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Occurrences and Antibiogram Pattern of *Listeria monocytogenes* in Vegetables Sold within Sokoto Metropolis, Nigeria

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Authors' contributions:

This work was carried out in collaboration among all authors. Author KTM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AA and AB managed the analyses of the study. Author AA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Listeriosis ranks third in mortality among food-borne bacterial pathogens. The evaluation of antibiogram of *Listeria monocytogenes* in 5 different areas within Sokoto metropolis was performed in the study. A total of 50 (fifty) samples from cabbage, spring onion, tomatoes, lettuce, and salad samples were obtained from different locations within the metropolis. The isolation was performed using *Listeria* selective media base incorporated with *Listeria* selective Supplement. Identification and confirmation of *Listeria* and other bacteria were performed using biochemical characterization. Antibacterial sensitivity test was performed using 8 (eight) different antibiotics namely Norfloxacin, Ceftazaime, Cefuroxine, Erythromycin, Ofloxacin, Augmentin, Cloxillicin and Gentamycin. Result of the studies demonstrated that *Listeria monocytogenes* was present in all the vegetables sold within the selected areas of Sokoto metropolis. *Listeria monocytogenes* was resistant to 6 of the 8 antibiotics used, these are Norflaxcin, Augmentin, Cefuroxine, Ceftazaime, Gentamycin and Cloxicillin. In conclusion, *Listeria monocytogenes* is present in vegetable sold within the selected areas of study. *Listeria monocytogenes* were resistant to multiple antibiotics commonly administered during listeriosis management. The implication of multi-drug resistance is that Listeriosis will be problematic to treat. The implications of these findings are herein discussed.

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1. INTRODUCTION

Listeria monocytogenes is the species of bacteria that causes Listeriosis. It is an anaerobic bacterium that survives in the presence or absence of oxygen. It can grow and reproduce inside the host's cells and is one of the pathogens. *Listeria monocytogenes* is a Gram-positive bacterium. It grows at low temperature, thereby increasing its ability to evade control in human foodstuffs. Human gastrointestinal tracts may be colonized by *Listeria monocytogenes* [1].

Nevertheless, clinical diseases due to *Listeria monocytogenes* are frequently diagnosed by veterinarians, especially as meningoencephalitis in ruminant's animals. Frequent pathogenicity, causing meningitis (acquired transvaginally), pregnant mothers are often advised not to eat soft cheeses which may be contaminated and permit the growth of *Listeria monocytogenes* [2]. It is the third-most-common cause of meningitis in newborns. *Listeria monocytogenes* can infect the brain, spinal cord membranes and/or the bloodstream of the host through the ingestion of contaminated food such as some pasteurized dairy or raw foods. It is catalase-positive and oxidase-negative and expresses a beta hemolysin, which causes the destruction of red blood cells. This bacterium exhibits characteristic tumbling motility under light microscope [3]. *L. monocytogenes* is actively motile by means of peritrichous flagella at room temperature (20−25°C), the organism does not synthesize flagella at body temperatures (37°C) [1]. Both *L. ivanovii* and *L. monocytogenes* are pathogenic in mice, but only *L. monocytogenes* is consistently associated with human illness [4].

The 13 serotypes of *L. monocytogenes* can cause disease, but more than 90% of human isolates belong to only three serotypes: 1/2a, 1/2b, and 4b. *L. monocytogenes* serotype 4b strains are responsible for 33 to 55% of sporadic human cases worldwide and for all major foodborne outbreaks in Europe and North America since the 1980s. Listeriosis in adults would later be associated with patients living with compromised immune systems, such as individuals taking immunosuppressant drugs and corticosteroids for malignancies or organ transplants, and those with HIV infection [5]. An outbreak of Listeriosis in Halifax, Nova Scotia, involving 41 cases and 18 deaths, mostly in pregnant women and neonates, was epidemiologically linked to the consumption of coleslaw-containing cabbage that had been contaminated with *L. monocytogenes*-contaminated sheep manure. Since then, a number of cases of foodborne Listeriosis have been reported, and *L. monocytogenes* is now widely recognized as an important hazard in the food industry, Invasive infection by *L. monocytogenes* causes the disease Listeriosis [6]. When the infection is not invasive, any illness as a consequence of infection is termed febrile gastroenteritis.

The adverse effects of Listeriosis include septicemia, meningitis (or meningoencephalitis) encephalitis, corneal ulcer, pneumonia, and intrauterine or cervical infections in pregnant women, which may result in spontaneous abortion at second to third trimester or stillbirth. Surviving neonates of feto maternal *Listeriosis* may suffer granulomatosis infantiseptica, symptoms including persistent fever, usually precede the onset of the aforementioned disorders. Gastrointestinal symptoms, such as nausea, vomiting, and diarrhea, may precede more serious forms of Listeriosis or maybe the only symptoms expressed. Gastrointestinal symptoms were epidemiologically associated with use of antacids or cimetidine. An early study suggested that *Listeria monocytogenes* is unique among Gram-positive bacteria in that it might possess lipopolysaccharide, which serves as an endotoxin [7]. *Listeria* cell walls are made up of lipoteichoic acids in which a glycolipid moiety, such as a galactosyl-glucosyl-diglyceride, is covalently linked to the terminal phosphomonoester of the teichoic acid. This lipid region anchors the polymer chain to the cytoplasmic membrane. These lipoteichoic acids resemble the lipopolysaccharides of Gram-negative bacteria in both structure and function, being the only amphipathic polymers at the cell surface [8]. *Listeria. monocytogenes* has D-galactose residues on its surface that can attach to D-galactose receptors on the host cell walls. These host cells are generally M cells and Peyer's patches of the intestinal mucosa. Once attached to this cells, *Listeria. monocytogenes* can translocate past the intestinal membrane and into the body. The infective dose of *Listeria. monocytogenes* vary with the strain and with the susceptibility of the victim. *Listeria. monocytogenes* may invade the gastrointestinal epithelium. Once the bacterium enters the host's monocytes, macrophages, or polymorphonuclear

leukocytes, it becomes blood borne (septicemic) and can grow. Its presence intracellularly in phagocytic cells also permits access to the brain and probably transplacental migration to the fetus in pregnant women. Depending on the location of the bacterium within the host organism, different activators up-regulate the virulence genes [9]. Little is known about how this bacterium switches between sacting as a saprophyte and a pathogen; however, several noncoding RNAs are thought to be required to induce this change. [10].This research aims to determine the occurrence and antibiogram pattern of *Listeria monocytogenes* in vegetables within Sokoto metropolis, Nigeria.

2. MATERIALS AND METHODS

2.1 Study Area

Sokoto state, northwestern Nigeria. Bordering the Republic of Niger to the north, it also shares boundaries with Kebbi state to the west and south, and Zamfara to the south and east. Sokoto state occupies an area of short-grass savanna vegetation in the south and thorn scrub in the north. A generally arid region that gradually merges into the desert across the border in Niger republic, it has limited rainfall from mid-May to mid-September and is subjected to the Sahara's harmattan (dry, dust-laden wind) from November to March. It is drained by the Sokoto (Kebbi) River and its tributaries, the Sokoto being itself a major tributary of the Niger River. Sokoto state is located to the extreme Northwestern part of Nigeria between the longitudes 4º 8'E and 6º 54'E and latitudes of 12ºN and 13º58'N.

2.2 Sample Collection of the Samples at Different Market in Sokoto Metropolis

Different vegetables such as Spring onion, Cabbage, Lettuce, Tomatoes and ready to eat salad were collected in new polythene bag throughout the research from Kasuwar Daji, kasuwar kure and Gawo nama area Sokoto State. All the vegetables mentioned above was collected in 10 places inside an ice box and were immediately transported to the research laboratory were the analyses were carried out at the Microbiology Departments Research Laboratory Usmanu Danfodio University, sokoto state, Nigeria.

Plate 1. Map of Nigeria highlighting where Sokoto is located

2.3 Isolation of *Listeria monocytogenes*

Listeria.monocytogenes from different vegetable samples was isolated as per USFDA/BAM/CFSAN method. Ten g each of vegetables was weighed and macerated with a sterile mortar and pestle and a gram was weighed and serial dilution was carried out. Diluent 10^4 and 10^5 was plated on *Listeria* Oxford agar containing selective agent 0.5% (w/v) acriflavin and nalidixic acid and 1.0% (w/v) cycloheximide) and incubated at 37°C for 24 hours. The standard methods described by [11] were adopted for the identification of *L. monocytogenes*.

2.4 Purification of *Listeria monocyto-genes* Isolates for Identification and Confirmation

The purification for *Listeria monocytogenes* and other microbial isolates was with *Listeria* selective agar and on-sub-culture onto the agar plates (Oxford and Nutrient). From the solid media, the following procedure, macroscopic examination, morphological (Gram reaction), physiological and biochemical reactions were performed as enumerated below.

2.5 Macroscopic Examination of Culture Plates

Attention was paid to Microorganisms that showed the cultural and evidence of aesculin hydrolysis or black – halo formation on Oxford *Listeria* agar based incorporated with *Listeria* agar supplement plates. The colonial morphology of the Listeria species on the solid media (Oxford *Listeria* agar) was observed after a period of 24, 48 and 72 hours incubation at 37°c. The presumptive identification of Listeria species was based on colors, sizes and growth temperatures, colonial morphology (Macroscopic) patterns and the appearance on the solid media compared with reference existing stock cultures of *Listeria* species. However, other microorganism that grew on the agar plates were also identified and characterized as described previously by [11] the details of presumptive identification of *Listeria* species on the culture plates were recorded accordingly.

2.6 Confirmation Tests for *Listeria monocyogenes* Isolates

To confirm the *Listeria monocytogenes* isolated from different vegetables obtained within Sokoto metropolis, the following tests/ analyses were performed which are Gram stain reaction, catalase reaction, motility at room temperature through TSI (Triple sugar) and Hanging drop method, sugar fermentation (Glucose, Lactose, Sucrose and Mannitol), and polymerase chain reaction.

2.7 Gram's Stain

The Gram staining was performed as described by [11]. The following reagents were used for the staining; Crystal violet stain, Lugol's iodine, Acetone-alcohol decolorizer and Neutral red. The detailed chemical composition and preparation of the reagents: Crystal violet stain, Lugols iodine, Acetone-alcohol decolorizer and Neutral red. All the reagents were prepared according to the manufacturer's instructions. Suspected *Listeria monocytogenes* bacteria colonies on any of the agar plates were emulsified in Normal saline on the slide to form a smear. The smear was allowed to air dry completely. The slide, (with the smear uppermost) was fixed by rapidly passing through flame of a Burnsen burner. The smear was allowed to cool. The fixed smear was covered with crystal violet stain for 30 seconds-1minute. The stain was rapidly washed off with clean running tap water. The smear was again covered with Lugol's iodine for another 30 seconds 1 minute and washed off with clean tap water and was rapidly decolourized (few seconds) with acetone-alcohol. This was washed immediately with clean tap water. The smear was then covered with neutral red stain for 2 minutes and washed off with clean tap water. The stained slide was placed in a draining rack and the smear allowed to air dry. The smear was examined microscopically using oil immersion objective lens (×100).

2.8 Microscopy

Listeria species that appeared in the Gram-stained slides were tentatively confirmed due to their Gram-positive stain morphology like rods, arranged singly, in short chains, in pairs at V-form angles and in groups that were parallel to each other along the long axis [11].

2.9 Haemolysis on Sheep Blood Agar (SBA)

The characteristic beta (α) haemolysis was tested with the standard procedure as described by [11]. *Listeria. monocytogenes* isolates was

streaked on 5% sheep blood agar plates and incubated at 35°C. Complete clear zones of haemolysis after 24 hours was observed.

2.10 Catalase Test

A drop of 3% of hydrogen peroxide (H_2O_2) was placed on a clean greese- free slide. Then a colony of the Listeria growth on nutrient agar medium for 18 to 24 hours was placed on the drop of hydrogen peroxides (H_2O_2) using the edge of another slide. Observation of bubbles and no bubbles were recorded [11].

2.11 Motility Test

The optimum growth temperature for *Listeria* is 28°C to 37°C. After Gram's stain and catalase reactions, cells revealing characteristics of Listeria were further subjected to motility in motility agar [11]. Using straight wire loop suspected colonies were stabbed into the Centre of the tube containing motility test medium to a depth of 5mm and incubated at room temperature for 24 hours to one week. The organisms in the tube that showed umbrella shape on the motility test medium near the microaerophilic.

2.12 Phenotypic Identification of Other/Non *Listeria* Isolate from the Vegetable Samples

Gram-negative bacteria were completely inhibited. However, some unwanted Gram-positive bacteria was not inhibited was identified by their reactions using Physical morphology, gram staining, catalase reaction, Mannitol test only. The organism included *Staphylococcus aureus*.

2.13 Identification and Confirmation of Other Microbial Isolate

Staphylococcus aureus (Slide Coagulase Test) Gram- positive cocci organisms that were in cluster form, were emulsified in a loop full of normal saline on grease-free slide and allowed to stand for 30 seconds. This was to observe for auto-agglutination. However, when no auto-agglutination was observed, a loopful of human plasma was put, mixed and observed for agglutination. The sugar fermentation and the slide agglutination of the bacterial suspensions were recorded as positive for *Staphylococcus aureus*.

2.14 Antibiogram of the Isolated *Listeria monocytogenes* (Antibiotics Sensitivity)

Pattern profile of these *Listeria monocytogenes* isolates was obtained by testing the susceptibility to commonly used antibiotics by disc diffusion as method described [12,13]. In this study, a total of 50 *Listeria monocytogenes* strains that were isolated from Vegetable (which includes Cabbage, spring onion, lettuce, tomatoes and ready to eat salad) were tested against eight antimicrobial discs that are Cefuroxime, Gentamycin, Erythromycin, oflaxicin, Augmentin, ceftaziamine, Norfloxacin, (NB) and Cloxicillin. Antimicrobial susceptibility of the isolates was tested using disc diffusion assay. *Listeria monocytogenes* strains from an overnight Tryptone soy agar (TSA) were transferred into 10 ml of Mueller Hinton broth (MHB). Tubes were incubated for 24 hours at 37°C until visibly turbid. Then the cultures were swabbed onto Mueller Hinton agar (MHA) in triplicate using sterile cotton swab. Discs contained each antimicrobial agent were placed onto MHA using antimicrobial susceptibility test system (Oxoid). The plates were incubated for 24 hours at 37°C and then the diameter of clear zone was measured using a ruler. The diameter of clear zone was measured in millimeter (mm) and only the disc with a diameter of 6 mm was used in this study. Results obtained were then analyzed according to National Committee for Clinical Laboratory Standards (NCCLS) and Performance Standards for Antimicrobial Disc Susceptibility Testing. A susceptibility category of each data was assigned based on breakpoints criteria for *L. monocytogenes.*

2.15 Statistical Analysis

All analyses were done using National Committee for Clinical Laboratory Standards guideline and Performance Standards for Antimicrobial Disc Susceptibility Testing.

3. RESULTS AND DISCUSSION

The results of this study in Fig. 1 shows established the occurrence of *Listeria monocytogenes* in the vegetables samples obtained within the study area. This is ascribed to the proximity of the agricultural land areas in which the vegetables are cultivated and grown, to sewage sludge and human faeces, most notably in Kofar dundaye. This sewage effluent at Kofar dunkaye constituted the major source of

contamination for most of the vegetables sold in the areas under study. This result of sewage sludge and human wastes contamination is in line with the findings of [14]. The potential implication of the utilization of fecal materials as agricultural fertilizers was linked to the outbreak of human Listeriosis in Nova Scotia in Canada. The environmental wastes that are used as fertilizers in agricultural practices are contaminants to these vegetables. This led to the outbreak of *Listeria monocytogenes* strain in South Africa in March 2018 in an area in Polokwane, causing 200 deaths and non-fatal injuries in 1000 confirmed cases. Also in February 2018 the outbreak of Listeriosis caused the death of 164 persons and infected a further 872 [15]. These wastes are thus potential sources of human infections in Nigeria. Consumption of the vegetable by humans must have aided the spread of *Listeria monocytogenes* isolated in the experimental specimens. The applied microbiology approach to studies of microorganisms of interest (like in this study) is much the same way as the clinical microbiology approach in culturing and studying of human pathogens.

Table 1 shows the resistance ability of *Listeria monocytogenes* to 6 antibiotic out of 8 tested. The degrees of resistance of *Listeria monocytogenes* to this drugs (antibiotics) were judged by comparison of the zone of inhibition produced for each antibiotic. This was 00 mm except for Cloxicillin in which it ranged from 00 mm to 14 mm. The implication of this finding is that the wide range of antibiotics available to administer when Listeriosis is diagnosed will not have positive effects on the infected patient(s), culminating in improper treatment of the patient(s). Therefore, Listeriosis infection will be so problematic to treat after diagnoses, Finding is similar to [16]. Table 2 is the interpretation of the zone of inhibition measured by the diameters. In line with NCCLS standard, 0 mm to 16 mm is resistant, 16 mm to 18 mm is intermediate and above 18 indicates susceptible [17].

Table 1. Diameter of clear zone of Isolated *Listeria monocytogenes* strains against 8 antibiotics tested by disc diffusion assay in (mm)

Isolates	NB	CAZ	CRX	CTR	ERY	CXC	OFL	CN
LM1	4	0	0	0	11	0	25	15
LM2	15	0	0	0	12	0	24	13
LM3	15	0	0	0	25	11	26	16
LM4	14	0	0	0	21	10	25	12
LM5	13	6	0	0	20	0	24	11
LM6	15	0	0	0	21	11	23	15
LM7	14	0	0	0	20	06	25	16
LM8	13	0	9	0	21	09	21	12
LM9	16	0	0	0	20	0	23	13
LM10	12	0	0	0	20	0	23	12
LM11	4	0	0	0	18	11	26	11
LM12	16	0	0	0	20	12	23	12
LM13	13	0	0	0	26	14	21	10
LM14	12	0	0	0	21	0	22	11
LM15	11	0	0	0	23	0	23	15
LM16	08	0	0	0	19	0	21	14
LM17	12	0	0	0	20	07	23	12
LM18	12	0	0	0	23	5	26	12
LM19	13	0	0	0	26	7	21	11
LM20	11	0	0	0	21	8	23	10
LM21	11	0	0	0	22	4	24	11
LM22	10	0	0	0	19	7	24	12
LM23	12	1	0	0	22	12	26	11
LM24	11	2	0	0	20	10	23	16
LM25	09	0	0	0	21	0	21	14

Keys: NB; Norfloxacin 5 µg, CAZ: Ceftazaime 30 µg, CRX: Cefuroxime 30 µg, ERY; Erythromycin 5 µg, 0FL: Oflaxicin 5 µg, AUG; Amoxocillin/Clavulanate 30 µg, CTR; Ceftriaxone, CXC; Cloxicillin 5 µg, LM1-25; Listeria monocytogene. The table one below shows the diameter of the clear zone in mm of the isolated Listeria monocytogenes strains to different antibiotics

Table 2. Distribution pattern of resistant *Listeria monocytogenes* strains against eight antibiotics according to the number of isolate

Isolates	NB	CAZ	CRX	CTR	ERY	CXC	OFL	CN
LM1	R	R	R	R	R	R	S	R
LM2	R	R	R	R	R	R	S	R
LM3	R	R	R	R	S	R	S	R
LM4	R	R	R	R	S	R	S	R
LM5	R	R	R	R	S	R	S	R
LM6	R	R	R	R	S	R	S	R
LM7	R	R	R	R	S	R	S	R
LM8	R	R	R	R	S	R	S	R
LM9	I	R	R	R	S	R	S	R
LM10	R	R	R	R	S	R	S	R
LM11	R	R	R	R	S	R	S	R
LM12	I	R	R	R	S	R	S	R
LM13	R	R	R	R	S	R	S	R
LM14	R	R	R	R	S	R	S	R
LM15	R	R	R	R	S	R	S	R
LM16	R	R	R	R	S	R	S	R
LM17	R	R	R	R	S	R	S	R
LM18	R	R	R	R	S	R	S	R
LM19	R	R	R	R	S	R	S	R
LM20	R	R	R	R	S	R	S	R
LM21	R	R	R	R	S	R	S	R
LM22	R	R	R	R	S	R	S	R
LM23	R	R	R	R	S	R	S	R
LM24	R	R	R	R	S	R	S	R
LM25	R	R	R	R	S	R	S	R

Keys: R=Resistance 0 mm-16 mm, S=susceptible 17 mm above, I=Intermediate 16 mm -17 mm, LM 1-25 Listeria monocytogenes

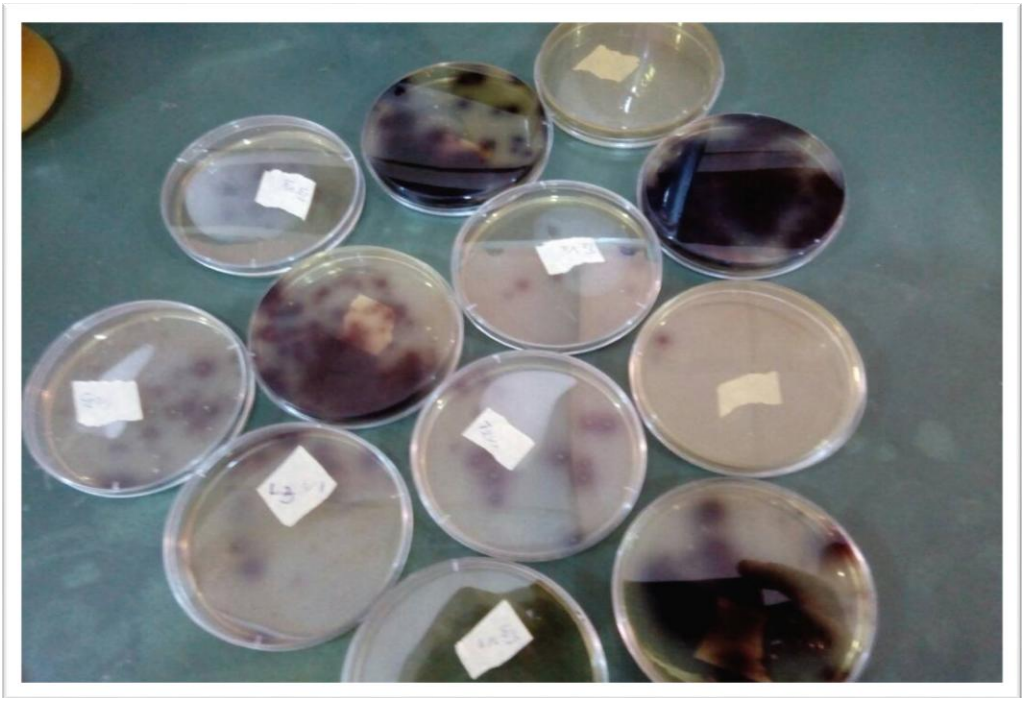

Fig. 1. Morphology features typical of *Listeria monocytogenes* colonial growth on selective agar

4. CONCLUSION

Based on the data collected and analyzed in this research, *Listeria monocytogenes* is present in most of the vegetables sold within Sokoto metropolis. The antibiogram pattern of *Listeria monocytogenes* isolated within the study area showed resistance to six antibiotics from a pool of eight (8) chemical agents. We recommend that environmental wastes which are sources of contamination to these vegetables should be treated before being used as fertilizer, and also proper diagnosis should be carried out on patients infected with Listeriosis to avoid multi-drug resistant issues. Human subjects infected with Listeriosis should as a matter of medical urgency get adequate diagnosis for proper treatment to avoid multi-drug resistance issues.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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