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Antibiotic resistant profiles of food (fresh raw milk) and environmental (abattoir effluents) isolates of *Listeria monocytogenes* from the six zones of Nigeria

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The prevalence of *Listeria monocytogenes* in fresh raw milk and abattoir effluents in the six zones of Nigeria was determined. Antibiotic resistant profile of the isolates was examined using the Bauer- Kirby disc diffusion assay. A total of 626 food and environmental samples were cultured on selective media out of which 54 (8.6%) were positive for *L. monocytogenes*. Chloramphenicol was the most effective antibiotic against the isolates with the least resistance (3.70%) while nalidixic acid proved to be least effective with resistance of 90.74%. The multiple-antibiotic resistant pattern of the isolates showed nalidixic acid/cloxacillin (35.2%), nalidixic acid/colistin (31.5%) and cloxacillin/colistin/nalidixic acid (29.6%) to be most prominent. The least value was observed in chloramphenicol/nitrofurantin/cotrimoxazole with 5.6%. The modal values of the minimum inhibitory concentrations (MICs) of the antibiotics to the isolates range between 4.0 and >16.0 µg/ml. Chloramphenicol, nitrofurantin and gentamycin recorded the highest MIC compared with other antibiotics. This study has demonstrated that a wide and rapidly expanding range of undesirable and, in some cases, multi-resistant determinants is currently present in *L. monocytogenes*.

Key words: *Listeria monocytogenes*, fresh raw milk, abattoir effluents, antibiotic resistance.

INTRODUCTION

The genus *Listeria* is a group of closely related Gram-positive, facultative anaerobic, non-spore forming, rod shaped, motile bacteria. The genus includes 10 species: *Listeria monocytogenes, Listeria ivanovii, Listeria inocua, Listeria welshimeri, Listeria seeligeri, Listeria grayi, Listeria marthii, Listeria rocourtiae, Listeria fleischmannii and Listeria weihenstephanensis* (Zhang et al., 2007; Halter et al., 2012) but *L. monocytogenes* is the principal pathogen in humans and animals. *L. ivanovii* is a pathogen of animals but is occasionally implicated in human disease. The other *Listeria* spp are generally considered non-virulent (Volokhov et al., 2002; Liu, 2006). *L. monocytogenes*, the causative agent of listeriosis is ubiquitous in the environment and has been recognized as animal pathogen since the 1920s but in the past two decades, it has been implicated in several outbreaks of food-borne illness in humans (Yucel et al., 2005; Pintado et al., 2005). Contamination of silage leads to infection of farm animals resulting in possible human infection by way of the food chain (Bockserman, 2000; Ramaswamy et al., 2007; Sleator et al., 2009) while it has been established that food-borne transmission constitutes the main acquisition route of listeriosis (Aureli et al., 2000; Churchill et al., 2006); most healthy humans are not significantly affected by the intake of small numbers of *L. monocytogenes* in foods. However,

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Table 1. Representative samples collected for listeria species isolation and identification from the six geo-political zones of Nigeria.

S/N	Source of sample	Number/type of sample	
		Fresh raw milk (FRM)	Abattoir Effluent (AE)
1	Southwest	80	80
2	Southeast	49	52
3	Southsouth	36	40
4	Northwest	27	28
5	Northeast	54	58
6	Northcentral	59	63
Total		305	321

certain sections of the population are predisposed to the development of listeriosis due to presence of existing chronic illness, suppression of the immune system, pregnancy, or extreme youth or age (under 1year or over 60y ears) (Lorber, 1990; Ramaswamy et al., 2007). This presents a significant public health problem because in such population, listeriosis is fatal in up to 30% of cases (Aurora et al.,2008) which may increase up to 75% in high risk groups such as pregnant women, neonates and immunocompromised persons (Jalali and Abedi, 2008); it far exceeds other common food-borne pathogens, such as *Salmonella enteritidis* (with a mortality of 0.38%), *Campylobacter* species (0.02-0.1%) and *Vibro* species (0.005-0.01%) in terms of disease severity (Allekruse et al., 1997; Mead et al., 1998).

In the past, those individuals who develop listeriosis have usually been treated with penicillin or ampicillin in conjunction with an aminoglycoside (Charpentier and Courvalin, 1999), although tetracycline, erythromycin or chloramphenicol alone or in combination have also been used (Hof, 1999). Current therapy of choice for all forms of listeriosis is a combination of ampicillin and gentamycin (Lorber, 1997; Schlech, 2000). *Listeria* spp. have been reported as susceptible to antibiotics active against Gram-positive bacteria (Hawkins et al.,1984) but more recently, reports of resistance in Listeria spp. have been published (Franco Abuin et al.,1994; Abraham et al.,1998). Such increases in antibiotic resistance among *Listeria* spp. are in line with a general worldwide pattern of an increasing prevalence of antibiotic resistance, including multiple antibiotic resistance among many groups of bacteria. Many pathogens are developing resistance to most currently used antibiotics and there are increasingly frequent reports of pathogens which are resistant to almost all available antibiotics. Antibiotic resistance in bacteria has been linked to over-use of antibiotics in animals and humans (Davies, 1998) since these therapeutic compounds were identified almost 60 years ago. Such resistance may arise from a mutation in an intrinsic chromosomal gene, or by acquisition of exogenous genetic material carrying single or multiple resistance determinants (Levy, 1994). It is now clear that such transfer is possible between unrelated bacterial species (Kruse and Soum, 1994), and that these interactions are a frequent and important means of genetic exchange among microorganisms.

It is evident that antibiotic resistance is becoming more and more widely reported in all bacteria, not just pathogens, and that the occurrence of antibiotic resistance in non-pathogens poses major, risks to human health. While many antibiotic-resistant bacteria in foods are currently saprophytic or commensal in habit, their resistance genes can be transferred to other food-borne bacteria, including pathogenic species within the gastrointestinal tract (Perreten et al., 1997). This process may have undesirable clinical implications for the host, and for the wider population coming into contact with derived antibiotic-resistant pathogens. This study examined the antibiotic susceptibility profiles of 54 strains of *L. monocytogenes* isolated from fresh raw milk and abattoir effluents in the six geo-political zones of Nigeria.

MATERIALS AND METHODS

Collection of samples

Samples of fresh raw milk from the teat of lactating cattle and abattoir effluents were randomly and aseptically collected into sterile McCartney bottles from the six geo-political zones comprising southwest, southeast, southsouth, northwest, northeast and north central. The samples that included 305 fresh raw milk and 321 abattoir effluents (Table 1) were quickly transported in an ice pack to the laboratory for immediate microbial analysis.

Processing of samples

Strains were isolated in accordance with United States Department of Agriculture and Association of Analytical Chemist/International Diary Federation (USDA, 1999; AOAC, 1995; IDF, 1995) method 993.12, modified by using Brilliance Listeria Chromogenic Agar (Oxoid) to obtain colonies. 25 ml of each sample was aseptically added to 225 ml Buffered Listeria Enrichment Broth Base and incubated at 30°C for 24 h. A portion of 1 ml of primary enrichment was transferred to 9 ml of Buffered *Listeria* Enrichment Broth with *Listeria* Selective Enrichment Supplement (with cycloheximide) (Oxoid) and incubated at 30°C for 24 h. Secondary enrichment of

Table 2. Rate of prevalence of *Listeria monocytogenes* in fresh raw milk and abattoir effluents in six geo-political zones of Nigeria.

Source	Type/number examined fresh raw milk (FRM)	Number positive	%	Type/number examined abattoir effluent (AE)	Number positive	%
Southwest	80	7	8.75	80	10	12.50
Southeast	49	3	6.12	52	5	9.62
Southsouth	36	3	8.11	40	3	7.50
Northwest	27	2	7.41	28	4	12.29
Northeast	54	3	5.56	58	5	8.62
Northcentral	59	5	8.47	63	4	6.35
Total	305	23	7.54	321	31	9.65
National prevalence:	626			54 (8.6%)		

0.1 ml of each sample was plated on Brilliance Listeria Chromogenic agar (BLCA) (Oxoid) containing Brilliance *Listeria* selective Supplement (Oxoid) and Brilliance *Listeria* Differential Supplement (Oxoid) and incubated at 37°C for 24 h. The suspected colonies were characterized by Gram-stain. Gram-positive colonies were tested for haemolysis on 7% sheep blood agar, and catalase. Species identification was done with API *Listeria* test kit mono (Oxoid).

Antibiotic sensitivity test

The antibiotic susceptibility pattern of the isolates was determined by using the disk diffusion method of Clinical and Laboratory Standard Institute (CLSI) (2005) on Mueller-Hilton agar. The following antibiotics (Oxoid) were used; amoxicillin (25 µg), chloramphenicol (30 µg), cloxacillin (5 µg), cotrimoxazole (25 µg), erythromycin (15 µg), gentamycin (10 µg) tetracycline (30 µg), nitrofurantin (200 g), ampicillin (10 µg), streptomycin (10 µg), colistin (25µg) and nalidixic acid (30 µg). The inoculum was standardized to the 0.5 McFarland turbidity standards, inoculated on nutrient agar and incubated at 37°C for 18 h. The diameter of the zone of inhibition (including the diameter of the disk) was measured to the nearest whole millimeter and interpreted based on CLSI guideline (CLSI, 2005).

The results of Minimum Inhibitory Concentration (MIC) were determined using the method of Stroke and Ridgway (1980).

RESULTS

Prevalence of *L. monocytogenes* in fresh raw milk and abattoir effluentl samples

The prevalence of *L. monocytogenes* in the examined samples is shown in Table 2. The identification of the isolates was based on cultural and morphological appearances and were further confirmed by biochemical tests. The results were interpreted according to Seelinger and Jones (1986). Results of this study reveal that the pathogen was present in the samples investigated; fresh raw milk and abattoir effluents. It was observed that abattoir effluents from the southwest zone of Nigeria had the highest occurrence of the pathogen (12.50%) and abattoir effluents from the northwest showed highest incidence of *L. monocytogenes* (Table 2).

Antibiotics resistance of the isolates and minimum inhibitory concentrations (MICs)

Table 3 shows the *in vitro* resistance of the isolates to common antibiotics. Nalidixic acid appeared to be most ineffective. 90.74% of the isolates were resistant to it. This was followed by colistin (85.19%). Chloramphenicol on the other hand appeared to be most effective as only 3.70% of the isolates were resistant to it. The number of resistant strains from fresh raw milk ranged from 4.35-91.30%, while resistant strains from abattoir effluents ranged from 3.23-90.32%. The minimum inhibitory concentrations (MICs) and multiple antibiotic resistant pattern of the isolates are shown in Tables 4 and 5. 19 isolates (35.2%) were resistant to the combination of nalidixic acid and cloxacillins while nalidixic acid/colistin had 31.5%.The cloxacillin/colistin/nalidixic acid resistant isolates were 29.6%. The least value (n=3) was observed in chloramphenicol/nitrofurantin/cotrimoxazole with 5.6%. The modal values of the Minimum Inhibitory Concentrations (MICs) of the antibiotics to the isolates ranged between 4.0 and >16 µg/ml. Chloramphenicol, nitrofurantin and gentamycin recorded the highest MIC compared with other antibiotics.

DISCUSSION

Reports from developed countries, like the United States of America, have implicated foods as a major vehicle of transmission for listeriosis (WHO, 2000; Yucel et al., 2005; Pintado et al., 2005; Robin et al., 2006). *L. monocytogenes,* the species predominantly responsible for listeriosis, is found in the soil, water, sewage, and effluents. Animals can carry the bacterium without appearing ill and can contaminate foods of animal origin such as meats, raw milk or non-pasteurized milk and other diary products (Bockserman, 2000; Yucel et al., 2005; Pintado et al., 2005). Our findings in this study show highest incidence of *L. monocytogenes* in abattoir effluents. This is very possible because at the time of slaughter, the animals that have been carriers contaminate

Table 3. *In vitro* antibiotics resistance of food and environmental isolates of *Listeria monocytogenes.*

Antibiotic	Fresh raw milk(FRM)		Abattoir effluents (AE)		Total	
	n = 23	%	n = 31	%	n = 54	%
Nalidixic acid (30µg)	21.0	91.30	28.0	90.32	49.0	90.74
Colistin (25µg)	20.0	86.96	26.0	83.87	46.0	85.19
Cloxacillin (5µg)	18.0	78.26	25.0	80.65	43.0	79.63
Amoxicillin (25µg)	16.0	69.57	24.0	77.42	40.0	74.07
Ampicillin (10µg)	14.0	60.87	20.0	64.52	34.0	62.96
Chloramphenicol (30µg)	1.0	4.35	1.0	1.23	2.0	3.70
Nitrofurantin (200µg)	1.0	4.35	2.0	6.45	3.0	5.56
Cotrimoxazole (25µg)	4.0	17.39	3.0	9.68	7.0	12.96
Erythromycin (5µg)	6.0	26.09	7.0	22.58	13.0	24.07
Tetracycline (10µg)	8.0	34.78	6.0	19.36	14.0	25.93
Gentamycin (10µg)	8.0	34.78	10.0	32.26	18.0	33.33
Streptomycin (10µg)	9.0	39.13	11.0	35.48	20.0	37.04

The data are modal values of three determinants.

Table 4. The minimum inhibitory concentrations (MICs) of some antibiotics against food and environmental isolates of *L. monocytogenes* (µg/ml).

Antibiotic	Fresh raw milk (FRM)	Abattoir effluent (AE)
Chloramphenicol	>16.0	16.0
Nitrofurantin	8. 0	>16.0
Cotrimoxazole	16.0	16.0
Erythromycin	16.0	8.0
Tetracycline	8.0	8.0
Gentamycin	16.0	16.0
Streptomycin	4.0	8.0
Ampicillin	8.0	8.0
Amoxicillin	8.0	4.0
Cloxacillin	8.0	16.0
Colistin	8.0	4.0
Nalidixic acid	4.0	8.0

The data are modal values of three determinants.

the abattoirs and its effluents. Though meat is thoroughly cooked before consumption, the chances of cross-contamination of ready-to-eat (RTE) food products in the refrigerator or kitchen, still exists. Considering the ability of *Listeria* species to grow at refrigeration temperature, their presence on any food is very undesirable and should be controlled if not eliminated. Other workers (Ikeh et al., 2010) have reported very high incidence (100%) of *Listeria* species from the butchers tables and abattoirs in southeast zone of Nigeria. This is indicative of poor hygiene by the abattoir managers on one hand and the butchers on the other hand. The results of this study reveal that the pathogen was present in samples of fresh raw milk examined (7.54%). This is not surprising as previous workers (Sanaa, 1996; Bhilegoankar et al., 1997; Prentice, 1997) have reported that the occurrence of the organism could be as high as 5% in some milk samples. Sanaa (1996) and Prentice (1997) attributed the contamination of fresh raw milk samples to mastitis caused by the organism or of faecal origin. This is similar to those of MacGowan et al. (1994) and tend to agree with the suggestion of Bockserman (2000) that the usual habitat of *L.monocytogenes* is the intestinal tract of animals and birds from where the organism enters the soil via animal droppings.

The results of this study suggest that the overall incidence of antibiotic resistance in *L. monocytogenes* is relatively high (Table 3). The study does confirm that since the first report of antibiotic-resistant strains of *L. monocytogenes* (Poyart-Salmeron et al., 1990), there has

Table 5. Multiple antibiotic resistance in food and environmental isolates of *Listeria monocytogenes.*

Antibiotic	Fresh raw milk		Abattoir effluent		Total
	n = 23	%	n = 31	%	%
Nal + Col	7.0	30.4	10.0	32.3	31.5
Nal + Clx	8.0	34.8	11.0	35.5	35.2
Amx + Amp	6.0	26.1	5.0	16.1	20.4
Chl + Nit	2.0	8.7	3.0	9.7	9.3
Cot + Ery	3.0	13.0	4.0	12.9	13.0
Tet + Gen	4.0	17.4	9.0	29.0	24.1
Chl + Nit + Cot	1.0	4.4	2.0	6.5	5.6
Ery + Tet + Gen	3.0	13.0	8.0	25.8	20.4
Strep + Amp + Amx	4.0	17.4	7.0	22.6	20.4
Clx + Col + Nal	6.0	26.1	10.0	32.3	29.6
Strep + Gen + Ery	2.0	8.7	3.0	9.7	9.3
Tet + Cot + Nit	2.0	8.7	2.0	6.5	7.4

Nal = Nalidixic acid (30 µg); Col = Colistin (25 µg); Clx =Cloxacillin (5 µg); Amx = Amoxicillin (25 µg); Amp = Ampicillin (10 µg); Strep = Streptomycin (10 µg); Gen = Gentamycin (10 µg); Tet = Tetracycline (30 µg); Ery = Erythromycin (15 µg); Cot = Cotrimoxazole (25 µg); Nit = Nitrofurantin (200 µg); Chl = Chloramphenicol (30 µg).

been a pattern of emergence of strains of *L. monocytogenes* isolated from food, the environment or from clinical cases of listeriosis, which are resistant to one or more antibiotics (Arpin et al., 1992; Charpentier et al., 1995). The range of antibiotics to which resistance has been acquired is wide as exemplified in this study. It is of concern that this expanding range now includes a number of antibiotics used to treat listeriosis, for example erythromycin, ampicillin, tetracycline,and gentamycin.

Thus, while the relative proportions of antibiotic-resistant *L. monocytegenes* were low, it is clear that a number of mechanisms are operating to facilitate the introduction of undesirable antibiotic-resistant genes into *L. monocytogenes.* Previous workers (Perez-Diaz et al., 1982; Flamm et al., 1984; Doucet-Populaire et al., 1991*)* have experimentally demonstrated a number of these mechanisms. *In vitro* and *in vivo* studies have shown conjugative transfer of antibiotic-resistance that receipt of enterococcal and streptococcal plasmids into the genus *Listeria* spp. and re-transfer of such plasmids within the genus including *L. monocytogenes.*

Such transfers have been reported to confer a number of the resistance noted in this study. Increasing recognition of the mechanisms by which antibiotic resistance, including multiple antibiotic resistance of the types observed in this study, can move into and among *Listeria* spp. suggests that such resistance will become an increasing frequently observed characteristic of *L. monocytogenes*.

Although antibiotic resistance in *Listeria* spp. was noted in relatively recent times (Gellin and Broome, 1989) as rare, the number of reports of antibiotic resistance in *L. monocytogenes* has been increasing (Poyart-Salmeron et al., 1990; Rota et al., 1996) to a point where as with many other pathogens, the list of effective clinical options is becoming disconcertingly small. This study has demonstrated that a wide and rapidly expanding range of undesirable and, in some cases, multi-resistant determinants is currently present in *Listeria monocytogenes*. More comprehensive and continuous monitoring of the course and nature of the acquisition and dissemination of antibiotic resistance by this pathogen is warranted.

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