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Genetic relatedness among isolates of *Plesiomonas shigelloides* from fish, vegetable and surface water in Southwest Nigeria

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Abstract

Background: Water-borne and food-borne outbreaks of diseases triggered by *Plesiomonas shigelloides* have been reported in many parts of the world. Water has always been the major source or route of contaminant implicated in many disease outbreaks.

*Methodology***:** We conducted this study to establish the prevalence and genetic variability of *P. shigelloides* from fish, vegetable, and water sources from Southwest Nigeria. Fish, vegetables, pond water, irrigation water, and surface water samples were collected from Southwest Nigeria and cultured on Inositol brilliant green bile salt agar after enrichment procedure. Simplex PCR was used to screen *P. shigelloides* isolates using 23SrRNA primers with the expected band size of 628bp. The confirmed isolates were further characterised by RAPD typing using OPA primers and RAPD patterns analysed with NTSYS software.

Results: A total of 36 isolates were confirmed positive for *P. shigelloides*. OPA 4 was the best primers for *P. shigelloides* detection producing the best polymorphic bands. Genetic variability was observed among the *P. shigelloides* strains. The results obtained indicated that irrigation, pond, and surface water may be the transmission route of *P. shigelloides* as they are closely related to strains obtained from vegetables and fishes.

Conclusion: Since fish and vegetable farming rely majorly on surface water such as rivers for irrigation and pond water supply, *P. shigelloides* from surface water may pose serious infection risk compared to other environmental sources in this region.

Keywords: *P. shigelloides, RAPD, surface water, fish, vegetable*

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Résumé

Contexte : Des épidémies d'origine hydrique et alimentaire déclenchées par Plesiomonas shigelloides ont été signalées dans de nombreuses régions du monde. L'eau a toujours été la principale source ou voie de contamination impliquée dans de nombreuses épidémies.

Méthodologie : Nous avons mené cette étude pour établir la prévalence et la variabilité génétique de P. shigelloides à partir de poissons, de légumes et de sources d'eau du sud-ouest du Nigeria. Des échantillons de poisson, de légumes, d'eau d'étang, d'eau d'irrigation et d'eau de surface ont été collectés dans le sud-ouest du Nigéria et cultivés sur gélose aux sels biliaires vert brillant d'inositol après la procédure d'enrichissement. La PCR simplex a été utilisée pour cribler les isolats de P. shigelloides en utilisant des amorces 23SrRNA avec la taille de bande attendue de 628 pb. Les isolats confirmés ont été davantage caractérisés par typage RAPD à l'aide d'amorces OPA et de modèles RAPD analysés avec le logiciel NTSYS.

Résultats **:** Au total, 36 isolats ont été confirmés positifs pour P. shigelloides. OPA 4 était les meilleures amorces pour la détection de P. shigelloides produisant les meilleures bandes polymorphes. Une variabilité génétique a été observée parmi les souches de P. shigelloides. Les résultats obtenus ont indiqué que l'irrigation, les étangs et les eaux de surface peuvent être la voie de transmission de P. shigelloides car ils sont étroitement liés aux souches obtenues à partir de légumes et de poissons.

Conclusion : Étant donné que la pisciculture et la culture maraîchère dépendent principalement des eaux de surface telles que les rivières pour l'irrigation et l'approvisionnement en eau des étangs, P. shigelloides provenant des eaux de surface peut présenter un risque d'infection grave par rapport à d'autres sources environnementales dans cette région.

Mots clés : *P. shigelloides, RAPD, eau de surface, poisson, légume*

Introduction

Plesiomonas shigelloides, in the family Enterobacteriaceae, is the only specie belonging to the genus *Plesiomonas. P. shigelloides* is a Gram-negative, oxidase-positive, fermentative, non-spore forming and facultative anaerobic rod, which is associated predominantly with surface water. Its recovery from river estuaries, seawater, in addition to aquatic and land animals have been reported [1-6] The isolation of this organism from humans, animals, and the environment, makes it a one health concern. Anthropogenic influence on the environment has continued to be a major concern especially pollution from sources such as agricultural waste, treatment plant effluents, industrial and hospital effluents [7-11]. *P. shigelloides* has been established as an emerging agent of gastroenteritis due to their implication in gastroenteritis diseases. Reports from other studies have also found this bacterium as the major causative organism of food-borne and water-borne diarrhea and gastroenteritis outbreaks in different countries across the globe with associated symptoms such as headaches, diarrhea fever, and vomiting [12-15].

There are some case reports in the literature confirming illness caused by *P. shigelloides* following the consumption of oysters [16], fish [17] and, crabs [18]. Other modes of transmission include contaminated drinking water [19], vegetables [20] as well as contact with aquatic and terrestrial animals [21-24]. *P shigelloides* have also recently been reported as an underappreciated cause of travellers' diarrhea among visitors from developed to developing countries [25]. It is also well known as an important agent of many opportunistic infections [26]. The frequent occurrence of *P. shigelloides* has been linked to its intrinsic ability to grow under a wide temperature range [27, 2, 28].

Many molecular typing methods such as Random Amplified Polymorphic DNA (RAPD), Pulsed Field Gel Electrophoresis (PFGE), and Multi Locus Sequence Typing (MLST) has been used to investigate the strain diversity and the relationship among *P. shigelloides* to differentiate between circulating strains of this bacterium, among different hosts [15,26,29, 30]. However, RAPD has been considered to be a fast, reliable, inexpensive typing method for *P. shigelloides* compared to ribotyping, multilocus electrophoresis (MEE), and restriction endonuclease analysis (REA) [30,26]. RAPD is a form of PCR in which segments of the genomic DNA of a pathogen are randomly amplified using a single 10-nucleotide primer without prior knowledge of the entire genomic DNA sequence.

In Nigeria, in the last three decades, *P. shigelloides* has been reported to be associated with recorded cases of diarrhea in the Northern and Southern parts of the country [31-33] and likewise, in the Eastern part of the country [34]. In our recent study, we reported the public health risks posed by surface water as a reservoir and source of *P. shigelloides* diarrhea in Southwest Nigeria [35]. However, there is a dearth of information on the molecular epidemiology of *P. shigelloides* across the different ecological hosts of the pathogen. Such information is needed for establishing genetic relatedness of strains and understanding mode of transmission of *P. shigelloides* in the country. The objective of this study, therefore, is to examine for the first time the genetic diversity of *P. shigelloides* in different environmental hosts (surface waters, irrigation water and vegetable, fish, and the pond water) in Southwest Nigeria.

Material and method

Sample source description

A total of 74 presumptively confirmed strains of *P. shigelloides* were randomly selected from a pool of samples in the Microbiology Laboratory of Obafemi Awolowo University Ile Ife, previously collected from different sites within Osun and Oyo state, Southwest Nigeria for this study. The demographics of the isolates which consist of strains from fish, pond water, vegetables, irrigation water, and surface water are as shown in Table 1.

Isolation of P. shigelloides from the different samples

Briefly, the sampling procedures are as follows: Fish and vegetable samples were collected in sterile plastic bags with aeration and immediately transported to the laboratory. Surface water, pond water, and irrigation water were collected also using 1 L sterile bottles. Processing of samples was done within 6 h of collection, following the procedure recommended by the American Public Health Association [36].

The fish intestines and gills were aseptically removed and homogenised in sterile physiological saline after which it was enriched in alkaline peptone water for 24 h at 37 °C. The vegetable samples were rinsed once with sterile distilled water to remove impurities. Then the vegetables were shaken in 250 mL of the 0.85% sterile physiological saline, and the resulting wash water was enriched in alkaline peptone water for 24 h at 37 °C. Likewise pond water, irrigation water, and surface water samples were enriched in peptone water (pH 8.6) and all the

solutions were incubated at 37 °C for 24 h after which all the different enriched samples were subjected to 10-fold serial dilution in 0.85% sterile physiological saline. Thereafter, 0.1 ml each of the dilution was placed at the centre of well labelled dried plates of Inositol Brilliant Green Bile Agar (Conda Pronadisa, Spain) and spread evenly all over the plate using a sterile glass spreader. The plates were then incubated at 37 °C in an inverted position for 24 h. Pinkish colonies, suggestive of *P. shigelloides* were selected on each plate. The isolates were further confirmed using Gram staining and oxidase test and only Gram-negative, oxidase-positive isolates were selected as presumptive *P. shigelloides* and sub-cultured on purity plate before being stored on 25% glycerol for further analysis.

Genomic DNA extraction

The extraction of DNA was done using the boiling method [29, 37]. Presumptive *P. shigelloides* colonies were selected and subcultured on LB agar plates for 18–24 h at 37 °C. Pure and distinct colonies selected were placed in 200 µl sterile distilled water, vortexed, boiled for 15 mins at 100 °C, and centrifuged for 10 mins at 15,000 rpm. The resultant supernatants were stored in Eppendorf tube at "80 °C until ready for use.

Molecular confirmation of Plesiomonas shigelloides isolates

The presumptive *P. shigelloides* identified were confirmed by 23SrRNA gene amplification by polymerase chain reaction (PCR) technique, using primer sets PS-F-52 - GCAGGTTGAAGGTTGGGTAA-32 and PS-R-52 -TTGAACAGGAACCCTTGGTC-32 developed by [38] which amplifies a 628 bp sequence of the 23SrRNA gene of *P. shigelloides.* The thermal condition for the PCR run was initial denaturation for 5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min; annealing, 68 °C for 1 min; extension, 72 °C for 2 min and final extension at 72 °C for 10 min. Electrophoresis was completed by filling 5 µl aliquots of the amplicons into 2% agarose gel in which 5 µl ethidium bromide stain has been added. DNA ladder of 100-bp was used for molecular size calibration on the gel and the electrophoresis running condition was 100 v for 45 min. PCR products were visualized with gel documentation equipment

Molecular typing by RAPD

The RAPD-PCR was done using 3 different random primers (OPA-03, 5'AGTCAGCCAC 3'; OPA-4, 5'AATCGGGCTG 3'; OPA-05, 5'AGGGGTCTTG 3'). The PCR cocktail is a 20 uL reaction, comprising 4 uL of 5× PCR master mix (Promega, Germany), containing 2.5 mM $MgCL_2$, 50 ng of genomic DNA of each isolate and 30 pmol of the primer. Amplification condition was denaturation at 95 °C for 6 min; 35 cycles of denaturation at 94 °C for 90 s, annealing at 32 °C for 60 s, and extension at 72 °C for 90 s with a final extension at 72 °C for 7 min. The RAPD-PCR assay was repeated thrice to check for repeatability, that is, the possibility of obtaining reproducible bands [39]. The RAPD bands produced were analysed by electrophoresis on 2% ethidium bromide pre-stained agarose gel, using 100 bp DNA ladders for sizing the bands and *E. coli* ATCC25922 as a reference strain The RAPD patterns obtained were analysed by NTSYSpc software (version 2.1, USA). The presence or absence of each RAPD

Table 1: Isolate Source Description and coordinates of sampling points

Isolates source	Location	No of randomly selected isolates	No of positive isolates	Coordinates
River	Erinle river, Ede, Osun State	6	3	N7° 462 23.73 E4° 272 58.33
River	Asejire river, Osun State	6	2	N7° 212 46.93 E4° 072 51.53
River	Dandaru river, Ibadan, Oyo State	6	2	N7° 262 25.03 E3° 492 57.53
River	Ona river, Ibadan, Oyo State	6	2	N7° 262 17.13 E3° 522 42.23
Pond water /fish	Ede road area, Ile Ife	20/6	12/2	N7° 502 30.93 E4° 512 33.43
Vegetable/ irrigation water	Opa area, Ile Ife	14/10	7/6	N7° 522 78.03 E4° 572 72.23

polymorphic band was scored as 1 and 0, respectively.

Cluster analysis and creation of dendrogram were performed by unweighted pair group method with arithmetic averages (UPGMA), using a genetic similarity cut off of 80% as previously described by Idil and Bilkay [40].

Genomic Diversity of Plesiomonas shigelloides Isolates

All the isolates yielded 36 composite band profiles which were arbitrarily numbered, and each band converted into binary numbers was used to produce a single dendrogram. The dendrogram generated from the RAPD profile produced 2 major clusters A and B which further delineated into seven sub-clusters (Fig 3). Cluster 6 contains the highest number of strains (9) which consist of 6 strains from pond water, one strain each from vegetable, surface water, and fish. Cluster 3 and 4 were the clusters with the lowest number of strains formed with only two strains each. The nine isolates from surface water yielded 9 RAPD types and displayed significant genetic variability. Among the 9 surface water isolates, 4 showed the closest relationship with isolates as well from surface water (PS09-PS04; PS06-PS01), 2 isolates each also

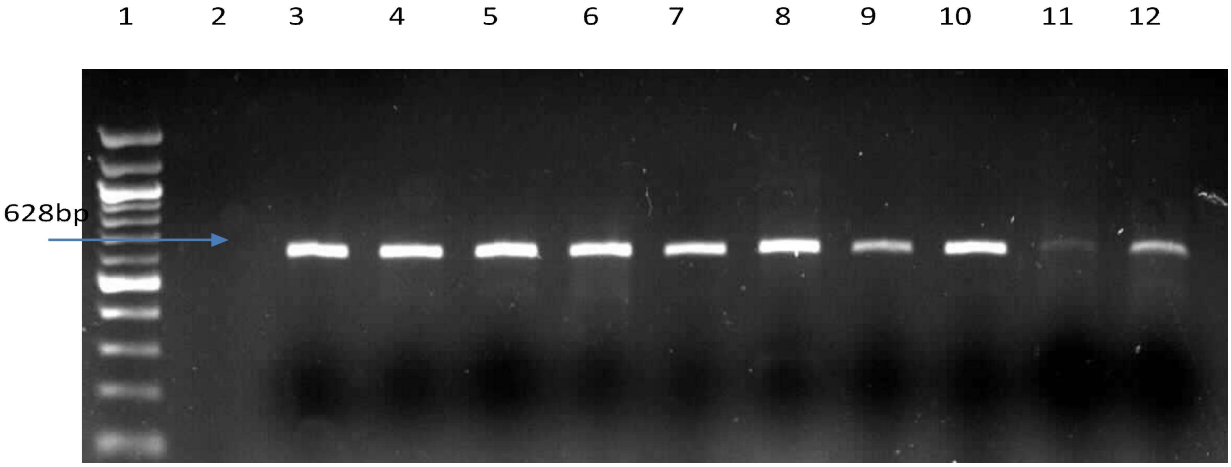

Fig.1: *Plesiomonas shigelloides* gene conûrmation. Lane 1: Molecular weight marker (100 bp); lane 2, negative control (ATCC 14029); lane 3: positive control; lane 4 to 12 *Plesiomonas shigelloides* positive isolates

Results

Confirmation of P. shigelloides isolates by PCR

All 36 *P. shigelloides* strains (fish, pond water, irrigation water, vegetable, and surface water) produced the expected band size of 628bp specific for *P. shigelloides* (Fig. 1). The reference strain also yielded the expected band size while the negative control which was made up of the reaction mixture without DNA showed no band. High prevalence of confirmed *P. shigelloides* was recorded in the isolates from irrigation water (60%) and pond water (60 %) (Fig 2).

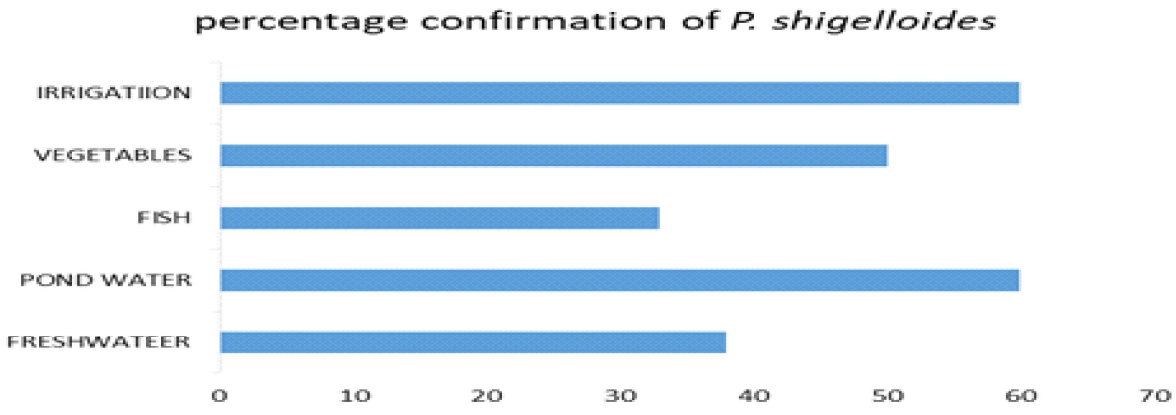

Fig.: 2: Percentage of PCR confirmed *P. shigelloides* isolates from different sources

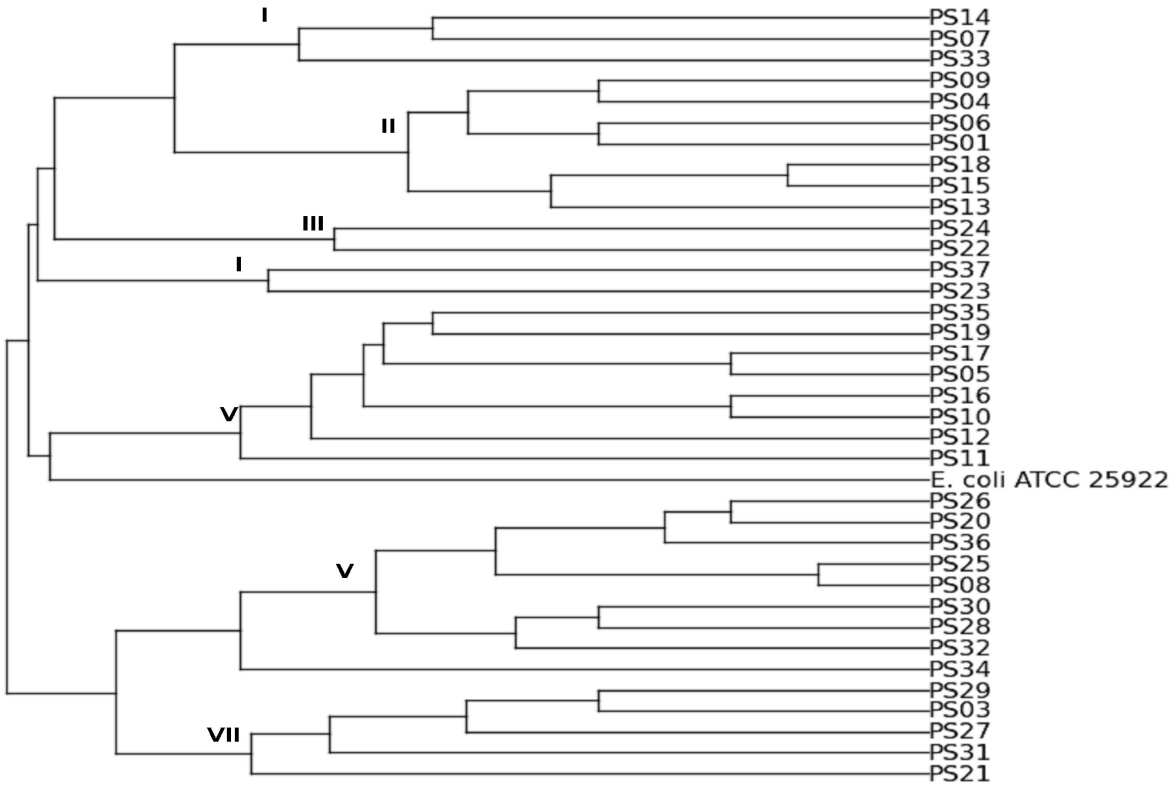

Fig. 3: Genetic variability between *P. shigelloides* isolates from surface water, fish, pond water, vegetables, and irrigation water derived from combined RAPD profiles

Table 2: Profile of *Plesiomonas shigelloides* strains

Surface W* Isolates (n=9) Strain no	Irrigation W* Isolates (n=6) Strain no	Vegetables Isolates (n=7) Strain no	Pond W* isolates (n=12) Strain no	Fish Isolates (n=2) Strain no
PS	PS	PS	PS	PS
01	011	012	024	036
03	013	014	025	037
04	017	015	026	
05	021	016	027	
06	022	018	028	
07	023	019	029	
08		020	030	
09			031	
010			032	
			033	
			034	
			035	

PS = P. shigelloides: W = water*

exhibited close association with isolates from pond water (PS25-PS08; P29-PS03) and vegetable (PS14-PS07; PS16-PS10) while only one isolate showed close association with isolates from irrigation water (PS17-PS05). This result revealed that 7 of the surface water isolates were closely related to isolates from other water sources (irrigation and pond water). Interestingly, many of the surface water isolates originating did not form a homogenous cluster. Conversely, they were interspersed with one another in the cluster analysis indicating no association between the RAPD patterns and the geographic origin of the isolates.

The 12 isolates from pond water yielded 12 RAPD types (Table 2) and also showed substantial genetic variability. PS33 showed the closest relationship with the group composed of PS14 (vegetable) and PS07 (water). Isolate PS24 showed close association with isolate PS22 from irrigation water. Two Pond water isolates PS35 and PS26 were closely related to vegetable isolates PS19 and PS20 respectively as shown by their clustering pattern. Likewise, 2 pond water isolates (PS25 and PS29) were also closely related to surface water isolates PS08 and PS03 respectively. Pond water isolate PS30 had the closest relationship with another pond water strain PS28. Also, pond water strain PS32 showed close affiliation with the group composed of strains PS30 and PS28, both from Pond water while PS34 had the closest association with isolates PS30, PS28 and PS32. Likewise, isolates PS27 and PS31 showed the closest relationship with the group comprising isolates PS29 (from pond water) and PS03 (from water). The clustering pattern of the 12 isolates from Pond water showed that 9 of these isolates exhibited notable close relationship with isolates from other water sources.

None of the six irrigation water isolates were clonally identical rather they displayed a clonal relationship with other isolates from fish, vegetable, surface water, and pond water. The irrigation water Isolate PS13 showed close relationship with the group composed of PS15 and PS18 both from vegetables. Similarly, other strains obtained from irrigation water clustered as follows: PS22 clustered with isolate PS24 (pond water), PS23 with PS37 (fish), PS17 with PS05 (surface water) while PS11 exhibited close association with the group consisting of strains from vegetable (PS12, PS16) and surface water (PS10). Likewise, isolate PS21 exhibited close association with the group comprising pond water (PS31, PS27, PS29) and surface water (PS03) respectively.

The two fish isolates considered in this RAPD typing did not exhibit the same RAPD pattern. Isolate PS37 had closest relationship with PS23 (irrigation water) while isolate PS36 had closest relationship with the clustered group composed of PS20 (vegetable) and PS26 (pond water).

Discussion

Plesiomonas shigelloides is well known as a causative agent of fish disease and opportunistic human infection. However, this organism has advanced to a status of "emerging" pathogen among humans, implicated in traveller diarrhea cases and outbreaks of gastroenteritis. DNA fingerprinting analysis of environmental isolates of *P. shigelloides* strains observed in this study using gel imaging and clustering analysis showed significant genetic heterogeneity among the strains. The 36 *P. shigelloides* isolates (2 from fish, 12 from Pond water, 6 from irrigation water, 7 from vegetable, and 9 from surface water) (Table 3) were subjected to RAPD typing with OPA primers in which OPA 4 primer was employed as it was more discriminatory for this specie and yielded 36 profiles. The dendrogram generated from the RAPD profile produced 2 major clusters A and B while cluster B

Table 3: Profile of the seveb RAPD cluster

Cluster 1	Cluster2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 7
P14-vegetable	P09- surface W*	P22-irrigation W*	P37-fish	P35-pond W*	P26-pond W*	P29-pond W*
P07-surfaceW*	P04- surface W*	P24-pond W*	P23-irrigation W*	P19-vegetable	P20-vegetable	P03- surface W*
P33-pond W*	P06- surface W*			P17-irrigation W*	P36-fish	P27-pond W*
	P01- surface W*			P05- surface W*	P25-pond W*	P31-pond W*
	P18-vegetable			P16-vegatable	P08- surface W*	P21-irrigation W*
	P15-vegetable			P10- surface W*	P30-pond W*	
	P13-irrigation W*			P12-vegetable	P28-pond W*	
				P11-irrigation W*	P32-pond W*	
					P34-pond W*	

W = water*

The 7 vegetable isolates also produce 7 RAPD types and displayed some level of genetic variability. Two of the vegetable Isolate (PS14 and PS16) showed close relationships and clustered with isolates from surface water (PS07 and PS10). Another 2 isolates PS19 and PS20 also clustered with pond water isolates PS35 and PS26 respectively. Only two vegetable isolates, PS18 and PS15 showed closest relationship with each other and clustered together while isolate PS12 clustered with isolate PS11 from irrigation water. Interestingly, five of the vegetable isolates had the closest relationship and clustered with isolates from different water sources while only 2 of the vegetable isolates exhibited association with each other.

further delineated into several sub-clusters (Fig. 3). However, none of the *P. shigelloides* strains isolated from the different environmental hosts produced genetically homogenous clusters. The observation in this study revealed a substantial genetic variability among the strains tested which is in agreement with the results obtained by Gu et al. [26] and Gonzalez-Rey *et al*. [3]. According to Gu *et al*. [26], RAPD typing was also used to study genetic diversity among 26 *Plesiomonas shigelloides* isolated from fish, human clinical sources, and freshwater and found high genetic variability among most of the isolates while none of the isolates had the same composite RAPD profile. Also, Shigematsu *et al.* [27], in their study using PFGE typing tool to study the genetic diversity among *P. shigelloides* isolates from Japanese travellers, reported that the molecular ûngerprints of a selection of 39 isolates and 3 reference strains of *P. shigelloides* were highly variable.

Some of the surface water, pond water and irrigation water isolates were found to show relatedness with vegetable and fish isolates in this study which suggests that *P. shigelloides* from water may pose a serious infection risk compared to other environmental sources of this isolate in Southwest Nigeria. In the earlier report by Adesiyan *et al.,* [35], high incidence of *P. shigelloides* was recorded in surface water resource of Southwest Nigeria which suggests that rivers may be an important reservoir of this organism. Since fish and vegetable farming rely majorly on surface water sources such as rivers for vegetable irrigation and pond water supply, the close relatedness observed among some of the isolates may reveal the common transmission route of *P. shigelloides* in this environment.

Considering the genetic relatedness observed among the isolates which are from different sample sources and different locations within Southwest Nigeria, there is a possibility that the isolates might have evolved from the same ancestral population. Moreover, strains having the same sequence type do not always come from the same host [29]. In this study, it could be observed from the RAPD result that water was the major driver of *P. shigelloides* spread and transmission as isolates from fish and vegetables were found clustered with either pond water, surface water, or irrigation water isolates. This observation portends that this organism is transmitted across the different sampling sources through different water pathways in this environment. Overall, the grouping pattern showed *that P. shigelloides* have a high degree of variants in its genomic organization, possibly due to transposon activity, recombination shuffling, or horizontal gene transfer [41]. Horizontal gene transfer phenomena have been used to explain why strains from different environmental host cluster together in epidemiological studies. It has also been noted that adaptation factors of different strains could be transferred repeatedly amongst genomic families [29].

The result obtained in this study further lay credence to the submission that *P. shigelloides* have some level of nucleotide diversity [3, 29] which results from frequent homologous recombination in housekeeping genes which affects its alleles and nucleotides 7 and 77 times more frequently than mutations. Furthermore, among the Enterobacteriaceae family recombination is found to occur more frequently in *P. shigelloides* compared to other species in this family [15,42,43].

Strains of bacteria forming clonal complexes are believed to be connected with specific virulence properties which may have epidemic tendencies. Therefore, there is a need to use other typing tools such as MLST to further evaluate the route and mode of transmission of *P. shigelloides* in different environment of Southwest Nigeria. Hence further studies will characterise the gene to establish their mode of transmission, virulence, and determine their ability to tolerate the acquisition of other genes.

This study reports for the first time the genetic diversity of *P. shigelloides* in different environmental sources in Southwest Nigeria and the RAPD analysis of the selected strains revealed a diverse population indicating the prevalent distribution of several strains of this *P. shigelloides* in Southwest Nigeria.

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References

1. Tsukamoto T, Kinoshita Y, Shimada T and Sakazaki R. Two epidemics of diarrhoeal disease possibly caused by *Plesiomonas shigelloides*. J Hyg. 1978;80(02):275-280.
2. Janda J., Abbott S. and McIver C. *Plesiomonas shigelloides* Revisited. *Clin Microbiol Rev* 2016. 29, 349-374. https://doi.org/10.1128/CMR.00103-15

3. Gonzalez-Rey C, Siitonen A, Pavlova A, *et al*. Molecular evidence of *Plesiomonas shigelloides* as a possible zoonotic agent. Folia Microbiol. 2011; 56(2):178-184.
4. Escobar J, Bhavnani D, Trueba G, *et al*. *Plesiomonas shigelloides* Infection, Ecuador, 2004–2008. Emerg Infect Dis. 2012;18(2):322-324.
5. Salerno A., Čižnár I., Krovacek K., *et al*. Phenotypic characterization and putative virulence factors of human, animal and environmental isolates of *Plesiomonas shigelloides*. *Folia Microbiol. (Praha)*. 2010.55, 641-647. https://doi.org/10.1007/s12223-010-0104-8
6. Schuetz A. N. Emerging agents of gastroenteritis: Aeromonas, Plesiomonas, and the diarrheagenic pathotypes of *Escherichia coli*. In Seminars in diagnostic pathology. WB Saunders. 2019.
7. Jian, H., Zhou R., Yang Y., *et al*. Characterizing the antibiotic resistance genes in a river catchment: Influence of anthropogenic activities. J. Environ. Sci. (China) 2018; 69: 125–132. https://doi.org/10.1016/j.jes.2017.08.009
8. Harnisz M. and Korzeniewska E. The prevalence of multidrug-resistant *Aeromonas* spp. in the municipal wastewater system and their dissemination in the environment. Sci. Total Environ 2018; 377–383. https://doi.org/10.1016/j.scitotenv.2018.01.100
9. Proia L., Anzil A., Subirats J., *et al*. Antibiotic resistance along an urban river impacted by treated wastewaters. Sci. Total Environ. 2018: 628–629; 453–466. https://doi.org/10.1016/j.scitotenv.2018.02.083
10. Fang H., Han L., Zhang H., *et al*. Dissemination of antibiotic resistance genes and human pathogenic bacteria from a pig feedlot to the surrounding stream and agricultural soils. J. Hazard. Matter. 2018: 357; 53–62. https://doi.org/10.1016/j.jhazmat.2018.05.066
11. Karkman A., Do T.T., Walsh F. and Virta M.P.J. Antibiotic-Resistance Genes in Waste Water. Trends Microbiol. 2017: 26; 220–228. https://doi.org/10.1016/j.tim.2017.09.005
12. Kain K and Kelly M. Clinical features, epidemiology, and treatment of *Plesiomonas shigelloides* diarrhea. J Clin Microbiol. 1989; 27(5):998-1001.
13. Graciaa, D.S., Cope, J.R., Roberts, V.A., *et al*. Outbreaks associated with untreated recreational water — United States, 2000–2014. Am. J. Transplant. 2018:18; 2083– 2087. https://doi.org/10.1111/ajt.15002
14. Wouafo M, Pouillot R, Kwetche PF, *et al*. An acute foodborne outbreak due to Plesiomonas shig- elloides in Yamounde, Cameroon. Foodborne Pathog 2006: 3; 209–211. http: // dx.doi.org/10.1089/fpd.2006.3.209.
15. Petrušić M., Vidaković D.O., Lazić S., Radnović D. and Knežević P. Prevalence and genetic variability of *Plesiomonas shigelloides* in temperate climate surface waters of the Pannonian Plain. Arch Biol Sci. 2018; 70(1):99-108.
16. Rutala W, Sarubbi F, Finch C, Maccormack J and Steinkraus G. Oyster-associated outbreak of diarrhoeal disease possibly caused by Plesiomonas shigelloides. Lancet. 1982; 319(8274):739.
17. Ingram C, Morrison A and Levitz R. Gastroenteritis, sepsis, and osteomyelitis caused by *Plesiomonas shigelloides* in an immunocompetent host: case report and review of the literature. J Clin Microbiol. 1987;25(9):1791-1793
18. Claesson B, Holmlund D, Lindhagen C and Mätzsch T. *Plesiomonas shigelloides* in acute cholecystitis: a case report. *J Clin Microbiol*. 1984; 20: 985-7. 49.
19. Van Houten, R., Farberman, D., Norton, J., Ellison, J., Kiehlbauch, J., Morris, T., Smith, P. *Plesiomonas shigelloides* and *Salmonella* serotype Hartford infections associated with a contaminated water supply - Livingston County, New York, 1996. Infect. Med. (1998): 15; 495–497.
20. Monge R, Arias-Echandi M, Utzinger D. Presence of cytotoxic A*eromonas and Plesiomonas shigelloides* in fresh vegetables. Rev Biomed. 1998; 9:176-80. 51.
21. Jagger T.D. *Plesiomonas shigelloides*: a veterinary perspective. *Infect Dis Rev.* 2000: 2; 199–210.
22. Huber I., Spanggaard B., Appel, K.F., Rossen, L., Nielsen, T., Gram, L. Phylogenetic analysis and in situ identification of the intestinal microbial community of rainbow trout (Oncorhynchus mykiss, Walbaum). J Applied Microbiol. 2004: 96; 117-132. https://doi.org/10.1046/j.1365-2672.2003.02109.x
23. Alexander S., Fazal M.-A., Burnett E., *et al*. Complete genome sequence of *Plesiomonas shigelloides* Type Strain NCTC10360. Genome Announc., 2016: 4; e01031-16. https://doi.org/10.1128/genomeA.01031-16.
24. Pan L., Liu S., Cheng X., *et al*. Isolation, identification and genomic analysis of

Plesiomonas shigelloides Isolated from diseased *Percocypris pingi* (Tchang, 1930). Am. J. Biochem. Biotechnol. 2017: 13; 226–232. https://doi.org/10.3844/ajbbsp.2017.226.232.

25. Bodhidatta L., McDaniel P., Sornsakrin S., *et al*. Case-control study of diarrheal disease etiology in a remote rural area in Western Thailand. Am. J. Trop. Med. Hyg. 2010: 83 (5); 1106–1109.
26. Gu W, Gonzalez-Rey C, Krovacek K and Levin R. Genetic variability among isolates of *Plesiomonas shigelloides* from Fish, Human Clinical Sources and Fresh Water, determined by RAPD Typing. Food Biotechnol. 2006; 20(1): 1-12.
27. Shigematsu M, Kaufmann M, Charlett A, Niho Y and Pitt T. An epidemiological study of *Plesiomonas shigelloides* diarrhea among Japanese travellers. Epidemiology and Infection. 2000; 125(3):523-30
28. Chen X., Chen Y., Yang Q., *et al*. (*Plesiomonas shigelloides* Infection in Southeast China. PLoS One 2013: 8; https://doi.org/10.1371/journal.pone.0077877
29. Salerno A, Deletoile A, Lefevre M, *et al*. Recombining Population Structure of *Plesiomonas shigelloides* (*Enterobacteriaceae*) Revealed by Multilocus Sequence Typing. J Bacteriol. 2007; 189(21):7808-7818.
30. Gonzalez-Rey C, Svenson S, Eriksson L, Ciznar I and Krovacek K. Unexpected finding of the "tropical" bacterial pathogen *Plesiomonas shigelloides* from lake water north of the Polar Circle. Polar Biol. 2003;26(8):495-499.
31. Kwaga J, Adesiyun A, Bello C and Abdullahi S. Occurrence of *Plesiomonas shigelloides* in humans and water in Zaria, Nigeria. Microbiologica 1988; 11:165-167.
32. Alabi S.A., and Odugbemi T. Occurrence of Aeromonas species and *Plesiomonas shigelloides* in patients with and without diarrhoea in Lagos, Nigeria.. J Med Microbiol. 1990; 32(1):45-48.
33. Eko FO, and Utsalo SJ. Occurrence of *Plesiomonas shigelloides*—associated diarrhoea in Calabar, Nigeria. East Afr Med J. 1991 Jul; 68(7):562-566
34. Nwokocha A.R.C. and Onyemelukwe N.F. *Plesiomonas shigelloides* diarrhea in Enugu area of south eastern Nigeria: incidence, clinical and epidemiological features. IOSR J. Dent. Med. Sci. (IOSR-JDMS) 2014: 13; (4), 68–73.
35. Adesiyan I. M., Bisi-Jo hnson M. A., Ogunfowokan A. O. and Okoh A. I. Incidence and antimicrobial susceptibility fingerprints of Plesiomonas shigelliodes isolates in water samples collected from some freshwater resources in Southwest Nigeria. *Science of the Total Environment*. 2019: *665*; 632-640.
36. APHA AW.WEF, 2012. Standard Methods for the Examination of Water and Wastewater. 2012 p. 22.
37. Jeong S., Kim J.O., Jeong S.H., Bae I.K. and Song,W. Evaluation of peptide nucleic acidmediatedmultiplex real-time PCR kits for rapid detection of carbapenemase genes in gram-negative clinical isolates. J. Microbiol. Methods. 2015: 113; 4–9.
38. Weimin Gu and Robert E. Levin. Quantification of *Plesiomonas shigelloides* in Pure Culture and Clams by Competitive PCR. Food Biotechnology. 2007: 21; 1, 17-31, DOI: 10.1080/089054307011910
39. Morandi S, Brasca M, Lodi R, *et al*. Biochemical profiles, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and multilocus variable number tandem repeat analysis (MLVA) for typing *Staphylococcus aureus* isolated from dairy products. Res Vet Sci. 2010: 88;427–435
40. Idil N and Bilkay IS. Application of RAPD-PCR for determining the clonality of methicillin resistant *Staphylococcus aureus* isolated from different hospitals. Braz Arch Biol Technol. 2014: 57; 548–553
41. Martins A.F.M., Pinheiro T.L., Imperatori A., *et al*. *Plesiomonas shigelloides*: A notable carrier of acquired antimicrobial resistance in small aquaculture farms. Aqua (2018), doi:10.1016/.aquaculture.2018.10: 040
42. Santos J. A., Rodríguez-Calleja J. M., Otero A., and García-López M. L. *Plesiomonas*. In Molecular Medical Microbiology. 2015: pp. 1111-1123. Academic Press.

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