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Molecular characterization of Multi-drug resistant *Vibrio* and *Listeria* species from unpasteurized cow milk (*Nunu*)

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Abstract

Nunu is an unpasteurized cow milk voraciously consumed in Northern Nigeria. The process of collection, processing, storage and marketing of this protein rich dairy product is prone to contamination. This study investigated the molecular characterization and prevalence of antibiotics resistant *Listeria* and *Vibrio* species isolated from *Nunu* in Owerri, Imo state.One hundred (100) samples of unpasteurized milk (*Nunu*) collected from different locations were examined for the presence of *Listeria* and *Vibrio* species using standard methods and identification manuals. The Unpasteurized milk from the studied locations showed a high percent prevalence of both organisms in the order; Mbaise (34.0% *Listeria monocytogenes* and 32.9% *Vibrio cholerae*), Obinze (41.3% *Listeria monocytogenes* and 46.9% *Vibrio cholerae*), Ama-Hausa (50.0% *Listeria monocytogenes* and 40.1% *Vibrio cholerae*), Okigwe (42.3% *Listeria monocytogenes* and 14.3% *Vibrio cholerae*) and Mgbirichi (61.5% *Listeria monocytogenes* and 33.3% *Vibrio cholerae*). All the isolates exhibited multiple resistant to more than one antibiotics with reference to the manual of Clinical and Laboratory Standards Institute. The presence of *Listeria* and *Vibrio* species portends serious public health importance as they have been implicated in listeriosis and cholera respectively. Poor hygiene in sample collection, processing, packaging and marketing could be the major vehicle of contamination. Adequate processing is therefore recommended as the panacea to reduce the menace of infection from consuming *Nunu*.

Keywords: *Nunu, Listeria monocytogenes, Vibrio cholerae*, antibiotic resistance

Introduction

Milk is described as a whole, fresh, clean, lacteal secretion obtained from the complete milking of healthy milch animalcontaining the minimum prescribed levels of fat and solids non-fat.Mammals' secrets this fluid for the nourishment of their offspring (Muthulakshmi *et al*., 2018). Since humans began to domesticate lactating animals, milk and milk products have been part of the human diet (James *et al*., 2005). Milk and milk products occupy a more significant role in the human food profiles and is considered one of the most complete sources of nutrients for human beings because of its diverse components, such as proteins, vitamins, and minerals that are important in human nutrition (Balthazar *et al*., 2017).

Due to the high nutritional value, neutral pH, and high water activity of raw milk, it serves as an excellent growth medium for different microorganisms, whose multiplication depends mainly on temperature and on competing microorganisms and their metabolic products (Adams and Moss, 2008). Raw milk also creates good growth conditions for a variety of spoilage and potentially pathogenic microorganisms, such as Shiga toxin-producing *Escherichia coli* (STEC), *Listeria monocytogenes*, *Salmonella enterica*, *Campylobacter* spp., *Yersinia* spp., *Vibrio* spp and others (Hill *et al*., 2012; Castro *et al*., 2017).

In the dairy industry, many problems associated with *Listeria* and *Vibrio* spp. contamination are related to minimally processed or post pasteurization contamination from plant environments, as well as the harbouring of disease causing organisms by the milk-producing animals (European Food Safety Authority, 2009; Latorre *et al*., 2009;Olszewska *et al*., 2015).

*Listeria monocytogenes*is ubiquitous in the environment and has been recognized as animal pathogens implicated in several outbreaks of food-borne illness in humans resulting in chronic illness and suppression of the immune systems of immune-compromised persons (Chambers, 2002; Jay *et al*., 2005; Pintado, 2005; Ramaswamy *et al*., 2007; Jalali and Abedi, 2008; Clark *et al*., 2010).

Vibrio species are associated with gastrointestinal illness andsepticemia. The majority of the food-borne illness is caused by *Vibrioparaheamolyticus, Vibrio vulnificus, Vibrio fluvialis* and *Vibrio cholerae. V.vulnificus* is responsible for 95% of sea food related deaths while immune suppressed individuals are most susceptible to other *Vibrio* infection (Jay *et al*., 2005; Dickinson *et al*., 2013; Miyoshi, 2013; Lutz *et al*., 2013; Islam *et al*., 2013).

V. cholerae is continuously becoming resistance to a variety of antimicrobial agents, necessitating use of newer drugs which are more expensive and have more adverse effect. According to Dunstan *et al*.(2013), the spread of cholera epidemics worldwide has been associated with the emergence of multiple drug resistance among a large number of *V. cholerae* strains.

This study reports on the multi-drug resistant *Vibrio* and *Listeria* species from unpasteurized cow milk (Nunu).

Materials and Methods

Description of sample locations

Unpasteurized milk (*Nunu*) samples were collected from five locations, namely, Obinze, Ama Hausa, Mgbirichi and Okigwe cattle market dominated by Fulani and cattle herdsmen in Imo State.

Collection of Samples

Samples were bought from Fulani young girls and women hawkers. Samples were collected into calabash and polyethylene materials and transported to the laboratory preserved in an iced box chest. One hundred sample were randomly collected according to the method of Kothari (2003).

Preparation of samples and inoculation

Ten milliliters (10 ml) of samples were serially diluted in 90 ml of sterile physiological saline and swirled to mix thoroughly to obtain 10^{-1} dilution. Further dilution was carried out until the desired dilution was obtained. Aliquot portion (0.1 ml) of appropriate dilution was inoculated into the pre sterilized and surface dried media (TCBS Agar and Listeria agar). Inocula were spread evenly to ensure uniform and countable colonies. Plates were incubated at ambient temperature for 24-48 h for heterotrophic bacteria (Beishir, 1987; Cheesbrough, 2000; Benson, 2005; Oyeleke and Manga, 2008).

Determination of microbial population and identification

Colony counts obtained on the media were counted and expressed as colony forming units per gram (CFU/g) of the total population and characterized with reference to standard manual (Harrigan and McCance, 1990; Buchanan and Gibbon, 2000).

Standardization of inoculum and Determination of Multi-Drug Resistant

Cells of twenty four hour old pure cultures of *Vibrio* and *Listeria* species were washed in sterile distilled water and standardized using McFarland method with cell turbidity equivalent to 1.5 x 10^8Cfu/ml (Cheesbrough, 2000; Benson, 2005).

Standardized pure cultures of test isolates were spread evenly on a freshly prepared and surface dried Mueller Hinton Agar medium and allowed to stand for 30 mins. Five commercial antibiotics (oxoid) of known concentrations were placed at equal distance on the medium previously seeded with the test organisms and incubated at 37^0C for 24-72h. Zone of inhibition was measured in triplicates and the mean recorded (Cheesbrough, 2000; Benson, 2005).

Toxicity Testing of Isolates

This was done by growing the isolates on blood agar. The degree of heamolysis determines the level of toxicity of the isolates (Cheesbrough, 2000).

Pure cultures of isolates from samples were streaked on freshly prepared Colombia blood agar and incubated at 37^{0}C for 24-48 h. The degree of heamolysis was recorded as beta-hemolysis (total/complete hemolysis), alpha-heamolysis (partial hemolysis) of gamma-heamolysis (no heamolysis).

Molecular Characterization of Isolates from Samples

Molecular characterization and extraction of DNA from the selected bacteria isolates was carried out at Everight molecular laboratory, Owerri, Imo State. The extractions were done according to the standard protocol (Liu, 2006; Maheshwari *et al*., 2011; Tamura *et al.,* 2013).

Preparation of samples for DNA isolation

The isolates were prepared by adding 200 – 500µl of the isolates to an equal volume of M-acetyl cystein 1NaOH and were vortexed gently to mix. The mixture was incubated for 250 mins at room temperature with shaking and the volume was adjusted to 25ml with sterile water. The sample mixture was centrifuged for 30mins at 4000xg and the supernatant was discarded.

The DNA pellet was resuspended in 0.5 – 1ml Buffer T1 and 200µl of the mixture was transferred to a new micro centrifuge tube. Lysis was carried out by adding 180µl Buffer T1 and 25µl proteinase K, followed by addition of 200µl Buffer B_2. The mixture was vortexed vigorously and incubated at 70°C for 10mins. DNA Binding conditions was adjusted by the addition of 210µl ethanol (96 – 100%) to the sample mixture and DNA was bound for each sample by placing one nucleo spin column into a collection tube. The silica membrane was washed twice using 500µl Buffer BW and 600µl B5, followed by centrifugation for 1min at 11,000xg. The flow was discarded and the column was placed back into the collection tube. The membrane was dried by placing the nucleo spin tissue column into a 1-5ml micro centrifuge tube and 100µl of buffer BE was added, followed by incubation at room temperature for 1min and centrifugation at 11,000xg(Tamura *et al.,* 2013).

PCR

A 25µl of PCR Master Mix for the reaction was prepared in a microtube on a cold block according to the description in and distributed equally to the fresh sample microtubes placed on the clock block (Tamura *et al.,* 2013).

Protocol Table of PCR Master Mix Preparation

Reagents	Volume
PCR Master Mix	16 µl
Listeria Forward primer	1 µl
Listeria Reverse primer	1 µl
Vibrio Forward primer	1 µl
Vibrio Reverse primer	1 µl
Template DNA	5 µl
Deionized water	5 µl
Total	30 µl

Amplification Program

The Qiagen Rotor Gene Q was used to perform the amplification process for the polymerase chain reaction to detect the *Vibrio cholerae* and *Listeria* genes from the isolated plasmid DNA as given below(Tamura *et al.,* 2013).

1 cycle of initial denaturation at 95^{0}C for 5mins
30 cycle of

Denaturation at 94°C for 45secs
Primer annealing at 55°C for 2 mins
Extension at 72°C for 60 secs

1 cycle of
Final extension at 72°C for 10 mins

Protocol Table of Gene amplification program

Temperature	Time	Cycle
94^0C	5 Mins	1X
94^0C 94^0C 94^0C	45 Secs 40 Secs 60 Secs	32X
72^0C	10 Mins	1X
4^0C	Hold	

Agarose Gel Electrophoresis

The PCR products underwent agarose gel electrophoresis to separate the fragments of DNA and visualize the target genes which moved forward according to their molecular weight (Tamura *et al.,* 2013). The steps of gel running and visualization were as follows.

Gel Preparation

The Erlenmeyer flask was heated and cleaned with water to remove any remnants of previously prepared gel.1g agarose powder was then measured and dissolved in 100ml of 1 X TAE taken in the Erlenmeyer flask. The mixture was heated in the microwave oven for 5 mins at low-medium temperature. The flask was removed every 30 secs and twirled gently to dissolve any leftover agarose on the walls. The solution was left to partly cool and 2µl of Ethidium bromide was added carefully and the cooled solution was poured into the gel cast with combs set and left to solidify for 30 mins at room temperature. When the gel was set, it was carefully removed from the cast and submerged in 2-6mm of 1 x electrophoresis buffer in the gel tank(Tamura *et al.,* 2013).

Gel running

The PCR amplicons were brought to room temperature. The sample and loading dye were taken in 2:8 ratio, mixed well and loaded into the wells using a 20µl pipette 05µl of the control DNA ladder (Molecular Weight Maker). The gel was run at 100 Volts and 400mA for 40 mins (Tamura *et al.,* 2013).

Gel Analysis

The gel electrophoresis apparatus was switched off after the run. The gel was carefully taken out and placed onto the UV transilluminator Gel Doc XR+. The picture was captured using Image Lab Software under Nucleic Acid Protocol (Ethidium Bromide with Filter position). The gel position was laid out within the frame and protocol was run(Tamura *et al.,* 2013). The picture obtained was adjusted for brightness and annotations marked for sample number, band size, and the molecular weight maker.

Results and Discussion

The microscopic and biochemical characteristics of the bacterial isolates on TCBS and Listeria Agar are shown in Table 1 and 2. Antibiotic susceptibility Test for *Vibrio* and *Listeria* species are shown in Table 3and 4 respectively.

Figs 1 and 2 shows pure cultures of *Vibrio cholerae* and *Listeria monocytogenes* respectively.

Fig 3 shows the brightness and annotations marked for sample number, band size, and the molecular weight maker. *Listeria monocytogenes and Vibrio cholera* were isolated from the unpasteurized dairy products (*Nunu).*

Table 1 Colonial, Microscopic and Biochemical Characteristics of Bacterial isolates on TCBS Agar

Colonial characteristics	Microscopic morphology	Cat	MR	Mn	Ar	Suc	Lac	Na+	LOC	AD	KIA	EH	Identity of isolates
Large moist and shiny yellow colonies	Small slender Gram negative rods in comma or sickle shape	+	+	+	+	+	-	+	+	+	+	+	*Vibrio cholera*
Colonies blue to green center	Small slender Gram negative rods in comma or sickle shape	+	-	+	-	+	-	+	+	+	+	+	*Vibrio sp*
Greyish-green to bluish green	Small slender Gram negative rods in comma or sickle shape	+	-	-	-	+	-	+	+	+	-	-	*Vibrio* sp

Cat, catalase; MR, methyl Red Reduction Test; Mn, mannitol; Ar, Arabinose; Suc, Sucrose; Lac, Lactose; Na+, Sodium; LOC, Lysine and Ornithine decarboxylase assay; AD, Arginine dihydrolase Test; KIA, Kligler Iron Agar, Production of Hydrogen Sulphate; EH, Esculin Hydrolysis Test

Table 2: Colonial, Microscopic and Biochemical Characteristics of Bacterial isolates on Listeria Agar

Colonial characteristics	Microscopic morphology	Cat	Oxi	MR	VP	NO_3	XYL	Mann	Rha	Lac	CAMP	HEAM	Identity of isolates
Blue-green colonies with opaque halo	Small Gram negative rods, non-spore forming, motile with peritrichous flagellation	+	-	+	-	-	-	-	+	+	+	+	*Listeria monocytogenes*
Gray-green colonies with black halo	Small Gram negative rods, non-spore forming, motile with peritrichous flagellation	+	-	+	-	+	-	-	+	+	+	+	*Listeria sp*
Circular moist and shiny yellow colonies	Large smooth Gram negative non-spore forming, non-motile rods	+	-	+	+	+	+	+	+	-	-	+	*Vibrio sp*

Cat, catalase; MR, Methyl Red Reduction Test; VP, VogesProskaeur; Mn, mannitol; Rh, Rhafinose; CAMP, Christie Atlkins Munch Peterson; HAEM, Heamolysis Test; Lac, Lactose; XYL, Xylose; OXI, Oxidase

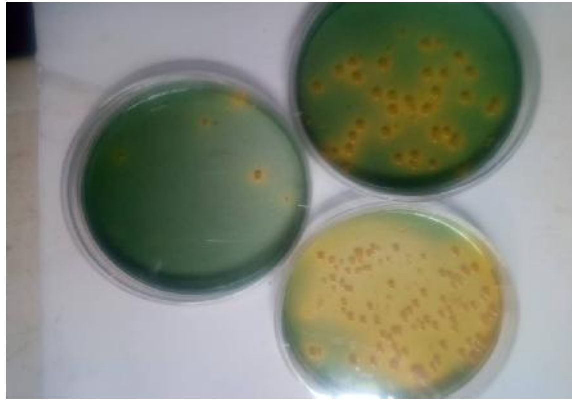

Fig 1: pure cultures of *Vibrio cholerae* on TCBS agar

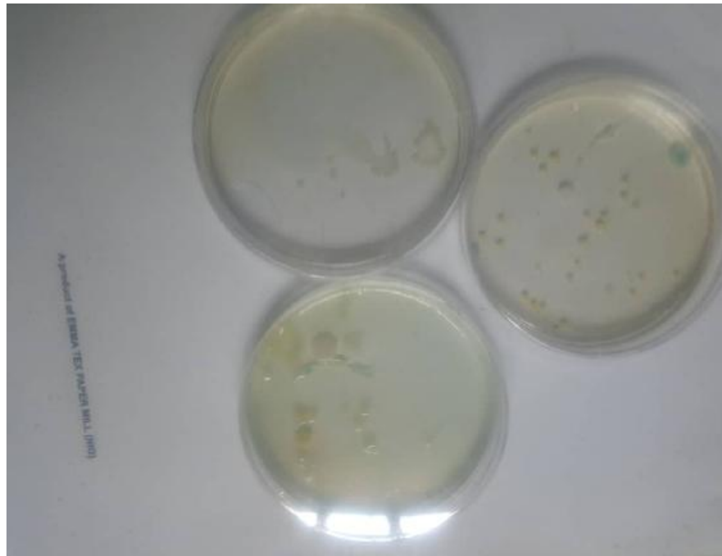

Fig 2: pure cultures of *Listeria monocytogene* on Listeria agar

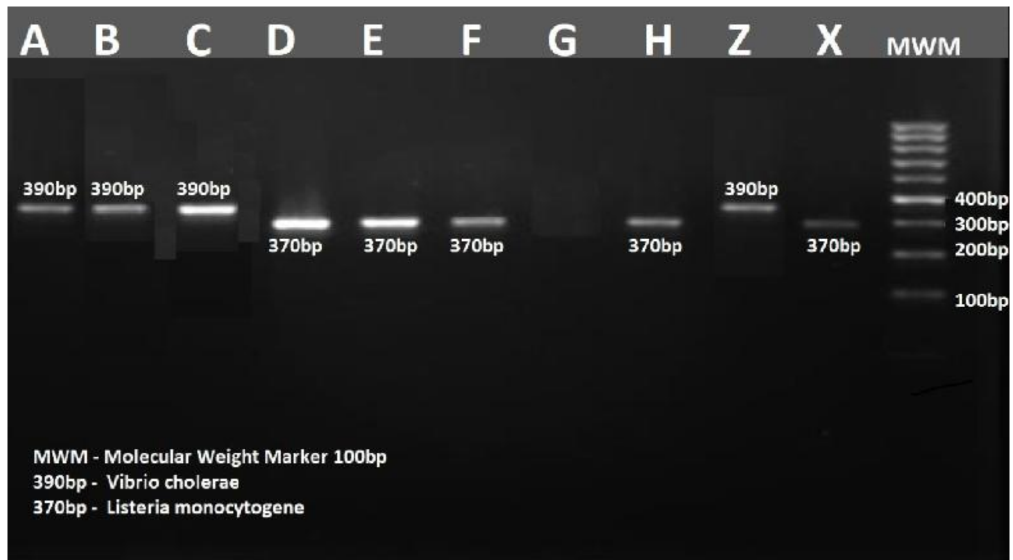

Fig 3: molecular weight maker of *Listeria monocytogene* and *Vibrio cholerae*

Table 3: Antibiotic susceptibility Test (*Vibrio* sp)

Bacterial isolates	TE	AK	CN	E	AMC	RD	S	OFX
Vibrio cholera	0	0	12	10	0	12	0	0
Vibrio sp	12	14	12	0	0	14	12	0
Vibrio sp	10	14	1	6	0	0	0	16

TE, Tetracycline, AK, Amikacin; GEN, Gentamycin; E, Erythromycin; AMC, Amoxicillin Clavulanate; RD, Rifampin; S, Streptomycin; OFX, Ofloxacin

Table 4: Antibiotic susceptibility Test (*Listeria* sp)

Bacterial isolates	TE	AK	CN	E	AMC	RD	S	OFX
Listeria sp	0	0	10	0	0	0	12	0
Listeria monocytogenes	0	8	8	0	0	0	12	0
Listeria sp	0	12	8	0	0	8	18	0

Milk and its product can be contaminated through many ways in the milk production process neither through the dairy animal which shed the pathogen in milk or during collection, transportation or could be subjected to time-temperature abuse as reported by Goh *et al*. (2017), Amagliani *et al*. (2012 and Lomonaco *et al*. (2015)The unclean hands of workers, poor quality of milk, unhygienic conditions of the manufacturing units, inferior quality of material used, water supplied for washing the utensils and adulteration of the final product could as well be the source of accelerating the bacterial contamination of milk products in addition to post manufacturing contamination (Noorlis *et al*., 2011).

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The overall high prevalence of *Listeria*428(44.5%)) in all the samples studied shows that *Listeria monocytogenes* is a common and constant contaminant of milk and its products in the area studied. This report is similar to the findings of Gaffa and Ayo (2002) in ready-to eat (RTE) dairy products. This finding further corroborate previous reports that *Listeria monocytogenes* is an important food-borne pathogen and is widely distributed in food, environmental and clinical samples (Oliver *et al*., 2005; Graves *et al*., 2010).

All the isolates exhibited multiple resistant to more than one antibiotics with reference to the Clinical and Laboratory Standards Institute and these isolates were molecularly characterized using the appropriate primers. Prevalence of antibiotic resistance by *Listeria* spp has been reported by Yucel *et al*.(2005) Resistant strains of these organisms may have been transferred to cattle from poor farm practices and poor handling during milking. The incidence of cholera and listeriosis causing pathogen in dairy milk and their drug resistance pattern demands immediate attention and good processing and manufacturing practices (Jay *et al*., 2005; Goh *et al*., 2017) . The results of this research warns the need for more strict preventive measures (Amagliani *et al*., 2012). For this, regular sterilization of dairy equipment, washing of utensils, adequate washing of milking workers hands, udders, and pasteurization of milk is required before collection and distribution to consumers (Chambers *et al*., 2002; James *et al*., 2005).Domestic and commercial handlers of raw milk should strictly follow the rules and guidelines of hygiene (Amagliani *et al*., 2012).

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