Characterization and antibiotic susceptibility of *Listeria monocytogenes* isolated from poultry and red meat in Morocco

Hayat Ennaji[1,2]
Mohammed Timinouni[2]
My Mustapha Ennaji[3]
Mohammed Hassar[1]
Nozha Cohen[1]

[1]Laboratoire de Microbiologie et Hygiène des Aliments et de l 'Environnement, Institut Pasteur du Maroc., Casablanca, Morocco; [2]Laboratoire de Microbiologie et Biologie Moléculaire, Institut Pasteur du Maroc., Casablanca, Morocco; [3]Laboratoire de Virologie et Hygiène and Microbiologie., Faculté des Sciences et Techniques - Mohammedia, Université Hassan II, Mohammedia, Morocco

Abstract: This study was carried out on 426 samples of raw meats collected from butcheries and supermarkets in Casablanca, Morocco. The samples were examined for the occurrence of *Listeria* species. Strains of *Listeria monocytogenes* were characterized by several biochemical tests and confirmed by polymerase chain reaction (PCR). β-hemolytic cultures and nonhemolytic isolates were tested for biochemical properties with the *Listeria* API test. Among the 43 *Listeria* species isolates; we identified 10 strains for *L. monocytogenes* (23.3%), 31 strains for *L. innocua* (72.1%) and 2 strains for *L. welshimeri* (4.6%). Strains of *L. monocytogenes* were separated by multiplex PCR; two serogroups IIb and IVb were thus differentiated. Antibiotic susceptibility of *L. monocytogenes* to 21 antibiotics was determined by the disk diffusion method. All isolates were susceptible to a wide range of the tested antibiotics with the exception of nalidixic acid, colistine and cephalosporins second and third generation for which they were all resistant.
Keywords: antibiotic susceptibility, *Listeria monocytogenes*, meat, PCR

Introduction

Listeria monocytogenes has long been acknowledged as a significant human and animal pathogen (Schuchat et al 1991; Nightingale et al 2004) and it is often implicated as sources of human listeriosis cases and outbreaks (Aureli et al 2000; De Valk et al 2001). However, sporadic listeriosis remains the most frequent manifestation of the illness (Gilot et al 1996).

The following individuals are at great risk for listeriosis: pregnant women (and their unborn children), new-born, immunocompromised persons (Walter 2000), and the elderly. This infection is regularly reported in Europe and North America, but in Africa and other developing countries (where the food industry is not very developed) only a few sporadic cases have been reported (Boukadidda et al 1994). In Morocco, the incidence of human listeriosis is rare (Benomar et al 2000).

L. monocytogenes often lives in the cold and moist environment found in refrigerators and it is present in all categories of food (Farber and Peterkin 1991; Azevedo et al 2005). Meat, poultry, and meat products have frequently been shown to be contaminated with this pathogenic bacterium (Lawrence and Gilmour 1994; Jay 1996). A number of antibiotics have been suggested for the treatment of *L. monocytogenes*. Unfortunately, failures have been reported for all therapeutic programs because there is no consensus among various authors as to which antibiotic regimen is the most effective (McLauchlin et al 1991). The mains goals of our work were: i) to detect *L. monocytogenes* by conventional method and application of a chromogenic medium for the rapid confirmation ii), to confirm and to serotype the strains isolated by polymerase chain reaction (PCR) and multiplex PCR, respectively, iii) and to determine *in vitro* the activity of various antibiotics against strains of *L. monocytogenes* isolated from red meat and poultry.

Correspondence: Nozha Cohen
Laboratoire de Microbiologie et d'Hygiène des Aliments et de L'Environnement, Institut Pasteur. 1, Place Louis Pasteur, Casablanca 20100, Morocco
Tel +212 6186 9299
Fax +212 2298 5063
Email nozha.cohen@pasteur.ma

Materials and methods

Sample collecting

A total of 426 samples: (a) raw meat (n = 112), (b) meat product (n = 240), and (c) poultry (n = 74) were collected from butcheries and supermarkets in Casablanca, Morocco. The samples were transported in iceboxes to our laboratory and analyzed immediately upon arrival using the ISO 11290-1 method.

Bacteriological analysis

Twenty-five grams of each sample were weighed into sterile stomacher bags, diluted with 225 ml of *Listeria* pre-enrichment Frazer broth (Biokar Diagnostics, BK115HA) homogenized and incubated at 30 °C for 24 h. A 1 ml portion from this pre-enrichment culture was transferred to 9 ml enrichment broth (Complete Fraser broth, BIO-RAD), and incubated at 37 °C for 24 h. A loopful of the enrichment culture was streaked onto Palcam, Oxford (AES Laboratory) and RAPID'L. mono (BIO-RAD, France). Plates were incubated at 37 °C for 24 h to 48 h. A great advantage of the chromogenic agar, RAPID'L. mono, that it allows for the differentiation *L .monocytogenes* from other *L*. spp. by a simple color change reaction even after 24 h; colonies appear blue. For identification of *L. monocytogenes* suspect colonies selected from Oxford, Palcam, and RAPID'L. mono agars were streaked on trypticase soya agar plate with 0.6% yeast extract (TSAYE; Biokar Diagnostics) and tested for Gram coloration, motility, oxydase, CAMP test, and hemolysis test. Typical colonies were characterized biochemically by the *Listeria* API commercial kit (Biomerieux, Mercy l'Etoile, France).

Molecular analysis

Total genomic DNA and PCR amplifications were used as described previously (Holoko et al 2002). For *L. monocytogenes*-specific identification, two primers pairs derived from the *L. monocytogenes* gene: hly1: 5'-CGGAGGTTCCGCAAAAGATG-3' and hly2: 5'- CCTCCAGAGTGATCGATGTT-3' were used. PCRs were carried out in a thermocycler (GeneAmp PCR system 2700, Applied Biosystems) in a 50 µl reaction volume containing: 5 µl target DNA, 5 µL 10 × Tp PCR, 200 µM dNTP, 1.5 mM $MgCl_2$; 0.4 µM of each primer, and 1 Unite of Taq DNA polymerase.

Amplification started with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles (94 °C for 1 min, 56 °C for 45 s, and 72 °C for 45s). Final extension was performed at 72 °C for 10 min. The PCR products were separated in a 1.2% agarose gel and stained with ethidium bromide at 0.5 µg/ml.

Serotype identification by multiplex PCR

Serogroups and serovars determinations were performed by multiplex PCR, according to the method described by Doumith and colleagues (2004). These analyses were practiced in the Centre National de Référence des *Listeria*, Institut Pasteur, Paris.

Antibiotics susceptibility

Fresh bacterial colonies of *L. monocytogenes* isolates were separately grown at 37 °C in brain heart infusion broth (BHI) (Merk V4324393-947) for 24 hours and each inoculum was applied on Muller Hinton (MH) agar (BIO-RAD 2M2121). Antibiotic susceptibility was determined by the disk diffusion method. Standard discs were applied using a disc dispenser and the plates were incubated at 37 °C for 24 h to 48 h. Then the size of inhibition zone was determined as previously described (Soussy 2005). The Antibiotics tested were selected considering i: the most frequently used in treatment, of farm animals (Johnston 1998). ii: those used to prevent and control infectious diseases in poultry production (Abiola et al 2005). iii: the most important drugs used for the treatment of human infections (Wiggins et al 1978; Winslow and Pankey 1982).

Results

Occurrence of *L. monocytogenes* in meats

Out of 426 samples examined, 43 (10%) were found to be contaminated with *L.* spp. Table 1 presents the types, numbers, and source of the samples analyzed in this study. Among the 43 strains of *L. species* isolates, 10 strains for *L. monocytogenes* (23%), 31 strains for *L. innocua* (72.1%), and 2 strains for *L. welshimeri* (4.6%) were identified.

Confirmation by PCR

To confirm the identification of the ten strains characterized by biochemical tests, we have done PCR analyses. We used two primers derived from the virulence associated gene hly specifically those that amplifying the 234 bp region of the listeriolysin O gene. PCR amplification gives all amplicons with same molecular size as shown in Figure 1.

Serotyping

Analysis of serogroups and serovars distribution showed that *L. monocytogenes* was represented by two serogroups. The first (IIb) comprised strains of serovars 1/2b, 3b, and 7 and the second (IVb) comprised strains of serovars 4b, 4d, and 4e. The results are shown in Table 2.

Table 1 Occurrence of *Listeria monocytogenes* in red meat and poultry

Type of meat	Nb of samples	*L.* spp.	*L.* m.	*L.* in.	*L.* w.
Raw meats and meats products					
Meats	112	5 (4.5%)	1 (0.9%)	4 (3.5%)	–
Ground meat and sausages	240	23 (9.6%)	8 (3.3%)	15 (6.2%)	–
Raw poultry	74	15 (20.3%)	1 (1.3%)	12 (16.2%)	2 (2.7%)
Total	426	43 (10.1%)	10 (2.4%)	31 (7.3%)	2 (0.5%)

Antibiotics susceptibility

Table 3 summarizes activities of the 21 antibiotics tested against the 10 strains of *L. monocytogenes* isolated from meat and poultry. In general, all isolates are susceptible to a wide range of antibiotics especially for amoxicillin, ticarcillin, gentamicin, tobramycin, amikacin, chloramphenicol, penicillin, and ampicillin. These strains showed different resistance levels of certain antibiotics and were completely resistant to nalidixic acid, colistine, and cephalosprins second and third generations.

Discussion

In our study the incidence of *L. monocytogenes* was 2.4% and the rate of contamination in ground meat was 3.3%. Compared to that published by other authors, nearly the same incidences were found in raw minced meat 4.7% (Yucel et al 2005) and moderate incidences 10.6% have been reported in raw sausages (Cordano and Rocourt 2001). On the contrary, in Tunis the presence of *L. monocytogenes* was lower; out of a total of ground meat samples analyzed only one strain has been contaminated (Etttriqui et al 1995). In the similar study done in Morocco, the prevalence of *L. monocytogenes* was 16.4% in ground bovine meat, and 32% in raw bovine sausages (Kriem et al 1998). This difference regarding the occurrence between our results realized in Casablanca and those effected in Rabat, Morocco, can be explained by the fact that this study was carried out after the inauguration of the new slaughterhouse installed in Casablanca, on the level of which; legislatives texts governing the inspection of the meats and hygiene have been applied. It relates a whole of a very rigorous control at each key point of the meat production. For the raw meat; from all the samples analysed (112), *L. monocytogenes* was found in only 1 sample (0.9%). According to other studies, the incidence was variable; Yucel and colleagues (2005) and Hilbert and colleagues (2004) detected this organism in 5.2% and 12% in beef, respectively.

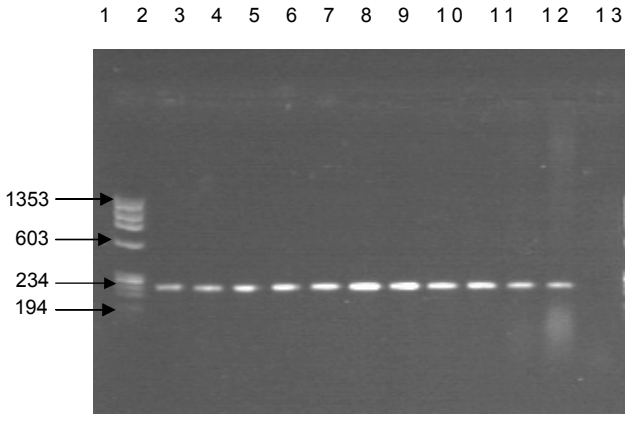

Figure 1 Identification by PCR of *Listeria monocytogenes*.
Notes: Lane: 1, molecular size marker $\phi \times 174$ diggers by Hae III; Lanes: 2–11, *Listeria monocytogenes* samples; Lane: 12, reference strain of *Listeria monocytogenes* (positive control); Lane: 13, negative control.

In Morocco, white meat is an important source of protein. In 2004, the population of Casablanca alone consumed 338,000 tons. Two kinds of poultry slaughtering are used in Morocco. One is an automated poultry slaughtering process established recently, whereby automated systems are used for scalding, plucking, eviscerating, rinsing, and packaging carcasses. Carcasses are then stored at 4 °C before sale to supermarkets. The second is traditional slaughtering which is commonly practiced in shops under poor hygienic conditions. More than 90% of poultry slaughtering in Morocco is done by traditional procedures (Cohen et al 2007). For this reason, we have also examined poultry for the occurrence of *L. monocytogenes* and the incidence rate was (1.3%). Other data on the prevalence of this bacterium have been reported by others authors in different countries: 11.5% (Yucel et al 2005) and 26% (Hilbert et al 2004) in chicken meat, and 32% in poultry meat (Capita et al 2001).

Concerning the antibiotic susceptibility, for gentamicin, all our isolates were susceptible to this antibiotic; our data are similar to those of Aureli and colleagues (2003). Likewise, both penicillin and ampicillin showed good activity against *L. monocytogenes*; these results are in agreement with those reported previously by Wong and colleagues (1990) who found 98.3% and 99.4% of susceptibility, respectively.

Table 2 Origin of isolates strains and identification of serogroups with multiplex PCR

N° of strains	Origin	Multiplex PCR fragment amplification					
		prs	*Lmo*1118	*lmo*0737	ORF2110	ORF2819	PCR Group
1	Sausages	+	–	–	–	+	IIb
2	Sausages	+	–	–	+	+	IVb
3	Sausages	+	–	–	–	+	IIb
4	Meats	+	–	–	–	+	IIb
5	Sausages	+	–	–	–	+	IIb
6	Ground meat	+	–	–	–	+	IIb
7	Sausages	+	–	–	–	+	IIb
8	Ground meat	+	–	–	+	+	IVb
9	Ground meat	+	–	–	–	+	IIb
10	Poultry	+	–	–	–	+	IIb

Abbreviation: PCR, polymerase chain reaction.

The isolates were all susceptible to chloramphenicol, similarly, Yucel and colleagues (2005) indicated that *L. monocytogenes* isolated from meat products were highly sensitive with a percentage of (100%). While Aureli and colleagues (2003), showed in their study that the various strains seem to be moderately susceptible (49.1%) in poultry and totally (100%) susceptible in meat. Of the other antibiotics, trimethoprim and trimethoprim in combination with sulfamethoxazole, except for one strain (meat) was resistant to both antimicrobial agents. In previous other findings, Yucel and colleagues (2005) indicated that the percentage of resistant strains of *L. monocytogenes* in meats was 66%.

Table 3 Activities of antimicrobial agents tested against the strains of *Listeria monocytogenes* isolated from meat and poultry

Antimicrobial agent	µg/disc	Nb that were susceptible		
		Resistant	Intermediate	Susceptible
Amoxicillin (AMX)	25	–	–	10
Ticarcillin (Tic)	25	–	–	10
Cephalotin (CF)	30	01	–	09
Cefoxitin (FOX)	30	10	–	–
Cefotaxime (CTX)	30	10	–	–
Ceftazidime (CAZ)	30	10	–	–
Cefazolin (CZ)	30	01	01	08
Amoxicillin + Clavulanic. Ac (AMC)	20/10	–	–	10
Cefixim (CFM)	30	10	–	–
Gentamicin (GM)	500	–	–	10
Tobramycin (TOB)	10	–	–	10
Amikacin (AN)	30	–	–	10
Trimethoprim (TMP)	5	01	–	09
Trimethoprim + Sulfamethoxaxol (SXT)	1,25/23,75	01	–	09
Nalidic acid (NA)	30	10	–	–
Colistin (CS)	10UI	10	–	–
Neomycin (N)	30UI	–	–	10
Teicoplanin (TEC)	30	–	–	10
Chloramphenicol (C)	30	–	–	10
Penicillin (G)	10UI	–	–	10
Ampicillin (AMP)	10	–	–	10

Finally, for the activity of the first generation cephalosporins tested, only one strain (meat) was resistant to cephalotin and cefazolin and one other strain had showed moderate susceptibility to cefazolin. Comparing to others studies realized in various foods (including meats), Wong and colleagues (1990) indicated highly susceptibility to cephalotin (99.4%). Similarly, Abiun and colleagues (1994) and Navratilova and colleagues (2004) showed that cephalotin displayed good activity. For the second and third generation cephalosporins, we have found resistance to cefotaxime, cefoxitin, cefixim, and ceftazidime. Because of the poor activity or complete resistance of the second and third generation cephalosporins against *L. monocytogenes*, Cormican and Jones (1995) confirmed that recent cephalosporins should not be used clinically for treating listeriosis.

In conclusion, in this study we have demonstrated that almost all strains of *L. monocytogenes* were susceptible to a wide range of antibiotics effective against Gram-positive bacteria, belonging to amino-glycosides group and glycopeptides and some strains among the isolates were resistant to one or more antimicrobial agent. Therefore veterinarians should be able to control the use of antimicrobial drugs in veterinary practice. On the other hand, the survey of this virulent bacterium in meat appears to be critically important from the viewpoint public health. Consistent with other countries, Morocco is experiencing newer consumer trends in the culinary traditions with an obvious tendency to adopt international practice of consuming processed foods, some of which are uncooked or undercooked. Such a tendency combined with incidence of *L. monocytogenes* contamination reported in meat, thereby increasing the risk of listeriosis for consumers (Cohen et al 2006). Where a suspect sample of poultry will be well cooked before consumption, the risks to the consumer are considered minimal. This is probably the reason why poultry and poultry products are rarely associated with listeriosis (Van Schothorst 1994). Results obtained in this study provide evidence that may be used by the Moroccan government to adopt regulations enforcing the application of the hazard analysis critical control points (HACCP) system as a means to identify and control the hazards in foods and especially in meat products. Furthermore, these results may promote the acceptance of programs such as HACCP by the meat industry in an attempt to provide safer and more wholesome products.

Acknowledgments

The authors are grateful to Dr Alban Le Monnier and Dr Alexandre LeClercq from Centre National de Référence des Listeria, Institut Pasteur Paris for their collaboration of the molecular serotyping. We also would like to thank the Casablanca authorities for their help.

References

Abiola FA, Diop MM, Teko-Agbo A, et al. 2005. Antimicrobial agents residues in liver and gizzard of broilers in the areas of Dakar and Thiès (Sénégal). *Rev Med Vet*, 156:264–8.

Abuin CMF, Fernandez EJQ, Sampayo CF, et al. 1994. Susceptibilities of *Listeria* species isolated from food to nine antimicrobial agents. *Antimicrob Agents Chemother*, 38:1655–7.

Aureli P, Fiorucci GC, Caroli D, et al. 2000. An outbreak of febrile gastroenteritis associated with corn contaminated by *Listeria monocytogenes. N Engl J Med*, 342:1236–41.

Aureli P, Ferrini AM, Mannoni V, et al. 2003. Susceptibility of *Listeria monocytogenes* isolated from food in Italy to antibiotics. *Int J Food Microbiol*, 83:325–30.

Azevedo I, Regalo M, Mena C, et al. 2005. Incidence of *Listeria* spp. in domestic refrigerators in Portugal. *Food Control*, 16:121–4.

Benomar S, Nejjari N, Lahbabi MS. 2000. Listériose néonatale: une infection exceptionnelle au Maroc. *Arch Pediatr*, 7:428.

Boukadidda J, Sboui H, Monastiri H, et al. 1994. La listériose humaine en Tunisie: deux nouveaux cas chez le nouveau- né. *Med Mal Infect*, 24:117–18.

Capita R, Alonso-Calleja C, Moreno B, et al. 2001. Occurrence of *Listeria species* in retail poultry meat and comparison of a cultural/immunoassay for their detection. *Int J Food Microbiol*, 65:75–82.

Cohen N, Ennaji H, Hassar M, et al. 2006.The bacterial quality of red meat and offal in Casablanca (Morocco). *Mol Nutr Food Res*, 50:467–590.

Cohen N, Ennaji H, Bouchrif B, et al. 2007. Comparative study of microbiological quality of raw poultry meat at various seasons and different slaughtering process in Casablanca (Morocco). *J Appl Poultry Res*, 16:502–8.

Cordano AM, Rocourt J. 2001.Occurrence of *Listeria monocytogenes* in food in Chile. *Int J Food Microbiol*, 70:175–8.

Cormican MG, Jones RN. 1995. Antimicrobial activity of cefotaxime tested against infrequently isolated pathogenic species (Unusual Pathogens). *Diagn Microbiol Infect Dis*, 22:43–8.

De Valk H, Vaillant V, Jacquet C, et al. 2001. Two consecutive nationwide outbreaks of listeriosis in France, October 1999-February. *Am J Epidemiol*, 154:944–50.

Doumith M, Buchrieser C, Glaser P, et al. 2004. Differentiation of the major *Listeria monocytogenes* serovars by Multiplex PCR. *J Clin Microbiol*, 42:3819–22.

Etttriqui A, Dhaou MA, Fliss I, et al. 1995. Research of *Listeria monocytogenes* in meat products in region of Tunis. *MHA*, 7:8–13.

Farber JM, Peterkin PI. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol Rev*, 55:476–511.

Gilot P, Genicot A, André P. 1996. Serotype and esterase typing for analysis of *Listeria monocytogenes* populations recovered from foodstuffs and from human patients with listeriosis in Belgium. *J Clin Microbiol*, 34:1007–10.

Hilbert F, Mayrhofer S, Paulsen P, et al. 2004. Antimicrobial resistance profile of five major food-borne pathogens isolated from beef, pork and poultry. *Int J Food Microbiol*, 97:23–9.

Holoko I, Urbanova J, Kantikova M, et al. 2002. PCR Detection of *Listeria monocytogenes* in milk products and differentiation of suspect isolates. *Acta Vet Brno*, 71:125–31.

Jay JM. 1996. Prevalence of *Listeria spp*. in meat and poultry products. *Food Control*, 7:209–14.

Johnston AM.1998. Use of antimicrobial drugs in veterinary practice. *Br Med J*, 317:665–7.

Kriem MR, EL-Marrakchi A, Hammama A. 1998. Prevalence of *Listeria* spp. on a variety of meat products in Morocco. *Microbiol Aliment Nutr*, 16:179–87.

Lawrence LM, Gilmour A. 1994. Incidence of *Listeria* spp. and *Listeria monocytogenes* in a poultry processing environment and in poultry products and their rapid confirmation by multiplex PCR. *Appl Environ Microbiol*, 60:4600–4.

McLauchlin J, Audurier A, Taylor AG. 1991. Treatment failure and recurrent human listeriosis. *J Antimicrob Chemother*, 27:851–7.

Nightingale KK, Schukken YH, Nightingale CR, et al. 2004. Ecology and transmission of *Listeria monocytogenes* infecting ruminants and in the farm environment. *Appl Environ Microbiol*, 70:4458–67.

Navratilova P, Schlegelova J, Sustackova A, et al. 2004. Prevalence of *Listeria monocytogenes* in milk, meat and foodstuff of animal origin and the phenotype of antibiotic resistance of isolated strains. *Vet Med-Czech*, 49:243–52.

Schlech III WF. 2000. Food-borne listeriosis. *Clin Infect Dis*, 31:770–5.

Schuchat A, Swaminathan B, Broome CV. 1991. Epidemiology of human listeriosis. *Clin Microbiol Rev*, 4:169–83.

Soussy CJ. 2005. Edition de Janvier 2005. Comite de L'antibiogramme de La Société Française de Microbiologie. *Communiqué* 2005.

Van Schothorst M. 1994. Choice of sampling plan and criteria for *Listeria monocytogenes*, International Commission on Microbiological Specifications for Foods. *Int J Food Microbiol*, 22:89–96.

Wiggins GL, Albritton WL, Feeley JC. 1978. Antibiotic susceptibility of clinical isolates of *Listeria monocytogenes*. *Antimicrob Agents Chemother*, 13:854–60.

Winslow DL, Pankey GA. 1982. *In vitro* activities of trimethoprim and sulfamethoxazole against *Listeria monocytogenes*. *Antimicrob Agents Chemother*, 22:51–4.

Wong HC, Chao WL, Lee SJ. 1990. Incidence and characterization of *Listeria monocytogenes* in foods available in Taiwan. *Appl Environ Microbiol*, 56:3101–4.

Yucel N, Çıtak S, Önder M. 2005. Prevalence and antibiotic resistance of *Listeria species* in meat products in Ankara, Turkey. *Food Microbiol*, 22:241–5.