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MOLECULAR IDENTIFICATION OF *Escherichia coli* ISOLATES FROM RIVER TAUGA AND SURROUNDING WELLS ASSOCIATED WITH ABATTOIR EFFLUENT IN KARU, FCT. ABUJA.

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ABSTRACT:

The high contamination levels in abattoir effluent is leading to the transmission of pathogens to human and diseases. Thus, detection and control of *Escherichia coli (E. coli)* in aquatic environments ensures that drinking and bathing water is bacteriological safe. Molecular biology has offered significant increase in detection and identification specificity of microorganisms as it can identify unique genetic properties that is encoded in genome of such organism. Polymerase chain reaction (PCR) based methods are applied most frequently to detect and identify particular bacterial species. This paper reports the use of molecular methods in the identification of *E. coli* isolates of River Tauga and three surrounding well waters associated with abattoir effluent inKaru, FCT, Abuja. DNA was extracted from 12 bacterial isolates from 12 samples sites in the sample area. Total genomic DNA yield and purity was between 12.0 (ng/µl) and 30.0 (ng/µl) for DNA yield and a genomic purity of between 1.9 to 2.5 (A260:A280). Bacterial 16S rRNA gene was amplified using the 16s sRNA universal primers. The amplified 16S rRNA gene sequence was compared with the sequence in NCBI sequence database using Basic Local Alignment Search Tool (BLAST) search program. All sequences queried showed >= 200 similarity indexwith E value of 0.0 and percentage similarity of sequences (btw 99.9 to 100%) with sequences deposited in the GenBank. This study has shown that molecular techniques are more reliable in the identification of bacteria than the traditional methods of identification. The results of in this study revealed a precise and definitive identification of the isolates. This showed that all the isolates were of *Escherichia coli* as an indicator of faecal contamination of water associated with the untreated abattoir effluent in the water samples of River Tauga and the three surrounding wells with close proximity of the wells. The presence of *E. coli* in well water, river water and abattoir effluent provided sufficient evidence of the possible presence of pathogenic organisms from the untreated abattoir wastewater in this study.

Key words: BLAST, *Escherichia coli*, GenBank, Molecular Identification, PCR 16s rRNA.

Introduction

The abattoir industry is a vital element of the livestock industry providing domestic meat as well as job creation within the industry in Nigeria (Nafarnda *et al.,* 2012). Most of the abattoirs in Nigeria are not well developed and amenities for the managing of abattoir solid waste and waste water are either absent nor not properly managed. This brings about pollution hence significantly intensifying the amounts of nitrogen, phosphorus, and total solids in the receiving water body (Akange *et al.,* 2016).
Bacteria from abattoir waste discharged into water columns can subsequently be absorb to sediments, and when the bottom stream is disturbed, the sediment releases the bacteria back into the water columns presenting long-term health hazards. Adie and Osibanjo (2007) have reported very high contaminant level in abattoir effluent leading to transmission of pathogens to human and diseases

such as *Bacillus*, salmonella infection, *Brucellosis* and *helminthic* disease (Keating 1994).
According to the American Public Health Association (APHA) (2012) 80% of all diseases and over one third of deaths are attributed to intake of contaminated water in developing countries of which these pathogens may include rotaviruses, hepatitis E virus, *Salmonella* spp., *E. coli* O157: H7, *Yersinia enterocolitica*, *Campylobacter* spp., *Cryptosporidium parvum* and *Giardia lamblia*

(Sobsey *et al.*, 2002; Nafarnda *et al.*, 2012; Ojo and Alamuoye, 2015; Ire *et al.*, 2017).

Again, the cost of contamination by pathogens originating from animal wastes comes in an array of either temporary morbidity to mortality, especially in individuals with low immunity. Owing to the problems of quantifying pathogens, indicators of fecal pollution, including coliform bacteria, fecal coliforms, *E. coli*, and/or Enterococci have been monitored in lieu of evident pathogens for more than 100 years (Byamukama *et al.*, 2005; Nafarnda *et al.*, 2012; Ire *et al.*, 2017).

As reported by the United States Environmental Protection Agency (USEPA) (1986), Epidemiological proof supports the link between the fecal indicator bacteria *E. coli*, *Enterococci*, and the occurrence of gastrointestinal illness.

The detection of *E. coli* in aquatic environments ensures that drinking and bathing water is bacteriological safe. Standard techniques used for detection of *E. coli* from water samples are based on cultivation on selective growth media in combination with conventional biochemical tests (Rompré *et al.,* 2002; Bernasconi *et al.,* 2006; Bozaslan *et al.,* 2016). Although the standard methods are still widely used they have many drawbacks such as low specificity (Rompré *et al.* 2002).Bozaslan *et al.* (2016) has equally reported the in-efficacy of the Mac Conkey agar on the growth of *E. coli* from effluent wastewater making it very necessary to provide effective, fast and more reliable methods for identification.

Progress made so far in methods of molecular biology has offered researchers a significant increase in specificity as microorganisms can be identified according to their unique genetic properties encoded in genomic DNA (Horakova *et al.,* 2008). Polymerase chain reaction (PCR) based methods are applied most frequently to detect and identify particular bacterial species. PCR protocols for detection of *E. coli* from environmental samples has been reported by Bej *et al.,*1991a,b,c; Cebula *et al.,*. 1995; Juck *et al.,* 1996; Franck *et al.,*1998; Frahm and Obst 2003, Chen, *et al.,*2006, Omar, 2007due to its high sensitivity and specificity.. Tekpor *et al.,* 2017 has equally reported the use of PCR techniques in the detection of opportunistic pathogenic bacteria (*Salmonella, entero-haemorrhagic Escherichia coli*, and *Aeromonas hydrophilia*) in raw and treated water. Compared to the traditional culture techniques, PCR methods has enhanced sensitivity, simplicity, and speed of Micro-organism identification (Abada *et al.,* 2019). Over the past few decades, the 16S rRNA gene has been generally accepted as a standard for identification and classification of prokaryotic species owing to its structure, containing conserved and variable regions, and occurrence in all organisms. Moreover, its relatively short length allows its easy sequencing (Coenye and Vandamme, 2003).

The structure of the 16S rRNA genes is defined by an alternation of highly-conserved and hyper-variable regions, reflecting the effects determined by function-related constraints in the former and those allowed by their absence in the latter (Smith *et al.*, 2007). The estimated substitution rate is ~7000 times higher for the hyper-variable regions than the highly-conserved ones and these genetic differences have been considered to reflect, for most bacteria, genome divergence (Ashelfold *et al.*, 2005), making the 16S sequence an ideal proxy to achieve a trustworthy level of taxonomic information. Widely recognized as the gold standard for bacterial identification due to these specific features, 16S rRNA gene sequencing has been the target of countless studies in which universal PCR primers have been applied and the resulting partial 16S gene amplicons, encompassing hypervariable regions, used to infer taxonomic identifications based upon bioinformatics alignments against sequence databases (Bartram *et al.*, 2011). In view of the significance which molecular identification plays in the identification of micro-organisms, this paper reports this use in identification of *E. coli* in River Tauga and surrounding wells associated with abattoir effluent from Karu abattoir in Karu FCT, Nigeria.

Materials and Methods

Study Area

Karu abattoir is located in Karu which is one of the satellite towns in Abuja Municipal Area Council (AMAC) of the Federal Capital Territory, Nigeria. It is located about 7 km North East of the Federal Capital City, off the Abuja-Keffi expressway. It lies between $8^{o}59^{1}38.6$N and $90^{o}01^{1}$ 39.6N and longitude $7^{o}33^{1}$ 17.19E and $7^{o}34^{1}49.61$ E (Adakayi, 2006). Karu has an area of about 275 square kilometers. It is bordered to the North by Nyanya, to the South by Jikoyi, to the West by Kugbo and to the East by Mararaba (in Nasarawa State). The abattoir is close to the residential area.

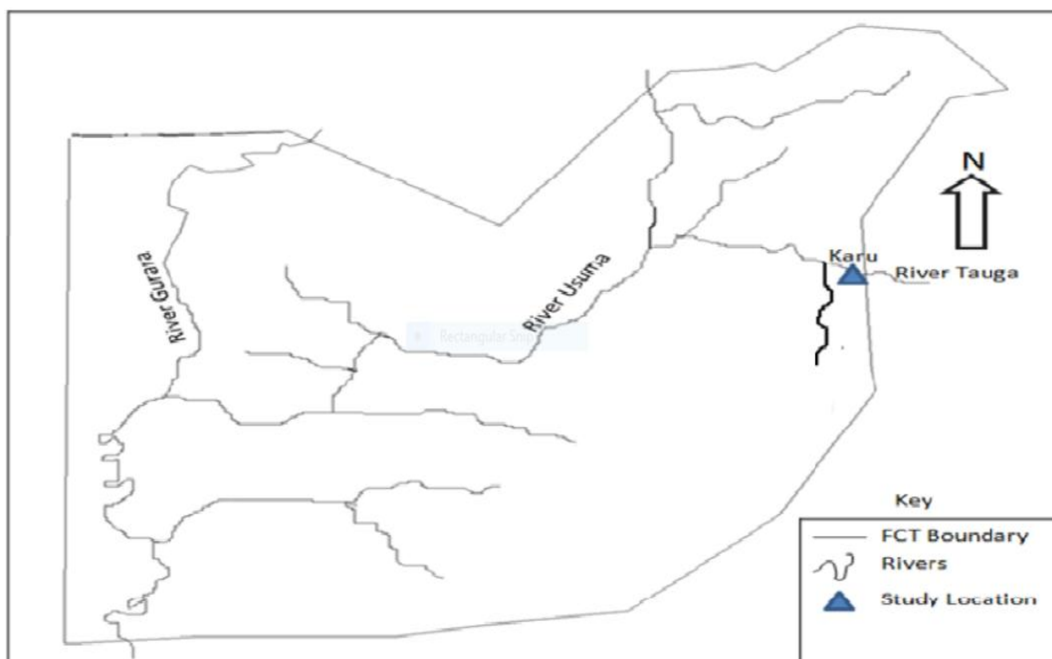

Figure 1 Location of the Study Area
Source: A.G.I.S. (2011)

Sample collection
Seven points were selected for sample collections. These were from the river water (RW), abattoir effluent (AE), the upstream of the river (UP), the receiving point of the abattoir effluent referred to as Midpoint (MP), 10 meters downstream after the receiving point (DS) and three different wells were selected at different distances around the abattoir environs Well 1 (W1) 100 meters away, Well 2 (W2) 200 meters and Well 3 (W3) 300 meters away from the abattoir. All samples were collected in sterile bottles and transported to the laboratory as described by Edward *et al.* (2017). Samples were collected during the rainy and dry seasons. The samples were collected thrice (2- week intervals) during each season. All samples were labeled appropriately according to sample and sample collection point.

Isolation and Purification of E. coli Isolate
E. coli was isolated using Eosin Methylene blue (EMB) as described by Isu and Onyeagba (1998). Bacterial colonies having green metallic sheen on Eosin Methylene blue agar were purified by repeated sub culturing on EMB and the discrete colonies were inoculated on Nutrient agar slant and stored at 4^0C for further identification.

DNA Extraction from E. coli Samples
The *E. coli* isolates from the samples was resuscitated by sub-culturing and inoculated into nutrient broth for DNA extraction. DNA from bacteria cells were extracted using the Zymo Quick-DNA Fungal/Bacterial Miniprep Kit following the manufacturers' protocols. Approximately 70mg (wet weight) bacteria cells that have been re-suspended in up to 200 µl of sterile distilled water where lysed in 750 µl Lysis Solution for about 30 mins using the ZR Bashing Bead™ Lysis Tube. They were then centrifuged at 10,000 x g for 1 minute. After which about 400 µl supernatant was collected to the Zymo-Spin™ IV Spin Filter (Orange Top) in a Collection Tube and centrifuge at 7,000 x g for 1 minute.
1,200 µl of Genomic Lysis Buffer was then added to the filtrate in the Collection Tube of which 800 µl of the mixture was then transferred into a Zymo-Spin™ IIC Columns in a Collection Tube which was then centrifuge at 10,000 x g for 1 minute. The flow through from the Collection Tube where discarded and step repeated again and flow through discarded. 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuge at 10,000 x g for 1 minute after which another 500 µl g-DNA Wash Buffer to the Zymo-Spin™ IIC Column and centrifuge at 10,000 x g for 1 minute. The Zymo-Spin™ IIC Column where then transferred into a clean 1.5 ml micro centrifuge tube and 100 µl DNA Elution Buffer added directly to the column matrix. This was then Centrifuge at 10,000 x g for 30 seconds to elute the DNA. DNA purity and quantified the extracted DNA samples was measured with the NanoDrop™ One/One C (Thermo Fisher Scientific). All DNA samples were stored at -20°C until further use for analysis.

DNA Electrophoresis

Agarose gel electrophoresis was used to determine the quality and integrity of the DNA by size fractionation on 1.0% agarose gels. Agarose gels were prepared by dissolving and boiling 1.0 g agarose in 100 mL 0.5 X TBE buffer solutions. The gels were allowed to cool down to about 45°C and 7 µl of 5 mg/mL GR Green (Biolabs, New England) was added, mixed together before pouring it into an electrophoresis chamber set with the combs inserted. After the gel had solidified, 5µL of the DNA mixed with 2 µl of 3X loading dye was loaded in the well created. Electrophoresis was done at 1200 V for 30 mins after which bands were visualized and photographed on gel documentation system (Edvotek, USA). The molecular size of the DNA was estimated using 1kb DNA ladder (Biolabs, New England). The extracted genomic DNA was used as template DNA for amplification of the 16S rRNA gene.

PCR Reaction of 16s Ribosomal Sub Unit and Bactria Identification
Amplification of the 16S ribosomal DNA was carried using the Universal 16s primers; 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3'). The final Polymerase chain reaction (PCR) protocol for the test samples was carried out in a volume of 28 µl containing 20 µl dream master mix (Biolabs, New England) containing 10x buffer (10 Mm TrisHCl], 2 mM MgCl2, 500 µM deoxynucleotide triphosphates (dNTPs), 0.5 units of Taq DNA polymerase), 1 µl bovine serum albumin (BSA) (Thermo Scientific, Lithuania). 2µl of the Forward and Reverse primers and 5 µl of total genomic DNA was also added to the PCR cocktail.
PCR reactions was carried out in the Peltier- based Thermal Cycler (LABKITS, Hong Kong.), with initial denaturation of 3 min at 94°C followed by 40 cycles of denaturation at 94°C for 1min, annealing at 40ºC for 1 min and extension at 72°C for 1 min, followed by a final extension for 10 min at 72°C.The amplified products were separated in 1% agarose gel electrophoresis using 1X TBE buffer stained with GR Green and Visualized on gel documentation system (Flour Shot LAB-KITS, Hong Kong). The molecular size of the amplification products was estimated using 1kb DNA ladder (Biolabs, New England).

Sequencing of the 16S Region in the PCR products
PCR products were cleaned using the nucleo spin1 extract II Kit (Macherey-Nagel, DuÈren, Germany), and sent for Sequencing. This was accomplished for both strands using 3730 DNA analyzer (Applied Bio-systems, Foster City, USA) at the bioscience laboratory of the Institute for Tropical Agriculture (IITA) Ibadan with the primers used for PCR. Sequences were manually edited for bad quality bases and assembled in contigs using Geneious Pro v9.2 (Biomatters, Auckland, New Zealand). They were aligned using the pairwise alignment algorithm implemented in Geneious Pro and the alignment was manually refined. Sequences were compared with known 16srRNA region sequences deposited in the GenBank (http://www.ncbi.nlm.nih.gov) and the percentage of similarity among the fragments was calculated using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Results
Total genomic DNA and PCR products Analysis
Total genomic DNA yield and purity is outlined in table 1. DNA samples showed a Genomic yield of between 12.0 (ng/µl) and 30.0(ng/µl) and a genomic purity of between 1.9 to 2.5 (A260:A280). This is considered to be good and purified DNA. The quality of DNA extracted was also confirmed on the 1.0% agarose gel by electrophoresis (Plate 1 and 2). Plates 3 and 4 show the PCR reaction of DNA isolates amplified using the Universal Primers for 16srRNA. Gel electrophoresis Pictures showed that the primers were able to amply the 16s rRNA region of the DNA samples.

Table 1: Total genomic DNA yield and purity of Bacteria Isolates

s/n	Samples	*DNA yield (ng/µl sample)	*DNA purity (A260:A280)
1.	Well 1(Dry Season)	12.8	1.84 ± 1.74
2.	Well 2 (Dry Season)	13.3	1.9.3 ± 1.24
3.	Well 3 (Dry Season)	14.9	1.80 ± 1.53
4.	Upstream (Dry Season)	15.9	1.97 ± 1.24
5.	Downstream (Dry Season)	16.8	1.94 ± 1.34
6.	Abattoir effluent (Dry Season)	29.7	1.81 ± 1.50
7.	Well 1 (Rainy Season)	30.1	2.12 ± 1.54
8.	Well 1(Dry Season)	29.5	2.52 ± 1.14
9.	Well 2(Rainy Season)	23.2	2.49 ± 1.14
10.	Well 3 (Rainy Season)	27.9	1.89 ± 1.94
11.	Upstream (Rainy Season)	29.8	1.94 ± 1.44
12.	Downstream (Rainy Season)	22.4	2.04 ± 1.24
13.	Abattoir Effluent (Rainy Season)	26.9	2.54 ± 1.34
14.	Well 2(Rainy Season)	27.0	1.89 ± 1.64
15.	Well 3 (Rainy Season)	30.4	1.90 ± 1.14
16.	Upstream (Rainy Season)	30.0	204 ± 1.44

*Value ± Mean Standard Error with n = number of each sample type.

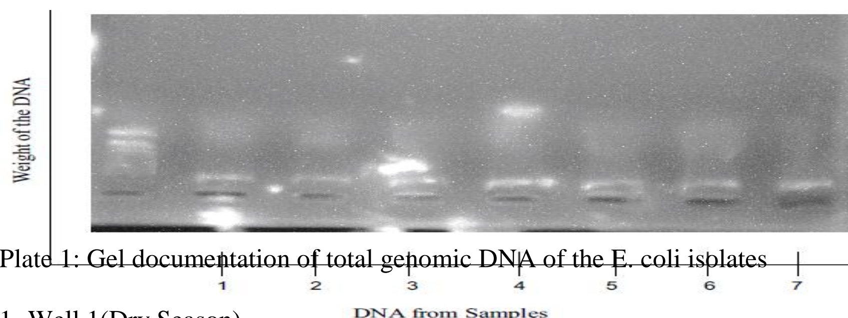

Plate 1: Gel documentation of total genomic DNA of the E. coli isolates

1- Well 1(Dry Season)
2 - Well 2 (Dry Season)
3 - Well 3 (Dry Season)
4 - Upstream (Dry Season)
5 - Downstream (Dry Season)
6 - Abattoir effluent (Dry Season)
7- Well 1 (Rainy Season)

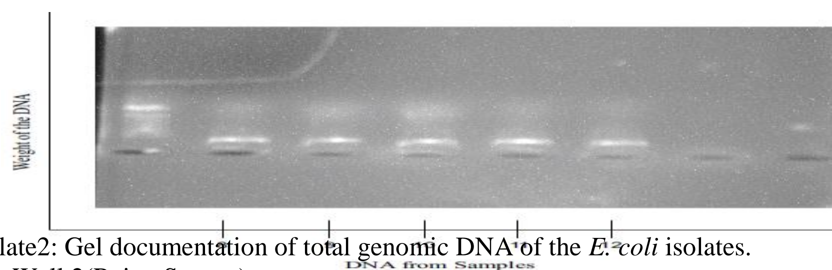

Plate2: Gel documentation of total genomic DNA of the *E. coli* isolates.
8- Well 2(Rainy Season)
9 - Well 3 (Rainy Season)
10 - Upstream (Rainy Season)
11- Downstream (Rainy Season)
12- Abattoir Effluent (Rainy Season)

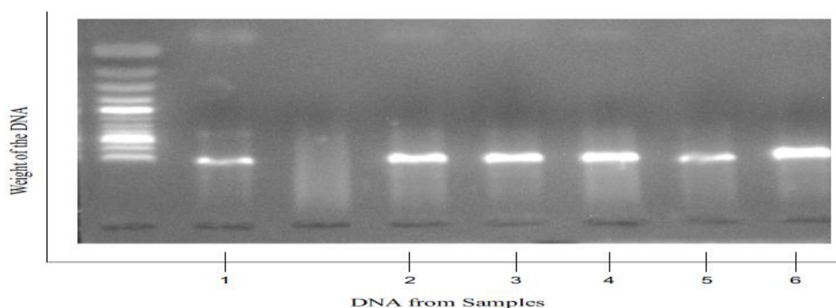

Plate 3: Gel documentation of PCR amplification of the 16s RNA region on DNA samples.
1- Well 1(Dry Season)
2 - Well 2 (Dry Season)
3 - Well 3 (Dry Season)
4 - Upstream (Dry Season)
5 - Downstream (Dry Season)
6 - Abattoir effluent (Dry Season

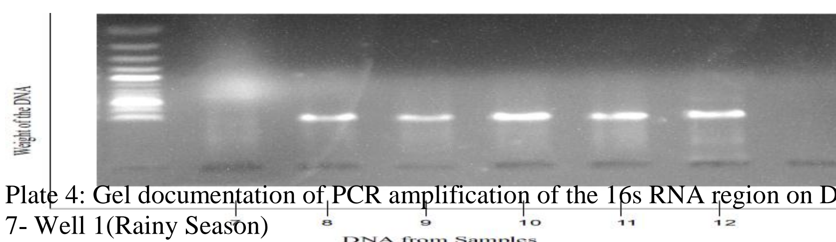

Plate 4: Gel documentation of PCR amplification of the 16s RNA region on DNA samples.
7- Well 1(Rainy Season)
8 - Well 2 (Rainy Season)
9 - Well 3 (Rainy Season)
10 - Upstream (Rainy Season)
11 - Downstream (Rainy Season)
12 - Abattoir effluent (Rainy Season)

16S rRNA sequence data

16S rRNA sequences were obtained for 12 individuals. The length of the analyzed 16s RNA fragments were about 1,550 base pairs (bp) long. However, after manual coding of the indels and removal of the repeated sequence motifs, the total length of the alignment was between 740 and 850 bp. newly generated sequences were deposited in the GenBank and GenBank numbers MT535587-MT535598, (www.ncbi.nlm.nih.gov/genbank/) have been assigned to all sequences deposited.

Identification of *E .coli* isolates

The 16S rRNA gene sequences generated compared with the sequence in National Centre for Biotechnology Information (NCBI) gene bank database by performing the Basic Local Alignment Search Tool (BLAST) search program. The BLAST web based program was able to align the search sequence to thousands of different sequences in a database and show the list of top matches. All sequences queried showed >= 200 similarity index with *E coli* 16S rRNA deposited in the GenBank (Figure 2). Majority of Sequences queried revealed similarities with the *E. coli* bacterium. All sequences queried had an E value of 0.0 and percentage similarity of sequences (btw 99.9 to 100%) with those in the GenBank (Figure 3).

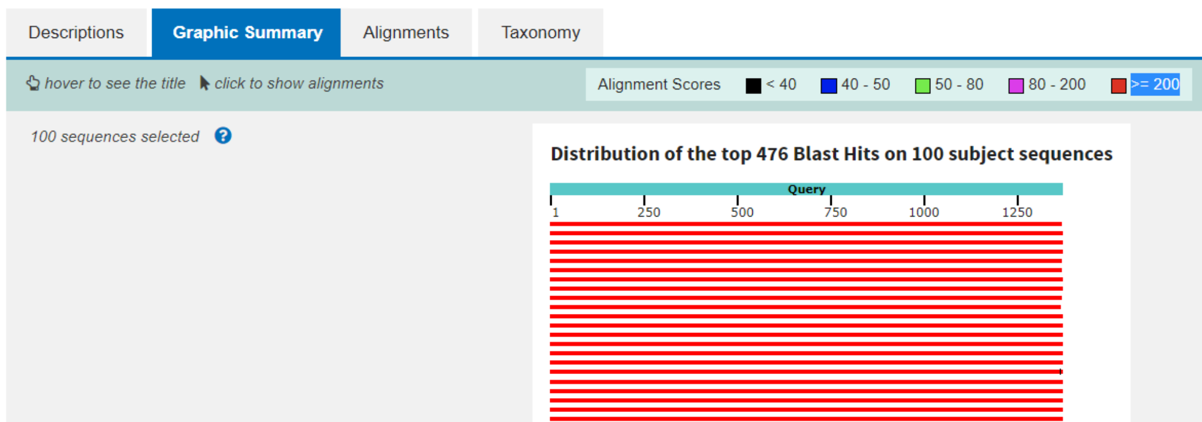

Figure 2: Basic Local Alignment Search Tool (BLAST) showing >= 200 similarity index with E coli 16S rRNA deposited in the GenBank.

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Select columns Show 100

select all 100 sequences selected GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Escherichia coli strain SA4 16S ribosomal RNA gene, partial sequence	Escherichia coli	2531	2531	100%	0.0	100.00%	1446	MT535590.1
Escherichia coli strain SA2 16S ribosomal RNA gene, partial sequence	Escherichia coli	2531	2531	100%	0.0	100.00%	1372	MT535588.1
Escherichia coli strain JCD07 16S ribosomal RNA gene, partial sequence	Escherichia coli	2531	2531	100%	0.0	99.93%	1446	MH532535.1
Enterobacteriaceae bacterium strain CAU2948 16S ribosomal RNA gene, partial seq...	Enterobacteriaceae bacterium	2525	2525	100%	0.0	99.85%	1387	MF428918.1
Enterobacteriaceae bacterium CCFM8315 16S ribosomal RNA gene, partial sequence	Enterobacteriaceae bacterium C...	2525	2525	100%	0.0	99.85%	1423	KJ803872.1
Escherichia coli strain C-X5B 16S ribosomal RNA gene, partial sequence	Escherichia coli	2525	2525	100%	0.0	99.85%	1408	KJ806497.1
Escherichia coli strain CAU1482 16S ribosomal RNA gene, partial sequence	Escherichia coli	2523	2523	100%	0.0	99.85%	1398	MF428898.1
Escherichia coli strain 54 16S ribosomal RNA gene, partial sequence	Escherichia coli	2521	2521	99%	0.0	99.85%	1410	MK621257.1
Escherichia sp. strain XS 10-1 16S ribosomal RNA gene, partial sequence	Escherichia sp.	2519	2519	100%	0.0	99.78%	1435	MT000013.1
Escherichia coli strain JCD08 16S ribosomal RNA gene, partial sequence	Escherichia coli	2519	2519	100%	0.0	99.78%	1446	MH532537.1
Escherichia coli strain Pk1 16S ribosomal RNA gene, partial sequence	Escherichia coli	2519	2519	100%	0.0	99.78%	1528	KX354348.1

Figure 3: Basic Local Alignment Search Tool (BLAST) showing E value and percentage similarity of sequences with those in the GenBank.

Discussions

During a BLAST hit, the E-values are the number of subject sequences that can be expected to be retrieved from the database that have a bit score equal to or greater than the one calculated from the alignment of the query and subject sequence, based on chance alone. According to Kerfeld and Scott, (2011), E-values for subject sequences that are very similar to the query sequence should be lower than 1. This refers to the assess of the confidence to which the subject sequence(s) and the query sequence are homo logs.

Molecular techniques have shown to be a reliable means for the identification of bacteria when compared to the traditional methods of identification (Olowe, *et al.,* 2017). Magray *et al.* (2011) reports the potentials of the ribosomal RNA (16S rRNA) sequence based analysis to be a distinctive method in understanding microbial diversity as well as in the identification of new strains. According to Sujatha *et al.* (2012), the assessment of the bacterial 16S rRNA gene sequence with a known bacterial sequence in a database is said to be an ideal genetic technique For proper bacterial identification. The results of in this study revealed a precise and definitive identification of the isolates. This showed that all the isolates were of *E. coli* as an indicator of faecal contamination of water associated with the untreated abattoir effluent in the water samples of River Tauga and the three surrounding wells with close proximity of the wells (100m, 200m and 300m) to the abattoir. The presence of *E. coli* in well water, river water and abattoir effluent provided sufficient evidence of the possible presence of pathogenic organisms from the untreated abattoir wastewater in this study. This has also been reported by Akpor (2011) and Ire *et al.* (2017).

Conclusion

The use of 16S rRNA gene sequencing in this study has contributed immensely in giving a decisive differentiation and identification *Escherichia coli* found in the River Tauga and surrounding wells associated with abattoir effluent. This is evidence

that *E. coli* of zoonotic origin can contaminate the environment as a result of the discharge of untreated abattoir effluent. The poor bacteriological quality of the studied wells may most likely be due to the nearness of the wells to the abattoir and the effect of the percolation of the abattoir effluents into the soil. it is therefore evident that proper hygiene and waste treatment and management systems are not employed in the abattoir.

The Authors have no conflict of interest.

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