counts of heterotrophic bacterial counts and total coliforms in various groundwater wells cited near pit latrines have been reported (Adelekan, 2010; Akinbile and Yusoff, 2011). Kiptum and Ndanbuki (2012) also reported an increase in the coliforms counts in the rainy season compared to dry season in which counts of less than 100Cfu/mL was observed, while Howard et al. (2003) also reported that rain fall can lead to heavy microbial contamination. It has been reported that feacal matter, domestic and wildlife animals are the natural reservoirs of many bacteria belonging to the family Enterobacteriaceae and they can be found in the groundwater either directly or through vectors via cross contamination of feaces with ground water (Ateba and Maribeng, 2011). Microbial contamination has been reported to be the greatest health risk that is associated with drinking water (Cabral, 2010).

Figure 3 shows the occurrence of bacterial isolates during rainy season in the well water samples, a total of

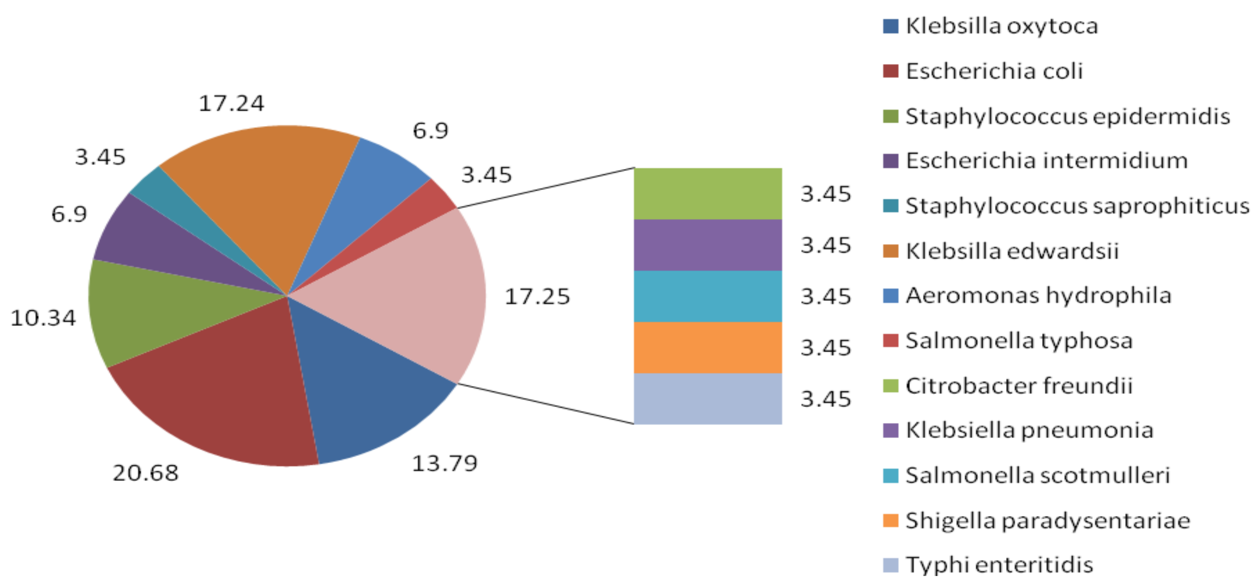

Figure 4. Frequency of occurrence of the identified microorganisms during dry season (Percentage).

thirty-one bacteria were isolated with *Citrobacter freundi* having the highest percentage of occurrence of 25.80%, while four bacterial isolates namely; *Shigella ceyclonesis*, *Enterobacter cloacae*, *Salmonella scotmulleri* and *Typhi enterifidis* had the least percentage of occurrence of 3.22%, during rainy season. Twenty-nine bacteria were isolated during the dry season, but *Escherichia coli* had the highest percentage of occurrence (20.68%), while seven bacteria showed the least percentage of occurrences of 3.4%, these isolates include, *Staphylococcus saprophiticus*, *Salmonella typhosa*, *Salmonella typhosa*, *Citrobacter freundi*, Klebsiella *pneumonia*, *Salmonella scotmulleri*, *Shigella paradysentariae* and *Typhi enterifidis* (Figure 4).

The Enterobacteriaceae are large family of Gram-negative bacteria that includes many harmless symbiots, many of the familiar pathogens such as *Salmonella*, *Escherichia coli*, *Yersina pestis*, *Klebsiella* and *Shigella*. Other disease causing bacteria in this family include *Proteus, Enterobacter, Serratia*, *Citrobacter* and others (Environmental protection Agency, 2002). Tairu et al. (2015) also reported 90% of *E. coli*, 89% *Staphylococcus* species, 72% of *Streptococcus* species, 56% of *Bacillus* species, 38% of *Pseudomonas* species, 23% of *Enterococcus* species present in the well water sampled around pit latrines in Igboora community, Nigeria. Bacterial isolates that include *E. coli*, *Salmonella typhilis*, *Streptococcus faecalis* and *Proteus* sp were also isolated from shallow wells in Makurdi (Isikwue et al., 2011). Many feacal or thermotolerant coliforms have been isolated from many wells in close proximity to pit latrines from many countries all over the world especially in the developing countries (Nwachukwu and Otokunefor, 2006; Kamanula et al*.,* 2014). The increase in the numbers of well water contaminated with feacal coliforms is a cause for alarm all over the world (WHO, 2008; EPA, 2009). Some of these isolated bacteria have been associated with several infections and some are opportunistic pathogen responsible for a wide range of acute and chronic infections (Adenodi et al., 2014).

Antibiotic resistance pattern of the isolated microorganisms

A total number of thirty one (31) organisms were isolated during rainy season and they were subjected to antibiotics sensitivity testing as shown in Figure 5. All the isolates showed 100% resistant to Cefuroxime, followed by Augumentin in which 30 isolates (96.77%) were resistant to it , while Ofloxacin had the least number of organisms that were resistant to it (11(35.48%)).. All isolates were found to be multi-antibiotic resistant (resistant to ≥3 antibiotics).

Twenty nine bacteria were isolated in the dry season, but all these isolates showed 100% resistant to three antibiotics; Augumentin, Ceftazidine and Cefuroxime (Figure 6). All isolates were also resistant to three or more antibiotics and they are therefore regarded to as multi drugs resistant isolates. Resistance by majority of the isolates to antibiotics in these wells can be attributed to wrong prescription and indiscriminate uses of antibiotics. Previous work on ground water in northern California found widespread occurrence of resistance to antibiotics among their isolates (Li et al., 2014).

A significant and apparently visible relationship was observed between the resistance pattern of Cephalosporin (Cefixime, Ceftazidime, Cefuroxime) and

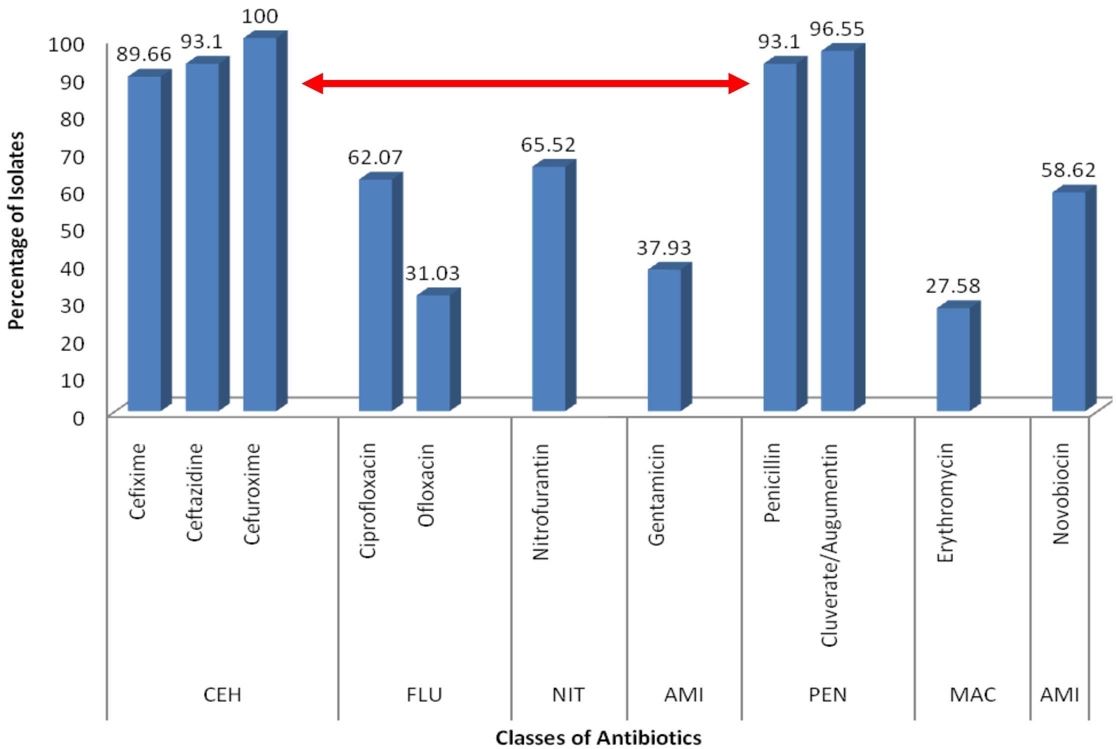

Figure 5. Percentage of resistant isolates during rainy season. CEP, Cephalosporin; PEN, Penicillin; FLU, Fluoroquinolones; MAC, Macrolides; NIT, Nitrofurans; AMI, Aminocoumanin; AMI- Aminoglycoside.

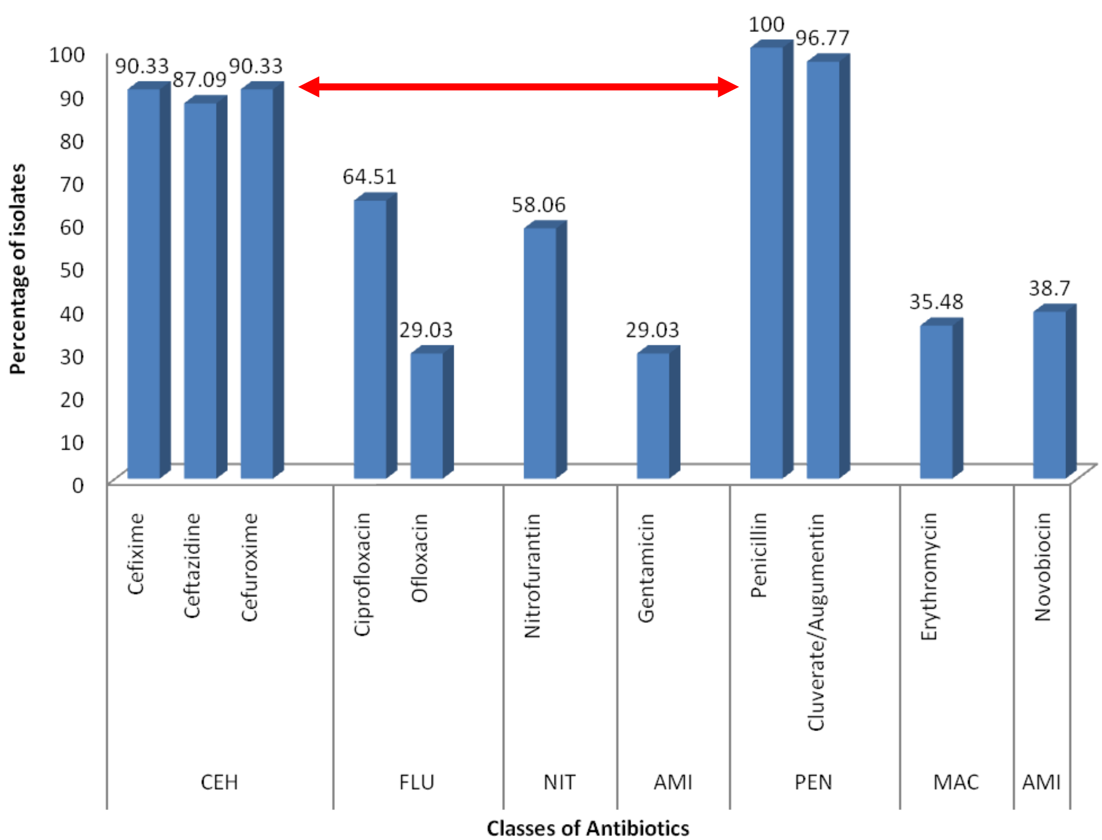

Figure 6. Percentage of resistant isolates during dry season.

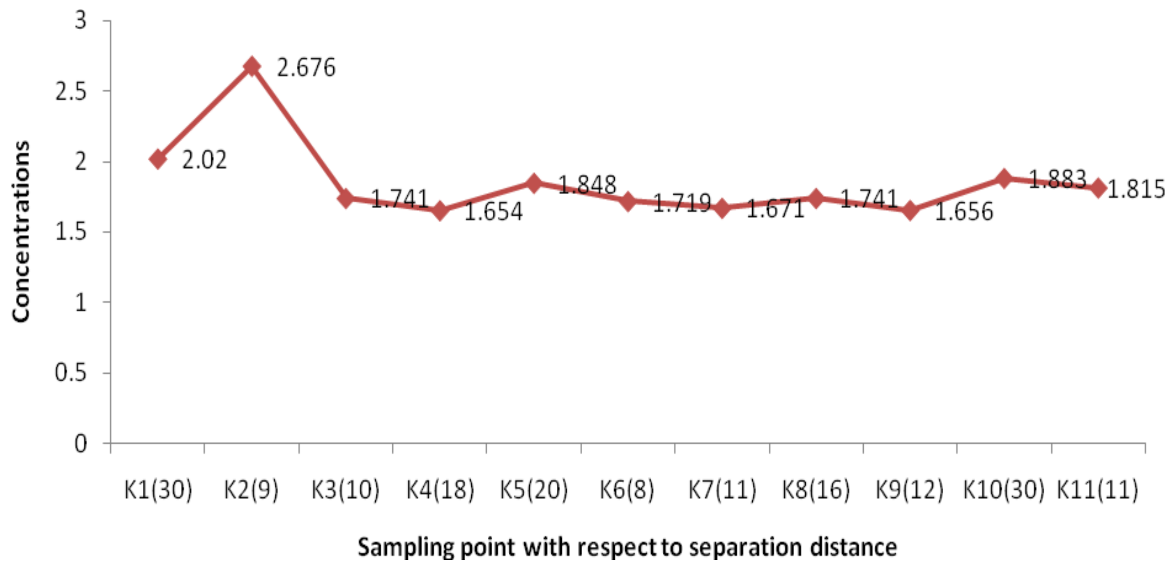

Figure 7. Concentration of the faecal sterol. K* = Sampling locations with reference to distance in meters.

Penicillin (Cluverate/ Augumentin and Penicillin) classes of antibiotics within the two season, The Frequency of isolates that are resistant to cephalosporin corroborate with the resistance pattern of Penicillins isolates. A common myth is that about 10% of patients with a penicillin allergy history will experience an allergic reaction if administered a cephalosporin. Flynn (2013) established this relationship to the chemical instability of the common B-lactam nucleus, the minor differences in chemical structures between the analogues, and the complex and relatively fast degradation of the compounds in aqueous solutions, however, it is also worthy to note that, both classes attacks the cell by inhibiting the synthesis of the cell wall. These two classes of antibiotics belong to the classes of most prescribed antibiotics, evidence from these research indicated that when such antibiotics are prescribed to patient or victims within the study location, the prescriptions might not be effective as expected.

Ayandiran et al. (2014) reported a 40 to 100% resistance to antimicrobial agents by the isolates used in their study. It was observed that Ofloxacin which is a broad spectrum drug was the most effective antibiotic among those tested during the rainy and dry seasons having the least resistance of 11(35.48%) and 12(41.37%) respectively, other fairly potent antibiotic during rainy season was Gentamicin (48.28 and 38.71%). Jiang et al. (2013) also reported the prevalence of antibiotic resistant bacteria from Huangpu river and drinking water sources in their study.

Occurrence of feacal sterol in the studied wells

This study showed that the correlation of absorbance and concentration of sterol shows a concerned variation and detectable concentrations of coprostanol were recorded in all water samples analysed, with values ranging from 1.654 to 2.676 abs (Figure 7). The optimum wavelength for the faecal sterol was observed at 226 nm. The maximum concentration was observed in K2 (2.676) with the well situated at 9 m away from Pit latrine while minimum was found to be K4 with 1.654 concentrations at a distance of 18 m. Overall, mean concentration was found to be 1.857. It was also noted that K1 had a concentration of 2.025 at a distance of 30 m. Gerardo et al. (2000) observed the highest spectra at 458 nm while Tonny et al. (2004) optimum wavelength of coprostanol was observed in ethanol at 250 nm. Previous studies had proposed that values greater than 1.000 abs to be an indication of faecal contamination. Obuseng et al. (2013) also reported that values greater than 1.5 should be considered as an indication of human derived faecal source. Therefore, the microbial contamination of these well waters is due to feacal concentration.

Relationship between different variables used in this work

A Pearson correlation coefficient was used to calculate the relationship between concentration of faecal sterol, distance of well from pit latrine and growth of Enterobacteriaceae (Table 5) for both the dry and rainy seasons, a non-significant weak negative relationship was found between growth of Enterobacteriaceae for dry season and distance of well from pit latrine (r (20) = -0.131, $p > 0.05$), while a weak positive, non-significant relationship was found between distance of well from latrine and the growth of Enterobacteriaceae for rainy

Table 5. Relationship between the concentration of sterol, distance of well from pit latrine and growth of enterobacteriaceae during the dry and rainy seasons.

Correlation coefficient		Growth of Enterobacteriaceae during dry season	Growth of Enterobacteriaceae during rainy season	Distance of well from pit latrine	Concentration of sterol
Growth on EMB for Dry season	Pearson correlation	1	0.677**	-0.131	-0.121
	Sig. (2-tailed)		0.001	0.563	0.591
Growth of Enterobacteriaceae during rainy season	Pearson correlation	0.677**	1	0.089	0.053
	Sig. (2-tailed)	0 .001		0.694	0.815
Distance of well from pit latrine	Pearson correlation	-0.131	0.089	1	0.012
	Sig. (2-tailed)	0.563	0.694		0.957
Concentration of sterol	Pearson correlation	-0.121	0.053	0.012	1
	Sig. (2-tailed)	0.591	0.815	0.957	

Correlation Significant at the 0.01 Level (2- tailed).

season (r (20) = 0.089, p > 0.05), both indicating non-significant linear relationships between distance of well from latrine and growth of Enterobacteriaceae. A weak positive relationship which is not significant was also found between distance of well from pit latrine and concentration of the faecal sterol (r (20) = 0.012, p > 0.05), indicating a non-significant linear relationship between distance of well from pit latrine and concentration of the feacal sterol (Table 3). Possible explanation for this is that the contaminants are of faecal sources and this also suggests that even when a well is reasonable separated from pit latrine structures, they are still prone to faecal contaminant. Muruka et al. (2012) reported in their study that a significant association existed between distances from dug wells to nearest pit latrine and that this association between level of dug-well faecal contamination and distance to nearest pit latrines was indirect. Studies have shown that sometimes no association might exist between dug-well water bacteriological quality and the distance of dug-wells to nearest pit latrines and they attributed this lack of association to many possible confounders, e.g. hygiene behavior, geomorphology of the area and the presence of other contamination sources. Other factors that can affect contamination of well water include; the environment where the well is cited, level of hygiene in terms of the use of drawers and the population of people using the well could be considered as other sources of contamination apart from pit latrines (Tairu et al., 2015). Studies have shown that until all these measures to control other sources of contamination are put in place, the role of latrines in the contamination of well water could not be quantified.

Conclusion

The study has revealed that well waters that are located in close proximity to sanitary pit latrines in Oko Township were highly vulnerable to bacteriological and finger print pollution. Despite the facts that both isolated organisms and sterol analysis indicated the contaminant to be of feacal origin, yet, a weak positive, non-significant statistical relationship was found between distance of well from latrine and the growth of Enterobacteriaceae organisms during rainy and dry season. This implies that safety of ground water are not guaranteed by distance but by consideration of hydrological, geomorphologic characteristics, hygienic conditions of the well, abstracting container and cross contamination from the well users, a justification from the fact that soil texture and topography constitute the rate at which ground water could be contaminated. The prevalence of multi antibiotic resistant bacteria from the studied well water is also high and this is a concern to human health. Therefore, the findings from this study will serve as a preliminary investigation on how to prevent the outbreak of waterborne diseases through ingestion of these multi-antibiotic resistant bacteria.

CONFLICT OF INTEREST

The authors declared that there was no conflict of interest whatsoever throughout the period of this research.

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