Detection of plasmid in *Listeria monocytogenes* isolated from fresh raw milk in Nigeria

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Abstract: Aims: To determine the presence of plasmids in *Listeria monocytogenes* strains isolated from fresh raw milk from healthy cattle in Nigeria and also determine its role in antibiotics resistance since this organism is a food borne pathogen of public health importance. Methods and results: Isolation of *Listeria monocytogenes* was done using selective enrichment and isolation protocol, as recommended by United States Department of Agriculture (USDA) and Association of Analytical Chemists/ International Dairy Federation (AOAC/ IDF). Identification was by phenotypic characterization and confirmed using β-haemolytic activity, phosphatidyl inositol phospholipase-C (PIPLC) assay and polymerase chain reaction (PCR) . Strains identified as *Listeria monocytogenes* from the six zones were determined and results expressed as rate of prevalence in graph. Twenty-three strains of *Listeria monocytogenes* isolated from 305 samples of fresh raw milk were screened for the presence of plasmid DNA using the method of Ehrenfeld and Clewel and agarose gel electrophoresis. One isolate was found to harbour one plasmid with molecular weight of 14.62kb. The plasmid was cured using different concentrations of acridine orange. Antibiotic sensitivity test was carried out on both the wild strain (with plasmid) and cured strain using disc diffusion method of Bauer-Kirby and it was found that the wild strain was resistant to seven of the twelve antibiotics used while the cured strain was resistant to only one. Conclusions: There is a possibility of the presence of strains of *Listeria monocytogenes* that can harbour plasmids. This could confer antibiotic resistance to the strains and thus make antibiotic therapy unsuccessful. The increase in the number of antibiotics which the cured strain of *Listeria monocytogenes* was susceptible to in this study showed that plasmid may play a significant role in antibiotics resistance. The Fulani herdsmen who take fresh raw milk without boiling or pasteurization may serve as carriers of resistant strains of *Listeria monocytogenes* and help in its spread in the environment through faecal pollution.

Keywords: Fresh Raw Milk, *Listeria monocytogenes*, Plasmid, Antibiotic Resistance

1. Introduction

Listeriosis caused by members of the genus *Listeria* occurs worldwide and in a variety of animals including man (1). They are a group of closely related rod shaped, Gram positive, facultative anaerobic, non-spore forming, motile bacteria. Ten species have been identified including *Listeria monocytogenes, Listeria ivanovii, Listeria seeligeri, Listeria innocua, Listeria welshimeri, Listeria grayi, Listeria marthii, Listeria rocourtiae, Listeria fleischannii and Listeria welhenstephanensis* (2,3). Among these species only *L.monocytogenes* and *L. ivanovii* are pathogenic, and the rest are non-pathogenic (4). *L. monocytogenes* is an intracellular foodborne pathogen that causes listeriosis and severe infections in humans with high mortality rate, mainly in high risk groups including pregnant women, neonates, the elderly, HIV/AIDS and cancer patients.*L.monocytogenes* has been isolated from soil, surface water, vegetation, the environment and various foods (5, 6).

In the United States of America, about 2500 cases of listeriosis occur each year with 20-30% mortality regardless of antimicrobial treatment (7). Thus, it indicates that the

prevalence of *Listeria monocytogenes* in foods poses a significant danger. The incidence of listeriosis in European Union in 2007 was reported to be 0.3 cases in every 100,000 population (8). In Nigeria, few sporadic cases of listeriosis have been reported (9), there is no data on outbreak of human listeriosis and the sources of contamination were unknown. Various food surveys conducted in Nigeria had reported on the detection of *L. monocytogenes* in different food products, including raw milk (10), smoked fish (11), beef, pork, goat meat, poultry, fish and vegetables (I2). *Listeria monocytogenes* has fair stability over antibiotic susceptibility, but in relatively recent time, reports of emergence of antibiotic resistant *Listeria monocytogenes* recovered from food and environment in Nigeria (10) and from sporadic cases of human listeriosis have remained of significant public health concern. Plasmids are small, circular, double stranded DNA molecules that are distinct from the cell's chromosomal DNA. They replicate independently of the genome, and are relatively stable. They are a key vector for horizontal gene transfer in bacteria through conjugation and thus are of significant importance in the prokaryotic world as they often provide the bacterial cell with a genetic advantage (13). Their presence has been linked with antibiotic resistance and other traits (14). This study examined the presence of plasmid in *Listeria monocytogenes* isolated from fresh raw milk from healthy cattle in Nigeria and its role in antibiotic resistance.

2. Material and Methods

2.1. Isolation and Identification

The samples which included 305 fresh raw milk from the six geo-political zones of Nigeria were aseptically collected into sterile McCartney bottles from the teat of lactating cattle. They were quickly transported in an ice pack to the laboratory, for immediate analysis for the presence of *Listeria monocytogenes* using selective enrichment and isolation protocol, as recommended by United States Department of Agriculture and Association of Analytical Chemists/International Dairy Federation (15, 16,17) method 993.12, modified by using Brilliance Listeria Chromogenic Agar (Oxoid) to obtain colonies. Briefly the samples (25ml each) were aseptically pre-enriched in 225ml of Buffered Listeria Enrichment Broth Base (Oxoid, England) at 30^0 C for 24h. After, 1ml of primary enrichment was inoculated into 9ml of Buffered Listeria Enrichment Broth with Listeria Selective Enrichment Supplement (with cycloheximide) and incubated at 30^0 C for 24h. Thereafter 0.1ml of each sample of the secondary enrichment was plated on Brilliance Listeria Chromogenic Agar (Oxoid) containing Brilliance Listeria Selective Supplement (Oxoid) and Brilliance Listeria Differential Supplement (Oxoid) and incubated at 37^0 C for 24h.. Suspected colonies were identified by Gram-stain. Gram-positive colonies were tested for haemolysis on 7% sheep blood agar Other confirmatory tests which included analytical profile index(API) test kit mono (Oxoid) and polymerase chain reaction (PCR) were performed. Molecular method which involved twenty-three Listeria-like isolates obtained was tested with a PCR assay targeting the iap gene which encodes the invasion associated protein to specifically identify *L. monocytogenes* isolates. Forward primers (List-univ 1) 5'-ATG TCA TGG AAT AA-3', and reverse primer (List-univ) 5'-GCT TTT CCA AGG TGT TTTT-3' (Inqaba Biotech, South Africa) define an 457bp fragment of the iap gene (18).

2.2. Rate of Prevalence

Milk samples were obtained from all the six geo-political zones and the number of positive samples (samples that had *Listeria* monocytogenes) from each zone was determined and the result expressed graphically.

2.3. Plasmid DNA Isolation

Plasmid DNA was isolated by the method of (19). One and half milliliter (1.5ml) of overnight broth culture of each isolate was spun for 1min. in a micro-centrifuge. Thereafter the cells were suspended in 200µl of solution A (100mM glucose, 50mM Tris hydrochloride (pH8), 10mM EDTA) containing 10mg of lysozyme per ml and incubated for 30 minutes at 37^0 C. Then 400µl of freshly prepared 1% solution of sodium dodecyl sulphate in 0.2N NaOH was added and mixed by inverting tubes. Exactly 300µl of a 30% potassium acetate solution (pH4.8) was added and mixed by vortexing. After incubating on ice for 5 minutes, the debris was removed by a 5 minutes centrifugation in a centrifuge. The supernatant was transferred and extraction was carried out once with a phenol-chloroform mixture (1:1). Equal volume of isopropanol was used to precipitate plasmid DNA. After allowing it to dry, plasmids DNA was dissolved in TBE buffer.

2.4. Agarose Gel Electrophoresis

The agarose gels were run in a horizontal electrophoretic apparatus for the detection of plasmid DNA using TBE (Tris, Boric Acid, EDTA, 0.8% agarose) buffer (20). The agarose gel in TBE buffer was boiled intermittently until the solution became clear. The solution was allowed to cool to 45 ^{0} C and 7µl of ethidium bromide was added. The function of ethidium bromide is to intercalate the basis of the DNA so that it fluores when viewed under the ultra violet (UV) light. The gel was then poured into the gel plate with comb in place and allowed to solidify. Subsequently, the gel tray and comb were removed and put into the tank containing the gel buffer, making sure that the buffer covered the gel completely. Two µl of the tracking dye (bromophenol blue) was mixed with 20µl of the sample and also loaded into the designated wells. Following the loading of the wells, the tank was covered, plugged into power and allowed to run from the negative to positive direction making sure it did not run more than ¾ of the gel. Finally, the gel was viewed on the UV transilluminator using protective goggle. Amplified plasmid DNA appeared as sharp band that fluores when excited with UV light. The molecular weight of isolated plasmid was

determined online at www.insilico.ehu.es using online molecular weight calculator as described by (21).

2.5. Curing of Plasmid

Nutrient broth (2ml) was dispensed in sterile universal bottles. Acridine orange at concentrations of 25, 50, 75, 100, 125 and 150µl/ml were added and inoculated with about 10^5 cells of overnight broth culture. The tubes were incubated in a shaker for 8h at 37 ^{0} C. Growth was assessed using the tubes with the highest concentration of acridine orange that allowed growth .They were serially diluted and plated on nutrient agar to give about 50-80 colonies per plate which were subsequently replicated as above and tested for haemolysis and antibiotic susceptibility.

## 2.6. Antibiotic Susceptibility Test

The susceptibility of wild and cured strains to antibiotics was tested by Bauer-Kirby disc diffusion assay of Clinical and Laboratory Standards Institute (22).The following antibiotics (Oxoid, England) were used: Amoxicillin 30µg (AMX), Erythromycin 5µg (ERY), Tetracycline 30µg (TET), Cloxacillin 5µg (CXC), Gentamcin 10µg (GEN), Cotrimoxazole 25µg (COT), Chloramphenicol 30µg (CHL), Ampicillin 25µg (AMP), Nalidixic Acid 30µg (NAL), Nitrofurantin 200µg (NIT), Colistin 25µg (COL) and Streptomycin 10µg (STR). The inoculum was standardized to the 0.5 McFarland turbidity standards, inoculated on nutrient agar (Oxoid, England) and incubated at 37 ^{0} C for 18h. The diameter of the zone of inhibition (including the diameter of the disc) was measured to the nearest whole milliliter and interpreted based on (22).
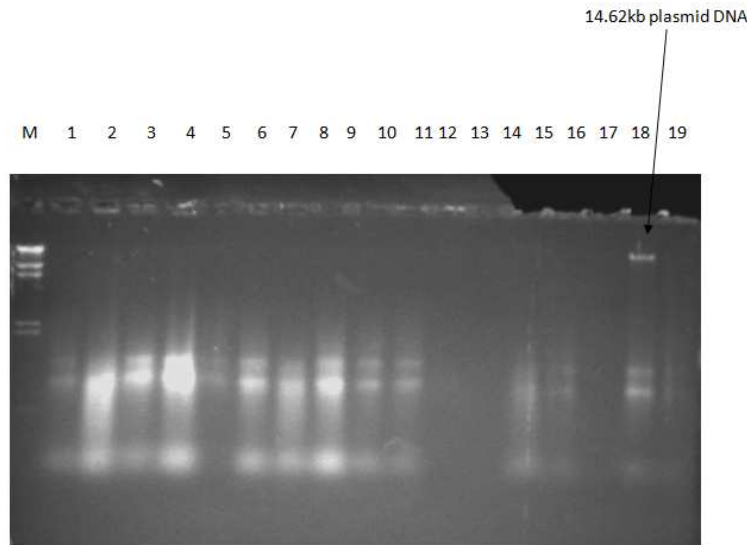
3. Results

3.1. Molecular Characterization

3.1.1. Detection of Virulence Gene (iap) in Listeria Monocytogenes
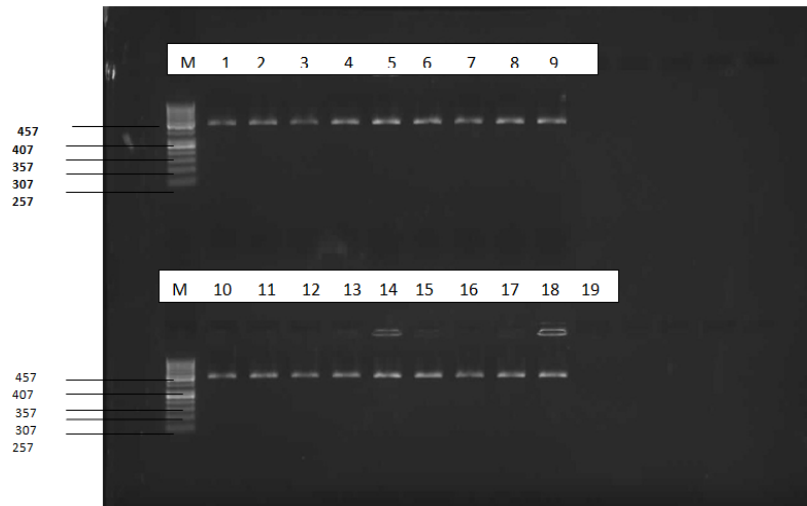
Plate 1. *PCR amplification of iap gene from the genome of L. monocytogenes strains. Lane M: 50bp molecular weight marker, serial number lanes 1-18 represent respective Listeria monocytogenes isolates.Lane 19 is control*

Plate 2. *Agarose (0.8%) gel electrophoretic analysis of plasmid DNA isolated from KFRM4 strain using the protocol of (18)*
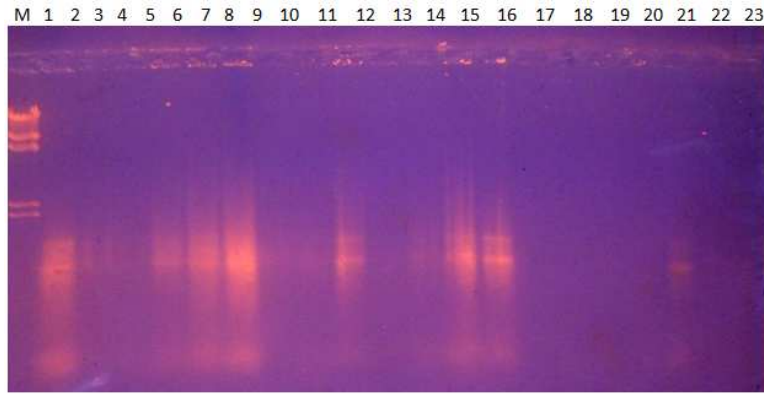
Plate 3. *Agarose gel electrophoresis plate showing no detectable plasmid after curing wild strain with 125μg/ml of acridine orange*
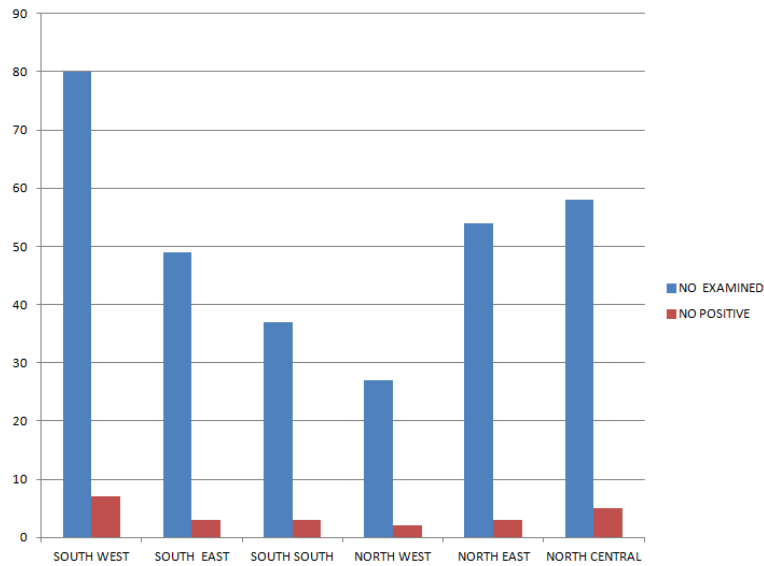

Fig 1. *Rate of Prevalence of Listeria mooncytogenes in fresh raw milk in Nigeria*

Table 1. *In vitro antibiotic susceptibility test on KFRM4 strain of Listeria monocytogenes before and after curing*

S/N Antibiotics			Wild strain of KFRM4		Cured strain of KFRM4	
			R	S	R	S
1	Chloramphenicol	(30μg)	--	+	--	+
2	Nitrofurantin	(200μg)	--	+	--	+
3	Cotrimoxazole	(25μg)	--	+	--	+
4	Erythromycin	(15μg)	--	+	--	+
5	Tetracycline	(30μg)	--	+	--	+
6	Gentamycin	(10μg)	+	-	--	+
7	Streptomycin	(10μg)	+	-	--	+
8	Ampicillin	(10μg)	+	--	--	+
9	Amoxicillin	(25μg)	+	--	--	+
10	Cloxacillin	(5μg)	+	--	--	+
11	Colistin	(25μg)	+	--	--	+
12	Nalidixic acid	(30μg)	+	--	+	--

KEY: R= resistant, S= sensitive

The primers were designed to amplify approximately 457bp iap gene which codes for invasion associated protein specific for *Listeria monocytogenes*. The gel electrophoresis of the PCR product as depicted in plate 1 showed a single band of amplicon. For the control where no target DNA was added there was no amplification (lane 19). The amplification of iap gene from the genome of all the 23 strains isolated in this study confirmed that they were all *Listeria monocytogenes*

3.2. Occurrence of Listeria Monocytogenes in Fresh Raw Milk in the Six Zones of Nigeria

Twenty-three strains of *Listeria monocytogenes* were obtained from three hundred and five fresh raw milk samples.Each of the six zones had positive samples as shown

in figure 1

3.3. Detection of Plasmid DNA

Twenty-three strains of *Listria monocytogenes* were screened for the presence of plasmid DNA.The strain designated KFRM4 in lane 18 was found to harbour one plasmid which has a molecular weight of 14.62kb (Plate 2)

3.4. Plasmid Curing

Listeria monocytogenes was found to have lost the plasmid after curing with 125μg/ml of acridine orange (Plate 3)

3.5. Haemolysis Test

The wild and cured strains produced β-haemolysis on 7% sheep blood agar.

3.6. Antibiotic Sensitivity Test

The cured strain was sensitive to eleven of the twelve antibiotics used compared to the wild strain which showed sensitivity to five of the twelve antibiotics (Table 1)

4. Discussion/Conclusion

Previous workers had reported the isolation of the organism from fresh raw milk (23,24,25) This supports the presence of this organism in food (raw milk) across the six zones in Nigeria..The presence of this organism in fresh raw milk in this study agreed with the reports of (26,27) who reported that the organism could be as high as 5% in some samples and attributed the contamination of fresh raw milk to mastitis caused by the organism or of faecal origin.

Plasmid profiles of all the strains of *Listeria monocytogenes* were examined and only one strain (KFRM4) was found to harbor 14.62kb plasmid. This strain was susceptible to eleven out of twelve antimicrobials (91.67%) used after curing with 125μg/ml of acridine orange. This result is consistent with the findings of (28) who reported the isolation of a strain of *Listeria monocytogenes* from a patient with meningitis that was resistant to chloramphenicol, streptomycim, erythromycin and tetracycline. The genes conferring resistance to these antibiotics were carried by a 37kb plasmid that was self transferable to other *Listeria monocytogenes* cells, to enterococci-streptococci and *Staphylococcus aureus*. These findings suggest that emergence of multiple antibiotic resistances in *Listeria* spp. is possible and can be due to acquisition of a replicon originating in enterococci-streptococci. It is known that microorganisms (bacteria) can either acquire, or transfer antibiotic resistance genes from plasmids and transposons of other bacterial species (including *Enterococci* spp) either *in vitro* or *in vivo* in the intestinal tract (29, 28). This is very important for their pathogenicity as bacteria that acquire new resistance are not disrupted by antibiotics during therapy. The high percentage of isolated wild strain of *Listeria monocytogenes* that showed antibiotic resistance towards nalidixic acid, colistin, cloxacillin, amoxicillin, ampicillin,

gentamycin and streptomycin is similar to a general worldwide pattern of an increasing prevalence of antibiotic resistance, including multiple antibiotic resistances among many groups of bacteria. This may be due to the fact that *Listeria* sp. can transfer resistance genes to other enteric bacteria that become resistant to these antibiotics normally used in treatment of its infections as ampicillin is one of the first choice drugs for the treatment of listeriosis (28). The results showed a high level of multiresistance and all isolated strains were susceptible to only one of the first choice antibiotics used in listeriosis therapy (tetracycline) and to cotrimoxazole used as second choice antibiotic in treatment of listeriosis especially in patients allergic to penicillin (30,31,32). *Listeria monocytogenes* is slowly becoming resistant because of the uptake of resistance genes from other Gram-positive bacteria such as *Listeria* spp., *Staphylococcus* spp. and *Enterococcus* spp..A continued surveillance of emerging antimicrobial resistance of this pathogen is therefore important to ensure an effective treatment of human listeriosis (28). Bacterial resistances to antimicrobials have been demonstrated from the pre-antibiotic era and from remote environments with no antimicrobial exposure (33). The development of resistance has largely followed the introduction and increasing availability of antimicrobial agents. Early studies that demonstrated this association and the possibility of the transfer of resistance between bacteria were undertaken in Japan in the 1950s. Studies with different bacterial pathogens showed that transfer of resistance was a common event (28). The developments in molecular biology enabled the genetic basis of this transfer, by extra chromosomal DNA (Rfactor or plasmids) to be explained (29). Bacteria have developed a wide range of mechanisms to avoid the action of antimicrobials, from antibiotic destroying enzymes to modifications of their own metabolic pathways. The situation is far from static, new and more complex, often interacting mechanisms of resistance are continually described, very often rapidly followed by the introduction of new antimicrobial agents (34). Control of the development and spread of antimicrobial resistance will require major initiatives by governments and pharmaceutical companies to improve and rationalize the use of antimicrobials.

References

[1] Low JC and Donachie W,(1997) A review of *Listeria monocytogenes* and listeriosis. Vet. J 153: 9-29.

[2] Zhang Y, Yeh E, Hall G, Crioe J,Bhaowat A.A, Meno J. (2007) Characterization of *Listeria monocytogenes* isolated from retail foods. Intl J. Food Microbiol. . 113 : 47-53

[3] Halter EL, Neuhaus K and Scherer S.(2012). *Listeia weihenstephanensis* sp. nov. isolated from the water plant *Lemna trisulca* of a German fresg water pond. Int. J. Syst. Evol. Microbiol. 63: 641-647.

[4] Liu D. (2006) Identification, subtyping and virulence determination of *Listeria monocytogenes* an important foodborne pathogen. J. Med.Microbiol. 55: 645-659

[5] Kuhn M, Scortti M and Vazquez-Boland JA (2008). Pathogenesis. In Liu, D (ed) Handbook of *Listeria monocytogenes,* CRC Press, New York. Pp 99-102.

[6] Liu, D (2008). Epidemioogy. In Liu, D (ed) Handbook of *Listeria monocytogenes,* CRC Press, New York, pp27-30.

[7] Mead PS, Slustker L, Dietz V and Bresee J. (1998). Food epidemic relaed illness ad the death in the United States. Emerg. Infect. Dis. 5 (5): 607-625.

[8] Lindback T, Secic I and Rorvik LM (2011). A contingency locus in *prfA* in a *Listeria monocytogenes* subgroup allows reactivation of the *prf*A virulence regulator during infection in mice. Appl. Environ. Microbiol. 77: 3478-3483.

[9] Chukwu OOC, Ogbonna CIC, Olabode AO, Chukwu AI, Onwuliri FC and Nwakiti OO (2006). *Listeria monocytogenes* in Nigerian processed mea and ready-to-eat-dairy products. Nigerian Journal of Microbiology 20 (2): 900-904.

[10] Enurah L.U., Aboaba O.O,Nwachukwu S.C.U, Nwosuh C.I. (2013) Antibiotic resistant profiles of food (fresh ra milk) and environmental (abattoir effluents) isolates of *Listeria monocytogenes* from the six zones of Nigeria. African Journal of Microbiology Research. 7 (34) 4373 4378.

[11] Salihu MD, Junaidu AU, Manga SB, Gulumbe ML, Magaji AA, Ahmed A, Adamu AY, Shittu A and Balarabe I (2008). Occurrence of *Listeria monocytogenes* in smoked fish in Sokoto, Nigeria. Afr. J. Biotechnol. 7: 3082-3084.

[12] Ikeh MAC, Obi SKC, Ezeasor DN, Ezeonu IM and Moneke AN (2010). Incidence and pathogenicity profile of *Listeria* sp. isolated from food and environmental samples in Nsukka, Nigeria. Afri. J. Biotechnol. 9 (30): 4776-4782.

[13] Clewell D.B. (1972) Nature of ColE1 plasmid replication in Escherichia coli in the presence of chloramphenicol. J. Bacteriol. 110. 667-676.

[14] Funnell B.E, Phillips G.J., editors,(2004) Plasmid Biology. ASM Press, Washington. DC, USA.

[15] USDA United States Department of Agriculture (1999) Listeria and food safety. Tips USD Consumer Publication List

[16] AOAC : Association of Analytical Chemists-International Methods of Analysis. 1995, 16th Edition

[17] IDF International Dairy Federation. Milk and milk products. Detection of *Listeria monocytogenes*. 43A, 1995. Hh//www.org/standardsEnglishhtw

[18] Wiedmann M, Bruce JL, Keating C, Johnson AE, McDonough PL and Batt CA.(1997) Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes*. J. Bacteriol. 180: 1350- 1356.

[19] Ehrenfeld EE and Clewell DB.(1987) Methods in microbiology. J. Bacterial. 169: 3473-3481

[20] Meyers JA, Sanchez D, Elwell LP and Falkow S.(1976) Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. 127: 1529 – 1537.

[21] Valery Terletskry, Valentina Tyshchenko, Hargi Martinez-Ballesteros, Javier Garaizai and Joseba Bikandi. (2010)Validation of double digest selective label database for sequenced prokaryotic genomes. Bioinformatcs. 26 (3) 417-418

[22] CLSI. (2005) Performance standard for antimicrobial susceptibility testing, fifteenth informational supplement, M100-S15, vol.25. No. 1. Clinical and Laboratry Standard Institute , Wayne Pa

[23] Prentice GA.(1997) The significance of pathogenic microorganisms in raw milk, *Listeria monocytogenes*. Mijekartvo. 47: 147-162.

[24] Onyemelukwe GC, Lawande RV, Egler LJ and Mohammed I (1983). *Listeria monocytogenes* in Northern Nigeria. *J. Infect. Dis.* 6: 141-145

[25] Aurora R, Parakash A, Parakash S, Rawool DB and Barbuddhe SB. (2008) Comparison of PILC based assay and PCR along with *invivo* pathogenicity tests for rapid detection of pathogenic *Listeria monocytogenes*. Food Cont. 19: 641 – 647.

[26] Bhilegonkar KN, Killshrestha SB, Kapoor KN, Ashok- Kumar, Rgarwal RK, Singh BR and Kumar A.(1997) Isolatio of *Listeria monocytogenes* from milk. Int. J. Food Sci. and Tech. Mysore 34: 248 – 250.

[27] Sanaa M. Listeriosis: contamination of milk and milk products. Summa. 1996, 13: 65-75.

[28] Pesavento G, Doci B, Nieri O, Comodo N and Lo Nostro A.(2010) Prevalevce and antibiotic susceptibility of *Listeria* spp. isolated from raw meat and retail foods. Food Control. 21: 708-713.

[29] Pourshaban M, Ferrini A, Cannoni V, Oliva B and Aureli P. (2002)Transferable tetracycline resistance in *Listeria monocytogenes* from food in Italy. J. Med. Microbiol. 51 (suppl 7). 564-566.

[30] David OM and Odeyemi AT. (2007) Antibiotic resistant pattern of environmental isolates of *Listeria monocytogenes* from Ado- Ekiti, Nigeria. African J. Biotech. 6 (18): 2135-2139

[31] Chao G, Zhou X, Jiao X, Qian X and Xu L. (2007) Prevalence and antimicrobial resistance of foodborne pathogens isolated from food products in China. Fooborne Pth. Dis. 4 (3): 277-284.

[32] Charpentier E, Gerbaud G, Jacquet C, Rocourt J and Courvalin P. (1995) Incidence of antibiotic resistance in *Listeria* spp. J. Infect Dis. 172: 277-281.

[33] Dancer SJ, Shears P and Platt D. (1997) Isolation and characterization of coliforms from glacial ice and water in Canadas High Artic. J. Appl. Microbiol. 82: 597-609.

[34] Finch RG. Antibiotic resistance. J. Antimicrob. Chem. (1998) 42:125-128