

Microbiological Assessment of Irrigation Water That Serves as Watershed for Wastewater in Osogbo and Its Impact on Fresh Vegetables

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Abstract: This study was designed to investigate the microbiological safety of irrigation water along with waste effluents and their impact on vegetables. Irrigation water samples situated amongst hospital, abattoir and petrol station were aseptically collected with fresh vegetables simultaneously. Physicochemical, microbiological and molecular studies were carried out using standard methods. The result of the physicochemical analysis shows that the irrigation water samples temperature ranged from 23.4-24.8°C while the pH ranged from 7.24-7.51. The bacterial count of isolates showed that the water samples were highly contaminated with microbial count ranging from 9×10^2 to 151×10^4 cfu/ml which has exceeded the WHO microbial count recommendation for potable water (10×10^3 cfu/ml). Organisms isolated from both water and vegetable samples were all pathogenic. Antimicrobial susceptibility test results showed that all the isolates were susceptible to cefepime and oxacillin while 60% of the isolates were multidrug resistant. *Aeromonas taiwanensis* was recovered from both the water and vegetable samples. This however calls for huge attention as *Aeromonas* species are potential pathogenic organisms that can be isolated from both clinical and polluted environmental samples such as wounds, petrol and abattoir litters. Therefore, it can be concluded that wastewater effluents should not be channeled directly into surface water bodies without prior safety treatments.

Keywords: *Aeromonas taiwanensis*, Effluents, Irrigation water, Multidrug-resistant organisms, Vegetables

I. Introduction

Vegetables can be defined as plant components that can be eaten as food by humans and animals [1]. It is most widely used to collectively refer to edible plant parts such as flowers, fruits, stems, leaves, roots and seeds [2]. The World Health Organization (WHO) recommends that vegetables that are to be consumed raw should only be disinfected to achieve a coliform amount of not more than 100 coliforms per 100 ml of the sample [3].

According to the reports of [4], China is the world largest producer of vegetables whereby

consumers can buy veggies that are grown in faraway countries through global trade of agricultural products. In addition, the production size of these vegetables ranges from subsistence farmers who supply vegetables for only their families to agribusinesses that farm a single vegetable plant on large acres of land.

The global production of fresh vegetables has been reported by [5] to have risen by 30% over the past years. This increase has been steady, thus increasing the export pace of fruits and vegetables.

Vegetables are very high in nutrients such as vitamins A, B, C, K and mineral elements such as calcium, potassium, magnesium as well as dietary fibres which aids in the prevention of chronic diseases such as diabetics, cancer, liver and kidney malfunctions, heart diseases, obesity, etc. [6] and they can either be consumed raw or cooked [7].

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However, most vegetables are widely exposed to microbial contamination through contact with soil, water, dust and the hand during harvesting and post-harvest processing. They can therefore serve as a vehicle for the transmission of a wide range of microorganisms that might be of plant or animal origin [8].

Irrigation can be simply defined as the artificial application of water to plant crops as plants such as vegetables require nutrients to be absorbed in liquid form. In addition, irrigation is an important component of anthropogenic demand for water, in which most of these irrigation water sources are subjected to contamination from point and non-point sources [9-10].

According to the report of [11], the soil on which these vegetables are grown can also be a source of water used for irrigation, application of insecticides and fungicides, manure composting and defecation which make the irrigation water serve as a potential threat when applied on the crops.

The likelihood of quite a large number of unreported cases of gastrointestinal infections from the consumption of plant vegetables as a result of contamination with irrigation water-related microbial contamination is very high [12]. Rivers, streams, creeks, lakes, etc have unreliable water quality due to the fact that upstream activities can drastically affect the level of contamination of the flowing water [13].

II. Materials and Methods

One litre (1L) of five irrigation water samples were aseptically collected upstream and downstream of vegetable fields into sterile containers by lowering the containers gently into the following water bodies from five different locations within Osogbo, Osun State, namely: Testing ground (N7.7871930

and E4.5762900), Elentere river, Ilobu (N7.7866500 and E 4.5762790), Ayepe river (N7.8381260 and E4.4801630), Lakata river (N7.8220310 and E4.4874110) and Erin river (N7.8356800 and E4.4762710) respectively and stored on ice packs and immediately transported to the Osun State University Microbiology laboratory for further analyses while vegetable samples: African spinach leaves (*Amaranthus cruentus*), jute leaves (*Corchorus olitorius*), pumpkin leaves (*Cucurbita pepo*), African eggplant leaves (*Solanum aethiopicum*) and African Lagos spinach leaves (*Celosia argentea*) were simultaneously collected inside a zip lock bag on icepacks from the five locations stated above.

A. Processing of Water and Vegetable Samples

One ml of each water sample was added to 9 ml of Ringer's solution. This was then aseptically serially diluted to 10^{10} . In addition, the vegetable sands were washed off and immersed in water with vigorous shaking for 15 minutes. 1ml of each homogenate was serially diluted into sterile tubes containing 9 ml of Ringer solution up to 10^{10} dilution factor. Appropriate dilutions were picked for both samples for further analyses. 0.1 ml aliquots were aseptically inoculated onto the surface of solidified plates of Thiosulfate-citrate-bile-salt (TCBS) and MacConkey agar using the spread plate method. The plates were incubated at 37°C for 24 hr.

B. Physicochemical Analysis of Irrigation Water Samples

The method described by [14] was used to determine the turbidity, pH, temperature, conductivity, salinity and dissolved oxygen. The pH of the samples was determined using an Orion pH meter (Model 310, Orion Research Inc, Beverly, MA) equipped with a glass electrode. The turbidity was measured directly using probes and expressed as

Nephelometric Turbidity Unit (NTU). Salinity, conductivity, dissolved oxygen (O₂) and temperatures were also measured using standard methods.

C. Microbial characteristics of isolated bacteria

The microbial profile of the water and vegetable samples were determined after 24 hr of incubation. Bacteria colonial count was done using nutrient agar, TCBS and MacConkey agar plates. *Vibrio* species (cfu/g) were identified using TCBS and MacConkey agar (Merck, Darmstadt, Germany). Four different colonies were randomly picked using visual assessment from the highest dilution factor of TCBS and MacConkey agar plates, purified and subjected to various biochemical tests such as Gram staining, citrate, catalase, coagulase, indole, oxidase, methyl-red, Vogues-Proskauer and sugar fermentation tests which include: glucose, lactose, mannitol, etc. according to the method employed by [15].

D. Antibiotics Susceptibility Testing

The antibiotic susceptibility testing was performed using the method of [16] following the CLSI standards. Colonies from 18-24 hr old culture were inoculated into 5ml of sterilized Ringer's solution to give a turbidity equivalent to 0.5 McFarland standards. Petri plates containing the Muller-Hinton sensitivity agar were seeded with 0.1 ml of each bacterial isolate and allowed to stand for 30mins to allow pre-diffusion of the inoculated species. Eight (8) commercially available antibiotics (kanamycin, aztreonam, cefepime, tigecycline, oxacillin, colistin-sulphate, doripenem and azithromycin) test disks were then aseptically placed on the surface of the inoculated MHA agar plates via an 8-place disc dispenser (Oxoid) and incubated for 18 to 24 hours. After the incubation period, diameters of inhibition zone were measured in millimeters.

The zones of inhibition were measured in mm and interpreted using EUCAST break point 2021[33].

E. Molecular Analysis of Multi-Drug Resistant (MDR) Pathogenic Isolates DNA Extraction

Bacteria chromosomal DNA was extracted from all resistant presumptive isolates using Zymo Research Genomic DNATM- Tissue MiniPrep Kit following the manufacturer's instructions and quantified using a UV-Vis Thermo ScientificTM Nanodrop Lite Spectrophotometer (model S-22, Boeco, Germany). The DNA samples were stored at -20° C for further analysis.

F. 16SrRNA Gene Detection and Gel Electrophoresis

The PCR reaction is a 25 µL volume comprising of 1.5µL of MgCl₂ (15Mm), 0.5µL of 1mM dNTP, 2.5µL of Taq buffer (5×), 0.5µL of each universal primer (2.5pM each); 16S rRNA Forward; AGAGTTGATCCTGGCTCAG, 16S rRNA Reverse, GGTTACCTTGTTACGACTT, as described by [31], 0.2µL of Taq DNA polymerase and 5µL of the DNA sample. PCR water was used to bring the final reaction volume to 25µL. The cycling conditions were; initial denaturation period at 94 °C for 5 minutes followed by 30 cycles of denaturation for 1 minute at 94 °C, annealing for 1 minute at 55 °C, elongation for 1 minute at 72 °C, and final elongation for 7 minutes at 72 °C and allowed to hold at 4 °C. The amplified DNA fragments on 2% agarose gel containing Safeview were run alongside a 100 bp ladder and visualized using a gel documentation unit (VilberTM).

G. Sequencing of Genomic Material

The PCR products were cleaned using EXOSAP protocol while sequencing was

done using applied biosystems BigDye Terminator v3.1 cycle sequencing kit (catalogue no 43374455) according to manufacturer’s instruction.

III. Results and Discussion

Table 1 presents the physicochemical properties of five vegetable irrigation water samples. It was observed that the turbidity values ranged from -0.17 to -0.19 indicating that the water is highly turbid. Neutral pH values were recorded in all the sampling sites (7.24-7.51) which are most suitable for the growth and proliferation of bacteria. The ambient temperature values obtained (23.4-24.8) created a conducive environment for bacteria growth.

In addition, the low salinity and moderate dissolved oxygen values: 0.07-0.30 and 6.7-12.0 respectively is one of the crucial parameters required to determine the survival of aquatic lives. The conductivity values of 0.17-0.60 also show a high level of risk if the water is used for consumption as high amount of ions are present in it. The reports documented in this study are in conformity to the claims of [17-19]. However, [20] recommends that dissolved oxygen level in potable water should not exceed 10mg/L.

The mean value of the bacteria colonial count of irrigation water and vegetable samples are represented in tables 2 and 3. The values ranged from 9×10^2 to 151×10^4 cfu/ml while that of vegetable samples ranged from 17×10^4 to 82×10^2 [21] recorded a close value of 2×10^5 cfu/ml in an irrigation water sample. This high microbial count could be due to the different anthropogenic activities within the streams and rivers. In addition, poor sanitation practices leading to high pollution in the water bodies is another source of contamination [22]. Site W1 seems to be the most highly contaminated when compared

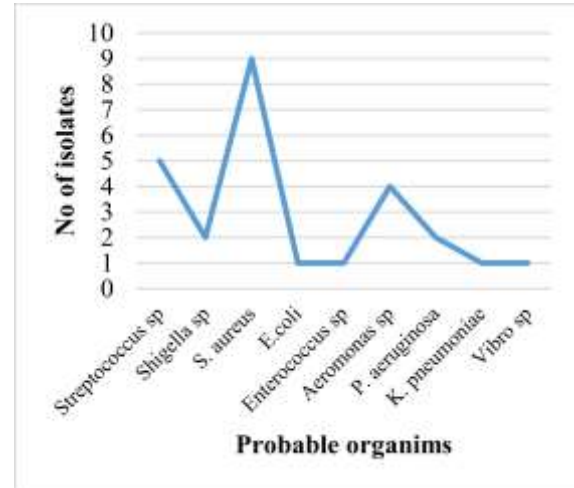


Figure 1: Number of bacteria species isolated from irrigation water samples

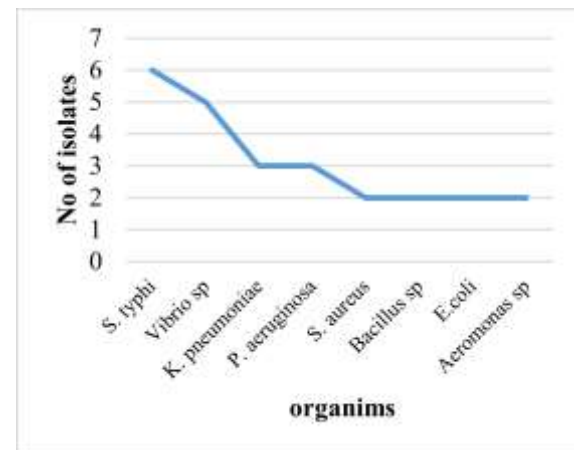


Figure 2: Number of bacteria species isolated from vegetable samples

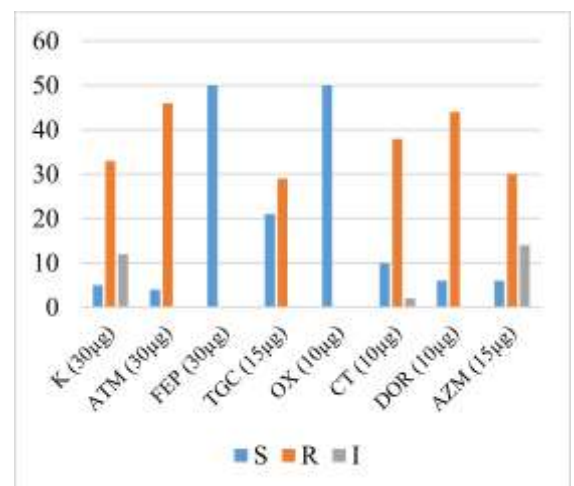


Figure 3: Antibiotics susceptibility test result of bacteria isolated from irrigation water and vegetable samples

Table 1: Physicochemical Properties of Irrigation Water Samples

Sample code	Turbidity (NTU)	pH	Temperature (°C)	Conductivity (ms)	Salinity (ppt)	Dissolved oxygen (mg/L)
A	-0.18	7.25	23.4	0.60	0.30	12.0
B	-0.17	7.37	24.8	0.34	0.17	9.0
C	-0.19	7.24	24.5	0.17	0.07	6.7
D	-0.17	7.50	24.4	0.49	0.25	7.6
E	-0.18	7.51	24.6	0.45	0.23	7.7

Table 2: Bacteria colonial count of irrigation water samples

Sample Code	NA (cfu/ml)			MAC (cfu/ml)			TCBS (cfu/ml)		
	10 ⁴	10 ⁴	Av	10 ³	10 ³	Av	10 ²	10 ²	Av
W1	272	30	151×10 ⁴	40	32	36×10 ²	150	100	125×10 ²
W2	184	28	106×10 ⁴	44	36	40×10 ²	132	114	123×10 ²
W3	200	36	118×10 ⁴	72	62	67×10 ²	98	32	65×10 ²
W4	132	14	59×10 ⁴	64	50	57×10 ²	60	40	50×10 ²
W5	164	38	101×10 ⁴	12	6	9×10 ²	140	64	102×10 ²

Key: NA= Nutrient agar, MAC= MacConkey agar, TCBS= Thiosulfate -citrate -bile salt sucrose agar

Table 3: Bacteria colonial count of vegetable samples

Sample Code	NA (cfu/ml)			MAC (cfu/ml)			TCBS (cfu/ml)		
	10 ⁴	10 ⁴	Av	10 ³	10 ³	Av	10 ²	10 ²	Av
1	40	38	39×10 ⁴	48	44	46×10 ³	48	50	49×10 ²
V2	16	18	17×10 ⁴	16	20	18×10 ³	18	22	20×10 ²
V3	72	70	71×10 ⁴	40	42	41×10 ³	64	60	62×10 ²
V4	60	62	61×10 ⁴	80	78	79×10 ³	84	80	82×10 ²
V5	28	80	29×10 ⁴	48	46	47×10 ³	36	40	38×10 ²

Key: NA= Nutrient agar, MAC= MacConkey agar, TCBS= Thiosulfate -citrate -bile salt sucrose agar

with other sites. This makes all the effluents coming out of these locations flow directly into the river. This, however, supports the earlier observation of [16-17,23].

The total number of each bacterium isolated from irrigation water and vegetable samples is shown in figures 1 and 2. The following bacterial organisms were isolated from irrigation water samples: *Streptococcus* sp, *Shigella* sp, *Staphylococcus aureus*, *Escherichia.coli*, *Enterococcus* sp, *Aeromonas* sp, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Vibro* sp while similar organism were also isolated from the vegetable samples except for *Salmonella typhi* and *Bacillus* sp. [24-27] have all documented the occurrence of the above listed pathogenic microorganisms from

irrigation water and vegetable food products except for *Aeromonas* sp which makes this report different from others. The survival of *Aeromonas* sp on the vegetables suggests that the waste water effluents are heavily contaminated with potentially infectious pathogenic organisms.

It was even noticed from one of the sample vegetable collection site that the seller had a wound sore in her hand which can most likely be passed onto the vegetables. All the microorganisms isolated from this research work are potentially opportunistic pathogens. This, however, makes their isolation from both food and water at high microbial counts of public health significance which in turn suggest that these vegetables are unsafe to be

consumed raw or undercooked [26]. However, the report from this study makes the stream and river water unsuitable for human consumption and unfit for vegetable irrigation without prior treatment as also documented by [17].

Key: K= Kanamycin (30 μ g), ATM = Aztreonam (30 μ g), FEP = Cefepime (30 μ g), TGC = Tigecycline (15 μ g), OX = Oxacillin (10 μ g), CT = ColistinSulphate(10 μ g), DOR = Doripenem (10 μ g), AZM = Azithromycin (15 μ g), I= Intermediate, R= Resistant, S= Sensitive

The antibiotics susceptibility test results for all the 50 bacterial isolates from irrigation water and vegetable samples shows that 100% of the isolates were susceptible to cefepime and oxacillin and about 60% of the isolates were multidrug-resistant. Cefepime belongs to the class of drugs known as cephalosporin antibiotics which has a very wide spectrum of activity against Gram positive and Gram negative bacteria. In addition, oxacillin, a beta-lactam antibiotic belongs to the second generation penicillin antibiotic that is also used to treat a wide range of bacterial infections. The high cost and restricted use of these two antibiotics might be responsible for its low drug exposure hence, reducing the occurrence of resistant bacteria pathogens. The high resistance of isolates to aztreonam, colistin sulphate and doripenem indicates that the organisms have been able to exhibit resistance by producing beta-lactamase. Another reason is drug abuse whereby people use antibiotics without proper prescription. Thus, leading to an antibiotic pressurized environment [28-29] also documented similar multidrug resistance of bacteria isolates from different samples.

The occurrence of *Aeromonas* sp from both irrigation water and vegetable samples indicates a cause for concern as they are potential pathogenic organisms that should not be found in foods. In view of this, since the previous studies documented the occurrence of all other bacterial pathogens isolated from this work except for *Aeromonas* sp, the isolate was further analyzed using molecular techniques.

Both extracted genomic DNA of the two multidrug-resistant *Aeromonas* sp were used as templates in the polymerase chain reaction (PCR) to amplify the 16S rRNA genes. The amplicons of PCR were subjected to agarose gel electrophoresis (1% agarose powder at 70 volts for 60 min) to determine the band size

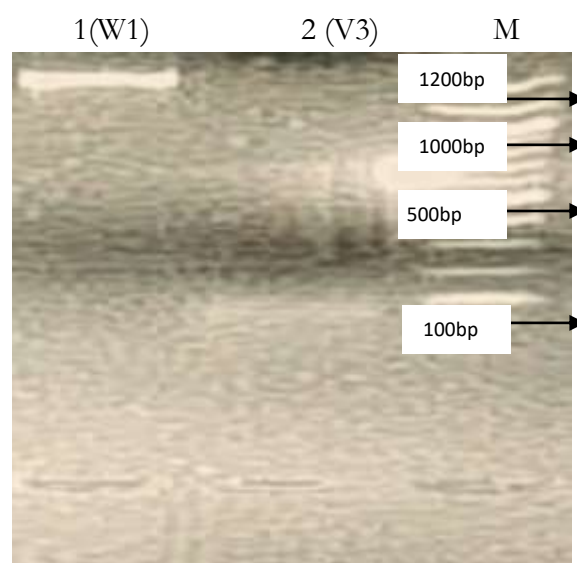


Figure 4: Agarose gel image of 16SrRNA gene fragments amplified from the multidrug resistant bacteria isolates (*Aeromonas* sp) showing bands at 1200bp.

Key: M= molecular marker/ladder, W1= irrigation water sample 1, V3= vegetable sample 3.

Table 4: DNA Quantification

S/N	Sample code	Nucleic Acid(ng/uL)	A260/A280	A260/A230	A260	A280	Nucleic Acid Factor	Baseline Correction (nm)	Baseline Absorbance
1	W1	132.60	2.035	1.047	2.652	1.303	50	340	0.293
2	V3	402.19	1.865	1.07	8.044	4.312	50	340	1.116

Table 5: Sequencing Result of *Aeromonas* sp

Isolate code	Scientific Name	Max Score	Total Score	E Value	Per. Ident	Acc. Len	Accession
TA03	<i>Aeromonas taiwanensis</i>	2001	2001	0.00	98.34%	1804	NZ_CDDD01000060.1

According to the report of [30] the primers are expected to bind at 1000-1500bp which is in line with the previous claims of [31-32]. This further confirms that the result from this work is in agreement with previous studies. The isolates were sequenced and blasted in NCBI (National Center for Biotechnology Information) by comparing the DNA sequence with other known sequences from GeneBank database using the Nucleotide Basic Local Alignment Search Tool (BLAST). The result showed that the *Aeromonas* sp was identical to *Aeromonas tawianensis* with 98.34% similarity which is a highly pathogenic organism of public health importance that originated from Taiwan [32].

IV. Conclusion

In conclusion, the result obtained from this study shows that the waste effluents from the nearby hospital, abattoir and petrol station might possibly be having a negative impact on the water bodies. It, therefore, has a public health significance on the irrigation water used for vegetable farming. Upstream and downstream point sources of effluents to the water bodies are creating a continuous influx of pathogenic microorganisms all year round. Therefore, it is expedient to recommend that irrigation water must be well treated before use and waste effluents should not be channelled directly into surface water bodies without prior safety treatments.

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