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Listeria species as contaminants of lettuce and its resistant genes in Benin city, Nigeria

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Abstract

This study was aimed at determining the level of Listeria contaminants and subsequently detects some resistance genes among the isolated *Listeria* species from lettuce sold in some vegetable markets in Benin City, Nigeria. Twenty-four lettuce samples were purchased from three vegetable markets in Benin City, Nigeria and examined using standard microbiological methods. Microbial characterization revealed *Listeria monocytogenes* and *L. grayi* as the predominant species isolated. Plate count analysis on Listeria selective agar revealed that lettuce sold in Oba market and Forestry market had the highest and lowest mean count of *Listeria* species 224.00 x 10^2 CFU/g and 83.00 x 10^2 CFU/g respectively. Most (63.75%) of the *Listeria* species isolates were found to be susceptible to Ofloxacin (5 µg), Ciprofloxacin (10 µg), Streptomycin (10 µg), Gentamycin (10 µg), Pefloxacin (5 µg) whereas species harbouring tetracycline (65%) and erythromycin (60%) resistant genes. The study provides an evidence of the colonization of *Listeria* species in lettuce sold in Benin City which may pose serious public health threat to the populace.

Keywords: *Listeria monocytogenes, Listeria grayi*, Lettuce, Resistance genes

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Introduction

Listeria is the causative agent for "Listeriosis" an infectious disease of public health significance with vulnerable groups as immunocompromised individuals (e.g. HIV/AIDS, patients on chemotherapy), pregnant women, new-born and the elderly (Farber, 1991). The genus *Listeria* comprises of 6 species: *L. monocytogenes*, *L. ivanovii*, *L. innocua, L. welshimeri, L. seeligeri, and L. grayi*. Though, only *L. monocytogenes* has been associated with outbreaks of human foodborne infection (Gilbert et al., 1999) and other life-threatening illnesses ranging from severe sepsis to meningitis/encephalitis. In December, 2017, a recent outbreak was reported in South Africa (WHO, 2018). Consequently, there have been threat alert to other African countries like Nigeria. The first documentation of *Listeria* in Nigeria by Eyo et al., (1969) emerged after Njoku-Obi and Njoku-Obi (1965) report using serological procedures among healthy blood donors. Subsequently, there emerged positive

reports of Listeriosis in Nigeria. The first confirmation of Neonatal Listeriosis in Nigeria associated with *L. monocytogenes* was reported by Onyemelukwe and Lawande (1982) with evidence of transmission from mother to new-born. Onyemelukwe et al., (1983) and Emele (2000) reported the association of *L. monocytogenes* in patients with meningitis and septicaemia from Nigeria. In 2002, *Shigella sonnei* and *Listeria monocytogenes* infection were linked to lettuce consumption in diet (salad) (Pelczar et al., 2006). Even though the incidence of Listeriosis is low in Nigeria with disparity in the prevalence (Mawak et al., 2008 and Ajayeoba et al., 2016), very high mortality rate ranging from 20% to 30% have been reported from other parts of the world (Mead et al., 1999). Although the main route of transmission is via consumption of contaminated food, other route such as mother to foetus transmission via placenta has been previously reported (Emele, 2000).

The intake of lettuce is increasing in several urban cities in Nigeria. Fresh lettuce is widely applied in dietary preparations like salads or sandwiches. Studies have shown that their plant tissue are internalised by some bacterial pathogens (Farber, 1991). Pondei and Ogbonna (2004) reported the isolation of *Listeria* from vegetables and irrigation water in Jos, Nigeria. Studies on food pathogens transmitted via fresh vegetables have reported the involvement of Gram-negative bacterial pathogens such as *Escherichia coli* and *Salmonella*. Consequently, there are few studies exploring the involvement of *Listeria* in contaminating fresh vegetables such as lettuce in Benin City, Nigeria. With evidence of the surfaces of raw vegetables contaminated with a range of microbes due to microbial population of the environment, mode of handling, time and condition of storage (Pelczar et al., 2006), it is therefore paramount to examine the microbial load of bacterial pathogens.

Antimicrobial resistance is currently the greatest challenge worldwide. It decreases the effectiveness of drugs that decrease morbidity and mortality associated with serious and life threatening infections and thus, compromising human health (Collignon et al., 2009). Food contamination with antimicrobial resistant bacteria can be a major threat to public health, as the antibiotic resistance determinants can be transferred to other bacteria of human clinical significance. Detection of resistance genes molecularly remains a mainstay however, there exist paucity in information on resistance genes among *Listeria*-contaminated lettuce in Benin City, Nigeria.

Therefore, this study is aimed at determining the microbial contaminants of *Listeria* species isolated from lettuce in vegetable markets in Benin City, Nigeria and their resistance genes using standard techniques.

Materials and Methods

Sample collection

Lettuce samples were purchased from three vegetable markets namely: Airport road market, Forestry market and Oba Market in Benin City, Edo State, Nigeria. Eight samples were collected from each market site for 4 weeks within the month of November 2015. Collected samples were collected in labelled sterile sample bags and transported in ice pack to the Microbiology Laboratory, Benson Idahosa University for analysis. A total of 24 lettuce samples were collected throughout the sampling period.

Identification and Serotyping of Listeria species

Isolation and Identification of isolates was done by cultural, morphological and biochemical test. Distinct colonies were picked from incubated plates and pure cultures were made following sub-culturing prior to biochemical test.

Serotyping was done using commercially prepared Listeria antiseria with Oxoid Listeria Test Kit (Oxoid, United Kingdom). A drop of saline was placed on a well of the reaction card, after which a distinct colony from the Listeria selective agar plate was collected using a sterile wire loop and emulsified in the drop. A drop of the test latex was added to the suspension and rocked for up to 2mins and examined for agglutination.

Principles: Polyvalent antisera are prepared against purified flagellin proteins from *Listeria monocytogenes* (antigens A, B and C) and *Listeria grayi* (antigen E) are used to coat latex particles. When mixed with a suspension containing *Listeria* species, the latex particles rapidly agglutinate to form visible clumps. The Oxoid Listeria Test Kit detects all motile strains of *Listeria* species.

Enumeration of Listeria species

Ten-fold serial dilution of each homogenized lettuce samples was made using 1% of sterile peptone water. This was serially diluted and were plated on Listeria selective agar (Oxoid, United Kingdom) using spread plate method and then incubated for 24-48 hours at 37^{o} C. Discrete Listeria colonies on each plate were counted and sub cultured onto freshly prepared agar plates after which pure cultures of the isolates were streaked onto tryptone soy agar.

Antibiotics susceptibility test

Antibiotics susceptibility test was performed on Listeria colonies by using the disc diffusion method as described by National Committee for Clinical Laboratory Standards (NCCLS, 2004). Antibiotics used include: Amoxycillin (AMX) 25µg, Ofloxacin (OFL) 5µg, Streptomycin (STR) 10µg, Chloramphenicol (CHL) 30µg, Ceftriaxone (CEF) 30µg, Gentamycin (GEN) 10µg, Pefloxacin (PEF) 5µg, Ciprofloxacin (CPX) 10µg, Erythromycin (ERY) 5µg-15µg.

The disc were transferred aseptically and placed unto Muller Hinton plates with a sterile forceps and then incubated at 37^{O}C for 24hours. Resistance was recorded when there were no clear zones of inhibition around the respective disc and sensitivity was recorded when there was presence of inhibition.

Extraction of DNA and detection of resistant genes

Genomic DNA of Listeria isolates were extracted after the bacteria was grown in tryptone soy agar for 24hours. The Listeria cells were harvested using 200µl nuclease free water after which it was subjected to denaturation by heating at 100°C for 10minutes then the supernatant containing the DNA was collected by centrifugation at 13,000x g for 1minute.

Genes coding for erythromycin resistance (EryB), daptomycin resistance (Mpr F), and tetracycline resistance (Tet M and Tet A) was carried out using PCR with the already published primers (Akortha and Egbule, 2008) and reaction outline listed in Table 1. The reaction mixture contained Inqaba PCR PreMix (Inqaba, South Africa) containing Tag DNA polymerase, dNTP, and $MgCl_2$. The reaction mixture was prepared in sterile 0.2 ml PCR tubes with 25 µl reaction volumes (12.5 µl PreMix, 8.5 µl nuclease free water, 0.5 µl forward primer, 0.5 µl reverse primer and 3.0 µl template DNA). Polymerase chain reaction products were separated in 1.5% agarose gel which was stained with ethidium bromide. Polymerase chain reaction (PCR) products on gel were visualized under ultra violet (UV) light.

Table 1: Primers and PCR reaction outline

Primer	Sequence (3^1-5^1)	Reaction
tetM forward	GTRAYGAACTTTACCGAATC	25 µl reaction volumes:
tetM reverse	ATCGYAGAAGCGGRTCAC	Initial denaturation: 94 °C for 4 mins
tetA forward	TTGGCATTCTGCATTCACTC	31 cycles of:
tetA reverse	GTATAGCTTGCCGGAAGTCG	Denaturation: 94 °C for 45 seconds
erm(B)-F	GAAAAGGTACTCAACCAAATA	Annealing:55 °C and 60 °C for 1 min
erm(B)-R	AGTAACGGTACTTAAATTGTTTAC	(Tet M/Erm B and Tet A/Mpr F)

mprF forward	TGCGGGTGGTCTTTACTTCC	Extension:68 °C for 1 minute
mprF reverse	CGCGAGCAAGTGTGTTGAAA	Final extension: 68 °C for 8 mins

Results

The results of *Listeria* species in some lettuce vegetables sold in Benin City is shown in Table 2. Among the three market locations sampled, Oba market had the highest microbial contaminated lettuce of *Listeria* species followed by Airport road market and Forestry market. *Listeria* species isolated in this study include: *Listeria monocytogenes* (90%) and *Listeria gravi* (10%). Biochemical characterization revealed the speciation of Listeria: *L. monocytogenes* (18) and *L. grayi* (2). All the *Listeria* species isolated were Gram positive rods, catalase positive, positive with Listeria antisera test kit, produced acid with fructose, glucose and xylose. All *Listeria species* were coagulase negative, oxidase negative while some produce acid with mannose and few were positive to OBIS mono test kit. Table 3 shows Antibiotic sensitivity pattern of *Listeria species* isolated from lettuce. Majority of the isolates were resistant to Amoxycillin, Chloramphenicol, Ceftriaxone, Erythromycin and Tetracycline but were sensitive to Ofloxacin (12mm-20mm) and Ciprofloxacin (13mm-25mm), Streptomycin (7mm-13mm), Gentamycin (8mm-15mm), Perfloxacin (12mm-20mm). The isolates demonstrated a high percentage resistance (45%-100%) to Amoxycillin, Chloramphenicol, Ceftriaxone, Erythromycin and Tetracycline [Figure 1].

Molecular study using polymerase chain reaction (PCR) showed the occurrence of antimicrobial resistant genes *in Listeria* species isolates from lettuce [Table 4]. Out of 20 *Listeria* species, 13 species representing 65%, were positive for Tetracycline genes: TetA and TetM respectively while 12 species representing 60% were positive for Erythromycin resistant gene (ErmB) and 2 species representing 10% were positive for Daptomycin resistant gene. Figure 2 shows the PCR band of the isolates after electrophoresis harbouring TET gene.

Table 2: Listeria counts from lettuce purchased from the different Vegetable Markets

		Listerial Mean Count (cfu/g)	
S/N	Location	Highest	Lowest
1	OM	224.00 X 10^2	13.33 X10^2
2	ARM	86.66 X 10^2	0.50 X10^2
3	FM	83.00 X 10^2	16.33 X10^2

Key: OM=Oba market; ARM=Airport road market; FM=Forestry market

Table 3: Antibiotic susceptibility pattern of *Listeria* species isolated from lettuce

	Number of *Listeria* species isolated		
Antibiotics (µg)	Sensitive	Intermediate	Resistant
Amoxycillin (25 µg)	0	0	20
Ofloxacin (5 µg)	16	2	2
Streptomycin (10 µg)	11	0	9
Chloramphenicol (30 µg)	2	1	17
Ceftriaxone (30 µg)	0	0	20
Gentamycin (10 µg)	12	0	8
Perfloxacin (5 µg)	12	2	6
Ciprofloxacin (10 µg)	16	2	2
Erythromycin (15 µg)	6	0	14
Erythromycin (5 µg)	2	0	18
Tetracycline (30 µg)	8	0	12

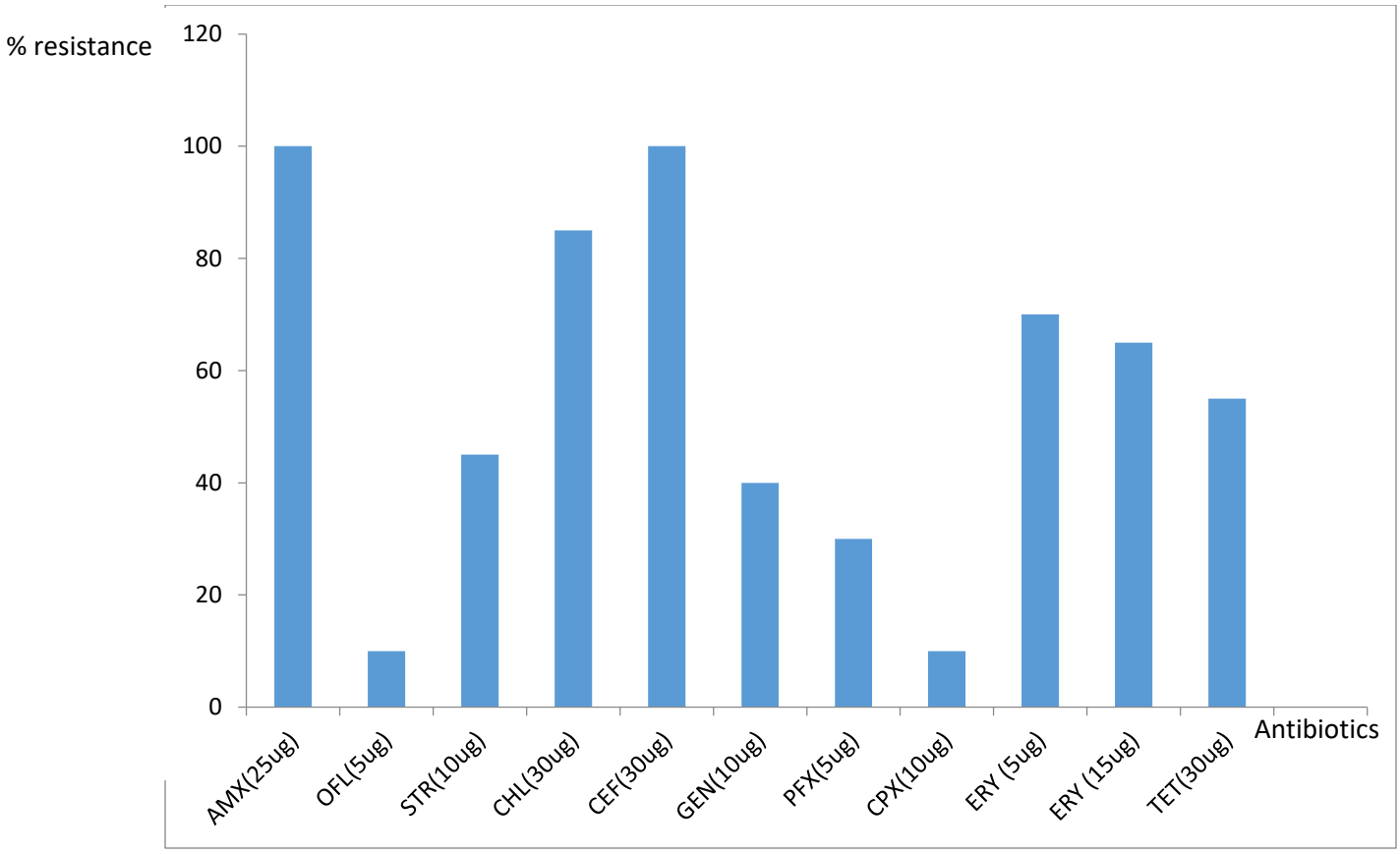

Figure 1: Percentage antibiotic resistance of *Listeria species* isolated from lettuce

KEY: Amoxycillin (AMX), Ofloxacin (OFL), Streptomycin (STR), Chloramphenicol (CHL), Ceftriazone (CEF), Gentamycin (GEN), Pefloxacin (PEF), Ciprofloxacin (CPX), Erythromycin (ERY 15-5µg), Tetracycline (TET)

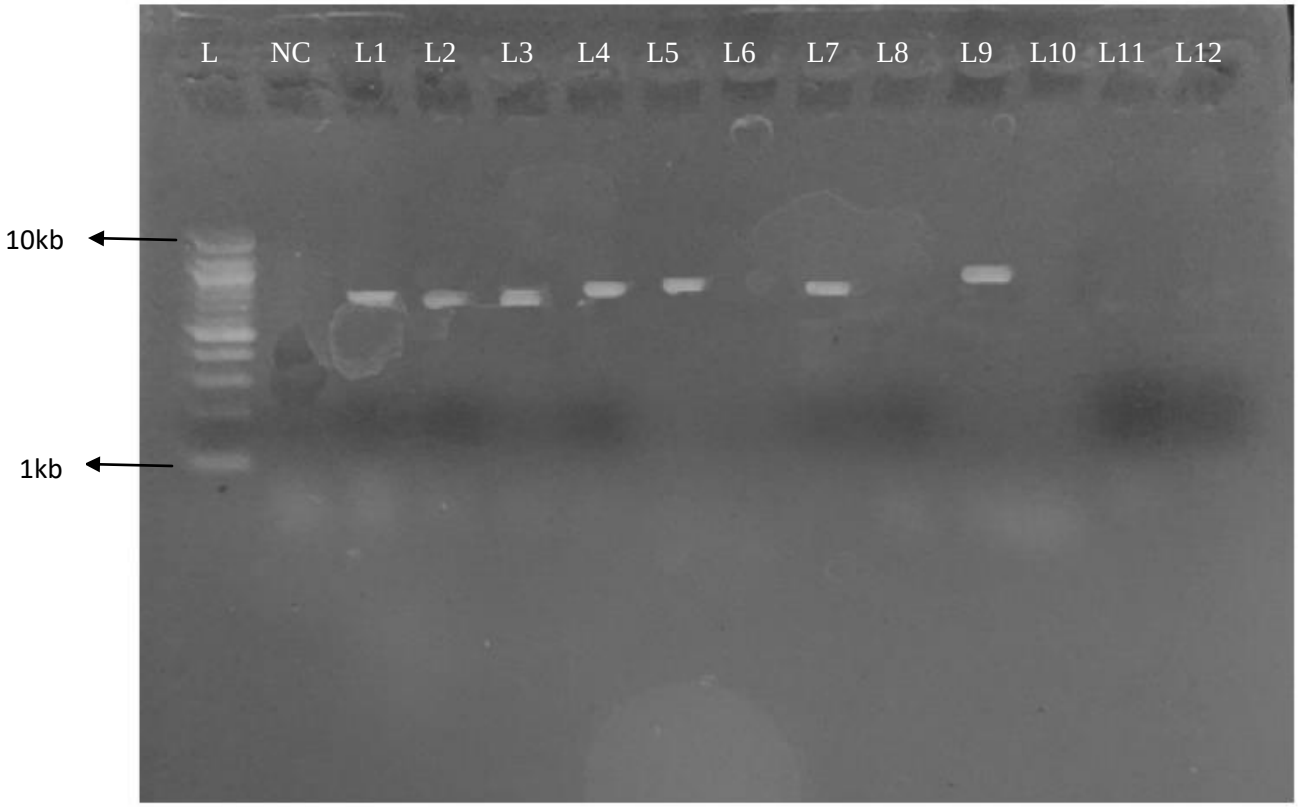

Figure 2: Polymerase chain reaction results for TET gene haboured by the isolates analyzed with 1.5% agarose gel electrophoresis stained with ethidium bromide. L (Ladder) is 1kb-10kb DNA ladder (molecular marker). Samples L1, L2, L3 L4, L5, L7 and L9 are positive for *Tet* gene. L6, L8, L10, L11 and L12 were negative for *Tet* gene. NC is a no DNA template control.

Table 4: Percentage occurrence of antimicrobial resistant genes among *Listeria* species isolated from Lettuce

Antibiotic resistant gene marker	No of *Listeria spp* tested	No of *Listeria spp* with genes
Tetracycline (Tet A)	20	13(65%)
Tetracycline (Tet M)	20	13(65%)
Erythromycin (Erm B)	20	12(60%)
Daptomycin (Mprf)	20	2(10%)

Discussion

The high microbial count documented in this study provides an evidence of high level of lettuce contamination by *Listeria* species. The standard limit of Listeria count for vegetables provided by the European Food Safety Authority has been reported as <100cfu/g (EFSA, 2014). The bacterial counts obtained in this study are significantly higher than the above standard. This is inconsistent with Ajayeoba et al. (2016) which reported *Listeria* species count below the standard limit which were isolated from lettuce sold in traditional markets in South-western

Nigeria. The discrepancy in both results could be due to contamination during handling and storage, since these lettuce are cultivated and transported from the Northern part of the country. Also, the practice of water application on the lettuce to sustain or keep the leaves fresh could contribute to the level of contamination. This present study collaborates the study of Rapeanu et al. (2008) who reported high *Listeria* species count from fresh lettuce sold in traditional markets and supermarkets in Romania with slightly similar identification and enumeration methods with our study. The recorded high microbial contamination of lettuce in this study could be attributed to low standard of personal hygiene, post-harvesting processing and poor environmental sanitation. Furthermore, contamination could have occurred during storage and transportation from Northern Nigeria (where lettuce are cultivated) or handling by lettuce vendor.

The high prevalence of *L.monocytogenes* in this study correlates with other studies done within and outside Nigeria on ready-to-eat vegetables (Little et al., 2007; Rapeanu et al., 2008; Ajayeoba et al., 2016). *Listeria* is considered to be intolerant to the temperature reached during processing (Rapeanu et al., 2008). Consequently, the presence of *Listeria monocytogenes* in lettuce samples investigated in this study could be due the temperature difference between lettuce and wash-water which permits movement of water into the plant tissue, unhygienic conditions during packaging, contamination from post-harvest processing and poor handling. Furthermore, the urban location of the markets could be a contributing factor which is consistent with study by Sauders et al. (2012) in New York which established an association between *L.monocytogenes* and urban environments. The unique feature of an urban city market is overcrowding which could justify the high distribution of *L.monocytogenes* in Oba market which attracts a large traffic of buyers being one of the oldest market in the city compared to other markets sampled. Baiyewu et al. (2007) associate overcrowding with high occurrence of *L.monocytogenes* in urban markets resulting from unhygienic handling of vegetables. Furthermore, the cosmopolitan nature of the organism serves as a contributing factor to its colonization of leaves and vegetables which could result from proper washing or cooking. To the best of our knowledge, there are no reports on *Listeria gravi* isolated from fresh lettuce sold in vegetables markets in Nigeria.

The drug resistance was low for the quinolones class of antimicrobial drugs (perfloxacin, ciprofloxacin, ofloxacin) in this study. This agrees with the report of Adamu et al. (2009) who observed a low resistance rate of isolates against quinolones. This depicts the fact that regional differences could influence the resistance profile of bacteria and further justifies the need to undertake regular antibiotic susceptibility studies on bacterial isolates from different geographical areas. The low resistance (45.1%) observed for Streptomycin in this study is in divergence with the high resistance rate (79%) reported by Iwaiokun et al. (2001) but substantiate the relative low resistance rate (54%) reported by Sheikh et al., (2003). The relative low level of resistance for Streptomycin antibiotic may perhaps be due to moderate use of the drug as it is currently recommended for the treatment of respiratory tract infections. This may have limited its misuse with subsequent decrease in resistance rate. The observed rate of resistance of isolates to gentamycin was quite low (40%). This correlates with the reports of Yah et al. (2007) and Akortha and Filgona (2009) who reported low resistance rates of 17.9% and 17.7% respectively for same antibiotic.

The relative low resistance rate observed for gentamycin could be attributed to the availability of the drug in ampules and thus intravenous form of administration thereby reducing its acceptability despite the fact that it is readily available over the counter unlike oral antibiotics. The results of this study confirms in vitro bacteriological efficacy of gentamycin as reported by Olukoya and Oni, (1990) and Iwaiokun et al. (2001). The high amoxicillin resistance rate (100%) observed in this study is comparable to that of other workers such as the resistance rate of 92.2% and 70% reported by Akortha and Egbule, (2008) and Diano and Akano (2009) respectively. The indiscriminate use of antibiotics with discontinuation of treatment course following disappearance of visible disease signs and symptoms prior to the

absolute eradication of the pathogen is some contributing factors that could promote the high rate of resistance. The difference in resistance pattern observed suggests the dynamic adaptation by bacteria in response to antibiotic treatment which occur readily.

Conclusion

Antimicrobial resistant *Listeria* species were readily detected with high microbial contamination on lettuce sold in vegetable markets in Benin City with the highest contamination from Oba market. Measures should be taken in educating food handlers and the general public on the hazard and risks associated with high microbial contamination of lettuce vegetables.

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