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PREVALENCE AND MOLECULAR CHARACTERISATION OF *LARIBACTER HONGKONGENSIS* FROM ENVIRONMENTAL WATER SOURCES IN OGHARA NEXUS

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ABSTRACT

The water nexus has been implicated as a potential source for the distribution of various microbial potential pathogens especially when such water sources harbors aquatic life and other water associated amphibians. In addition, human/animal anthropogenic activities including fishing, swimming, washing of clothes etc, are seen as potential drivers of pollution which may affect the safety/quality of such water sources. Suffice to say thattap, river, lake/swamp and well are the common water resource used in Oghara community and contamination compromisesthe safety of water usage. In recent times, domestic users of these water sources have shown potential gastroenteritis presumed to be associated with *Laribacter hongkongensis*after contact with such water sources both for washing, bathing, preparation of fruit cocktail and other uncooked food products. We present here the prevalence and molecular characterisation of *L. hongkongensis*from environmental water sources in Oghara nexus. Four water samples including tap, river,lake/swamp and well water were collected in Ogharefe community, Delta State for isolation of bacterial strains.The samples were analyzed using standard microbiological techniques while presumptive isolates were further confirmed usingmolecular technique, while antibiotic susceptibility testing (AST) was conducted on strains. Fourteenstrains of *L. hongkongensis*were obtained from the different water sources with results of tap water sample showing 0% of strain,river water harbor 35% strains, lake/swamp water sample harbor 57% of strains and well water harbor 7% of organism. The AST revealed that strain were highly resistant to amoxicilin and sensitive to amikacin with high multiple antibiotic resistance nature to the beta-lactam antibiotics.This study has shown that strains of *L. hongkongensis*inhabit water nexus with potential host been fresh water organisms. Observing such strains of *L hongkongensis*in water nexus posespotential health related risk to people who use the sampled water source for domestic purpose. It is therefore suggested that adroit application of interpersonal hygiene as well as adequate hygienic condition of environmental water sources be encouraged since such water sources has fallen below acceptable standard for human consumption.

Keywords:Laribacter hongkongenesis; environmental water sources; contamination; antibiotic susceptibility testing; hygiene

INTRODUCTION

Laribacter hongkongensis, a novel genus and specie was first isolated in Hong Kong in 2001 from the blood and empyema pus of a 54- year old Chinese man with alcoholism related cirrhosis, bacteraemia and empyema (Igere et al., 2021; Lau et al., 2009a). Phenotypically, it is a facultative anaerobic, motile, non- sporing, urease – positive, Gram negative, S-shaped bacillus (Igere et al., 2021). By phylogenetic analysis using 16S RNA gene sequence, it showed that*L. hongkongensis* belongs to the Neisseriaceae family of the β-subclass of proteobacteria (Bessong et al., 2009). It was subsequently discovered in the stool of six patients with commonly acquired gastroenteritis in Hong Kong and

Switzerland (Feng et al., 2011). Using cefoperazone MacConkey agar as the selective medium, it was confirmed that *L.hongkongensis* is associated with community acquired gastroenteritis and travelers' diarrhea (Raja et al., 2013). Furthermore, it is confirmed that freshwater fish and other water associated animals (amphibians) are a reservoir for *L.hongkongensis*. Patients who either resided in or had histories of recent travels to Asia, Europe, America and Africa have also been reported to harbor the organism. This implies that the bacterium is of global importance (Susanna *et. al*, 2009). *L. hongkongensis* is strongly associated with diarrhea. Suffice to say that diarrheal diseases are the second leading cause of death in children under five years old, and is responsible for killing around 525 000 children every year (Lau et al., 2009b). Diarrhea arising from *L. hongkongensis* infection may last several days, and can leave the body without the water and salts that are necessary for survival (Raja et al., 2013). In the past, most people experience severe dehydration and fluid loss which are the main causes of diarrhoea deaths (Raja et al., 2013). Now, other disease implications such as septic bacterial infections are likely to account for an increasing proportion of all diarrhea-associated deaths.Diarrhoea is usually a symptom of an infection in the intestinal tract, which can be caused by a variety of bacterial, viral and parasitic organisms. Infection by *L. hongkongensis* is spreading through contaminated fishes, drinking-water, or from person-to-person as a result of poor hygiene (Lau et al., 2007a,b). Globally, 780 million individuals lack access to improved drinking-water and 2.5 billion lack access to improved sanitation (Igere et al., 2020; 2021). These aforementioned concerns of water and environment are associated with diarrhea infection which is spreading throughout developing countries and under developed countries. (W.H.O, 2017). Water may be polluted by various pathogenic bacteria, viruses, protozoa, and helminths. According to the WHO (WHO, 2011), 80% of all diseases in the developing countries results from contaminated water. The major sources of infections include usage ofuntreated and improperly treated sewage, animal waste in fields and feedlots beside waterways, meat packing and tanning plants that release untreated animal waste into environmental water making the nexus in most underdeveloped and developing countries a major hub of microbial distribution (W.H.O, 2000).

Oghara communitybeing a home for fishermen with cultural practice of fishing and fish farming in nearby river has welcome diverse interest based personnel especially with the advent of the river resort for recreational activities located around the Ethiope East/West L.G.A. Although, there is only one major river in Oghara metropolis which is the Ethiope River, other rivers, ponds and lakes have been described as water bodies which channels water into the river. The river is a beacon to everyone far and wide; it is a recreational site for vast majority of people resident in communities nearby. This has made this water a potential source of distribution of potential pathogens amongst people who lack knowledge of bacterial associated health challenges.Since the practice of interpersonal hygiene and control of wastewater are not routinely organized in our immediate environment, individuals tend to imbibe whatever seem fit which posses a great risk to the large population that goes into the river.It is as a result of the aforementioned that we

embark on the study to determine the prevalence of *L. hongkongensis* isolates in environmental water sources within Oghara nexus.

MATERIAL AND METHODS

Study Area

This short term cross-sectional study was conducted in the Department of Microbiology and Biotechnology, Western Delta University Oghara, Delta State Nigeria from 15th of April to 13th of October, 2021. The study was conducted within the suburban area of the Western Delta region of the State and Western Delta University. Four communities of the region were selected for the study including Ajagbodudu, Oghara-efe, Oghara-eki and Otefe.These communities were selected based on their proximity and potential susceptibility distribution of *L. hongkongensis*.

Sources of Samples

The samples were collected from four (4) different water sources (namely tap water, river water, lake/swamp water and well water)within four communities in the Western Delta region of the Delta state.

Ethical Consideration

The ethical clearance was approved on the 7th February 2021 by the Department of Microbiology and Biotechnology Research Ethics Board with reference number WDUMCB/2020/ECCvol/140. This is to ensure that experimental activities during study does not compromise or fall below standard of microbiological practice and experiment does not encourage further spread of potential pathogen.

Sample Collection and Analysis

Sterile Nalgene bottles were used in collecting the water from the various sources, secured tightly to prevent further cross-contamination and kept in cooler box and transported to the laboratory for analysis.

Presumptive Isolation of Strains

Collected water samples were cultured onto glucose peptone water for 24 hrs and sub-cultured onto 16 - 32 µg/mL Cefoperazone (Cefobid) or Ceftriaxone supplemented MacConkey agar, incubated at 37 ^{o}C for 24 - 48 hrs, as previously described by Igere and other research colleagues (Igere et al., 2021; Woo et al., 2009; Lau et al., 2003) with few modifications that did not affect result. Single colony showing Gram negative and non-lactose fermenting with sea gull-shaped and motility positive strains were purified and stored in aliquot culture (660 µl) on sterile 2 ml graduated skirted Cryo preservation tubes (Starlabs, Milton Keynes, UK) containing a 0.4 ml mixture of normal saline and glycerol (1:1 or a 50 % glycerol) in 0.6 ml of sterile de-ionised water. The tube was vortexed briefly to ensure thorough mixing of contents and immediately stored frozen in a refrigerator.

Isolation of Pure Culture

Pure isolate were recovered by sub-culturing onto fresh nutrient agar plates using the wire loop. The media was then incubated for 24-48 hours.

Antibiotics Sensitivity Test

The sensitivity of an isolated organism was tested by placing antibiotic disc on culture plates then followed with the test organisms and by judging the degree of sensitivity by the size of inhibition zones resulting after 24 hours of incubation. The isolates were placed into peptone water then incubated at 37^{0}C for 3 – 4 hours. Sterile Mueller Hinton

agar plate was dried in hot air oven and then the plate was flooded with growth in peptone water. Antibiotic disc (multi-disc) was placed on the surface of the culture medium using the Kirby Bauer disc diffusion method and was incubated overnight at 37^0C. Zones of inhibition were measured around the antibiotic disc using a meter rule and the result was recorded in millimeter (mm). the results were interpreted using the clinical Laboratory Standard Institute guidelines (CLSI, 2017)

Motility, Colonial morphology and Biochemical tests

A needle inoculating wire loop was stabbed onto the medium in a straight line and incubated at 37^0C for 18-24 hours or until growth is evident. A positive motility test is indicated by the turbidity of the medium. Gram stain was carried out in order to determine whether the organism is a Gram negative or Gram positive bacterium. The sizes and shapes of strains were also noted. Other biochemical test conducted were catalase test, coagulase test, oxidase test, indole test,urease test and citrate utilization test.

Genomic DNA Extraction

Genomic DNA of the presumptive isolates was extracted using the crude DNA extraction method previously described by Igere and his colleagues (Igere et al., 2020). This method uses the heating of pure isolates harvested onto a microfuge tube, heated to boiling and centrifuge. The supernatant is collected and used as DNA template.

Agarose Electrophoresis

Agarose electrophoresis was conducted using a Sigma-based tris acetate-EDTA (TBE) of 50× (Sigma Aldrich, Dorset, UK), which was re-constituted to a 1X TBE running buffer. Gel was prepared by dissolving 1.5 g of agarose powder (Sigma Aldrich) in 100 mL of running buffer and heated to boiling. The prepared gel is casted on a minigel tray (Anachem, Dorset, UK), allowed to polymerize, placed carefully in an electrophoresis tank filled with 1 × TBE Buffer and electrophorese (electrophoresis machine CLS-AG100, Warwickshire, UK) at 100 V for 50 min. The gel was visualized on a Gel doc imaging system (Bio Rad Hercules, Califonia, USA).

RESULT

The results are as described in tables and figures. Table 2 shows the biochemical test results of isolates, it reveals the enzyme based characterization techniques of the various isolates. Table 3 shows the numbers of isolates recovered from the various water sources. This includes their major water sources and the confirmed isolates using PCR. Figure 1 shows the Gram reaction photograph of a seagull shaped bacilli. Figure 2 shows the agarose electrophoresis photo of isolates

Table 1: Description of the Sampling Points

S.P	ACTIVITIES	TIME	DATE
River sample	Swimming, bathing, washing	12:00am	14/8/21
Tap sample	Bathing, washing, cooking	1:00am	14/8/21
Swamp/ sample	Swimming, washing, cooking	2:00pm	14/8/21

Note: SP- sampling point

Table 2: Domestic activities, Water source and Disease pattern of the Populace

Domestic water sources	Total heterotrophic count	Total Presumptive isolates	Total PCR confirmed isolates
River	3.20 to 9.4 log 10	10 (35.7%)	5 (35.7%)
Lake/swamp	5.70 to 7.8 log 10	15 (53.6%)	8 (57.1%)
Tap	0.0	0	0
Well	1.1 to 2.1 log 10	3 (10.7%)	1 (7.1%)
Total		28	14

The above describes isolate of *L. hongkongensis* recovered from various water source

Table 3: Shows the Antibiogram of confirmed*L. hongkongenesis* to relevant antibiotics used

Isolates Nos	LEV-5C	PTZ-110C	SAM-20C	AMX-25C	AK-30C	GM-10C	OFL-5C	AUG-30C	NI-200C	CIP-5C
1	S	S	S	S	S	S	S	S	R	R
2	R	R	R	S	S	S	R	S	R	S
3	R	R	S	S	S	S	R	S	S	S
4	S	R	S	R	S	S	S	S	S	S
5	S	S	R	R	S	S	S	S	S	R
6	R	S	R	S	S	S	S	S	S	S
7	S	S	R	S	S	S	S	S	R	S
8	S	R	R	S	S	S	S	R	R	R
9	R	S	S	S	S	S	S	R	R	R
10	S	R	R	S	S	S	S	R	R	S
11	S	S	S	S	S	S	S	R	R	S
12	S	S	R	S	S	S	S	S	R	S
13	S	S	R	S	S	R	S	S	R	S
14	R	S	R	R	S	R	S	S	S	R

Key: "R" represents resistance and "S" represents sensitive as interpreted using CLSI guidelines while intermediate was reported as resistance or R

Fig 1: Seegull shaped Gram negative rods of *L. Hongkongensis*isolated during the study

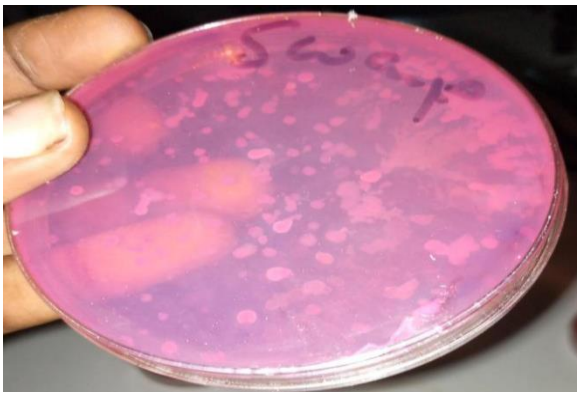

Fig 2: Showing the photo of isolates recovered from water sources on Ceftriaxone supplemented MacConkey agar

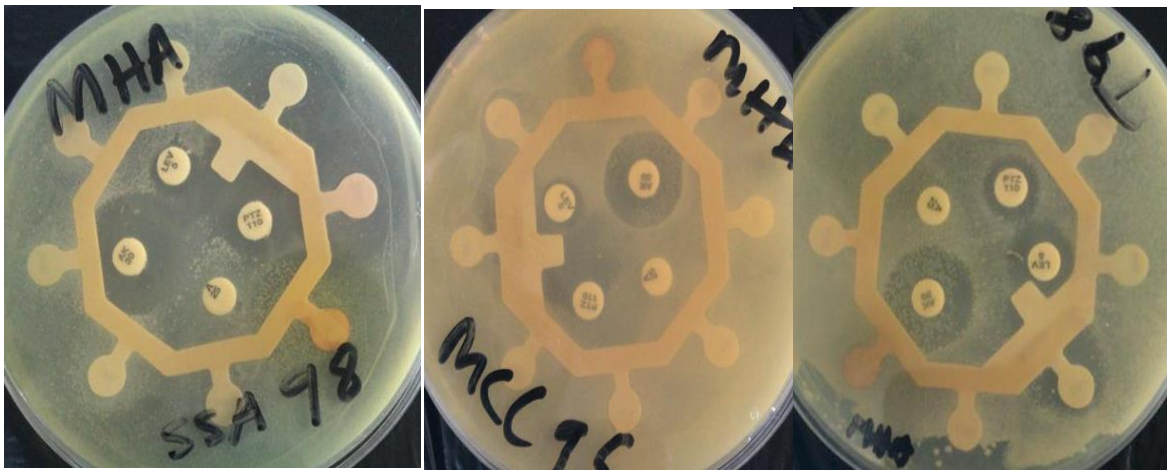

Figure 3: Antibiotic Susceptibility test plates of Isolates observed during study

Fig 4 shows the gel photo of detected strains. It reveals the 16S rRNA gene (320 bp) in some isolates, L represents a molecular marker of 1.2kb, while numbers 1-5 are positive isolates.

DISCUSSION

Recent investigators reports on poor implementation of inter-personal hygiene, water contamination and emerging environmental pollutants have shown that there is proliferation and distribution of potential pathogens especially in localities were such organisms are not endemic. Such has been the current situation as potential pathogens which were not reported in some localities are been observed in association with environmental contaminants and water pollution. The isolation and prevalence of *L. hongkongensis* from various water sources shows that the water nexus in Oghara harbors 50% (14/28) *L. hongkongensis*strains which pose health risk to the populace in the study environment. It was observed from the study that river water harbors 35.7% of isolates, lake/swamp harbors 57.1% and 7.1% in well water (Table 2). This is an indication that a high prevalence of *L. hongkongensis* was recorded in lake/swamp water during the study (Table 2). It is important to note that *L. hongkongensis*inhabit water environment where its possible hosts are fishes, toads and other fresh water organisms (Woo et al., 2004). In addition, it is also possible that the recently observed community-acquired gastroenteritis and diarrhea in the study area are associated with these potential pathogens as previously reported by some investigators (Woo *et al*., 2004; Woo et al.,

2009). Also, observing such strains in the water environment pose risk to those that uses the water for domestic purpose. Other previous studies have reported that *L. hongkongensis* has been found in up to 60% of intestines of commonly consumed freshwater fish of the carp family and fruit cocktails (Woo *et al*., 2004; Teng *et al*., 2005; Igere et al., 2021).

Fig 1 shows the sea gull shape Gram negative rod which reveals the presumptive occurrence of strains. The determination of isolates virulence determinants was not conducted; however the occurrence has indicated a potential health concern. The PCR detection technique further confirmed the isolates. Twenty eight isolates were presumptively recovered during the study. However fourteen (50%) isolates were further confirmed by the molecular detection technique which shows the relevance of molecular detection technique in the characterization of related isolates.

Following the CLSI interpretative guidelines for various *in vitro* antibiotics used, the bacterial strains were grouped into R (resistant strains), I (intermediate strains), S (sensitive strains) (CLSI, 2015). The intermediate (I) was reported as resistance (R) as shown in Table 3 above. The antibiotic susceptibility testing reveals that amykacin (AK), gentamicin (GM) and Ofloxacin (OFL) are potential antibiotics of choice since they are the most effective antibiotics as revealed by the antibiogram of strains. The Table 4 and figures 4 shows the antibiotic profile and the interpretation of ten antibiotics applied during the study. The resistant phenotypes observed among isolatesarerevealed as follow: Ampiclox (AMX-25C; 64.3%), Gentamycin (GM-10C; 85.7%), Augmentin (AUG-30C; 78.6%), Nitrofurantoin (NI-200C; 35.1%), Ofloxacin (OFL-5C; 85.7%), Sulbactam-Ampicillin (SAM-20C; 71.4%) etc as shown in Table 4 and figure 4 above. The figures 5 shows the antibiotics disc *invitro*-test on the isolates which were interpreted based on the CLSI interpretative guidelines. It is important to note that these reported antibiotics are routine antibiotics commercially applied in the treatment and management of infections implicated by the observed organisms. Observing such high level resistant phenotype is an indication of spread and sharing of these resistant genes within the environment. The isolates were shown to harbor multiple antibiotics resistant to the various groups of antibiotics such as β-lactam, β-lactam inhibitors, fluoroquinolone, aminoglycoside, macrolide and nitrofuran. The observation of these high resistant *L. hongkongensis*members affirms that the isolates have thrived in the environment with such multiple antibiotic resistance nature and are been distributed within various water environmental sources. This is a call for public health concern as it is suggestive that attention and interest towards limiting or eradicating such potential clinically relevant pathogens be instituted (Igere *et al*., 2021; 2020). The high resistant strains observed during the study may be associated with the poor awareness of the thriving tendency of resistant strains in the environment and other domestic sources. It may also be associated with inappropriateness in the spread or sharing of resistant phenotypes in the study area which inform the need for appropriate application of hygiene.

5.0 Conclusion

From the analysis of water sources conducted, it has been established that *L. hongkongensis*is prevalent in water sources especially water that harbors fresh water organisms such as fishes, toads etc which

may influence gastrointestinal tract infections. Such bacterial strains have been implicated ingastrointestinal tract (GIT) related infections such as enteric fever,diarrhea in humans amongst immuno-compromised individual. The level of contamination observed may also have negative health consequences to those who reside within the study area that probably use the water as domestic water sources.The study further revealed the high level of resistance to most of the antibiotics, indicating a possible indiscriminate use of drugs and high level of prior exposure of these bacterial species to the conventional antibiotics used in this study. From available literatures, this research is apparently the first research for the isolation and molecular characterization of *L. hongkongensis* of environmental water nexus and therefore contributes to scientific knowledge, with useful data set for future research work in the study area.

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Conflict of interest
Non was declared

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