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Occurrence, Antimicrobial Susceptibility and Biofilm Production in *Listeria monocytogenes* Isolated from Pork and other Meat Processing Items at Oko-Oba Abattoir, Lagos State, Nigeria

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ABSTRACT

Unhygienic slaughtering practices are associated with meat contamination by pathogens including *Listeria monocytogenes*. This study investigated the occurrence, bacterial load, antimicrobial susceptibility and biofilm formation of *L. monocytogenes* in pork, slaughter facilities and water from Oko-Oba abattoir, Lagos State, Nigeria. A total of 216 samples; 146, 30, 20 and 20 from pork, water, table swabs and knife swabs respectively were collected for bacteriological analysis. *Listeria* selective media was used to isolate and characterise *L. monocytogenes,* antimicrobial susceptibility of the isolated bacteria was also determined. Biofilm production by the isolates for periods of 12, 24 and 36 hours were evaluated. Glucose, ethanol and crystal violet binding assay were used as nutrient, stressor and indicator respectively. The occurrence of *L. monocytogenes* in pork, water samples, table and knife swabs were 64.5%, 75%, 90% and 95% respectively, while overall prevalence was 73.3%. The mean load per gram were 3.78 ± 2.85, 4.27 ± 2.54, 5.60 ± 1.95 and 5.52 ± 1.41 ($p < 0.05$) for pork, water table and knife swab samples, respectively. Fifty five percent of the isolates exhibited multidrug resistance (MDR) patterns to erythromycin (55%), tetracycline (55%), augmentin (85%), amoxicillin (90%), cotrimoxazole (95%), and cloxacillin (100%). The isolates produced biofilms at different concentrations of glucose and ethanol with the highest concentrations of biofilm produced at 36 hours. There were significant differences in mean biofilm values produced by the isolates. The occurrence of *L. monocytogenes* and the bacterial load in different samples are of economic and public health importance. Hygienic meat production from the abattoir could prevent contamination of pork by *L. monocytogenes*.

Keywords: Antimicrobial resistance; Biofilm; *Listeria monocytogenes*; Pork

INTRODUCTION

According to Food and Agricultural Organization and other reports, pork is the most commonly consumed meat worldwide, accounting for about 38% of meat production globally, although consumption varies widely from place to place (WHO, 2004; Shahbandeh, 2019). *Listeria monocytogenes* has been recognized as an important food-borne pathogen that causes listeriosis (Mead *et al*., 1999; Anonymous, 2000; Swaminathan and Gerner-Smidt, 2007; Tan *et al*., 2013). Listeriosis is rare, but its fatality rate is very high (about 16%) compared with 0.5% for *Salmonella*, making it the third leading cause of death by foodborne illnesses (WHO, 2004). *L. monocytogenes* is an opportunistic intracellular pathogen that has become an important cause of human food borne infections worldwide (Liu 2006; Williams *et al*., 2011). Pregnant women, new born and immuno-compromised persons are at great risk of listeriosis (Walter, 2000).

Listeria monocytogenes is the most important *Listeria* specie in terms of public health risk and frequency of appearance in foodstuffs (Kayode *et al*., 2019). It causes encephalitis, septicaemia and abortion in humans and animals (Jeffers *et al*., 2001; WHO, 2018). Environmental contamination of foodstuffs such as; meat, milk, fish, and cheese are the main transmission route of the bacteria (Vazquez Boland *et al*., 2001; Evans *et al*., 2004; Lappi *et al*., 2004; Kayode *et al*., 2020). Although not commonly isolated from swine faeces (Buncic, 1991; Lida *et al.,* 1998; Stein *et al*., 2018), pork meat products have been linked to human infections (De Valk *et al.,* 2001; Tchatchouang *et al*., 2019). Contamination of the slaughter and processing environment have been traced back to healthy carrier swine (Giovannacci *et al.,* 2000; Kayode *et al*., 2020).

Many bacteria are able to attach to and colonize environmental surfaces by producing three-dimensional matrix of extracellular polymeric substances (EPS) called biofilm (Poulsen, 1999; Dieltjens *et al*., 2020). Biofilms are a collection of microorganisms surrounded by amorphous secretion that is not responsive to antibiotics and host immune clearance (Chen and Wen, 2011; Vestby *et al*.,

2020). Bacteria have the ability to attach, colonize and form biofilms on a variety of surfaces (Donlan, 2002; Kassinger and van Hoek, 2020) this increases their resistance to antimicrobial/sanitizing agents, desiccation and UV light. Bacterial adhesion to surfaces is the initial step in biofilm formation. Bacterial cells in biofilms may be up to one thousand times more resistant to antibiotics than in their planktonic condition (Ouyang *et al*., 2012; Dieltjens *et al*., 2020). *Listeria monocytogenes* attaches to numerous surfaces and equipment and forms biofilms that serve as protective layers of protein and polysaccharide surrounding the bacteria (Kalmokoff *et al.,* 2001; Sharma *et al*., 2019; Kassinger and van Hoek, 2020). Biofilms can allow *L. monocytogenes* to persist in the food processing environment for a long period of time (Donlan, 2002; Kassinger and van Hoek, 2020). Although extensive research has been conducted on *L. monocytogenes* biofilms, the control of biofilms is still a major problem in the food industry (Giovannacci *et al*., 2000; Rothrock *et al*., 2019).

The most important aspect of *L. monocytogenes* in food hygiene is its ability to survive in a wide range of temperature and make biofilms on various environmental surfaces which serve as natural habitats or reservoirs (Adetunji and Isola, 2011; Iwu and Okoh, 2020). *Listeria* may be transmitted in ready to eat foods that have been kept properly refrigerated as a result of its survival ability at temperatures below freezing (-7ºC) (Optimal growth at -18ºC to 10ºC, a temperature range used for refrigeration), thereby presenting a major challenge in its control (Rothrock *et al*., 2019).

Listeria monocytogenes as well as other *Listeria* species, are susceptible to most antibiotics, except cephalosporins and fosfomycin (Iwu and Okoh, 2020). Antibiotic resistant and multidrug resistant strains were first reported in France in 1988 (Poyart-Salmeron *et al.,* 1990). Since then, *Listeria* species isolated from food, environment or sporadic cases of human listeriosis have shown resistance to one or more antibiotics (Adetunji and Isola 2011; Iwu and Okoh, 2020). The drug of choice for treating listeriosis is ampicillin or penicillin G combined with an aminoglycoside classically, gentamicin (Al-Mashhadany, 2019). The combination of trimethoprim with a sulfonamide, such as sulfamethoxazole in cotrimoxazole is considered to be a second choice of therapy (Charpentier and Courvalin, 1999; Kemnic and Coleman, 2019). Unfortunately, gentamicin-resistant clinical strains of *L. monocytogenes* were reported in 1997 (Walsh *et al.*, 2001). Clinical strains of *Listeria* resistant to streptomycin, erythromycin, kanamycin, sulfonamide and rifampin were also reported from around the world. Multiple drug resistance was also observed in strains isolated from foods in some countries (Poros-Gluchowska and Markiewicz, 2003; Prieto *et al*., 2016).

Unhygienic pig slaughtering environment and meat dressing surfaces and equipment harbouring bacterial biofilm could serve as sources of pork contamination which could pose public health threats to consumers of pork in Nigeria. This study aimed at determining the occurrence, antimicrobial susceptibility and biofilm formation of *Listeria monocytogenes* isolated from pork, slaughter facilities and water at Oko-Oba abattoir in Lagos State, Nigeria.

MATERIALS AND METHODS

Study Area

The sample location was Oko-Oba abattoir, the central meat processing abattoir in Lagos State on coordinates of 6º35´N; 3º45´E. It has facilities for food animal slaughtering with an average of 30 pigs slaughtered daily. The breed predominantly slaughtered was large white.

Sample Collection

A total of 216 samples comprising of 146 and 30 samples from pork and water including 20 samples each from table and knife swabs were collected randomly and used for the study. About one gram of pork, 10 ml water sample, table and knife swabs were aseptically collected for bacteriological analysis using sterile 10 ml sample collection bottles and swab sticks. Samples were transported to the Food and Meat Hygiene Laboratory of the University of Ibadan in insulated container on ice packs within 3-4 hours of collection.

Isolation and Phenotypic Characterization of *Listeria monocytogenes* from Pork, Water, Table and Knife swabs

The pork muscle tissue (1 g), 1 ml water sample, knife swab and table swabs were each inoculated into 9 ml buffered peptone water (BPW) (Oxoid Limited, Thermo Fisher Scientific Inc., UK) as pre–enrichment broth and incubated at 37 ºC for 18-24 hours. Thereafter, 0.1 ml each of the BPW was inoculated into 5 ml of *Listeria* Selective enrichment broth (LSEB) (Oxoid Limited, Thermo Fisher Scientific Inc., UK) and incubated at 37ºC for 18-24 hours (Jones and D'Orazio, 2013). This was later serially diluted to the 5-fold dilution containing 9 ml of normal saline using 1 ml of cultured *Listeria* Selective enrichment broth. After this, 0.1 ml from the serially diluted culture was plated on *Listeria* Selective Agar (Oxoid Limited, Thermo Fisher Scientific Inc., UK) and incubated at 37ºC for 18-24 hours. Distinct colonies of *L. monocytogenes* having brown coloured colonies with aesculin hydrolysis were subjected to gram stain and biochemical tests. Isolates that fermented glucose, lactose, rhamnose, and were positive for catalase, motility, Voges Proskauer and methyl red tests but did not ferment sucrose, araninose and mannitol in addition to been negative for oxidase and urease tests were stored in nutrient agar slants for antimicrobial sensitivity test.

Antimicrobial Susceptibility Test

Disc diffusion susceptibility method was used for antimicrobial sensitivity test (CLSI, 2016). Distinct *L. monocytogenes* colonies of each isolate from nutrient agar slants were sub cultured on *Listeria* Selective Agar**,** pure colonies in lysogenic broth solution was adjusted optically to 0.5 McFarland. The adjusted solution was inoculated on Mueller Hinton agar using sterile swab stick and gram positive antimicrobial discs (comprising commonly used antimicrobial agents in the pig industry in the study area: 10µg streptomycin, 10µg gentamicin, 10µg tetracycline,

10µg chloramphenicol, 5µg erythromycin 25µg cotrimoxazole, 5µg cloxacillin and 30µg augmentin), were placed aseptically on the surface of the plate and incubated at 37 ºC for 24 hours before the zones of inhibition were measured to the nearest millimetre using a transparent ruler. Inhibition zones were recorded and interpreted according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2016).

Biofilm Production and Quantification

One distinct colony of *L. monocytogenes* was inoculated into tryptone soy broth (TSB) and incubated at 37 ºC for 24 hours (Lee *et al*., 2013). Serial dilution of 1:40 of the 24-hour broth culture with glucose (1%, 5% and 10%) and ethanol (1%, 3% and 5%) were made. 50 µl of each dilution was added to the micro-titre plates in triplicates. Negative control was TSB without any test strain while TSB with confirmed *L. monocytogenes* (NGA 34) (Usman *et al*., 2016) was used as the positive control. Micro-titre plates were incubated at 37 ºC for 12, 24 and 36 hours (there were three different micro-titre plates for glucose and ethanol each for these hours of incubation). At 12 hours, cultures with different concentrations of glucose and ethanol were poured out of the plates and washed three times with distilled water to remove the unbound *Listeria* cells. The plate was then air dried and heat fixed by gently passing the plate over the flame quickly. Each well was stained with 100 µl of crystal violet for 20 - 30 minutes, excess stain was washed off with distilled water and allowed to air dry. One hundred microliter (100 µl) of 95% ethanol was added to the stained wells to remove the bound cells. Biofilm formation (Optical density) was determined using ELISA reader at the absorbance of 600nm wavelength. The procedure was repeated at 24 and 36 hours, respectively.

Data Analyses

Data were analysed and presented using One-Way Analysis of Variance (ANOVA) and Descriptive statistics. The confidence level was taken at $p < 0.05$.

RESULTS

Ninety-four (64.4%) of the 146 pork tissue samples collected were positive for *L. monocytogenes* which was 43.5% of the overall samples collected. Twenty-three (77%), 19 (95%) and 18 (90%) of 30, 20 and 20 water, knife and table swabs collected respectively were positive for *L. monocytogenes*. The occurrence of *L. monocytogenes* of 10.7%, 8.8% and 8.3% were recorded for water, knife and table swabs respectively while an overall occurrence of 71.3% was recorded for *L. monocytogenes* in the study (Table 1).

The 146 pork samples had between 0 - 6.92 log cfu/g *Listeria* load with a mean value of 3.78 ± 2.85 log cfu/g. Mean *Listeria* load in the 30 water samples was 4.27 ± 2.54 log cfu/g (0 - 6.28 log cfu/g). The mean *Listeria* load in table swabs was 5.60 ± 1.95 log cfu/g (0 - 6.78 log cfu/g) while the mean *Listeria* load in knife swabs was 5.52 ± 1.41 log cfu/g (0 - 6.49 log cfu/g) (Table 2).

Ninety five percent of the *Listeria monocytogenes* isolates were sensitive to gentamicin and 80% to chloramphenicol, 45% were sensitive to both erythromycin and tetracycline, 15% to augmentin, 10% to amoxicillin, and 5% to cotrimoxazole. All (100%) the isolates were sensitive to cloxacillin (Figure 1).

Table 1: Occurrence of *L. monocytogenes* Isolated from Pork Tissue, Water, Table and Knife swabs at Oko Oba abattoir, Lagos

S/No.	Sample	Number of Samples Collected (n)	Number / (%) positive for *L. monocytogenes* within the sample	Prevalence (%)
1.	Pork tissue	146	94 (64.4)	43.5
2.	Water	30	23 (77.0)	10.7
3.	Knife swab	20	19 (95.0)	8.8
4.	Table swab	20	18 (90.0)	8.3
	Total	216	154	71.3

Table 2: The Bacterial Load of *L. monocytogenes* Isolated from Pork Tissue, Water, Table and Knife swabs at Oko Oba abattoir, Lagos

S/No.	Samples	Number of samples collected	*Listeria monocytogenes* count (Log cfu/g) Mean±S.D.
1.	Pork tissue	146	3.78±2.85
2.	Water	30	4.27±2.54
3.	Table swab	20	5.60±1.95
4.	Knife swab	20	5.52±1.41
	Total	216	4.26±2.68

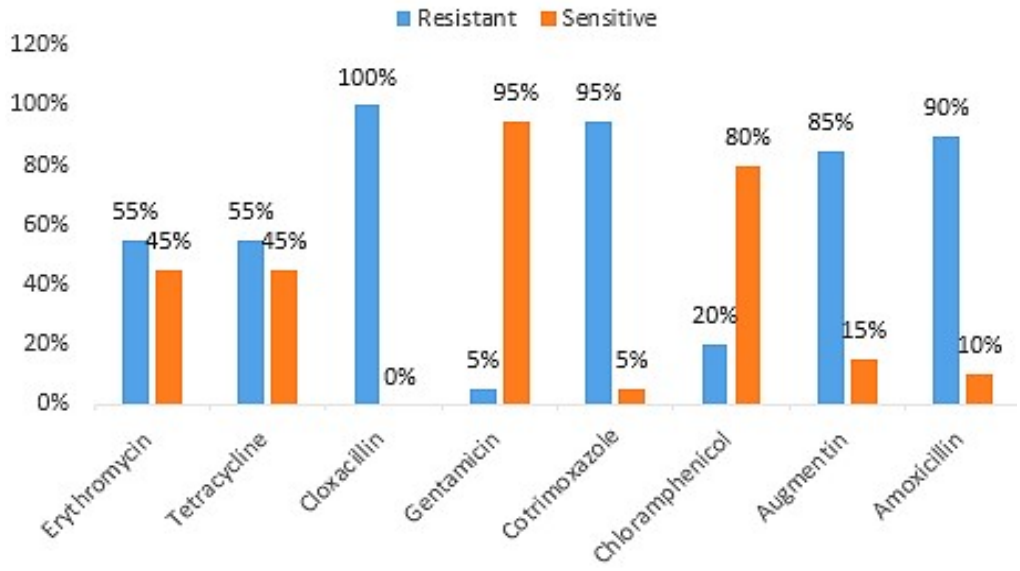

Figure 1: Susceptibility Profiles of *L. monocytogenes* Isolated from Pork Tissue, Water, Table and Knife Swabs at Oko Oba abattoir, Lagos

Table 3: Mean Values of Biofilm Formation in *Listeria monocytogenes* Isolated from Pork Tissue, Water, Table and Knife Swabs at Oko Oba abattoir, Lagos

Dilution Factor	Mean±S.D.					
	12 hours		24 hours		36 hours	
	Glucose	Ethanol	Glucose	Ethanol	Glucose	Ethanol
First	0.074 ± 0.015	0.064 ± 0.011	0.066 ± 0.010	0.066 ± 0.015	0.098 ± 0.034	0.096 ± 0.038
Second	0.066 ± 0.017	0.059 ± 0.017	0.077 ± 0.031	0.058 ± 0.023	0.094 ± 0.052	0.084 ± 0.024
Third	0.061 ± 0.018	0.054 ± 0.006	0.063 ± 0.024	0.087 ± 0.076	0.079 ± 0.046	0.109 ± 0.058
Control	0.058 ± 0.014	0.055 ± 0.010	0.055 ± 0.007	0.055 ± 0.007	0.063 ± 0.009	0.063 ±0.009

Listeria monocytogenes strains were observed to differ in the amount of biofilm production at 12, 24 and 36 hours respectively with crystal violet binding plate assay used to detect differences in biofilm-forming ability. The range of the mean values of biofilm formation for the first, second and third concentrations were; 0.064 at 12 hours to 0.098 at 36 hours, 0.058 at 24 hours to 0.094 at 36 hours and 0.054 at 12 hours to 0.063 at 36 hours respectively (Table 3). At 12 hours, biofilm formation was observed in all glucose and ethanol concentrations with 1% glucose and 1% ethanol showing the highest values. At 24 hours, an increase in biofilm value in 5% glucose was observed with 10% ethanol having the highest increase in biofilm values. At 36 hours, there was an increase in biofilm values in all sugar and ethanol concentrations. Crystal violet binding assay revealed that higher biofilms were formed with the availability of glucose and when the isolates were placed under the stress of varying concentrations of ethanol (Table 3).

DISCUSSION

The results obtained in this study showed that the muscle tissue could have been contaminated from the processing surfaces and water. Autio *et al.* emphasized equipment used as a point of cross contamination of *L. monocytogenes* (Autio *et al.,* 1999) which is similar to the findings of prevalence of processing surfaces in this study. The high occurrence of *L. monocytogenes* in the processing surfaces indicated poor hygienic and sanitary conditions in the slaughter slab, this could be due to insufficient public health education in addition to the poor attitude of abattoir workers to hygiene despite the provision of basic needs of the abattoir workers by the government. Weak enforcement of good operational practices by the government, government agencies and veterinarians could also contribute to the poor hygienic state of the abattoir. The prevalence of 20% reported for *L. monocytogenes* from pork carcasses by Hellstrom *et al.* (2010), this was lower than the 64.5% pork contamination in present study (Hellstrom *et al*., 2010).

The overall mean bacteria count of all the samples obtained ranged from 3.78 to 5.60 log cfu/g which is higher than the recommended international limit of 10^2 cfu of *L. monocytogenes* per g (2 log cfu/g) (Boes *et al.*, 2005).

Since the first report of antibiotic resistance of *Listeria* strains (Polyart-Salmeron, 1990), there has been a continuing emergence of multiple resistant *Listeria* strains isolated from food, meat, environment and clinical isolates (Charpentier *et al*., 1995). The findings of an earlier work done by Adetunji and Adegoke (2008) on *L. monocytogenes* isolated from local cheese (Wara) reported augmentin as a highly sensitive drug which differs from this study in which

the organism was 85% resistant to augmentin. The complete resistance (100%) shown by cloxacillin indicated that the antibiotics could be ineffective in the treatment of listeriosis, this is similar to reports from a study in Ado-Ekiti, Nigeria where environmental isolates were resistant to cloxacillin (David and Odeyemi, 2007). The low sensitivity of cloxacillin, cotrimoxazole and amoxycilin indicated the possibility of multidrug resistance in some of the *L. monocytogenes* isolated in this study. The zoonotic implication of resistant *Listeria* isolated from this study corroborates the report by Poyart-Salmeron et al. in which *L. monocytogenes* isolated from a patient with meningo-encephalitis was resistant to erythromycin, streptomycin and chloramphenicol although they were sensitive to gentamicin (Poyart-Salmeron *et al.*, 1990). The high rates of resistance found in this study can be explained by the widespread use of antimicrobial agents given to swine as prophylaxis, growth promoters or treatment (Williams and Nadel, 2001; Hao *et al*., 2014).

The biofilm assay revealed that *L. monocytogenes* is resistant to varying concentrations of ethanol as a result of the formation of biofilms which may be responsible for their persistence in pork and food processing surfaces and therefore requires rigorous and thorough disinfection. The *L. monocytogenes* strains that were observed to differ significantly in the amount of biofilm production at 12, 24 and 36 hours is similar to the findings of Adetunji and Odetokun that reported higher biofilms formation with the availability of nutrients particularly glucose and to some extent blood (Adetunji and Odetokun, 2012). This study further revealed that higher biofilms were formed when the isolates were stressed with varying concentrations of ethanol. The Department of Food and Environmental Hygiene of the University of Helsinki that investigated how *L. monocytogenes* can become a persistent contaminant in food processing plants and the possibilities for adherence to surfaces discovered that persistent *Listeria* strains adhered faster to surfaces like stainless steel than non-persistent strains (Lundén and Korkeala, 2004).

Conclusion

The occurrence of multidrug resistant strains *L. monocytogenes* in this study is of public health concern. Education of pig farmers by public health practitioners and the enforcement of laws controlling the use of antimicrobial agents in the livestock industry by the government and her agencies is recommended.

Conflict of Interest

The authors declare that they do not have any conflict of interest.

Authors Contributions

OIO, AJO and AOA designed the research, AOA, OIO and OOO carried it out while OIO, AJO and OOO were involved in the analyses, writing and proof reading of the manuscript.

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