

1 **Multidrug resistance and high prevalence of class 1**
2 **integrons in *Escherichia coli* isolated from waters and**
3 **vegetables in Nsukka and Enugu, Nigeria.**
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5 **Chinyere B. Chigor**^{1,2¶}, **Ini-Abasi I. Ibangha**^{1,3&}, **Nkechinyere O. Nweze**^{1,2¶},
6 **Chizoba A. Ozochi**^{1,3¶}, **Valentino C. Onuora**^{1,3&}, **Yinka Titilawo**^{4¶}, **Tatyana N. Chernikova**^{5¶},
7 **Peter N. Golyshin**⁵ and **Vincent N. Chigor**^{1,3*}

8
9 ¹Water and Public Health Research Group (WPHRG), University of Nigeria, Nsukka, Enugu State,
10 Nigeria.

11
12 ²Department of Plant Science and Biotechnology, Faculty of Biological Sciences, University
13 Nigeria, Nsukka, Enugu State, Nigeria.

14
15 ³Department of Microbiology, Faculty of Biological Sciences, University of Nigeria, Nsukka,
16 Enugu State, Nigeria.

17
18 ⁴Department of Biology/Microbiology/Biotechnology, Alex Ekwueme Federal University, Ndufu-
19 Alike Ikwo, Ebonyi State, Nigeria.

20
21 ⁵School of Natural Sciences, Bangor University, Bangor Gwynedd, United Kingdom

22
23 *Corresponding author

24 E-mail: vincent.chigor@unn.edu.ng (VNC)

25 "VNC and PNG are Joint Senior Authors"

26
27 **Keywords:** multidrug resistance; Integrons; pathogenic *E. coli*; wastewater-irrigated vegetables;
28 public health risks

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31 **Running title: Multidrug resistance and class 1 integrons in *Escherichia coli***
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40 **Abstract**

41 In spite of treated wastewater presenting itself as an attractive alternative to scarce quality water
42 in the developing countries, the associated contamination of fresh produce by irrigation waters
43 leading to outbreak of foodborne illnesses is on the rise. Horizontal transfer of integrons play
44 important role in the spread and maintenance of antimicrobial resistance among strains of
45 *Escherichia coli*. This study assessed the effluents from the University of Nigeria, Nsukka
46 Wastewater Treatment Plant (UNN-WWTP) as well as vegetables irrigated with the effluent, and
47 vegetables sold in selected markets from Nsukka and Enugu cities for the presence of *E. coli* and
48 determined the prevalence integrons in multidrug-resistant isolates. Isolation of *E. coli* was done
49 using eosin methylene blue agar and isolates subjected to Gram staining for identification of
50 presumptive colonies. Confirmation of *E. coli* was achieved by polymerase chain reaction (PCR)
51 technique, targeting beta-glucuronidase (*uidA*). Resistance to antibiotics was determined using the
52 Bauer-Kirby disk diffusion assay and the Clinical and Laboratory Standard Institute criteria.
53 Integrons were detected by multiplex PCR using primers specific for class 1 and 2 integrons. A
54 total of 178 *E. coli* isolates were obtained from WWTP effluent (41), and vegetables from
55 greenhouse (46), farms (55) and market (36). Multi-drug resistance was detected in all the isolates,
56 ranging from five-drug resistance in a single isolate to 16-drug resistance patterns in two different
57 isolates. Of the total isolates, class 1 integrons were abundantly detected in 175 (98.3%) and class
58 2 in 5 (2.8%). All the class 2 integrons were found in isolates that were positive for class 1. The
59 high detection of *E. coli* in the studied effluent and vegetables pose potential public health hazards
60 heightened by observed multidrug resistance in all the isolates and the high prevalence of class 1
61 integron. It is concluded that the vegetable samples are significant reservoirs for potentially
62 pathogenic *E. coli*. Therefore, vegetable irrigation farming with unsafe water should be
63 discontinued, while appropriate improvement strategies to ensure compliance should be facilitated
64 without further delay.

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66 **Introduction**

67 Pathogenic *Escherichia coli* causes significant morbidity and mortality worldwide [1-3]. Reported
68 risk factors in the developing countries and sub-Saharan African regions include poor hygiene,
69 unsafe water, improper disposal of waste and faeces, and contaminated food, local beverages and
70 vegetables [2, 4, 5]. Vegetables can become contaminated with pathogenic and commensal

71 bacteria from animals and humans, during growth, harvesting, distribution, storage and processing
72 [6]. Although the contamination of fresh produce by irrigation waters has led to outbreak of
73 foodborne illnesses, yet treated wastewater presents itself as an attractive alternative to scarce
74 quality water in the developing countries.

75 *E. coli* has been reported as an aetiological agent of diarrhoea in both the northern and south-
76 western parts of Nigeria [7-12]. In a study that detected *E. coli* in 119 (44.74%) of 270 diarrhoeal
77 stool samples in Enugu and Onitsha cities, south-eastern Nigeria [13], enterotoxigenic *E. coli*
78 (ETEC) was reported as the second most prevalent pathotype (21.57%) after enteropathogenic *E.*
79 *coli* (EPEC) (49.02%). Likewise, a study conducted in Nsukka, that involved watery stools,
80 drinking water, and some fruits and vegetables collected during the rainy periods (between April
81 and October) over 3 year sampling regime (1996 to 1998), [4] reported that enteropathogenic *E.*
82 *coli* (EPEC) was detected in 9 (1.8%) of 500 stool samples, whereas no enteric bacterial pathogen
83 was isolated from the fruits and vegetables. There appears to be no reports on ETEC prevalence in
84 humans and on irrigated vegetables in Nsukka.

85 Excessive and inappropriate usage of antimicrobials in preventing or treating human and
86 veterinary bacterial infectious diseases has led to increased antimicrobial and multidrug resistance
87 (MDR) and the risk of transmission of antibiotic resistant bacteria (ARB) and antibiotic resistant
88 genes (ARGs) from one country to another is a growing global challenge. [14-16] Attention should
89 be given to how anthropogenic activities might be causing evolution of antibiotic resistance in the
90 environment [16], and studies have shown that waste water treatment plants form a significant
91 reservoir of resistance genes and suggested that waste water disposal increases the reservoir of
92 resistance determinants in the environment either by the addition of resistance genes or input of
93 agents selective for resistant phenotypes [17].

94 Along with transposons and plasmids, integrons, genetic elements commonly found in
95 bacterial genomes that allow efficient acquisition and expression of exogenous genes, are central
96 in the dissemination of antibiotic resistance among Gram-negative bacteria [18,19]. Horizontal
97 transfer of integrons have been shown to play important role in the spread and maintenance of
98 antimicrobial resistance among strains of *E. coli* and ARB can be transferred across borders by
99 human travelers, animal and insect vectors, agricultural products and surface water [15,20]. Not
100 much is known about the risk factors in spreading across local borders.

101 It is thought that University towns, characterized by regular and significant demographic
102 changes arising from admissions and vacations, could play major role in dissemination of
103 resistance determinants locally, and even internationally where the institution has a good number
104 of internationals. Nsukka, in Southeast Nigeria, is the location of one of Nigeria's biggest
105 universities and one also in which the town developed around the university. This study assessed
106 the effluent from the University of Nigeria, Nsukka Wastewater Treatment Plant (UNN-WWTP)
107 as well as vegetables irrigated with the effluent and vegetables sold in selected markets for the
108 presence of *E. coli* and determined the prevalence integrons in multidrug-resistant isolates.

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110 **Methods**

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112 **Description of study area**

113 The university town of Nsukka (6.8429° N, 7.3733° E) is in Enugu State,
114 southeast Nigeria, with an area of 1,810 km² and a population of 309,633 (NPC 2006). The sewage
115 treatment facility (WWTP) in Nsukka, consisting of a screen, primary settling (Imhoff) tank,
116 sludge drying beds and two oxidation ponds, is situated at the northwest end of the University of
117 Nigeria, Nsukka. The final effluents have been widely utilized for fresh produce irrigation during
118 dry season.

119 **Cultivation of *Amaranthus* in the greenhouse**

120 The most commonly cultivated vegetables in the study area, during the dry season, include
121 the green leafy vegetable amaranth (*Amaranthus* spp), fluted pumpkin leaves (*Telfaria*
122 *occidentalis*), scarlet eggplant leaf (*Solanum aethiopicum*) and water leaf (*Talinum fruticosum*). In
123 this study, *Amaranthus* was chosen, being the second most produced and sold leafy vegetable,
124 after *Telfaria* [21], and it equally grows very easily and matures faster. Amaranths were grown for
125 10 weeks (July 26 to October 03, 2014) in earthen pots at the Soil Science Departmental
126 greenhouse. They were irrigated daily using the sprinkler method. A total of 60 earthen pots were
127 used for the cultivation of vegetables, 48 were irrigated with treated wastewater (final effluent of
128 the University of Nigeria, wastewater treatment plant (WWTP) and 12 with tap water. The pots
129 irrigated with tap water served as the control.

130 **Collection of Samples**

131 Samples collected for this study included treated wastewater and vegetables. Sampling was
132 done according to the standard procedure [22]. Effluents were collected with 10 L plastic cans for
133 irrigation of the green house vegetables. Samples of the WWTP effluent were collected using
134 sterile wide-mouthed, screw-capped 250-ml bottles. Vegetables were obtained from the green
135 house, irrigated gardens and local markets in Nsukka and Enugu metropolis, during December
136 2014. Samples of the major vegetables cultivated during the dry season include fluted pumpkin
137 leaves (*Telfaria occidentalis*), scarlet eggplant leaf (*Solanum aethiopicum*), water leaf (*Talinum*
138 *fruticosum*) and the green vegetable (*Amaranthus* Spp) were collected. All samples were
139 transported on ice to the laboratory and analysed within 6 h of collection.

140 **Isolation and identification of presumptive *E. coli***

141 This was carried out at the Water and Public Health Laboratory, University of Nigeria
142 Nsukka. Exactly 5 g of each vegetable sample was homogenized in a clean porcelain mortar, and
143 1 g of the homogenate diluted into 9ml normal saline [23, 24]. Serial dilutions (10-fold) were made
144 by pipetting out 1ml stock solution into successive 9ml of sterile normal saline bottles. A 1 ml
145 working sample dilution (10^{-1} and 10^{-2}) was spread-plated onto eosin methylene blue (EMB) agar
146 (Oxoid, UK), incubated at 44 °C for 18-24 h. Raised, entire colonies with dark greenish metallic
147 sheen, typical *E. coli* colonies were subjected to Gram-staining [25] and standard biochemical
148 tests (IMViC). All presumptive *E. coli* isolates were sub-cultured in tryptic soy broth (Oxoid, UK)
149 and then stored at -20 °C for further investigations. All media were prepared following the
150 manufacturers' instructions.

151 **Extraction of genomic DNA**

152 Genomic DNA were extracted from a pure culture of each isolate grown overnight on
153 nutrient agar at 37°C, by the conventional boiling method, as described [28]. Briefly, one loopful
154 of bacterial cells was suspended in 1ml of sterile distilled water. The bacterial suspensions were
155 then heated for 5 min at 100°C, cooled to room temperature and centrifuged at 12,000 xg for 5 min
156 to remove the debris. The supernatant was stored at -20°C and used as the template DNA for PCR
157 analysis.

158 **Detection of beta-glucuronidase (*uidA*) gene for confirmation of *E.*** 159 ***coli***

160 The confirmation of *E. coli* was achieved by polymerase chain reaction (PCR) detection of
161 the target beta-glucuronidase (*uidA*). This was done at the School of Natural Sciences, Bangor
162 University, United Kingdom, following the procedures described [29, 30]. The extracted DNA
163 were cleaned using QIAGEN (QIAEX®II) gel extraction kits and kept at -20°C. PCRs were carried
164 out with BIORAD DNA Engine Tetrad®Peltier Thermal Cycler (BIORAD, USA). The PCR
165 reaction mixtures consisted of 25 µl of PCR Master Mix (Thermo Scientific, (EU) Lithuania), 0.5
166 µl each of oligonucleotide primers (Eurofins Genomics, Ebersberg Germany), 10 µl of template
167 DNA and 14 µl of nuclease free water to constitute a total reaction volume of 50 µl. The PCR
168 cycling conditions, with some modifications, were in accordance with the protocols prescribed
169 elsewhere [31]. *E. coli* strain (NCTC 13353) and *Enterobacter aerogenes* (NCTC 10006) were
170 used as positive and negative controls respectively for *E. coli* genus identification. The
171 oligonucleotide sequence of primers used, target genes and expected amplification products are
172 given in Table 1. For gel electrophoresis, 3 µl of DNA ladder (1 Kb Plus DNA Ladder; Invitrogen),
173 6 µl of positive control and 10µl of template DNA were ran on 2.5% (w/v) agarose gels in 1x-TBE
174 buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 8.0) at 100V for 25-30 min. The gels were
175 viewed and photographed with BIORAD Molecular Imager® Gel Doc™ XR Imaging System
176 (BIORAD, USA).

177 **Antibiotic susceptibility testing**

178 Isolates were subjected to antibiotic susceptibility testing using the Kirby-Bauer disc
179 diffusion test [26]. Evaluation of results was based on the standards of the Clinical Laboratory
180 Standards Institute (CLSI) [27]. Briefly, isolates grown on nutrient broth were suspended into
181 sterile normal saline (0.9% (w/v) NaCl) with the aid of a sterile wire loop until the turbidity
182 equivalent of 0.5 McFarland standard was reached. Sterile non-toxic cotton swabs were dipped
183 into the standardized inoculum and used to smear the entire surface of the Muller-Hinton agar
184 (Thermo Fisher Scientific, USA) plates. Antibiotic discs were placed aseptically using sterile
185 forceps. All plates were incubated at 35±2oC for 16 to 18 h. The following antibiotics were
186 employed for the test: Amoxycillin (AMX) 10µg, Ampicillin (AMP) 10µg, Metronidazole (MTZ)
187 5µg, Rifampicin (RIF) 5µg, Vancomycin (VAN) 30µg, Cloxacillin (COX) 5µg, Penicillin G
188 (PNG) 10iu, Streptomycin (STR) 10µg, Erythromycin (ERT) 15µg, Clarithromycin (CLR) 15µg,

189 Cefuroxime (CXM) 30µg, Chloramphenicol (CHL) 30µg, Imipenem (IPM) 10µg, Tetracycline
190 (TET) 30µg, Ciprofloxacin (CIP) 5µg, Trimethoprim (TMP) 5µg, Norfloxacin (NOR) 10µg,
191 Sulphamethoxazole (SMZ) 25µg. The *E. coli* ATCC 25922 strains was used as control for
192 antibiotic susceptibility testing. Zones showing complete inhibition around the discs were
193 measured and classified as resistant (R), intermediate (I) and susceptible (S) according to the
194 diameters of the zones recorded to the nearest millimetres.

195 **Detection of integrons**

196 The isolates were screened for class 1, 2 and 3 integrons by a multiplex PCR procedure as
197 described by Machado *et al.* [32] and Karger *et al.* [33]. The PCR reactions (a total volume of 50µl
198 reaction mixture) each consisted of consisting of 10 µl Buffer of 5x MyTaq Reaction Buffer
199 (Bioline, with dye), 0.75µl of each the primers *intI1*, *intI2* and *intI3* (Eurofins Genomics,
200 Ebersberg, Germany.), 27.25µl nuclease free water (Sigma-Aldrich), 0.25µl MyTaq DNA
201 polymerase (Bioline) and 5µl DNA template. For gel electrophoresis, 3µl of DNA ladder (1 Kb
202 Plus DNA Ladder; Invitrogen), 6 µl of positive control and 10 µl of samples were ran on 1.5%
203 (w/v) agarose gels in 1x-TBE buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 8.0) at 100 V
204 for 25 min. The gels were viewed and photographed with BIORAD Molecular Imager® Gel Doc™
205 XR Imaging System.

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209 **Table 1: Primers for the detection of *E. coli* and integrons**

Genetic marker	Primer name and sequence (5' to 3')		Amplicon size (bp)	Thermocycling conditions	Reference
<i>uidA</i>	UAL-754	AAAACGGCAAGAAAAAGCAG	147	Initial activation at 95°C for 3 min, followed by 40 cycles consisting of denaturing at 94°C for 1 min, annealing at 65°C for 1 min, extension at 70°C for 1 min and final elongation at 72°C for 7 min	[29, 30]
(beta-glucuronidase)	UAR-900	ACGCGTGGTTACAGTCTTGCG			
<i>intI1</i> (Class 1 integron)	Int1-F Int1-R	GGTCAAGGATCTGGATTTTCG ACATGCGTGTAATCATCGTC	436	Initial activation step at 94°C for 5 min, followed by 32 cycles consisting of denaturing at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 min and final elongation at 72°C for 10 min	[32, 33]
<i>intI2</i> (Class 2 integron)	Int2-F Int2-R	CACGGATATGCGACAAAAAGGT GTAGCAAACGAGTGACGAAATG	788		
<i>intI3</i> (Class 3 integron)	Int3-F Int3-R	AGTGGGTGGCGAATGAGTG TGTTCTTGTATCGGCAGGTG	600		

211 **Results and Discussion**

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

217 In the present study, the total number of samples collected were 288 including WWTP
218 effluents (60), greenhouse (60), farm (84) and market (84) vegetables. A total of 178 *E. coli* isolates
219 were confirmed by PCR amplification of the β -glucuronidase *uidA* gene (Fig 1), with 41 isolates
220 from the WWTP effluents, 46 greenhouse from the effluent irrigated vegetables, 55 from
221 vegetables collected from gardens that produce vegetables sold in local markets and 36 from
222 vegetables bought from selected markets in Nsukka and Enugu (Table 2).

223

224 **Fig 1. PCR products for *E. coli* confirmation by *uidA* gene amplification.**

225 This is the Fig 1 legend: M: Molecular weight marker (1 KB), W: water, NC: Negative control
226 (*Enterobacter aerogenes*), PC: Positive control (*E. coli*; NCTC 13353), Lanes 1-15: Positive
227 isolates

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229 **Table 2: Isolation of *E. coli* in effluent wastewater and vegetable samples.**

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Sample type	No. of samples	No. of <i>E. coli</i> isolates
WWTP effluent	60	41
Greenhouse vegetables	60	46
Farm vegetables	84	55
Market Vegetables	84	36
Total	288	178

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232 Table 3 shows the antibiotics susceptibility profiles of the 178 *E. coli* isolates tested with
233 18 different antibiotics. Generally, higher resistance percentages were observed in *E. coli* from
234 market vegetables compared with others. This could be attributable to further contamination of
235 vegetables by clinical *E. coli* strains arising directly from handling by sellers. All the *E. coli*
236 isolates, showed susceptibility to imipenem and only 5.6% (10/178) of all the isolates were
237 resistant to cefuroxime (a cephalosporin). Chloramphenicol, ciprofloxacin and norfloxacin were
238 very effective. The most significant resistance phenotypes were detected among
239 sulphamethoxazole (58.4%), amoxicillin (52.8%), tetracycline (47.2%), trimethoprim (44.9%) and
240 streptomycin (37.1%), as these antibiotics are commonly used in the studied communities (Table
241 2).

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260 **Table 3: Antibiotics susceptibility profile of *E. coli* isolates**

S/N	Antimicrobial Agent (disc concentration)	Class (Subclasses)	Code	Number of isolates resistant (%)				
				Effluent (n = 41)	Greenhouse (n = 46)	Farm (n = 55)	Market (n = 36)	TOTAL (n = 178)
1	Cloxacillin (5µg)	β-Lactam (Penicillins)	COX	41 (100)	46 (100)	55 (100)	36 (100)	178 (100)
2	Metronidazole (50µg)	Nitroimidazoles	MTZ	41 (100)	46 (100)	55 (100)	36 (100)	178 (100)
3	Vancomycin (30µg)	Glycopeptides	VAN	41 (100)	46 (100)	53 (96.4)	36 (100)	176 (98.9)
4	Rifampicin (5µg)	Rifamycins	RIF	41 (100)	45 (97.8)	52 (94.6)	34 (94.4)	172 (96.6)
5	Penicillin (10U)	β-Lactam (Penicillins)	PNG	39 (95.1)	45 (97.8)	54 (98.2)	34 (94.4)	172 (96.6)
6	Erythromycin (15µg)	Macrolides	ERT	40 (97.6)	46 (100)	50 (90.9)	32 (88.9)	168 (94.4)
7	Clarithromycin (15µg)	Macrolides	CLR	40 (97.6)	37 (80.4)	47 (85.5)	36 (100)	160 (89.9)
8	Ampicillin (5µg)	β-Lactam (Cephalosporins)	AMP	34 (82.9)	38 (82.6)	50 (90.9)	36 (100)	158 (88.7)
9	Sulphamethoxazole (25µg)	Sulphonamides	SMZ	23 (56.1)	27 (58.7)	26 (47.3)	28 (77.8)	104 (58.4)
10	Amoxicillin (10µg)	β-Lactam (Penicillins)	AMX	23 (56.1)	24 (52.2)	26 (47.3)	21 (58.3)	94 (52.8)
11	Tetracycline (30µg)	Tetracyclines	TET	25 (61.0)	20 (43.5)	16 (29.1)	23 (63.9)	84 (47.2)
12	Trimethoprim (5µg)	Dihydrofolate Reductase (DHFR) inhibitors	TMP	27 (65.9)	20 (43.5)	12 (21.8)	21 (58.3)	80 (44.9)
13	Streptomycin (10µg)	Aminoglycosides	STR	15 (36.6)	21 (45.7)	16 (29.1)	14 (38.9)	66 (37.1)
14	Chloramphenicol (30µg)	Phenicols	CHL	4 (9.8)	6 (13.0)	6 (10.9)	9 (25.0)	25 (14.0)
15	Ciprofloxacin (5µg)	Fluoroquinolones	CIP	5 (12.2)	7 (15.2)	6 (10.9)	7 (19.4)	25 (14.0)
16	Norfloxacin (10µg)	Fluoroquinolones	NOR	2 (4.9)	7 (15.2)	9 (16.4)	5 (13.9)	23 (12.9)
17	Cefuroxime (30µg)	β-Lactam (Cephalosporins)	CXM	2 (4.9)	2 (4.4)	2 (3.6)	4 (11.1)	10 (5.6)
18	Imipenem (10µg)	β-Lactam (Carbapenems)	IMP	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

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264 Multidrug resistance (MDR) has been frequently reported in Nigeria among *E. coli* isolates
265 obtained from human specimens [12, 37, 42, 43], animal sources [36] and environmental samples
266 [38, 42, 44, 45]. In the present study, all the isolates were multidrug resistant, ranging from 5-drug
267 to 16-drug resistance patterns. Although some studies have reported a high removal efficiency for
268 total ARGs in wastewater [46], our data suggest that sewage treatment process at UNN is not
269 effective in reducing ARGs as all the *E. coli* isolated from the effluent were MDR. The spread of
270 AMR often limits the availability of therapeutic options to only a very few efficacious antibiotics
271 [47]. The last-resort drugs, the carbapenems such as imipenem (used in this study) and meropenem,
272 are themselves not only increasingly challenged by emerging resistance, as evident from the data
273 presented here, but are not affordable in the developing regions.

274 Multidrug resistance (MDR) was detected in all *E. coli* isolates, and although this study
275 did not determine the full virulence potentials of all the isolates subjected to antimicrobial
276 susceptibility testing (AST), irrigational use of WWTP effluent represents a pathway for human
277 exposure to antibiotic-resistant commensal and pathogenic bacteria. Vegetable farming at the site
278 should therefore be discontinued as it presents significant threat to the health of consumers of such
279 vegetables.

280 It is known that AMR and MDR in *E. coli* are acquired by the transfer of mobile genetic
281 elements, such as plasmids, transposons and integrons [15, 18, 20]. In the present study, of the 178
282 *E. coli* isolates, class 1 integrons were detected (Fig 2) in 175 (98.3%), and class 2 in 5 (2.8%). All
283 the class 2 integrons were found in isolates that were positive for class 1. Such co-carriage has
284 been previously published on *E. coli* from meat turkeys in Italy, [34].

285

286 **Fig 2. Multiplex PCR products for detection of class 1 and 2 integrons**

287 This is the Fig 2 legend: M: Molecular weight marker (1 KB Plus ladder), W: water, Lanes 1-15:
288 *E. coli* isolates

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290 The integron carriage rate for the 137 vegetable isolates was 97.8%, whereas the rate for
291 41 effluent isolates was 100%. Considering that all the isolates were MDR, the detected high

292 prevalence of class 1 integron is not surprising and compares with a previous study that reported
293 that MDR phenotypes were observed in 96.8% of the integron-positive isolates [35]. These rates
294 portend serious public health risks as it is known that class 1 integron could carry diverse antibiotic
295 resistance genes (ARGs) and conduct horizontal gene transfer among microorganisms [20].

296 The data presented here shows that class 2 integrons were less frequently detected 5 (2.8%).
297 Similar data have been published for Enterobacteriaceae in Nigeria [36, 38] and elsewhere [34, 39,
298 40]. Ramírez et al [39], reported that unlike the widespread distribution of class 1 integron within
299 Gram-negative bacilli, only *Acinetobacter baumannii* and *Enterobacter cloacae* harboured class 2
300 integrons at a high frequency. However, in an earlier study in China [41], Class 2 integrons were
301 present in 25 (80.6%) of the *Shigella sonnei* isolates and 29 (87.9%) of the *S. flexneri* isolates
302 whereas class 1 integrons were found in only 6 (9.4%) of *Shigella* spp. isolates.

303 **Conclusions**

304 The present study revealed high detection of *E. coli* in the studied effluent and vegetable
305 samples and represent potential public health hazards intensified by observed multidrug resistance
306 in all the isolates and the high occurrence of class 1 integrons. It is concluded that UNN-WWTP
307 is a significant reservoir for diarrheagenic *E. coli*. Vegetable farming at the site should therefore
308 be discouraged as it presents significant threat to the health of consumers of such vegetables.

309 **Acknowledgments**

310 The authors are also grateful to Mr. Uchenna Oyeagu of the Water and Public Health
311 Laboratory, University of Nigeria, Nsukka, Nigeria, for laboratory assistance.

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313 **References**

- 314 1. Stacy-Phipps S, Mecca JJ, Weiss JB (1995) Multiplex PCR Assay and Simple Preparation
315 Method for Stool Specimens Detect Enterotoxigenic *Escherichia coli* DNA during Course
316 of Infection. *J Clin Microbiol* 33: 1054–1059.
- 317 2. Okeke IN (2009) Diarrheagenic *Escherichia coli* in sub-Saharan Africa: status,
318 uncertainties and necessities. *J Infect Dev Ctries* 3: 817–842.
- 319 3. Brown JW, Badahdah A, Iticovici M, Vickers TJ, Alvarado DM, Helmerhorst EJ,
320 Oppenheim FG, Mills JC, Ciorba MA, Fleckenstein JM. Bullitt E (2018) A Role for

- 321 Salivary Peptides in the Innate Defense Against Enterotoxigenic *Escherichia coli* J Infect
322 Dis. 217:1435–1441
- 323 4. Nzeako B, Okafor N (2002) Bacterial enteropathogens and factors associated with seasonal
324 episodes of gastroenteritis in Nsukka, Nigeria. Br J Biomed Sci 59: 76–79.
- 325 5. WHO/UNICEF [World Health Organization and the United Nations Children’s Fund]
326 (2017). Progress on drinking water, sanitation and hygiene: 2017 update and SDG
327 baselines. Geneva; 2017. Licence: CC BY-NC-SA 3.0 IGO.
- 328 6. FAO/WHO [Food and Agriculture Organization of the United Nations and World Health
329 Organization]. (2008). Microbiological hazards in fresh leafy vegetables and herbs:
330 Meeting Report. Microbiological Risk Assessment Series No. 14. Rome. 151pp.
- 331 7. Ogunsanya TL, Rotimi VO, Adenuga A (1994) Study of the aetiological agents of
332 childhood diarrhoea in Lagos Nigeria. J Med Microbiol 40: 10–14.
- 333 8. Okeke IN, Fayinka ST, Lamikanra A (2000) Antibiotic Resistance in *Escherichia coli* from
334 Nigerian Students, 1986–1998. Emerg Infect Dis 6: 393–396.
- 335 9. Olorunshola ID, Smith SI, Coker AO (2000) Prevalence of EHEC O157: H7 in patients
336 with diarrhea in Lagos, Nigeria APMIS 108: 761–763.
- 337 10. Okeke IN, Steinruck H, Kanack KJ, Elliott SJ, Sundstrom L, Kaper JB, Lamikanra A
338 (2002) Antibiotic-resistant cell-detaching *Escherichia coli* strains from Nigerian children.
339 J Clin Microbiol 40: 301–305.
- 340 11. Chigor VN, Umoh VJ, Smith SI, Igbinsosa EO, Okoh AI (2010) Multidrug resistance and
341 plasmid patterns of *Escherichia coli* O157 and other *E. coli* isolated from diarrhoeal stools
342 and surface waters from some selected sources in Zaria, Nigeria. Int J Environ Res Public
343 Health 7: 3831–3841.
- 344 12. Onanuga A, Igbeneghu O, Lamikanra A (2014) A study of the prevalence of diarrhoeagenic
345 *Escherichia coli* in children from Gwagwalada, Federal Capital Territory, Nigeria. Pan Afr
346 Med J 17: 146.
- 347 13. Nweze EI (2010) Aetiology of Diarrhoea and Virulence Properties of Diarrhoeagenic
348 *Escherichia coli* among Patients and Healthy Subjects in Southeast Nigeria. J Health Popul
349 Nutr 28:245–252.
- 350 14. Okeke IN, Edelman R (2001) Dissemination of antibiotic-resistant bacteria across
351 geographic borders. CID 33: 363–369.
- 352 15. Gaze WH, Zhang L, Abdouslam NA, Hawkey PM, Calvo-Bado L, Royle J, Brown H,
353 Davis S, Kay P, Boxall ABA, Wellington EMH (2011) Impacts of anthropogenic activity
354 on the ecology of class 1 integrons and integron-associated genes in the environment. The
355 ISME J 5: 1253–1261.
- 356 16. Wellington EMH, Boxall ABA, Cross P, Feil EJ, Gaze WH, Hawkey PM, Johnson-
357 Rollings AS, Jones DL, Lee NM, Otten W, Thomas CM, Williams AP (2013) The role of
358 the natural environment in the emergence of antibiotic resistance in Gram-negative
359 bacteria. Lancet Infect Dis 13: 155–165.
- 360 17. Amos GCA, Zhang L, Hawkey PM, Gaze WH, Wellington EM (2014) Functional
361 metagenomic analysis reveals rivers are a reservoir for diverse antibiotic resistance genes.
362 Vet Microbiol 171: 441–447.
- 363 18. Gillings MR (2014) Integrons: Past, Present, and Future. Microbiol Mol Biol Rev 78: 257–
364 277.

- 365 19. Strugeon E, Tilloy V, Ploy MC, Da Re S (2016) The stringent response promotes antibiotic
366 resistance dissemination by regulating integron integrase expression in biofilms. *mBio*
367 7(4):e00868–16.
- 368 20. Ma L, Li AD, Yin XL, Zhang T (2017) The Prevalence of Integrons as the Carrier of
369 Antibiotic Resistance Genes in Natural and Man-Made Environments. *Environ Sci*
370 *Technol* 51: 5721–5728.
- 371 21. Asadu A, Enwelu I, Ifejika P, Igbokwe E (2016) Urban crop production in southeast
372 Nigeria: Potentials and constraints. *Afr J Agric Res* 11: 4646–4653.
- 373 22. American Public Health Association (APHA) (2005). Standard Methods for the
374 Examination of Water and Wastewater. 21st Edn. American Public Health Association,
375 American Water Works Association, Water Environment Federation, Washington DC,
376 USA.
- 377 23. Falomir MP, Gozalbo D, Rico H (2010) Coliform bacteria in fresh vegetables: from
378 cultivated lands to consumers. In: A. Mandez-Vilas (Ed.). Current Research, Technology
379 and Education Topics in Applied Microbiology and Microbial Biotechnology.
380 FORMATEX; Badajoz Spain, pp. 1175–1181.
- 381 24. Saeed AY, Mazin H, Saadi A, Hussein SO (2013) Detection of *Escherichia coli* O157 in
382 vegetables. *IOSR J Agric Vet Sci* 6: 16–18.
- 383 25. Claus D (1992) A standardized Gram staining procedure. *World J Microbiol Biotechnol*
384 8:451–452.
- 385 26. Bauer A, Kirby W, Sherris J, Turck M (1966) Antibiotic susceptibility testing by a
386 standardized single disk method. *Am J Clin Pathol* 45:493–496.
- 387 27. CLSI (2012) Performance Standards for Antimicrobial Disk Susceptibility Tests;
388 Approved Standard—Eleventh Edition. Vol 32, No.1.
- 389 28. Vankerckhoven V, Van Autgaerden T, Vael C, Lammens C, Chapelle S, Rossi R, Jabes D,
390 Goossens H (2004) Development of a Multiplex PCR for the Detection of *asa1*, *gelE*, *cylA*,
391 *esp*, and *hyl* Genes in Enterococci and Survey for Virulence Determinants among European
392 Hospital Isolates of *Enterococcus faecium*. *J Clin Microbiol* 42: 4473–4479.
- 393 29. Bej AK, Dicesare JL, Haff L, Atlas RM (1991) Detection of *Escherichia coli* and *Shigella*
394 spp. in Water by Using the Polymerase Chain Reaction and Gene Probes for *uid*. *Appl*
395 *Environ Microbiol* 57: 1013–1017.
- 396 30. Horakova K, Mlejnkova H, Mlejnek P (2008) Specific detection of *Escherichia coli*
397 isolated from water samples using polymerase chain reaction targeting four genes:
398 cytochrome bd complex, lactose permease, b-D-glucuronidase, and b-D-galactosidase. *J*
399 *Appl Microbiol* 105: 970–976.
- 400 31. Lopez-Saucedo C, Cerna JF, Villegas-Sepulveda N, Thompson R, Velazquez FR, Torres
401 J, Tarr PI, Estrada-García T (2003) Single multiplex polymerase chain reaction to detect
402 diverse loci associated with diarrhoeagenic *Escherichia coli*. *Emerg Infect Dis* 9: 127–131.
- 403 32. Machado E, Canton R, Baquero F, Galan JC, Rollan A, Peixe L, Coque TM (2005) Integron
404 Content of Extended-Spectrum--Lactamase-Producing *Escherichia coli* Strains over 12
405 Years in a Single Hospital in Madrid, Spain. *Antimicrob Agents Chemother* 49: 1823–
406 1829.
- 407 33. Kargar M, Mohammadalipour Z, Doosti A, Lorzadeh S, Japoni-Nejad A (2014) High
408 Prevalence of Class 1 to 3 Integrons Among Multidrug-Resistant Diarrheagenic
409 *Escherichia coli* in Southwest of Iran. *Osong Public Health Res Perspect* 5: 193–198.

- 410 34. Piccirillo A, Giovanardi D, Dotto G, Grilli G, Montesissa, C, Boldrin C, Salata C,
411 Giacomelli M (2014) Antimicrobial resistance and class 1 and 2 integrons in *Escherichia*
412 *coli* from meat turkeys in Northern Italy. *Avian Pathol* 43: 396–405.
- 413 35. Vasilakopoulou A, Psychogiou M, Tzouvelekis L, Tassios PT, Kosmidis C, Petrikkos G,
414 Roma ES, Charvalos E, Passiotou M, Avlami A, Daikos GL (2009) Prevalence and
415 characterization of class 1 integrons in *Escherichia coli* of poultry and human origin.
416 *Foodborne Pathog Dis* 6: 1211–1218.
- 417 36. Chah KF, Agbo IC, Eze DC, Somalo S, Estepa V, Torres C (2010) Antimicrobial
418 resistance, integrons and plasmid replicon typing in multiresistant clinical *Escherichia coli*
419 strains from Enugu State, Nigeria. *J Basic Microbiol* 50: S18–S24.
- 420 37. Odetoyin BW, Labar AS, Lamikanra A, Aboderin AO, Okeke IN (2017) Class 1 and 2
421 integrons in faecal *Escherichia coli* strains isolated from mother-child pairs in Nigeria.
422 *PLoS ONE* 12: e0183383.
- 423 38. Adelowo OO, Helbig T, Knecht C, Reincke F, Mäusezahl I, Müller JA (2018) High
424 abundances of class 1 integrase and sulfonamide resistance genes, and characterisation of
425 class 1 integron gene cassettes in four urban wetlands in Nigeria. *PLoS ONE* 13: e0208269.
- 426 39. Ramírez MS, Pineiro S, Argentinian Integron Study Group, Centron D (2010) Novel
427 Insights about Class 2 Integrons from Experimental and Genomic Epidemiology.
428 *Antimicrob Agents Chemother* 54: 699–706.
- 429 40. Malek MM, Amer FA, Allam AA, El-Sokkary RH, Gheith T, Arafa MA (2015) Occurrence
430 of class I and II integrons in Enterobacteriaceae collected from Zagazig University
431 Hospitals, Egypt. *Front Microbiol* 6: 601.
- 432 41. Pan JC, Ye R, Meng DM, Zhang W, Wang HO, Liu KZ (2006) Molecular characteristics
433 of class 1 and class 2 integrons and their relationships to antibiotic resistance in clinical
434 isolates of *Shigella sonnei* and *Shigella flexneri*. *J Antimicrob Chemother* 58: 288–296.
- 435 42. Chigor VN, Umoh VJ, Smith SI, Igbiosa EO, Okoh AI (2010) Multidrug resistance and
436 plasmid patterns of *Escherichia coli* O157 and other *E. coli* isolated from diarrhoeal stools
437 and surface waters from some selected sources in Zaria, Nigeria. *Int J Environ Res Public*
438 *Health* 7: 3831–3841.
- 439 43. Okeke IN, Lamikanra A, Czczulin J, Dubovsky F, Kaper JB, Nataro JP (2000b)
440 Heterogeneous Virulence of Enteroaggregative *Escherichia coli* Strains Isolated from
441 Children in Southwest Nigeria. *J Infect Dis* 181: 252–260.
- 442 44. Smith SI, Bello OS, Goodluck HA, Omonigbehin EA, Agbogu VN, Odeigah P (2009)
443 Prevalence of EHEC O157:H7 from human and environmental samples from Lagos and
444 Zaria. *Pakistan J Med Sci* 25: 398–403.
- 445 45. Titilawo Y, Sibanda T, Obi L, Okoh A (2015a) Multiple antibiotic resistance indexing of
446 *Escherichia coli* to identify high-risk sources of faecal contamination of water. *Environ Sci*
447 *Pollut Res* 22: 10969–10989.
- 448 46. Yang Y, Li B, Zou S, Fang HHP, Zhang T (2014) Fate of antibiotic resistance genes in
449 sewage treatment plant revealed by metagenomic approach. *Water Res* 62: 97–106.
- 450 47. Juhas M, Widlake E, Teo J, Huseby DL, Tyrrell JM, Polikanov YS, Ercan O, Petersson A,
451 Cao S, Aboklaish AF, Rominski A, Crich D, Bottger EC, Walsh TR, Hughes D, Hobbie
452 SN (2019) In vitro activity of apramycin against multidrug-, carbapenem- and
453 aminoglycoside-resistant Enterobacteriaceae and *Acinetobacter baumannii*. *J Antimicrob*
454 *Chemother* 74: 944–952.
- 455

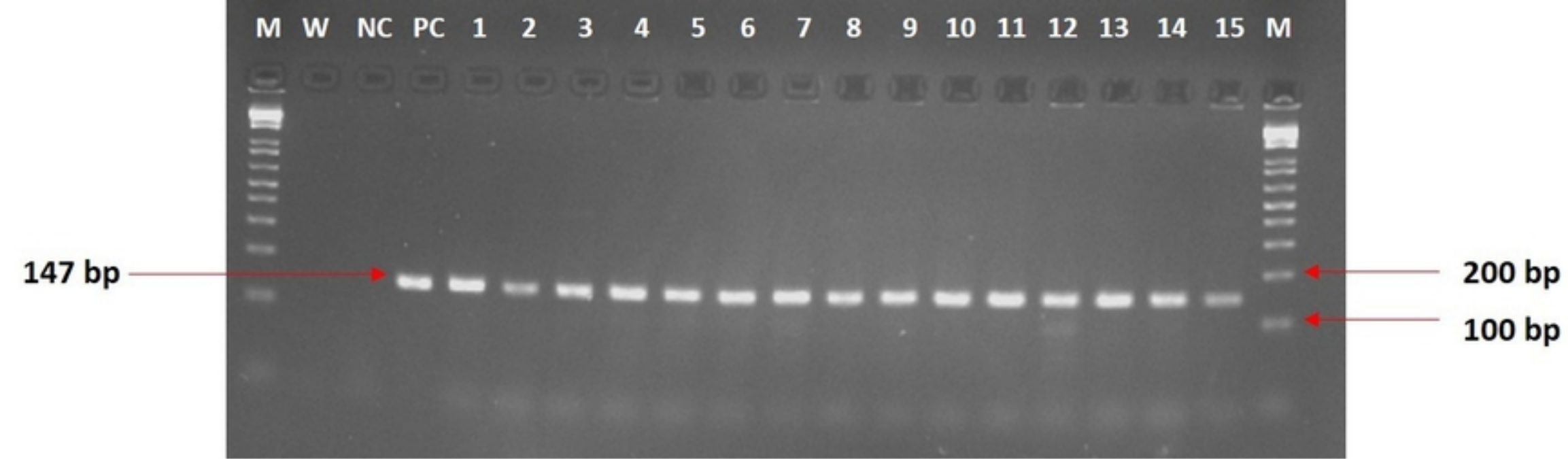


Figure 1

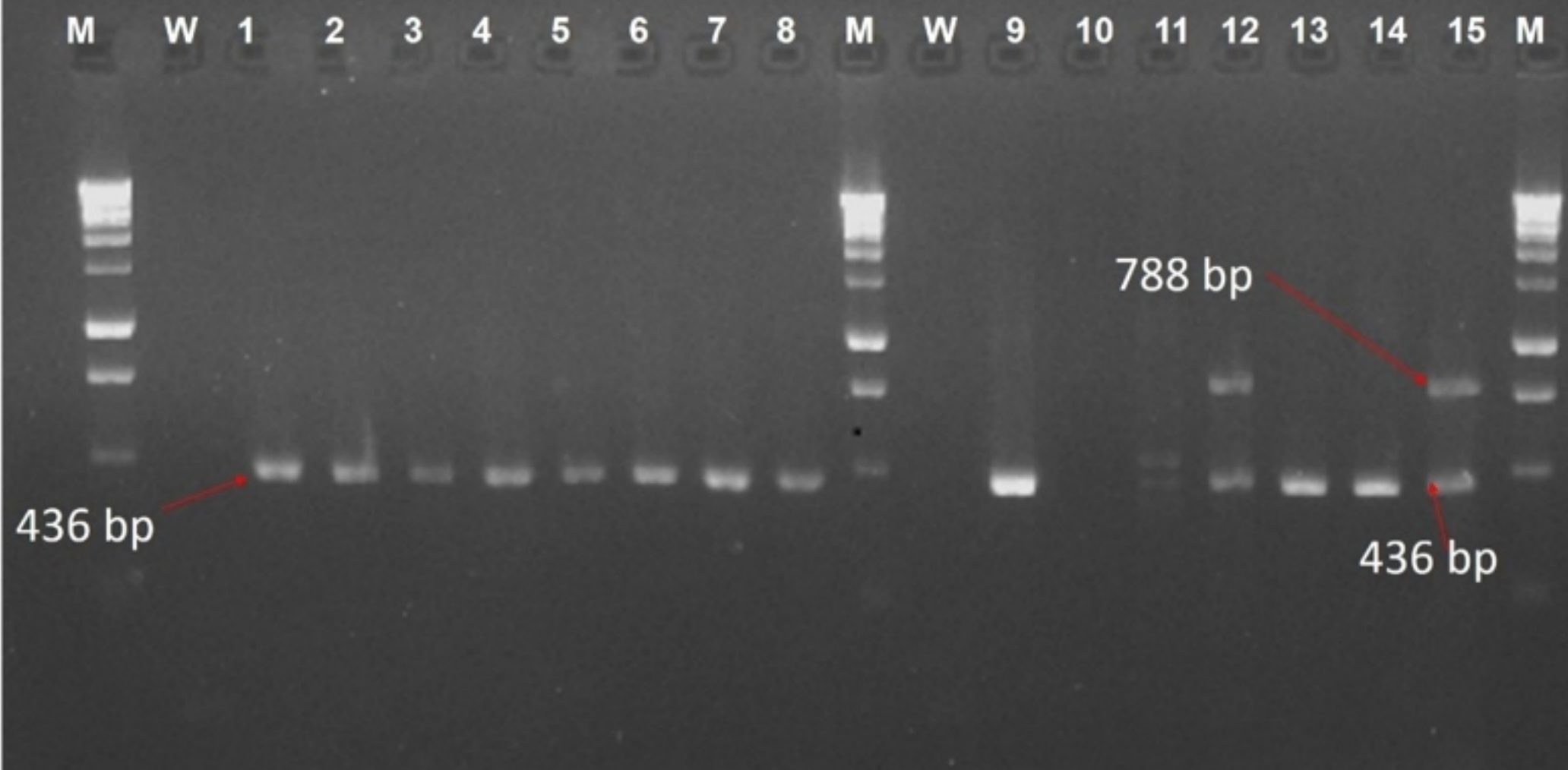


Figure 2