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Full Length Research Paper

Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) fingerprinting reveals intra-serotype variations among circulating *Listeria monocytogenes* strains

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Forty-five presumptive *Listeria monocytogenes* isolates were confirmed by multiplex polymerase chain reaction (PCR) and characterized for antimicrobial susceptibility and tolerance to commonly used disinfectants. Isolates were also serotyped by PCR and characterized by enterobacterial repetitive intergenic consensus ERIC-PCR fingerprinting. All of the isolates showed PCR products of 938 bp (genus) and of 750 bp (species). Antimicrobial susceptibility was 100% for ampicillin, amoxicillin/ clavulanic acid, vancomycin and chloramphenicol, whereas for trimethoprim/ sulfamethoxazole it was 98, azithromycin 96, erythromycin 91, tetracycline 82, penicillin 97.8 (2.2% no susceptible), ciprofloxacin 84.4, rifampin 64.4, meropenem 71.1 and clindamycin 22.2%, respectively. All the isolates were resistant to cephalosporins. 71% of the isolates showed a MIC ≤ 200 ppm/10 to15 min for sodium hypochlorite and 98% a MIC ≤ 1.5%/2 to15 min for Tego-51. 58% of isolates were serotyped as 4b/4d/4e, 16% as ½b/3b, 7% as ½a/3a, and 4% as ½c/3c. ERIC-PCR showed 28 polymorphic bands ranging from 100 to 2810 bp that did not cluster according to any phenotype. ERIC-PCR fingerprinting revealed intra-serotypic variations and proved that different *L. monocytogenes* strains were circulating in the country during the isolation period.

Key words: *Listeria monocytogenes*, molecular serotyping, antimicrobial susceptibility, disinfectant tolerance, enterobacterial repetitive intergenic consensus- polymerase chain reaction (ERIC-PCR).

INTRODUCTION

Genus *Listeria* includes eight species (Orsi et al., 2011): *Listeria monocytogene*, *Listeria ivanovii*, *Listeria innocua*, *Listeria welshimeri*, *Listeria seeligeri*, *Listeria grayi* and two new species recently reported named *Listeria marthii* (Graves et al., 2010) and *Listeria rocourtiae* (Leclercq et al., 2009). Only *L. monocytogenes* is a human and animal pathogen, being the causal agent of listeriosis. In humans the disease has two forms, the invasive one that can affect the central nervous system (CNS) leading to death or leaving neurological sequels, while the non-invasive form of the illness causes gastrointestinal syndrome. Listeriosis can occur in apparently healthy

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people and there are risk groups such as infants, pregnant women (Salazar et al., 2001), the elderly and immunocompromised people (Torres et al., 2004); the mortality rate within the risk groups is about 20 to 30% (Korkeala and Siitonen, 2003).

The clinical forms of the disease vary according to the susceptibility of the infected patient. The most common manifestations are meningitis, meningo-encephalitis, septicaemia, abortion, prenatal infection and gastroenteritis (Torres et al., 2005). In sporadic outbreaks and epidemics a wide variety of foods act as vehicles: milk, cheese, pate, beef, pork, poultry meat, vegetables, and seafood (Kells and Gilmour, 2004; Torres et al., 2004).

Significant differences between the virulence of clinical strains and foods depending on the serotypes have been reported (Norrung and Andersen, 2000). Currently 13 serotypes of *L. monocytogenes* have been described; however, three of them (½a, ½b and 4b) have been isolated in more than 90% of the cases from human and animal listerioses (Low et al., 1993; Torres et al., 2004; Orsi et al., 2011). Serotypes such as ½c have been frequently found contaminating food (Espaze et al., 1991; Orsi et al., 2011).

Four evolutionary lineages with different but overlapping ecological niches have been identified for *L. monocytogenes*: group or division I which includes serotypes ½b, 3b, 3c and 4b, commonly associated with human clinical cases (Piffaretti et al., 1989, Orsi et al., 2011), group or division II including serotypes ½a, ½c and 3a commonly found in foods and widespread in farm environments and responsible for causing animal listeriosis and sporadic human clinical cases (Piffaretti et al., 1989; Orsi et al., 2011), group or division III is smaller and comprises serotypes 4a, 4b and 4c (Rasmussen et al., 1995; Wiedmann et al., 1997) and a newly named fourth group or division IV consisting of serotypes 4a, atypical 4b and 4c. Members of lineages 3 and 4 are rare and isolated predominantly from animals (Roberts et al., 2006, Ward et al., 2008, Orsi et al., 2011).

Apparently there are geographical differences in the overall distribution of serotypes; for instance, serotype 4b predominates in Europe and serotypes ½a, ½b and 4b predominate in Canada and the United States. It is known that strains of serotype 4b were the source of most outbreaks reported in Europe and North America (Comi et al., 1992; Schmid et al., 2003; Torres et al., 2004).

In Colombia there are few published studies on *L. monocytogenes* typing (Medrano et al., 2006), either molecular serotyping or conventional serotyping (Vanegas-López and Martínez-León, 2008). A few other publications have been made in which the serotype was related to the type of food and there are few published papers analyzing the antimicrobial susceptibility pattern of *Listeria* spp. (Gallegos et al., 2008).

The purpose of our study was to detect any ERIC-PCR fingerprint relationships among origin, serotype, disinfectant tolerance and antimicrobial susceptibility of *L. monocytogenes* from different cities of Colombia, and hence make a contribution to the knowledge on the behaviour and spread of this pathogen.

MATERIALS AND METHODS

Isolates

DNA of 45 presumptive *L. monocytogenes* isolates collected from foods, humans and animals were used. Food isolates were from: poultry (n=3; 6.6%) from Bogotá; 4 cheese isolates from Bogotá and 7 from Pamplona (Colombia) (n=11; 24.4%); lettuce (n=5; 11.1%) from Funza; spinach (n=15; 33.3%) from Funza; 3 cow raw milk isolates from Madrid (Colombia) and 2 from Bogotá (n=5; 11.1%). Human isolates (n= 5; 11.1%) were distributed as follows: 4 from Bogotá and 1 from Cali. Animal isolates (n=1; 2.2%) were from Bogotá. All of them were stored at -70°C in fresh culture media supplemented with 20% (v/v) of glycerol for cryopreservation (Meza et al., 2004).

Genomic DNA purification, quantification and visualization

Biochemically presumptive *L. monocytogenes* isolates were cultivated in BHI supplemented with 0.5% (w/v) glucose during 12 h at 37°C and 250 rpm. One millilitre of culture was taken for DNA purification using the Wizard Genomic DNA Purification Kit (Promega). DNA purity and concentration were determined with a Biospec 1601 Shimadzu spectrophotometer ($\lambda_{260}/\lambda_{280}$ nm) with background correction set at λ_{320} nm (Sambrook and Russell, 2001).

L. monocytogenes PCR identification

Two sets of primers were employed: L1/U1 and LF/LR (Bansal, 1996; Poutou et al., 2005). The PCR final reaction volume was of 35 µl, composed of 1X Green PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM dNTPs, 20 pmol of primers and 2 µ of GoTaq Flexi DNA polymerase (Promega). Five µl of DNA were used for thermal cycling. Cycling temperature was controlled in a C1000™ Thermal Cycler (BioRad). Amplification cycles and temperatures are listed in Table 1. *L. monocytogenes* (ATCC 19115) and *L. innocua* domestic isolates (L5) were used as PCR controls.

Antimicrobial susceptibility test (AST)

For the antimicrobial susceptibility testing of isolates, a broth microdilution technique (MicroScan system) was employed. A cell suspension equivalent to 0.5 on the McFarland scale prepared in Müeller-Hinton medium supplemented with lysed horse blood was inoculated into the MICroSTREP plus 3 (SIEMENS) panel that included penicillin (PEN), ampicillin (AM), cefotaxime (CFT), cephradine (CFR), cefepime (CPE), chloramphenicol (C), trimethoprim/sulfarnethoxazole (TMP/SMX), cefuroxime (CRM), rifampin (RIF), meropenem (MER), amoxacillin/clavulanic acid (AOX/CLAV), clindamycin (CD), tetracycline (TET), azithromycin (AZI), erythromycin (E), vancomycin (VA), and ciprofloxacin (CP). Panels were incubated following the manufacturer's recommendations. *S. pneumoniae* (ATCC 49619) was used as a control for ASTs (Clinical and Laboratory Standards Institute, 2008). Software Whonet 5.6 (2010) was used for descriptive statistical analysis (Fasehun, 1999; Miranda et al., 2006b).

Table 1. Sets of successive amplifications used for the taxonomic identification, the serotype detection and the molecular characterization of *L. monocytogenes* isolates.

Primer set	Forward sequence	Reverse sequence	Product size (bp)	Thermocycling conditions hot start; (clycling details) $_{\text{number of cycles}}$; final elongation	Specificity	References
L1/U1$^{\nabla}$	CTCCATAAAGGTGACCCT	CAGCMGCCGCGGTAA TWC	938	95°C x 1´; (94°C x 30s, 51°C x 20s, 72°C x 30s) $_{40}$; 72°C x 8´	Genus (16S rDNA)	(Bansal, 1996, Poutou et al., 2005)
LF/LR$^{\nabla}$	CAAACGTTAACAACGCAGTA	TCCAGAGTGATCGATGTTAA	750		Species (*hly*A)	
D1*	CGATATTTTATCTACTTTGTCA	TTGCTCCAAAGCAGGGCAT	214	95°C x 3´; (95°C x 30s; 59°C x 30s; 72°C x 1´) $_{25}$; 72°C x 10´	Division I or III	
D2*	GCGGAGAAAGCTATCGCA	TTGTTCAAACATAGGG CTA	140	95°C x 3´; (95°C x 30s; 59°C x 30s; 72°C x 1´) $_{25}$; 72°C x 10´	Division II	(Borucki and Call, 2003)
*Fla*A*	TTACTAGATCAAACTGCTC^{b}C	AAGAAAAGCCCCTCGTCC	538	95°C x 3´; (95°C x 30s, 54°C x 30s; 72°C x 1´) $_{25}$; 72°C x 10´	Serotypes 1/2a and 3a	
GLT*	AAAGTGAGTTCTTACGAGATTT	AATTAGGAAATCGACCTTCT	483	95°C x 3´; (95°C x 30s, 45°C x 30s; 72°C x 1´) $_{25}$; 72°C x 10´	Serotypes 1/2b and 3b	
MAMA-C*	CAGTTGCAAGCGCTTGGAGT	GTAAGTCTCCGAGGTTGCAA	268	95°C x 10´; (95°C x 30s, 55°C x 1´, 72°C x 1´) $_{40}$; 72°C x 10´	Serotypes 4a and 4c	(Rasmussen et al., 1991, 1995; Jinneman and Hill, 2001)
ERIC 1R/ERIC 2 ♦	ATGTAAGCTCCTGGGGATTCAC	AAGTAAGTGACTGGG GTGAGCG	Several	95°C x 2´; (94ºC x 30s, 92ºC x 30s, 50ºC x 30s, 52ºC x 1´, 65ºC x 8´) $_{35}$, 65° x 8´	Enterobacterial Repetitive Intergenic Consensus	(Jersek et al., 1999; Chung-Hsi and Chinling, 2006)

$^{\nabla}$:1% (w/v) agarose gel in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8.0 ± 0.2), 120 volts, 1h.*: 1.5% (w/v) agarose gel in 1X TAE buffer. ♦: 1% (w/v) agarose gel in 1X TAE buffer at 4volt/cm ^{b}: The underlined nucleotide is a mismatch that was introduced by (Borucki and Call, 2003) to increase primer specificity. Gels for DNA or PCR products were stained with etidium bromide (5 µg/ml) and visualized directly under UV light.

Preliminary determination of disinfectant tolerance of *L. monocytogenes* isolates

We employed two commonly used disinfectants in the national food industry: sodium hypochlorite (Merck) and Tego-51 (Merck). For this analysis we performed a McFarland calibration curve by measuring the $OD_{600\ nm}$ in a Genesys 10 UV spectrophotometer (Thermo Spectronic) and correlating it with the cells·ml^{-1} of each tube of the scale (Equation 1). On the other side, we took into account the equivalence given by Manzano et al. (1997) (Equation 2).

$$y = 0.51267\times10^{-3}X + 0.03768; \quad R^2 = 0.9875 \qquad (1)$$

$$OD_{600nm} = 0.2 \Rightarrow 1\times10^{7} UFC \qquad (2)$$

Isolates of *L. monocytogenes* were grown in BHI broth supplemented with 0.5% (w/v) glucose at 37°C, 100 rpm, for 24 h $OD_{600\ nm}$ was then measured and a cell suspension equivalent to 0.5 on the McFarland scale was prepared in saline solution (0.85% (w/v) NaCl). 300 µl of the suspension were inoculated into 2.7 ml (1/10) of the disinfectant in order to obtain the desired concentration and the mixture was then incubated at room temperature for different exposure times.

After each exposure time, 20 µl of the suspension were inoculated into BHI broth supplemented with 0.5% (w/v) of glucose (1/150) and incubated for 24 h at 35°C.

The $OD_{600\ nm}$ was measured after the incubation, and using the equivalence in cells·ml^{-1} obtained from the McFarland calibration curve, the effect of the disinfectants on the cell population was analyzed comparing it with the

inoculated population of cells·ml^{-1}. If the population of cells·ml^{-1} decreased, we considered this observation as a result of the exposure to the disinfectant and therefore we interpreted it as the minimum inhibitory concentration (MIC) of the disinfectant and expressed it in terms of the concentration of disinfectant and the exposure time in minutes. If the population of cells·ml^{-1} was maintained or increased we considered this observation as a result of "tolerance" and interpreted it as the necessity to increase the concentration of disinfectant and/or the exposure time to find the point in which the microorganism concentration decreases to report a MIC value (MIC/time).

Molecular serotyping

Sorting by divisions

The isolates of *L. monocytogenes* were serotyped by PCR. All the amplifications were performed in a Thermal Cycler C1000 TM (BioRad). Different sets of primers were employed: the first set was D1 which yields a product of 214 bp and classifies isolates into division 1 (serotypes ½b, 3b, 4b, 4d and 4e) or division III (serotypes 4a and 4c). Isolates that did not amplify the 214 bp band were further amplified with the primer set D2, which yields a product of 140 bp and classifies the isolates into the division II (serotypes ½a, ½c, 3a and 3c), (Borucki and Call, 2003).

Serotyping

The isolates classified into division II were subtyped using the FlaA primer set to generate a product of 538 bp that is characteristic of serotypes ½a and 3a; the absence of amplification indicated the presence of serotypes ½c or 3c (Borucki and Call, 2003). Isolates grouped into divisions I and III were subtyped with the GLT primer set to obtain a product of 483 bp that identifies serotypes ½b and 3b; Isolates that did not amplify the band of 483 bp were considered serotype 4 and thus further subtyped with primers MAMA-C (LM4/LMB) yielding an amplified product of 268 bp that identifies serotypes 4a and 4c. In this way the strains that did not amplify were considered serotype 4 (b, d or e), (Jinneman and Hill, 2001), (Table 1). The 100-bp ladder (Promega or Invitrogen) was used as molecular size marker and *L. monocytogenes* (ATCC 19115) was used as PCR control.

Serotyping reaction mixture

Primer sets D1 and D2 were used for classifying into divisions, and primer sets FlaA and GLT were used for PCR subtyping; the reaction mixture consisted of: 25 µl reaction volume, 50 pmol/µl of each primer, 1U of GoTaq Flexi DNA polymerase, 1X of Green PCR Buffer, 0.2 mm of each dNTP, 2.5 mM $MgCl_2$ and 5 µl of sample DNA (Borucki and Call, 2003). For PCR subtyping of serotype 4 of division 3, the primer set MAMA-C was used; the reaction mixture consisted of: reaction volume of 50 µl, 0.5 µmol of each primer, 2U TaqDNApol, 1X PCR buffer, 200 µM of each dNTP, 2.0 mM $MgCl_2$ and 2 µl of sample DNA (Rasmussen et al., 1991; Rasmussen et al., 1995; Jinneman and Hill, 2001). Cycles and temperatures of the amplifications are listed in Table 1.

ERIC-PCR reaction mixture

ERIC1R/ERIC2 primers were used for the ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus) of all the isolates (Table 1). For the amplification mixture we used 75 ng of template DNA in 25 µl of a solution containing 25 pmol of each primer, 0.25 mM of each dNTP, 2.5 mM $MgCl_2$, 2% (v/v) DMSO and 1µ of GoTaq Flexi DNA polymerase (Promega), (Jersek et al., 1999; Chung-Hsi and Chinling, 2006).

Analysis of ERIC-PCR products

The gel was photographed under UV light in the Geldoc (BioRad), which allowed the standardization of gel alignments involving internal reference bands. The similarities between DNA fingerprints were calculated using the Jaccard's coefficient (Sj). The proportion of bands common to two strains, A and B, is defined as:

$$Sj = \frac{\eta AB}{(\eta A + \eta B - \eta AB)}$$

Where: ηAB is the number of bands common to A and B, and ηA and ηB are the total number of bands for A and B respectively. The Jaccard's coefficient is represented as a value between 0 and 1, where 1 represents 100% of similarity (presence and position) of all the bands in the comparison of the two DNA fingerprints, and 0 is the total absence of band similarity (Jaccard, 1901; Jersek et al., 1999; Poutou et al., 2000; Chung-Hsi and Chinling, 2006). Based on the banding, we constructed a dendrogram to analyze the similarities among isolates and to identify clusters, using the UPGMA algorithm and the NTSYSpc software version 2.20 b.

RESULTS

Genus and species identification

PCR of gDNA (Figure 1A) of biochemical and phenotypic presumptive *L. monocytogenes* isolates using sets of primers U1/L1 - LR/LF showed the presence of two PCR products, 938 bp and 750 bp, confirming the genus and species respectively in all the isolates studied. Both *L. monocytogenes* (ATCC 19115) and *L. innocua* (L5) strains used as controls amplified the expected bands (Figure 1B).

Antimicrobial susceptibility evaluation

The antimicrobial susceptibility testing of *L. monocytogenes* isolates was carried out simultaneously to the disinfectant tolerance test. Susceptibility, resistance and intermediate patterns can be seen in Table 1. Considering that *L. monocytogenes* is resistant to cephalosporins from 3rd to 6th generation and that resistance to cephalosporins included in the panel has been reported (Charpentier and Courvalin, 1999; Troxler et al., 2000), the results of antibiotic susceptibility to CFT, CPE, CFR and CRM were excluded from Table 2.

Based on breakpoints established by CLSI (Clinical and Laboratory Standards Institute, 2008; 2010) for *Staphylococcus* spp. and *Enterococcus* spp., 35.5% (16/45) of isolates were classified as multi-resistant. Among the phenotypes of multidrug resistance we found RIF, CD, AZI, E, MER, TMP/SMX and CP. Only 7% (3/45) of multi-resistant isolates showed simultaneous

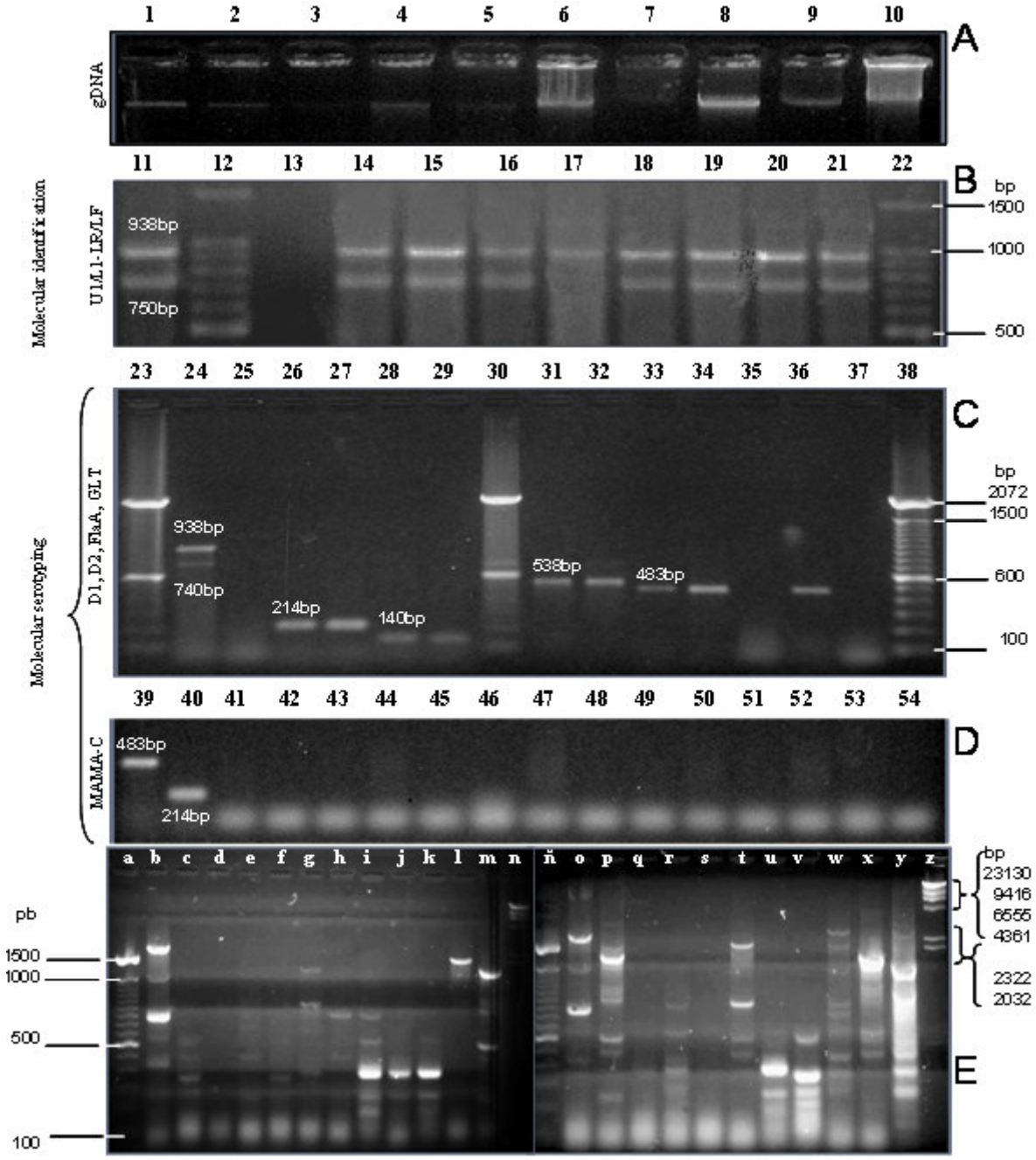

Figure 1. Agarose gels in 1X TAE, processed in Quantity One V. 4.6.9. (BioRad). Electrophoresis. A: Extraction of DNA from different isolates, B: PCR for genus and species identification, C and D: PCR for serotyping, E: ERIC-PCR of the isolates Controls: Lanes B-11, C-24, E-b and E-o: *L. monocytogenes* (ATCC 19115), Lane B-17: *L. innocua* (L5); Lanes B-12 and B-22: 100 bp molecular size marker (Invitrogen), Lanes C-23, C-30, C-38, E-a and E-ñ: 100 bp molecular size marker (Promega); Lanes E-n and E-z: λ-Hind III molecular size marker (Promega). Lanes B-13, C-24, D-54, Ed and Es: PCR Reagents control. Electrophoresis B: Lane 14: LMA-PUJ-13 (cow's milk, Bogotá), Lane 15: LMA-PUJ-39 (cheese, Pamplona), Lane 16: LMA-PUJ-139 (spinach, Funza); Lane 18: LMA-PUJ-118, Lane 19: LMA-PUJ-128, Lane 20: LMA-PUJ-130, Lane 21: LMA-PUJ-133.Electrophoresis C: Lane 24: LMA-PUJ-175 vs. U1-L1/LF-LR primers, Lane 26: LMA-PUJ-58 and Lane 27: LMA-PUJ-62, vs. D1 primers, Lane 28: LMA-PUJ-71 and Lane 29: LMA-PUJ-74, vs. D2 primers, Lane 31: LMA-PUJ-71 and Lane 32: LMA-PUJ-74, vs. FlaA primers, Lane 33: LMA-PUJ-54 and Lane 34: LMA-PUJ-62 vs. GLT primers, Lane 35: LMA-PUJ-196 vs. MAMA-C primers, Lane 36: LMA-PUJ-142 vs. GLT primers, Lane 37: LMA-PUJ-226 vs. MAMA-C primers. Electrophoresis D: Lane 39: LMA-PUJ-54 vs. GLT primers, Lane 40: LMA-PUJ-58 vs. D1 primers. Isolates that did not amplify with the MAMA-C primers, Lane 41: LMA-PUJ-17, Lane 42: LMA-PUJ-55, Lane 43: LMA-PUJ-58, Lane 44: LMA-PUJ-130, Lane 45: LMA-PUJ-151, Lane 46: LMA-PUJ-156, Lane 47: LMA-PUJ-161, Lane 48: LMA-PUJ-163, Lane 49: LMA-PUJ-169, Lane 50: LMA-PUJ-169, Lane 51: LMA-PUJ-191, Lane 52: LMA-PUJ-192, Lane 53: LMA-PUJ-226.

Table 2. Antimicrobial susceptibility of *L.* monocytogenes isolates.

Antimicrobial	Breakpoint (mg/ml)			MIC Range	MIC_{50}	MIC_{90}	Isolates # (%)			
	R	I	S				R	I	S	NS
PEN	-	-	≤ 2	0.25 – 8	1	4	0(0)	0(0)	44 (97.8)	1 (2.2)
AM	-	-	≤ 2	0.25 – 2	0.5	2	0(0)	0(0)	45 (100)	0 (0)
TMP/SMX	≥ 4/76	1/19 - 2/38	≤ 0.5/9.5	0.125 – 1	0.25	0.25	0(0)	1(2.2)	44 (97.8)	0 (0)
AMOX/CLAV	≥ *32*	*4*	≤ *8*	0.125 – 1	0.5	0.5	0(0)	0(0)	45(100)	0 (0)
MER	≥ 16	8	≤ 4	0.12 – 8	4	8	0(0)	13(28.9)	32(71.1)	0 (0)
RIF	≥ 4	2	≤ 1	0.5 – 4	0.5	4	15(33.3)	1(2.2)	29(64.4)	0 (0)
CP	≥ 4	2	≤ 1	0.12 – 2	0.5	2	0(0)	7(15.6)	38(84.4)	0 (0)
CD	≥ 4	1-2	≤ 0.5	0.25 – 8	4	4	33(73.3)	2(4.4)	10(22.3)	0 (0)
AZI	≥ 8	4	≤ 2	0.125 – 8	0.25	2	2(4.4)	0(0)	43(95.6)	0 (0)
E	≥ 8	1-4	≤ 0.5	0.125 – 8	0.25	1	1(2.2)	1(2.2)	43(95.6)	0 (0)
C	≥ 32	16	≤ 8	4 – 8	4	4	0(0)	0(0)	45(100)	0 (0)
TET	≥ 16	8	≤ 4	0.1 – 8	1	8	0(0)	8(17.8)	37(82.2)	0 (0)
VA*	≥ 16	4-8	≤ 2	0.25 – 4	1	2	0(0)	0 (0)	45(100)	0 (0)
VA ◊	≥ 32	8-16	≤ 4	0.25 – 4	1	2	0(0)	0(0)	45(100)	0 (0)

R: resistant, I: intermediate, S: susceptible, NS: non susceptible. *L. monocytogenes* breakpoints were used for PEN, AM and TMP / SMX. For the other antimicrobials we used breakpoints for *Staphylococcus* spp. and *Enterococcus* spp.*: Breakpoints for *Staphylococcus* spp. ◊: Breakpoints for *Enterococcus* spp. (Clinical and Laboratory Standards Institute, 2008, Clinical and Laboratory Standards Institute, 2010).

resistance to CD and E (Figure 2).

The geographic provenance of multi-resistant isolates was as follows: 19% (3/4) from Madrid (Colombia), 71.4% (5/7) from Pamplona (Colombia), 69% (9/13) from Bogotá, 25% (5/20) from Funza, and 100% (1/1) from Cali.

Sodium hypochlorite and Tego-51 tolerance evaluation

Tolerance to two commonly used disinfectants in the domestic industry was evaluated. Isolates showed tolerance variability with different concentrations and exposure times to the disinfectants sodium hypochlorite (halogen) and Tego-51 (amphoteric disinfectant), as evidenced by the MICs obtained (Table 3).

The variability in the MICs found for sodium hypochlorite was: 71% (32/45) isolates had values ≤ 200 ppm/10-15 min (Table 4) and only 29% (13/45) had MICs > 200 ppm/5-10 min. The distribution by sample type or origin of the isolates with MICs > 200 ppm of sodium hypochlorite is shown in Table 4.

Molecular serotyping

Molecular serotyping showed that 73% (33/45) of the isolates were 4b/4d/4e (division I), 16% (7/45) were ½b/3b (division I), 7% (3/45) were ½a/3a (division II) and 4% (2/45) were ½c/3c (division II). There were no isolates belonging to division III. *L. monocytogenes* ATCC 19115 was serotyped ½b (division I), (Figures 1C, 1D) as expected.

ERIC-PCR

The ERIC-PCR analysis showed a total of 28 polymorphic bands ranging from 100 bp to 2810 bp (Figure 1E). These results generated a set of zeros and ones (0s and 1s) that were the data used to construct a dendrogram using the Jaccard's coefficient to show the genetic similarities among isolates (Figure 2).

Only 84.4% (38/45) of the isolates amplified with the ERIC primers. The dendrogram showed three major groups (1, 2 and 3), (Figure 2), separated at Sj = 20%; this first cluster grouped isolates irrespectively. Additionally, there were 3 clusters with 100% of similarity: A, B and C.

The cluster A grouped 3 isolates, one from spinach (serotype 4b/4d), one from lettuce (serotype 4b/4d) and one from milk (serotype ½ a/3a), all of them of division I, with varying antimicrobial resistance patterns and a high geographical proximity.

The cluster B 2 isolates from vegetables (spinach and lettuce), both 4b/4d serotype, from the same geographical area and with resistance to CD. The cluster C is much more distant from other isolates, grouped 2 isolates from spinach, and presented the same characteristics as the cluster B.

DISCUSSION

Genus and species identification

The identification of the genus was done with the primers L1/U1 that detect a sequence of the 16S rDNA and amplify a 938-bp fragment. On the other side, primers

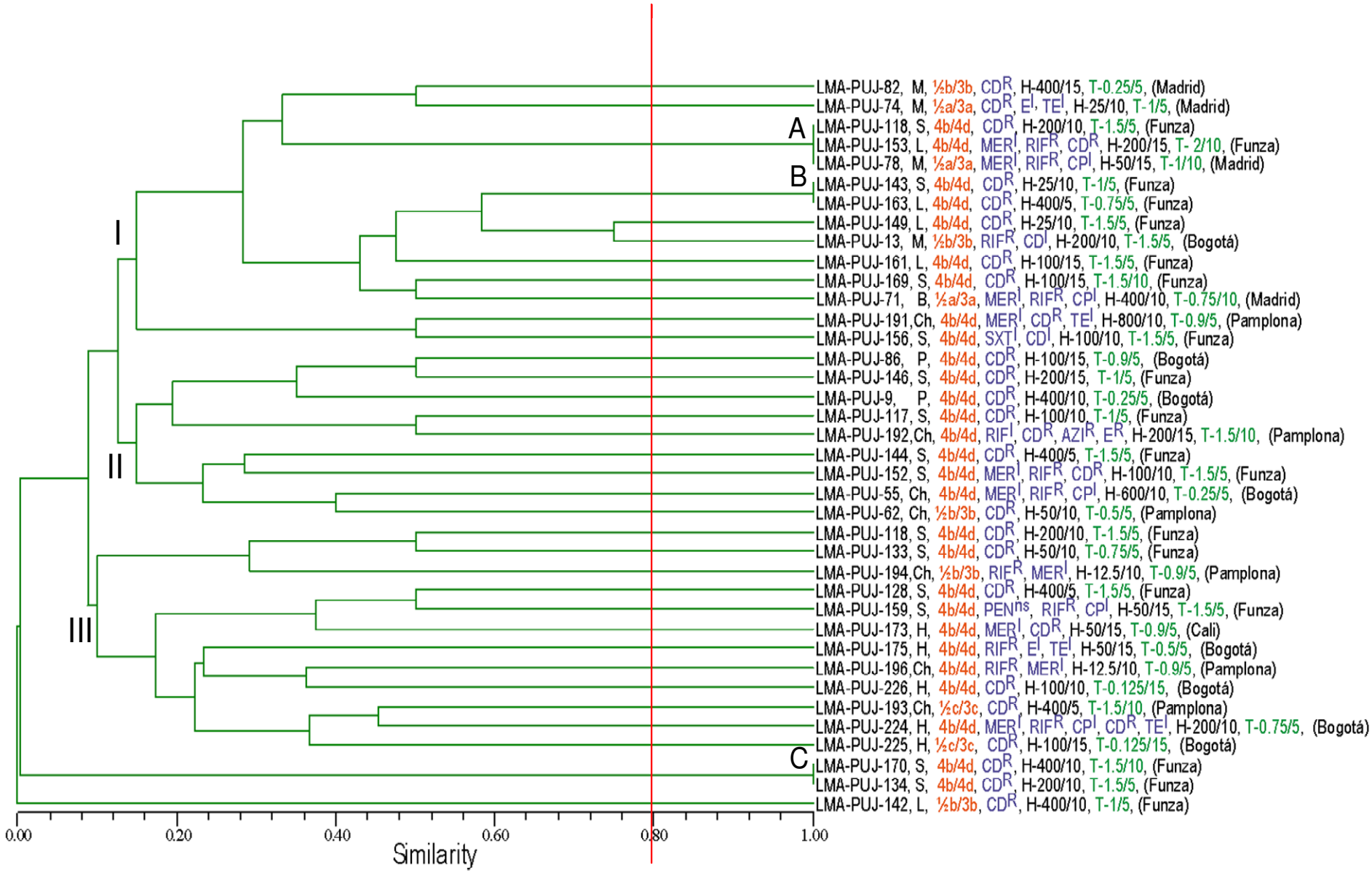

Figure 2. Dendrogram resulting from analysis of ERIC-PCR performed on isolates of *L. monocytogenes* obtained from different sources. The labels of amplified samples are coded as follows: isolate code (black font); source (black font) (M: milk, S: spinach, L: lettuce, V: cow, Ch: cheese, P: poultry, H: human); serotype code (red font); antimicrobial susceptibility patterns (blue font); tolerance to disinfectants sodium hypochlorite (H) (black font) and Tego-51 (T) (green font); geographic provenance of the isolate (black font, in parenthesis).

Table 3. Tolerance to sodium hypochlorite and Tego-51.

Disinfectant	MIC	# isolates (%)
Hypochlorite (ppm/min)	12.5/10	2 (4.4)
	25/10	3 (6.7)
	50/10	3 (6.7)
	50/15	4 (8.9)
	100/10	4(8.9)
	100/15	6 (13.3)
	200/10	7 (15.6)
	200/15	3 (6.7)
	400/5	5 (11.1)
	400/10	5 (11.1)
	400/15	1 (2.2)
	600/10	1 (2.2)
	800/10	1 (2.2)
Tego-51 ((% v/v) /min)	0.125/15	2 (4.4)
	0.25/5	4(8.9)
	0.5/5	2 (4.4)
	0.75/5	4(8.9)
	0.75/10	1 (2.2)
	0.9/5	6 (6)
	1/5	7 (15.6)
	1/10	1 (2.2)
	1.5/5	14 (31.1)
	1.5/10	3 (6.7)
	2/10	1 (2.2)

Bold font: minimum and maximum recommended concentrations for use in the food industry.

Table 4. Distribution of *L. monocytogenes* isolates with a MIC higher than the recommended for sodium hypochlorite.

Origin	Source distribution, origin and tolerance to sodium hypochlorite. # Isolates (MIC ppm/min), (%)				
(# of isolates)	Bogotá	Cali	Madrid (Colombia)	Funza	Pamplona (Colombia)
Spinach (15)	0(0), (0)	0(0), (0)	0(0), (0)	2 (400/5), (13.3%) 1 (400/10), (6.7%)	0(0), (0)
Milk (5)	0(0), (0)	0(0), (0)	1 (400/15), (20%)	0(0), (0)	0(0), (0)
Lettuce (5)	0(0), (0)	0(0), (0)	0(0), (0)	1 (400/5), (20%) 1 (400/10), (20%)	0(0), (0)
Poultry (3)	1 (400/5), (33.3%) 1 (400/10), (33.3%)	0(0), (0)	0(0), (0)	0(0), (0)	0(0), (0)
Cheese (11)	1 (600/10), (9.1%)	0(0), (0)	0(0), (0)	0(0), (0)	1 (400/5), (9.1%) 1 (400/10), (9.1%) 1 (800/10), (9.1%)
Animal (1)	0(0), (0)	0(0), (0)	1 (400/10), (100%)	0(0), (0)	0(0), (0)
Human (5)	0(0), (0)	0(0), (0)	0(0), (0)	0(0), (0)	0(0), (0)

LF/LR detect a region of the *hly*A gene coding for hemolysine O (LLO) and amplify a 750-bp band (Figure 1B). *L. monocytogenes* ATCC 19115 showed the same banding result as expected, whereas *L. innocua* only showed the 938-bp band (Figure 1B).

Antimicrobial susceptibility evaluation

The therapy choices for effective treatment of human listeriosis are penicillin, ampicillin and trimethoprim/sulfamethoxazole (Clinical and Laboratory Standards Institute, 2008). Our *L. monocytogenes* isolates displayed between 97.8% and 100% of susceptibility to those antimicrobials (Table 2). Only 2.2% (1/45) of the isolates were non susceptible to penicillin with a MIC of 4 µg ml^{-1} (Table 2). Recent studies have reported high levels or intermediate levels of resistance to penicillin compared with our results (Santos Mantilla et al., 2008; Chen et al., 2010; Pesavento et al., 2010).

The penicillin MIC_{90} was 4 µg ml^{-1}, exceeding the susceptibility breakpoint (Table 2). This finding suggests an antimicrobial resistance increase of some *L. monocytogenes* isolates when compared with reports on clinical isolates (Martínez-Martínez et al., 2001) that showed MIC_{90} = 1 µg ml^{-1}.

In our study, only one isolate was intermediate (MIC 1 µg ml^{-1}) for TMP/SMX, (Table 2). Our results agree with recent publications that report resistance levels between 0.6% and 1.6% (Lyon et al., 2008; Conter et al., 2009); however, some authors have reported up to 66% of resistance (Yücel et al., 2005). A MIC_{90} lower than the TMP/SMX susceptibility breakpoint proves the antibiotic effectiveness (Table 2).

The majority of isolates were susceptible to one or another antimicrobial of therapeutic primary choice, which is an encouraging result in terms of possible treatment.

All the other antimicrobials except vancomycin have common breakpoints with *Staphylococcus* spp. and *Enterococcus* spp. (Clinical and Laboratory Standards Institute, 2008, 2010); for this reason, they were used for interpreting the susceptibility pattern.

Meropenem shows that 28.9% of isolates are intermediate (Table 2). Apparently it has been used few times for antimicrobial susceptibility testing o treatment; however, failure of clinical treatment against *L. monocytogenes* has been reported before (Stepanović et al., 2004).

As a wide-spectrum antimicrobial, rifampin acts on the RNA polymerase in Mycobacteria and Gram-positives (Wehrli, 1983). RIF resistance has been documented before (Facinelli et al., 1991; Morse et al., 1999; Conter et al., 2007; Santos Mantilla et al., 2008; Conter et al., 2009).

In our study we found 33.3% of resistance intermediates (Table 2), a finding that should be considered as alarming because rifampin is the major choice in therapeutic treatment against *Mycobacterium tuberculosis* when no multidrug-resistance pattern is detected (Tobón, 2001; Miranda et al., 2006a), additionally, it is not convenient to have RIF^R strains that could exchange genetic material indirectly with *M. tuberculosis* through other microorganisms.

Ciprofloxacin MIC_{90} values (2 µg ml^{-1}) similar to that found in our present study (Table 2) have been reported for clinical isolates of *L. monocytogenes* (Martínez-Martínez et al., 2001). Although the frequency of 15.6% of intermediates found in our study is not comparable with lower frequencies found by other authors (1.6%-2.4%), (Li and Logue, 2006; Conter et al., 2009), our results agree with the recently reported reduction on susceptibility to ciprofloxacin (De Nes et al., 2010).

Clindamycin, erythromycin and chloramphenicol interfere with the protein synthesis by binding to the bacterial 50 S ribosomal subunit, and this is why a cross-resistance among them can sometimes be detected (Depardieu et al., 2007). In our study, 73% of isolates displayed a strong resistance against clindamycin. A 2.2% of resistance for erythromycin was found, whereas 100% of isolates were chloramphenicol-susceptible.

An inducible-clindamycin resistance phenomenon has been described in *Staphylococcus* spp. clinical isolates (Clinical and Laboratory Standards Institute, 2010) through the expression of the *erm*M for erythromycin resistance which provokes therapeutic failure when an E^R/CD^I or an E^R/CD^S strain causing infection is being treated with clindamycin (Clinical and Laboratory Standards Institute, 2010). In spite that it is not yet possible to extrapolate this phenomenon to *L. monocytogenes*, it is important to note that in our present study only 3 out of 45 isolates (7%) displayed both clindamycin and erythromycin resistance, suggesting a possible modification of the 23S rRNA in these isolates (Brisson-Noel et al., 1988; Davis and Jackson, 2009).

Tetracycline resistance in *L. monocytogenes* varies widely and it is frequently found in strains from different sources (Harvey and Gilmour, 2001; Pourshaban et al., 2002; Conter et al., 2007; Ruiz-Bolivar et al., 2008). Considering that intermediates have the possibility of becoming into resistant, our results (17.8% intermediates) agree with those of Li et al. (2006), who found 18.6% of resistance (Table 2). Despite some other reports on susceptibility, tetracycline is not considered a primary choice drug for listeriosis treatment and its use is not recommended in children and pregnant women.

Disinfectant tolerance evaluation

The cell wall of Gram-positives consists essentially of peptidoglycan and teichoic acid. Neither compound appears to act as an effective barrier against the entry of antiseptics and disinfectants. Large molecules can easily cross the cell wall, which may explain the sensitivity of these organisms to many antibacterial agents. However, the plasticity of the bacterial cell envelope is a

phenomenon that can be affected by the rate of growth, nutrients that affect the physiological state of cells, the thickness and the loss of peptidoglycan (McDonnell and Russell, 1999).

In this study we evaluated the tolerance of *L. monocytogenes* isolates from different origins to sodium hypochlorite (NaOCl) and Tego-51 ($C_{18}H_{40}ClN_3O_2$), two commonly used disinfectants in the domestic industry. Sodium hypochlorite neutralizes the amino acids forming salt and water, leading to the formation of chloramines that interfere with cell metabolism, and breaks down fatty acids generally affecting the integrity of the plasma membrane, causing irreversible enzyme inhibition and altered phospholipid metabolism (Estrella et al., 2002). Moreover, Tego-51 is an amphoteric surfactant that reduces surface tension of the membrane affecting the permeability and the exchange of substances and nutrients. The properties of the membranes of microorganisms differ depending on their chemical composition; in this way, the effect of disinfectants will not be the same in Gram-positives and Gram-negatives (Copello et al., 2008).

In general, it has been shown that several of the sanitizing agents and disinfectants used in the food industry are effective against *L. monocytogenes* in cell suspension, but the formation of biofilms and the presence of organic matter significantly decrease the effectiveness of disinfectants (Norwood and Gilmour, 1990; Seok and Schraft, 2000; Aarnisalo et al., 2007; Kastbjerg and Gram, 2009).

Sodium hypochlorite tolerance

This study proposes a dilution test as an alternative methodology to assess the tolerance of *L. monocytogenes* strains to disinfectants, based on the increase in optical density ($OD_{600\ nm}$) and by calculating the concentration of cells ml^{-1} from a McFarland calibration curve and Makino equivalence. Our results showed variation in susceptibility to disinfectants among isolates, taking into account that different MICs were found for the disinfectants tested (Table 3).

Only 28.8% (13/45) of the isolates showed MICs higher than those commonly used in industry, which is an encouraging finding, but it is known that *L. monocytogenes* tends to form biofilms that increase its tolerance to hypochlorite up to 2,500 ppm (Lundén et al., 2003).

Biofilm resistance to biocide action seems to depend on its structure. As the biofilm gets older and thicker, resistance will be lost as the biofilm structure disassembles during the disinfection procedures. Consequently, the effectiveness of disinfection will be directly related to the ability of pre-cleaning to remove and break down the extra-cellular matrix. Similarly, it was found that sodium hypochlorite and anionic sanitizers are better than quaternary ammonium compounds and iodine in cleaning stainless steel surfaces to eliminate extra-cellular polymeric substances excreted by *Listeria* (Herrera, 2004).

One of the 2 previous officially unpublished studies showed that 1,000 ppm of hypochlorite were required for inhibiting the growth of *L. monocytogenes* cell suspensions. The second one showed that concentrations of sodium hypochlorite of about 250 and 500 ppm inhibited the growth of *L. monocytogenes*, although that study included only 5 isolates and tested only 5 concentrations.

Another recent study has evaluated 25 *L. monocytogenes* isolates by comparing their response to sodium hypochlorite and Tego-51 besides other disinfectants. Tolerance was found between 100 and 200 ppm for sodium hypochlorite. In the case of Tego-51, the sensitivity found was of 0.25% (Molina-Moreno et al., 2009).

Results obtained in our study are consistent with those of previous reports (Molina-Moreno et al., 2009), although methodologies used to determine the tolerance or sensitivity differed among studies.

Tego-51 tolerance

It is encouraging that only one isolate showed a Tego-51 tolerance above the manufacturer's recommended concentration (Table 3). Our study included 21 isolates that were studied in a parallel research that tested the Tego-51 tolerance by other methods (Molina-Moreno et al., 2009).

Our results agreed with those of 76.2% (16/21) of isolates and only 23.8% (5/21) did not coincide. Human isolates from all patients showed MICs within the limits employed in the industry, but the recommendation for the use of Tego-51 in hospitals is 0.01% for 30 s; however, to ensure the effectiveness against pathogens such as *P. mirabilis* or *B. subtilis*, concentrations above 0.1% (v/v) are recommended for an effective disinfection of hospital instruments and floors (Suk et al., 1997).

Romanova et al. (2006) showed that MRDL, one of the two efflux pumps identified in *Listeria*, is involved in the adaptation to benzalkonium chloride (Romanova et al., 2006). Other efflux pumps confering resistance to disinfectants have been described in *S. aureus* and *S. epidermidis* (McDonnell and Russell, 1999).

On the other hand, plasmids carrying genes for resistance to disinfectants have been found in *S. aureus* (MRSA), *Corynebacterium jeikeium*, *Enterococcus faecium* and *Streptococcus mutans*. Considering that *L. monocytogenes* exchanges genetic material either directly or indirectly with some of those microorganisms, it is appropriate to think about the possibility of acquiring molecular mechanisms of resistance to disinfectants; however, we did not find any MICs values in our study that could raise suspicion about the presence of any of

the above mechanisms of resistance to disinfectants.

Molecular serotyping

Only certain serotypes occur more often in food or in infecting humans or animals, which are differentiated by the antigenic determinants expressed on the cell wall from lipoteichoic acids of membrane proteins of the flagella and fimbriae (Graves et al., 1999). Although 12 serotypes can cause disease, only 95% of the *L. monocytogenes* isolated from human listeriosis cases corresponded mainly to 3 serotypes: ½a, ½b, and 4b (Kathariou, 2002). Apparently there are geographical differences in the overall distribution of serotypes, being the serotype 4b predominant in Europe and the serotypes ½a, ½b and 4b predominant in Canada and the United States (Torres et al., 2004; Taillefer et al., 2010). We also know that strains with serotype 4b were the source of all the outbreaks reported in Europe and North America during the past 25 years (Schmid et al., 2003; 2005; Orsi et al., 2011; Taillefer et al., 2010).

In Colombia, there are few published data or easily accessible documents with information about the presence and distribution of *L. monocytogenes* serotypes. This is one of the first studies that carries out a molecular serotyping of isolates from different sources. Our findings show that despite the non epidemiological distribution of the *n* the most frequent serotype is 4b/4d, which is present in isolates from all the different sources. Serotype ½c/3c was found in a human source and a food source (cheese), ½b/3b was found in food (lettuce, milk and cheeses), and ½a/3a was found in animal (cow) and food (milk) sources.

Strains with serotype 4b have presented incidences between 50% and 70% in clinical cases, and have been also identified in sporadic infections, are common sources of epidemics, and are representative of perinatal listeriosis showing the ability to cross the placental barrier (Marakusha et al., 1996).

Serotypes 4b and ½c have been found in sporadic cases of listeriosis and epidemics. Serotype ½b has been found in non-pregnant women with severe disease, corresponding in some studies to up to 10% of the cases (Torres et al., 2004). An investigation in Los Angeles discovered an incidence of 31% of serotype ½b, except those associated with episodes of foodborne diseases (FBD). This serotype was identified in 65% of patients infected with human immunodeficiency virus (HIV), considering a possible association with diet or sexual practices. Serotype ½b may be relatively non-infectious for individuals in other risk categories. This serotype seems to be genetically close to serotype 4b and very different from serotype ½a; however, both serotypes ½ and 4b represent distinct lineages that vary in the composition of their antigens and possibly in their virulence and ecological niches (Kathariou, 2002).

Our results revealed that in a small sample there are several serotypes circulating in an interchangeable way, a finding that deserves importance for the soon beginning of epidemiological studies.

ERIC-PCR fingerprinting

Different ERIC-PCR fingerprints were found (Figure 1E and 2). No isolates were grouped according to phenoltype, suggesting an extensive intra-serotypic variation and showing a wide dispersion of ERIC-PCR patterns. This consideration is supported by the fact that isolates formed interchangeable groups: group 1 (vegetables and milk: serotypes 4b/4d, ½b/3b and ½a/3a), group 2 (chicken, vegetables and dairy products: serotypes 4b/4d and ½b/3b), and group 3 (human, plant and dairy products: serotypes ½b/3b, 4b/4d and ½c/3c). Similarly, no significant association was found based on the source of all isolates (food, animal or human source).

The ERIC-PCR technique has been used to analyze isolates of *L. monocytogenes* in experimental conditions that allow the strains to be grouped according to the source of isolation with an Sj <20% (Jersek et al., 1999), but our study showed no similarity among strains isolated from humans or animals and strains isolated from food.

On the other hand, Chen et al. (2010) used the ERIC-PCR for genotyping the *tet*M gene in isolates of *L. innocua* from fish, finding in the strains that they investigated that the genotypes of this gene are unique and the similarity between them is low, which coincides to some extent with our results.

Conclusion

In a small sample without any epidemiological distribution, several *L. monocytogenes* serotypes that have been associated by other authors to different situations (outbreaks, foods, surfaces, etc.) were found to be in circulation in Colombia without any specific association to source or city. Our isolates revealed different degrees of tolerance to both disinfectants commonly used in industry, and showed that Tego-51 continues to be more effective, but noting that our test was performed in cell suspensions, which led to the assumption that biofilm tolerance may be higher. In terms of antimicrobial susceptibility, the antibiotics of choice are still effective; however, we must emphasize that *in vitro* resistance to RIF and CD, as well as intermediate susceptibility to MER, TET and CP was high.

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