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Isolation and Molecular Detection of *Listeria monocytogenes* from FRESH AND LOCALLY FERMENTED MILK (*Kindirmu*) in Maiduguri Metropolis, Borno State, Nigeria

[1]Adama A. Bwala, [2]Jasini A. Musa & [3] Aisha Issa
[1]Department of Science Laboratory Technology, Ramat Polytechnic, Maiduguri, Nigeria
[2]Department of Veterinary Microbiology, University of Maiduguri, Borno State
[3]Nigerian Institute of Medical Research, Maiduguri Outstation, Borno State

Abstract: *This study was carried out to isolate and detect Listeria monocytogenes and its associated virulence genes from fresh and locally fermented milk (kindirmo) in Maiduguri metropolis, Borno State, Nigeria. A total of 280 samples of fresh (120) and locally fermented (kindirmo) (160) milk were collected from three dairy farms and four points of sales. The samples were analysed using standard bacteriological techniques and molecular protocols. The detection of Listeria species using conventional biochemical test showed the distribution 2 (50.0%) from fermented milk and 10 (11.2%) from fresh milk giving the total 12% β-hemolysis on sheep blood agar, Catalase positive, Gram positive rods and Oxidase negative which are suggestive of L. monocytogenes and there was significant association between the milk sample types. Of all the 12% of presumptive L. monocytogenes subjected to multiplex polymerase chain reaction, only 67% of L. monocytogenes were found to be associated with one or more virulence genes of L. monocytogenes; Iap, InA, ActA, and hlyA at molecular weights of 131 bp, 255 bp, 268 bp and 702 bp respectively. This gives a detection rate of 2.9% of L. monocytogenes. The detection of L. monocytogenes associated virulence genes in milk is an indication that the isolates are pathogenic and therefore may pose a risk to public health. It is therefore recommended that adequate hygienic measures be observed during milking, preparation, retail and consumption of fresh and locally fermented milk in Maiduguri Metropolis, Borno State, Nigeria.*

Keyword: *Listeria monocytogenes, Listeriosis, Fresh milk, Pasteurization, Contamination*

INTRODUCTION

Listeria monocytogenes (*L. monocytogenes*) is of global public health concerns due to its ability to cause serious disease such as miscarriage in pregnant women and mortality in immune-compromised individuals associated with cancer, renal and heart diseases, as well as organ transplant patients (Jamali *et al*., 2013; Boujemaa *et al*., 2013; Usman *et al*., 2016a; Lennox *et al*., 2017). *Listeria monocytogenes* is a gram positive, non-spore forming rods, facultative anaerobic and catalase positive

that are sometimes arranged in short chains (Farber and Peterkin, 1991; Alzubaidy *et al*., 2013). Haemolytic activity on blood agar has been used as a marker to distinguish *L*. *monocytogenes* from other *Listeria* species. It is a bacterium that causes listeriosis, a disease that is highly fatal (Todar, 2012; FDA, 2013; David, 2017).

Listeriosis being an emerging infection worldwide associated with food borne outbreaks and significant risk of mortality and morbidity, prompted the Centre for Disease Control and Prevention (CDC) to evaluate the case of 2,500 and more than 500 deaths related to listeriosis in the United States of America (Beumer and Hazelzer., 2013; Gohar *et al.*, 2017). Although there were no reported cases of listeriosis in Nigeria, the prevalence case of 6.6% detection rate of *L .monocytogenes* in milk from Kaduna, Nigeria was reported by Usman *et al* (2016a), similarly, Faeji *et al.* (2016) also isolated 6.9% *L*. *monocytogenes* in fresh milk samples in Northern, Nigeria and 11% detection rate in milk from Jos, Nigeria. . More recently, in South Africa, there was an outbreak of listeriosis from January 2017 through March 2018, 879 laboratory confirmed cases were reported to National Institute of communicable Diseases (NICO) from all provinces. The outcome of illness is known for 674 patients of whom 183 (27%) of them died (WHO, 2018). Most of the cases are persons who have higher risks from severe disease outcome such as neonates, pregnant women, the elderly and immune-compromised persons (WHO, 2018). Another confirmed case of listeriosis was reported in Namibia, in this outbreak, 42% of the cases were neonates who are infected during pregnancy or delivery (WHO, 2018). A multi-state outbreak of *L. monocytogenes* affected nine States in the U.S.A, in 2016, nineteen of the infected persons were hospitalized and one person died of listeriosis from Michigan.

Listeria monocytogenes can be found throughout the environment and from domestic and wild animals, birds, soil, vegetation, fodder, water from floor drains and wet areas of food processing factories (Todar, 2012; Markey *et al*., 2013; Lakicevic *et al*., 2015). Additionally, it has been found in milk, processed foods, uncooked vegetables and fruits such as apples (FDA, 2013; Nwaiwu, 2015). Pasteurization and sufficient cooking is inimical to *Listeria*, however, contamination may occur after cooking and before packaging especially in processing plants producing ready to eat foods such as hot dogs and deli meats. (Alzubaidy *et al*., 2013; Nwaiwu, 2015)

The organism grows over a wide range of temperature from 1^{o}C-45^{o}C, with an optimum temperature around 30^{o}C to 37^{o}C. *Listeria monocytogenes* can grow at pH values between 4.4 and 9.4 and become more sensitive to acidic condition at higher temperature (FDA, 2013; Alzubaidy *et al.,* 2013; Bertrand *et al*., 2016). Like most bacterial species, *L. monocytogenes* grows optimally at a water activity of 0.90 and 0.97 with sodium chloride as solute. The bacterium is resistant to various environmental stresses, such as highly salty or acidic solutions which allows it to survive longer under stressful condition than non-spore forming bacteria of food borne disease. It has the ability to form biofilms which contribute to its ability to colonize food processing facilities (FDA, 2013; Lakicevic *et al.,* 2015). The organism also has a multi factorial virulence system, with the thiol activated haemolysin, listeriolysin O (LLO) being identified as a crucial role in the organisms' ability to multiply within host phagocytic cells and to spread from cell to cell (Farber and Peterkin, 1991; Markey *et al*., 2013). Extensive sanitation policies and procedure should be strictly observed as preventive measures to avoid *Listeria* contamination (Todar, 2012).

Symptoms may begin a few days after eating contaminated food, but it may take as long as 30 days or more before the onset of first signs and symptoms of infection begin, which include; headache, fever, muscle aches, nausea and diarrhea (Mayo Clinic, 2017). If *Listeria* infection spreads to the nervous system, signs and symptoms may include headache, stiff neck, confusion or changes in alertness, loss of balance and convulsions in pregnancy. *Listeria* infection is likely to cause only mild signs in the mother and the consequences may lead to the death of baby prior to parturition (Mayo Clinic, 2017, Zhu *et al*., 2017). It is unique among food borne pathogens, since the incubation time from ingestion

of *Listeria* cells to illness is at least seven days (Jones, 2010). Listeriosis is a rare disease with high mortality rate causing about 43% of food poisoning associated with death in the US (Zhu *et al*., 2017).

The common route of transmission of *L. monocytogenes* to human is via consumption of contaminated food. However *L. monocytogenes* can be transmitted directly from mother to child or from contact with animals and through hospital acquired infection (Nosocomial infection). Healthy individuals can be asymptomatic carriers of *L. monocytogenes* (Nwachukwu and Orji, 2012; FDA, 2013). Human to human transmission of *Listeria* infection is caused by ingestion of bacteria, most often through the consumption of contaminated food (Momtaz and Yadollahi, 2013; Marler, 2017).

Despite an increasing rate of outbreaks of listeriosis in recent years in the United States,

Canada, China and some African countries, the occurrence and prevalence of the sorganism in food borne diseases in Nigeria is scarcely reported. There is limited information on the status of food borne listeriosis caused by *L. monocytogenes* in Nigeria especially in Maiduguri Metropolis associated with fresh and locally fermented milk. Therefore, this study will provide information on isolation and molecular detection on *L. monocytogenes* from fresh and locally fermented milk (*kindirmu*) in Maiduguri Metropolis

MATERIALS AND METHODS

The Study Area

The study was conducted in Maiduguri the capital city of Borno State in North-eastern Nigeria. Maiduguri lies between Latitude 11.46^{o} N to 11.54^{o} N and longitude 13.04^{o} E to 13.14^{o} E (Figure 3.1) and situated at an elevation of 300 meters above sea level. Maiduguri has a population of 1,112,449 making it the biggest city in Borno State. The climate of Maiduguri is generally hot, dry, windy, and dusty for most part of the year with an average rainfall of 613 mm per annum (that starts in June and ends in September), and temperature range of 25.8^{o}C to 40^{o}C (NIMET, 2018). The major occupation of the people is farming, livestock rearing, trading and fishing.

Sampling Technique

A convenience sampling technique was employed in this study, where samples were collected based on availability. Fresh and locally fermented milk samples were obtained directly from farms and different sales points. Samples were collected on a weekly basis for the period of two months (May to June 2019). The samples were analysed using standard bacteriological techniques and molecular protocols.

Sample Collection, Packaging and Transportation

A total of 280 samples of fresh and locally fermented milk (*kindirmu*) were collected from three cattle dairy farms and four points of sales in Maiduguri Metropolis. Forty samples of fresh milk each from three different farms and forty samples of locally fermented milk (*kindirmu*) from four selected points of sales were collected. All samples were labeled accordingly, placed in cool box containing ice packs, and transported to the Veterinary Microbiology Laboratory, Department of Veterinary Microbiology, University of Maiduguri for processing

Isolation and Identification of *Listeria* species

Isolation of *Listeria* species on Laboratory Media

Listeria species were isolated according to the procedure described by Food and Drug Administration; Bacteriological Analytical Manual (FDA-BAM, 1997). One millilitre of milk sample was aseptically added to 9 ml of *Listeria* enrichment broth (LEB) containing selective *Listeria* enrichment supplement in a conical flask then incubated at 37°C for 24 hours. A loop full of this enrichment culture was streaked on to *Listeria* selective agar (Oxoid SR 141E) and incubated at 37°C for 24 hours. The culture plates were examined for grayish colonies with black halos and sunken centre as described by Ribeiro and Carminati (1996).

Haemolysis on Blood Agar

The *Listeria* species suspected isolates were grown on 5% sheep blood agar to obtain pure colonies and a clear zone of clearing around the colonies upon incubations at 37°C for 24 hours. The β-haemolysis was observed by producing narrow zones of haemolysis.

Phenotypic Identification of *Listeria* species

Gram Staining

Presumptive colonies based on colony morphology were subjected to gram staining based on the method of Cheesbrough (2000). A smear was made on a slide from the isolates and heat-fixed, crystal violate stain was then poured on the smear. The smear was allowed to stand for 1 minute and rinsed with tap water. Lugols iodine solution was added to the smear for 1 minute and rinsed with tap water before it was decolourized with 95% alcohol for 2 seconds and then counterstained with safranin for one minute. The slide was rinsed with water and allowed to air dry and viewed under the microscope using the oil immersion objectives lens at X100 magnifications.

Biochemical Tests

Conventional biochemical tests were carried out according to the method described by Alzudaiy *et al*. (2013**)**. Catalase, oxidase and sugar fermentation tests (Glucose, sucrose and maltose) were performed to identify *Listeria* species.

Catalase Test

Suspected isolates were tested for their ability to produce catalase enzyme. A drop of 3% hydrogen peroxide was placed on a slide and a colony of the suspected isolates was picked with a sterile wire loop and then suspended in the hydrogen peroxide. The outcome of the reaction was recorded.

Oxidase Test

Suspected *Listeria* isolates were tested for their ability to produce cytochrome oxidase. A sterile wire loop was used to aseptically transfer a colony of the suspected isolates unto the surface of the oxidase detection strip and observed for 3 minutes. The outcome of the reaction was recorded.

Sugar fermentation tests

All suspected *Listeria* like organisms were tested for their ability to ferment glucose, lactose and sucrose. The suspected isolates were inoculated into phenol red-peptone broth containing 1% of any the sugars for their fermentation tests and incubated at 37°C for 24 hours. A positive result was indicated by changing from red to yellow colour.

Data Analyses

The data obtained from this study were analysed using statistical packaged for solution service software (SPSS) version 16.0. The data were presented using chi square and descriptive statistics such as tables and figures

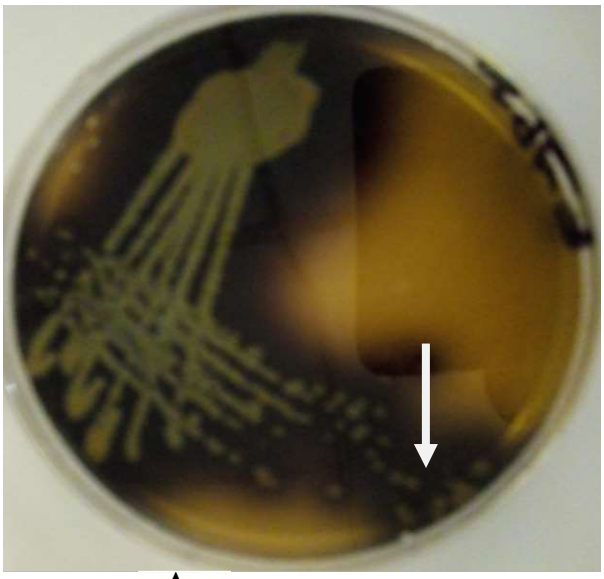
RESULTS

Isolation and Phenotypic Identification of *Listeria* species in Fresh and Locally Fermented Milk (*kindirmu*) in Maiduguri Metropolis

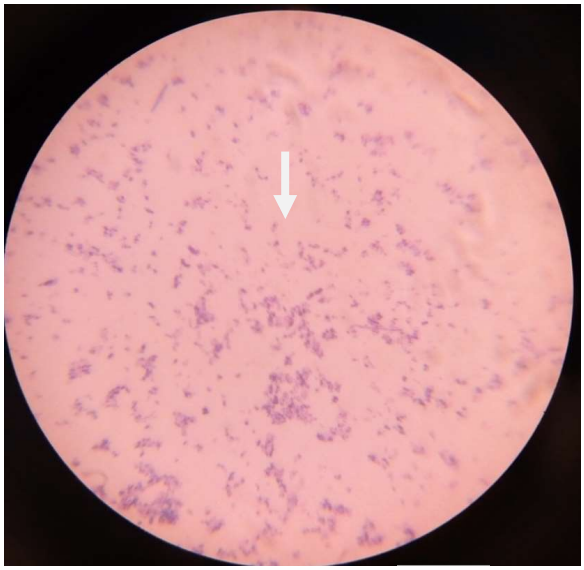
Isolates with grey-green appearance and sunken centres surrounded by black halos on *Listeria* selective agar were observed after incubation at 37°C for 24 hours. *Listeria* produces small transparent and raised shaped colonies with smooth borders, having entire edge after 24 hours incubation at 37°C (A). The microscopic appearance of *Listeria* species appeared purple red, arranged singly in short chains, rod-shaped and coccoid after staining with Gram stain (B). The *Listeria* species produces narrow zones of haemolysis that did not extend beyond the edge of the colony on sheep blood agar after 24 hours at 37°C (C). Out of 280 milk samples collected and analyzed, 93 (12%) were presumptive isolates of *Listeria* species.

Listeria organism growing on *Listeria* selective agar (A), showing Gram

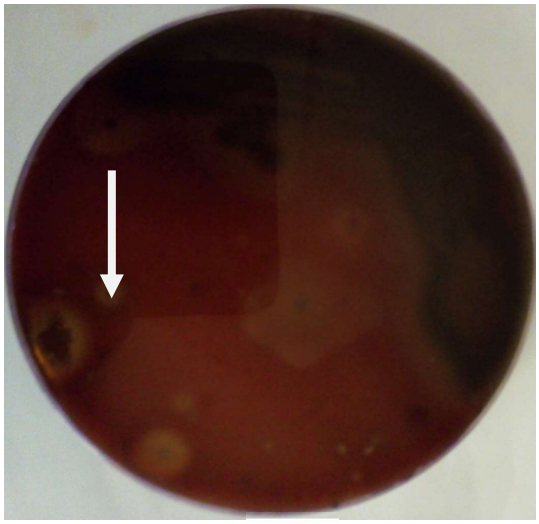
Positive short rods (B) and β- hemolysis on sheep blood agar (C)



A



B



C

Table 2**: Isolation and Phenotypic Identification of *Listeria* Species in Fresh and Locally Fermented Milk (*kindirmu*) in Maiduguri Metropolis**

Location	Type of Samples	Number of samples Collected	No. (%) of suspected *Listeria* isolates
Borno State Dairy Farm (DF)	Fresh milk	40	28 (70.0)
College of Agriculture Farm (CAF)	Fresh milk	40	30 (75.0)
University of Maiduguri Farm (UMF)	Fresh milk	40	33 (82.0)
Bulumkutu (BLF)	Fermented milk	40	2 (5.0)
Monday market (MMFM)	Fermented milk	40	0 (0.0)
Baga Road (BFM)	Fermented milk	40	0 (0.0)
Damboa Road (BFM)	Fermented milk	40	0(0.0)
Total		280	93 (12)

Table 2: Distribution of *Listeria* species isolated from milk in Maiduguri, Borno State

The distribution of *Listeria* species isolated from milk on *Listeria* selective agar showed that 2 (50.0%) were from fermented milk in Bulumkutu, while the lowest percentage of 2 (6.5%) was from Borno State Diary farm. The total distribution of positive samples was found to be 12 (85.8%) for presumptive *Listeria*. species after biochemical test. When the association between the isolation rate for *Listeria* species and the location of the sample were tested, there was no significant association between the two ($p< 0.082$). The distribution of *Listeria* species base on sample type and positive *Listeria* species of 2 (50.0%) were from fermented milk and 10(11.2%) were from fresh milk. When the association between the positive *Listeria* species and the sample type were tested, there was significant association between the two ($p< 0.0024$).

Table 3: Distribution of *Listeria* species isolated from Fresh and Locally Fermented milk (*kindirmo*) in Maiduguri Metropolis, Borno state

Variable Location	No. of sample	No. of positive (%)	X^2	P value
Borno State dairy Farm	31	2 (6.5)	6.695	0.082
Collegeof Agric.	36	4 (11.1)		
University farm	22	4 (18.2)		
Bulunkutu fermented milk	4	2 (50.0)		
Total	93	12 (85.8)		
Milk type				
Fermented milk	4	2 (50.0)	5.118	0.024
Fresh milk	89	10 (11.2)		

DISCUSSION

The colonies of presumptive *Listeria* appeared black with sunken centre surrounded by black halos on *Listeria* selective agar. This concur with the earlier report that *Listeria* can breakdown esculin in the medium to glucose and esculetin, which forms an olive green to black complex with ferric ions which stain the colonies (Chukwu *et al*., 2004; Gasanov *et al*., 2005). However, other organisms such as enterococci and bacilli can mimic the morphology of *Listeria* when growing on *Listeria* selective agar. Gram positive short rods of *Listeria* species arranged singly in short chain and in pairs were observed in this study. This is in agreement with the findings of Azubaidy *et al.* (2013) which showed that the organism could be rod shaped, coccoid or filamentous. This shape may depend on nutrients, environment and cultural characteristics or conditions.

The distribution of *Listeria* species isolates from fresh and locally fermented milk (*kindirmo*) samples in Maiduguri Metropolis showed that 4 (11.2%) from College of Agriculture in this study is similar to 11% *Listeria* species in milk from Jos, Nigeria by Chukwu *et al*. (20004), but lower than 35.1% in milk

reported by Usman *et al*. (2016b) in Kaduna, Nigeria. When the association between the distribution of *Listeria* species and the location of the sample were tested, there was no significant association between the two (p< 0.082). The distribution of *Listeria* isolates based on sample type showed 2 (50.0%) were from fermented milk and 10 (11.2%) fresh milk which gave an overall distribution of 12.9% in this study. When the association between the positive *Listeria* species and the sample type were tested, it was found to be significant. This is not in agreement with Usman *et al*. (2016a), who showed that there was no significant different between the sample type and positive *Listeria* species in milk sample collected from Kaduna. The differences in the isolation rates may likely be due to locations, identification methods, the health care given to the cows as well as precautions taken by milk handlers (Chukwu *et al*., 2004; Faeji *et al*., 2016). Also, the cattle rearing practice by the herdsmen in the study area that are likely to increase or decrease the isolation rate may be that the herdsmen have been health and hygiene conscious and have been consulting veterinary doctors for adequate health services and the use new technologies, another risk factor could be inadequate frequency of cleaning the exercise area, poor cow cleanliness and incorrect disinfection of towels between milking might be some contributing factors (Faeji *et al*, 2016). The *Listeria* detection rate in milk observed in this study is lower than the 60% detection rate in milk reported by Mugampoza *et al*. (2011) in Uganda. Such differences might be due to poor hygiene and sanitation activities in the milk production; processing and supply chains which may be mainly associated with faecal contamination of milk (Uhitil *et al.*, 2004)

The isolation of *Listeria* species from milk observed in this study could be due to faecal or environmental contamination during milking, storage and/or infected cows in dairy farms (Faeji *et al*., 2016). The low detection rate of *Listeria* species in fermented milk (*kindrimo*) observed in this study may be due to conversion of lactose during fermentation by lactic acid bacteria present in the locally fermented milk (*kindrimo*) leading to the development of low pH in the milk and hence the inhibition of *Listeria* species (Jamali *et al*., 2013; Usman *et al.,* 2016b). Although, 12% of all the isolates in this study gave colonial appearance typical of *Listeria* species, only 12% were found to be Gram positive rods, catalase positive, oxidase negative and showed β-haemolysis on sheep blood agar. This finding is suggestive of *Listeria monocytogenes* isolates. Haemolytic activity on blood agar has been used as a marker to distinguish *L. monocytogenes* from other *Listeria* species.

The associated haemolytic virulence factor is regarded as a member of pore forming cholesterol-dependent cytolysin toxin family (Gedde *et al*., 2000; Gelfand, 2011; Alzubaidy *et al*., 2013) which may possibly be related to the haemolytic factor recorded in this study. The 12% isolates of *Listeria* species observed in this study were found to ferment glucose, sucrose and maltose. Some *Listeria* species are known to harbour enzymes that are capable of fermenting sucrose, maltose, lactose and glucose (Alzubaidy *et al*., 2013; Jamshidi and Zeinali, 2019).

CONCLUSION

In this study, 12% of *Listeria* species was isolated from fresh and fermented milk in Maiduguri metropolis, Nigeria. Only twelve (12%) of the isolates were suggestive of *L. monocytogenes* based on colonial appearance, microscopy, biochemical and sugar fermentation tests

The study was able to isolate and detect 2.9% *L. monocytogenes* in fresh and locally fermented milk (*kindirmu*) in Maiduguri, Borno State; therefore, fresh milk (*kindirmu*) should be pasteurized or boiled properly before consumption so as to reduce the bacteria load as well as supply cold facilities should provided to the sellers of locally fermented milk.

High risk communities such as those living in the study area should be educated on how to maintain good hygiene, preparation and storage practice with respect to fresh and locally fermented milk (*kindirmu)*.

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Conflicting interest

Authors have declared that there is no conflicting interest

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