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Prevalence of integrons in multidrug resistant *Escherichia coli* isolates from waters and vegetables in Nsukka and Enugu, Southeast Nigeria

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Abstract

Irrigation of fresh produce with poorly treated wastewater or contaminated freshwater sources can lead to produce contamination and foodborne illnesses, as well to dissemination of antimicrobial resistance determinants. In this study, we assessed the presence of integrons in multidrug resistant *E. coli* isolated from the University of Nigeria, Nsukka Wastewater Treatment Plant effluent, tap water, vegetables from irrigated gardens, and vegetables sold in selected markets from Nsukka and Enugu cities. *E. coli* was isolated following standard laboratory procedure and confirmed through beta-glucuronidase (*uidA*) targeted polymerase chain reaction (PCR). The antibiotic resistance of the isolates was determined using Bauer-Kirby disk diffusion assay and multiplex PCR was used to determine the presence of class 1 and 2 integrons. Our result revealed a total of 188 *E. coli* isolates from WWTP effluent (n = 41), tap water (n = 10) and vegetables from greenhouse (n = 46), farms (n = 55) and market (n = 36). Multi-drug resistance was detected in all the isolates, ranging from three-drug resistance in a single isolate to 7-drug resistance patterns in two different isolates. Of the total isolates, class 1 integrons were abundantly detected in 175 (93.1%) and class 2 in 5 (2.7%). All the class 2 integrons were found in isolates that were positive for class 1. The abundance of multidrug resistant *E. coli* harbouring class 1 integrons in the effluent and vegetable samples is a potential public health risk. Therefore, the appropriate measures for the safe use of poorly treated wastewater for vegetable farm irrigation are required to be put in place to reduce the microbial load of the discharged effluent. Also, education of farmers and the community on the dangers of wastewater effluent-grown plants and proper methods for cleaning harvested vegetable is recommended.

Introduction

Pathogenic *Escherichia coli* causes significant morbidity and mortality worldwide (Stacy-Phipps et al. 1995; Okeke 2009; Brown et al. 2018). Reported risk factors in the developing countries and sub-Saharan African regions include poor hygiene, unsafe water, improper disposal of waste and faeces, and contaminated food, local beverages, and vegetables (Okeke 2009; Nzeako and Okafor 2002; WHO 2017). In many countries, surveillance on the microbiological quality of vegetables and fresh produce has also been reported (Saksena et al. 2020; Tango et al. 2018; Richter et al. 2021; Sair et al. 2017; Baloyi et al. 2021). Vegetables can become contaminated with pathogenic and commensal bacteria from animals and humans, during growth, harvesting, distribution, storage and processing (WH Organization 2008). Although, the contamination of fresh produce by irrigation waters might lead to an outbreak of foodborne illnesses, yet treated wastewater presents itself as an attractive alternative to scarce quality water in developing countries.

E. coli has been reported as an aetiological agent of diarrhoea in both the northern and south-western parts of Nigeria (Ogunsanya et al. 1994; Okeke et al. 2000a; Olorunshola et al. 2000; Okeke et al. 2002; Chigor et al. 2010; Onanuga et al. 2014). In a study that detected *E. coli* in 119 (44.74%) of 270 diarrhoeal stool samples in Enugu and Onitsha cities, south-eastern Nigeria (Nweze 2010), enterotoxigenic *E. coli* (ETEC) was reported as the second most prevalent pathotype (21.57%) after enteropathogenic *E. coli* (EPEC) (49.02%). Likewise, a study conducted in Nsukka, that involved watery stools, drinking water, and some fruits and vegetables collected during the rainy season, from 1996 to 1998 Nzeako and Okafor (2002) reported that enteropathogenic *E. coli* (EPEC) was detected in 9 (1.8%) of 500 stool samples, whereas no enteric bacterial pathogen was isolated from the fruits and vegetables. There appears to be no reports on the prevalence of ETEC and the other *E. coli* pathotypes in humans and on irrigated vegetables in Nsukka.

Excessive and inappropriate usage of antimicrobials either in preventing diseases or treating infected human and animals have led to increased antimicrobial and multidrug resistance (MDR) and the risk of transmission of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) from one country to another is a growing global challenge (Okeke and Edelman 2001; Gaze et al. 2011; Wellington et al. 2013). Attention should be given to how anthropogenic activities might be causing the evolution of antibiotic resistance in the environment (Wellington et al. 2013). Studies have shown that wastewater treatment plants form a significant reservoir of resistance genes and suggested that wastewater disposal increases the reservoir of resistance determinants in the environment either by the addition of resistance genes or input of agents selective for resistant phenotypes (Amos et al. 2014).

Along with transposons and plasmids, integrons, genetic elements commonly found in bacterial genomes that allow efficient acquisition and expression of exogenous genes are central to dissemination of antibiotic resistance among Gram-negative bacteria (Gillings 2014; Strugeon et al. 2016). Horizontal transfer of integrons have been shown to play an important role in the spread and maintenance of antimicrobial resistance among strains of *E. coli* and ARB can be transferred across borders by human travelers, animals and insect vectors, agricultural products, and surface water (Gaze et al. 2011; Ma et al. 2017). Not much is known about the risk factors in its spread across the local borders.

Nsukka, in Southeast Nigeria, is the location of one of Nigeria's biggest universities in which the town developed around it. Therefore, the focus of this study is to assess the microbiological safety of vegetables and the presence of integrons in multidrug resistant *E. coli* isolated from the University of Nigeria, Nsukka Wastewater Treatment Plants (WWTP) effluent, vegetables from irrigated gardens, and vegetables sold in selected markets from Nsukka and Enugu cities.

Methods

Description of study area

The university town of Nsukka (6.8429° N, 7.3733° E) is in Enugu State, southeast Nigeria, with an area of 1,810 km² and a population of 309,633 (Commission 2006). The sewage treatment facility (WWTP) in Nsukka, consisting of a screen, primary settling (Imhoff) tank, sludge drying beds, and two oxidation ponds, is situated at the northwest end of the University of Nigeria, Nsukka. The final effluent after the treatment have been widely utilized for fresh produce irrigation during the dry season. Enugu is is about 61.3 kilometers from Nsukka and is been supplied with fresh produce from farmers within and around villages surrounding it.

Cultivation of *Amaranthus* in the greenhouse

The most commonly cultivated vegetables in the study area, during the dry season, include the green leafy vegetable amaranth (*Amaranthus* spp), fluted pumpkin leaves (*Telfaria occidentalis*), scarlet eggplant leaf (*Solanum aethiopicum*) and water leaf (*Talinum fruticosum*). In this study, *Amaranthus* was chosen, being the second most produced and sold leafy vegetable, after *Telfaria* (Asadu et al. 2016), and it equally grows very easily and matures faster. *Amaranthus* plants were grown in a greenhouse for 10 weeks in truncated cone-shaped flower pots. Each of the pots contained about 3.8 liters of loam soil collected from a garden located within the premises of the Green House at the Department of Soil Science, University of Nigeria, Nsukka, as depicted in Figure S1. The dimensions of the flower pots were: depth of 20 cm, a base-of-pot diameter of 12 cm, and a top-surface diameter of 19 cm. The amount of soil potted was determined using Potting Soil Calculator (https://www.omnicalculator.com/biology/potting-soil-calculator). A total of 60 pots were used for the cultivation of vegetables, 48 were irrigated with treated wastewater (final effluent of the University of Nigeria, wastewater treatment plant (WWTP), and 12 with tap water. The

pots irrigated with tap water served as the control. They were irrigated daily using the sprinkler method.

Collection of Samples

Samples collected in this study include treated wastewater, tap water, and vegetables. Water sampling was done according to the standard procedure (APHA 2005). The WWTP effluent and tap water were collected in the morning (between 8 and 10am), with 10 L plastic cans for irrigation of the greenhouse vegetables. Simultaneously, samples of the effluent and tap water were collected using sterile wide-mouthed, screw-capped 250-ml bottles for bacteriological analysis. The major vegetables cultivated during the dry season in the study area include fluted pumpkin leaves (*Telfaria occidentalis*), scarlet eggplant leaf (*Solanum aethiopicum*), water leaf (*Talinum fruticosum*) and the green vegetable (*Amaranthus* spp). Vegetable samples were obtained from the greenhouse WWTP effluent-irrigated, potted-greenhouse amaranths, and from gardens raised by rural women that were irrigated with wastewater (including the WWTP effluent and water scooped from hostel drains) as well as from local markets in Nsukka and Enugu metropolis. The source of water for irrigation of the vegetables obtained from market are unknown. All samples were transported in a box containing ice to the laboratory and analysed within 6 h of collection.

Isolation and identification of presumptive *E. coli*

Analysis of the samples were carried out at the Water and Public Health Research Laboratory, University of Nigeria, Nsukka. Exactly 5 g of each vegetable sample was homogenized in a clean porcelain mortar, and 1 g of the homogenate diluted into 9ml normal saline (Falomir et al. 2010; Saeed et al. 2013). Serial dilutions (10-fold) were made by pipetting out 1ml stock solution into successive 9ml of sterile normal saline bottles. A 1-ml working sample dilution (10^{-1} and 10^{-2}) was spread-plated onto eosin methylene blue (EMB) agar (Oxoid, UK),

incubated at 44 °C for 18-24 h. Tap water and wastewater samples were analysed following Standard Methods (American Public Health Association, 2012). Water samples were processed via the membrane filtration technique (Forster and Pinedo 2015). Exactly 100 ml aliquots of the water samples or dilutions were filtered through a 47-mm diameter, 0.45-µm pore-sized membrane filters (Millipore, Ireland). The filters were incubated overnight at 44.5 °C on eosin methylene blue (EMB) agar (Oxoid, UK) plates. Raised, entire colonies with dark greenish metallic sheen, typical *E. coli* colonies were subjected to Gram-staining (Claus 1992) and standard biochemical tests (IMViC – indole, methyl red, Voges-Proskauer, in, citrate tests). All presumptive *E. coli* isolates were sub-cultured in tryptic soy broth (Oxoid, UK) and then stored at -20 °C for further investigations. All media were prepared following the manufacturers' instructions.

Extraction of genomic DNA

Genomic DNA was extracted from a pure culture of each isolate grown overnight on nutrient agar at 37°C, by the conventional boiling method, as described by Vankerckhoven et al. (2004). Briefly, one loopful of bacterial cells was suspended in 1ml of sterile distilled water. The bacterial suspensions were then heated for 5 min at 100 ^{o}C, cooled to room temperature and centrifuged at 12,000 x g for 5 min to remove the debris. The supernatant was stored at -80 ^{o}C and used as the template DNA for PCR.

Detection of beta-glucuronidase (*uid*A) gene for confirmation of *E. coli*

The confirmation of *E. coli* was achieved by polymerase chain reaction (PCR) detection of the target beta-glucuronidase (*uid*A) gene. This analysis was done at the School of Natural Sciences, Bangor University, United Kingdom, following the procedures described by Bej et al. (1991) and Horakova et al. (2008). The extracted DNA was cleaned using QIAGEN (QIAEX®II) gel extraction kits and kept at -80°C. Amplification of the target gene was carried out with BIORAD DNA Engine Tetrad®Peltier Thermal Cycler (BIORAD, USA). The PCR

reaction mixtures consisted of 25 µl of PCR Master Mix (Thermo Scientific, (EU) Lithuania), 0.5 µl each of oligonucleotide primers (Eurofins Genomics, Ebersberg Germany), 10 µl of template DNA, and 14 µl of nuclease free water to constitute a total reaction volume of 50 µl. The PCR cycling conditions, with some modifications, were in accordance with the protocols prescribed elsewhere (López-Saucedo et al. 2003). *E. coli* strain (NCTC 13353) and *Enterobacter aerogenes* (NCTC 10006) were used as positive and negative controls respectively for *E. coli* genus identification. The oligonucleotide sequence of primers used, target genes and expected amplification products are given in Table 1. For gel electrophoresis, the obtained PCR products were run on 2.5% (w/v) agarose gels in 1x-TBE buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 8.0) at 100V for 25 min and visualized with BIORAD Molecular Imager® Gel Doc^{TM} XR Imaging System (BIORAD, USA).

Antibiotic susceptibility testing

Isolates were subjected to antibiotic susceptibility testing using the Kirby-Bauer disc diffusion test (Bauer 1966). Evaluation of results was based on the standards of the Clinical Laboratory Standards Institute (CLSI) (Clinical and Institute 2012). Briefly, isolates grown on nutrient broth were suspended into sterile normal saline (0.9% (w/v) NaCl) with the aid of a sterile wire loop until the turbidity equivalent of 0.5 McFarland standard was reached. Sterile non-toxic cotton swabs were dipped into the standardized inoculum and used to smear the entire surface of the Muller-Hinton agar (Thermo Fisher Scientific, USA) plates. Antibiotic discs were placed aseptically using sterile forceps. All plates were incubated at 35±2 ^{o}C for 16 to 18 h. The following eight antibiotics were employed for the test: Sulphamethoxazole (SMZ) 25µg, Amoxicillin (AMX) 10µg, Tetracycline (TET) 30µg, Streptomycin (STR) 10µg, Chloramphenicol (CHL) 30µg, Ciprofloxacin (CIP) 5µg, Cefuroxime (CXM) 30µg, Imipenem (IPM) 10µg. The *E. coli* ATCC 25922 was used as a control for antibiotic susceptibility testing. Zones showing complete inhibition around the discs were measured and classified as resistant

(R), intermediate (I) and susceptible (S) according to the diameters of the zones recorded to the nearest millimeters.

Detection of integrons

The isolates were screened for class 1, 2 and 3 integrons by a multiplex PCR procedure as described by Machado et al. (2005) and Kargar et al. (2014) (Table 1). The PCR reactions (a total volume of 50µl reaction mixture) each consisting of 10 µl Buffer of 5x MyTaq Reaction Buffer (Bioline, with dye), 0.75µl of each the primers *int*I1, *int*I2 and *int*I3 (Eurofins Genomics, Ebersberg, Germany), 27.25µl nuclease free water (Sigma-Aldrich), 0.25µl MyTaq DNA polymerase (Bioline) and 5µl DNA template. For gel electrophoresis, the amplified samples were ran on 1.5% (w/v) agarose gels in 1x-TBE buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 8.0) at 100 V for 25 min. The gels were viewed and photographed with BIORAD Molecular Imager® Gel Doc^{TM} XR Imaging System

Results and Discussion

It is worthy of note that amaranths irrigated with wastewater effluent were of higher yields compared to the controls irrigated with tap water. This is attributable to the fact that the effluent is rich in nutrients. It also underscores the continued preference for WWTP effluent over the scarce treated water by the vegetable farmers. Despite a perceived understanding farmers have on the use of unsafe WWTP effluent, this knowledge seems not to bother them.

In the present study, the total number of samples collected were 110 water samples including WWTP effluent (60) and tap water (50) samples and 228 vegetable samples: greenhouse vegetable samples (60), farm vegetable samples (84), and market vegetable samples (84). A total of 188 *E. coli* isolates were confirmed by PCR amplification of the β-glucuronidase *uid*A gene (Fig. S2), with 41 isolates from the WWTP effluent, 10 from tap water, 46 from the effluent-irrigated greenhouse vegetables, 55 from vegetables collected from gardens that

produce vegetables sold in local markets and 36 from vegetables bought from selected markets in Nsukka and Enugu (Table 2). The source of water for irrigation of the vegetables obtained from market are unknown. Interestingly, tap water samples contained *E. coli* which might have arose as a result of contamination of the drinking water sources or wastewater draining into the tap water carrying pipes. In most cases, the inherent and wide use of underground septic tank and suck-away systems could contribute to the introduction of pathogens into the underground water table. For this reason, adequate measures should be put in place to secure the drinking water distribution systems from wastewater contamination. Standard treatment procedure must be maintained in the treatment of water to ensure it is safe for drinking and free from potential microbial pathogens. It was also observed from our study that fresh produce from the local markets were laden with *E. coli*. The source of this bacteria could likely originate from the water used for irrigation by the farmers or mishandling of the vegetables by the sellers. It could also be traceable to cow dung-contamination of the different freshwater sources used for irrigation since open grazing of cattle is a common practice in Nigeria.

Table 3 shows antibiotics susceptibility profiles of the 188 *E. coli* isolates tested with 8 different antibiotics. Generally, higher resistance percentages were observed in *E. coli* from market vegetables compared with others. This could be attributable to further contamination of vegetables by clinical *E. coli* strains arising directly from handling by sellers (Richter et al. 2021). In Nigeria and other African countries, for instance, were open market system is operated, majority of the traders who deal on vegetable produce often display it openly under the sun and frequently sprinkle it with water to reduce the rate of leaves becoming flaccid. The water used might likely be the potential source of the contamination. All the *E. coli* isolates showed susceptibility to imipenem and only 6.4% (12/188) of all the isolates were resistant to cefuroxime (a cephalosporin). Chloramphenicol, ciprofloxacin, and norfloxacin were very effective. The most significant resistance phenotypes were detected among sulphamethoxazole

(58.5%), amoxicillin (52.7%), tetracycline (46.8%), and streptomycin (36.7%), as these antibiotics are commonly used in the studied communities (Table 3).

Multidrug resistance (MDR) has been frequently reported in Nigeria among *E. coli* isolates obtained from human specimens (Onanuga et al. 2014; Odetoyin et al. 2017; Chigor et al. 2010; Okeke et al. 2000b), animal sources (Chah et al. 2010) and environmental samples (Adelowo et al. 2018; Chigor et al. 2010; Smith et al. 2009; Titilawo et al. 2015). In the present study, all the isolates were multidrug resistant, ranging from 3-drug to 7-drug resistance patterns (Table 4). This classification is based on the bacteria being resistant to more than one antibiotics in greater than or equal to three antibiotic categories as described by Magiorakos et al. (2012).

Although some studies have reported a high removal efficiency for total ARGs in wastewater (Yang et al. 2014), data presented here reveal that all the *E. coli* isolated from the effluent were MDR, suggesting that the sewage treatment process at UNN is not effective in reducing ARGs. The spread of AMR often limits the availability of therapeutic options to only a very few efficacious antibiotics (Juhas et al. 2019). The last-resort drugs, the carbapenems such as imipenem (used in this study) and meropenem, are themselves not only increasingly challenged by emerging resistance, as evident from the data presented here, but are not affordable in the developing regions.

Multidrug resistance (MDR) was detected in all *E. coli* isolates, and although this study did not determine the full virulence potentials of all the isolates subjected to antimicrobial susceptibility testing (AST), irrigational use of WWTP effluent represents a pathway for human exposure to antibiotic-resistant commensal and pathogenic bacteria. Our earlier report, Chigor et al. (2020) revealed that 26.4% (23/87) of *E. coli* isolates harboured the *lt* gene of the enterotoxigenic *E. coli* (ETEC) strain. Farmers should apply caution when cultivating their vegetable at the wastewater treatment site to ensure the safety of their produce for human

consumption since contaminated vegetable present a significant threat to the health of consumers.

Genes involved with AMR and MDR are carried and disseminated by genetic elements such as plasmids, transposons, and integrons. They confer AMR and MDR phenotypes on *E. coli* bearing those genes. (Gaze et al. 2011; Gillings 2014; Ma et al. 2017). In the present study, of the 188 *E. coli* isolates, class 1 integrons were detected (Fig. S3) in 175 (93.1%), and class 2 in 5 (2.7%). All the class 2 integrons were found in isolates that were positive for class 1. Such co-carriage has been previously published on *E. coli* from meat turkeys in Italy, (Piccirillo et al. 2014).

The integron carriage rate for the 137 vegetable isolates was 97.8%, whereas the rate for 41 effluent isolates was 100%. Considering that all the isolates were MDR, the detected high prevalence of class 1 integron is not surprising and compares with a previous study that reported that MDR phenotypes were observed in 96.8% of the integron-positive isolates (Vasilakopoulou et al. 2009). These rates portend serious public health risks as it is known that class 1 integron could carry diverse antibiotic resistance genes (ARGs) and conduct horizontal gene transfer among microorganisms (Ma et al. 2017).

The data presented here show that class 2 integrons were less frequently detected 5 (2.7%). Similar data have been published for *Enterobacteriaceae* in Nigeria (Chah et al. 2010; Adelowo et al. 2018) and elsewhere (Piccirillo et al. 2014; Ramírez et al. 2010; Malek et al. 2015). Ramírez et al. (2010) reported that unlike the widespread distribution of class 1 integron within Gram-negative bacilli, only *Acinetobacter baumannii* and *Enterobacter cloacae* harboured class 2 integrons at a high frequency. However, in an earlier study in China (Pan et al. 2006), Class 2 integrons were present in 25 (80.6%) of the *Shigella sonnei* isolates and 29

(87.9%) of the *S. flexneri* isolates whereas class 1 integrons were found in only 6 (9.4%) of *Shigella* spp. isolates.

Conclusions

The present study revealed high prevalence of *E. coli* in tap water, WWTP effluent, and vegetable samples. The high concentration of *E. coli* in the studied effluent and vegetables pose potential public health hazards heightened by observed multidrug resistance in all the isolates and the high prevalence of class 1 integron. It is concluded that the vegetable samples are significant reservoirs for potentially pathogenic *E. coli.* Therefore, appropriate improvement strategies need to be developed for wastewater effluent quality in vegetable irrigation farming to ensure the safety of human health. Tap water use for drinking should be properly treated and protected from contamination. Although, our study did not cover *E. coli* endophytism in vegetable, we recommend that future studies should examine the prevalence of *E. coli* in vegetable tissues as well as measure their decay rate or survival tendency on the phylloplane.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legend

Fig. S1 Amaranth plants grown for 10 weeks in the Greenhouse. A: Irrigated with WWTP effluent and B: Irrigated with tap water. The bigger growth observed in A is attributable to the manure in wastewater.

Fig. S2 PCR products for *E. coli* confirmation by *uid*A gene amplification.

M: Molecular weight marker (1 kb Plus Ladder, Invitrogen), W: water, NC: Negative control (*Enterobacter aerogenes*), PC: Positive control (*E. coli*; NCTC 13353), Lanes 1-15: Positive isolates

Fig. S3 Multiplex PCR products for the detection of class 1 and 2 integrons

M: Molecular weight marker (1 kb Plus Ladder Invitrogen), W: water, Lanes 1-15: *E. coli* isolates

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