Prevalence, characterization and antimicrobial resistance of *Listeria monocytogenes* isolated from beef meat in Meknes city, Morocco

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Abstract

Introduction *Listeria monocytogenes* is one of the most pathogenic bacteria related to the consumption of contaminated food. This study aims to determine the prevalence of *L. monocytogenes* in raw beef meat in Meknes city of Morocco, to evaluate its pathogenicity and resistance to antimicrobials.

Methods During four seasons, a total of 140 samples were collected from supermarkets, butcheries and Souk (weekly traditional market). The PCR method was used to examine the presence of specific and virulence genes in the isolated strains, and also to identify their serotypes. The antimicrobial resistance was determined.

Results The results show a prevalence of 7.14% which depends on retail sites and also on the season's variation. The majority of the strains were detected in butcheries (6 strains), and supermarkets (4 strains). Moreover, the majority of strains were detected during summer (50%). Concerning virulence genes, the seven researched genes were detected in 100% of isolated strains. The majority of strains were of the (1/2a, 1/2c, 3a and 3c) serogroup (70%), while two of them were of the (1/2b, 3b, 4b and 4d) serogroup (20%). All isolates were resistant to at least one antimicrobial, while three strains were resistant to nine tested antimicrobials. However, they were highly susceptible to amikacin, imipenem, gentamicin, sulfamethoxazole and chloramphenicol.

Conclusions According to results, isolated *L. monocytogenes* from analyzed beef meat shows a high level of pathogenicity and resistance to the most used antimicrobials in listeriosis therapy, which calls for the severe application of quality systems at the slaughterhouses and retail sites level.

Keywords *Listeria monocytogenes*, listeriosis, meat, hygiene, Morocco

Introduction

Listeriosis is a serious infection around the world. It results from consumption of food contaminated with *Listeria monocytogenes* and is considered among the major causes of death related to foodborne illnesses worldwide.¹ According to records, the infection risk is higher

for the immunocompromised, the pregnant women, the elderly and the newborns.² In Europe, the frequency of reported cases has increased from 1581 to 2480 cases during 2007-2017.³ In Morocco, despite the low frequency of detection and the lack of data and studies about

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this illness, pregnant women represent 1/3 of affected patients with listeriosis.⁴

L. monocytogenes is a Gram positive, facultative anaerobic, non-spore-forming, motile at 20-25°C, coccobacillus, and catalase-positive pathogenic bacterium. The optimal temperature for growth of this bacterium is between 30-37°C. However, its danger increases with its ability to survive and grow at low temperatures (refrigeration temperatures) that can reach -2°C.⁵ Three phylogenetic divisions of *L. monocytogenes* have been determined including 14 serotypes of which three (1/2a, 1/2b, 4b) are considered the most pathogenic on the human health and account globally for more than 90% of human infections related to germs worldwide.⁶

Beef meat occupies a large part of meat consumption around the world and the first position as the most consumed red meat in Morocco.⁷ Because of its richness in water and proteins that can enrich approximately 65% of the fresh meat's weight, and its high rate of water activity ($a_w=0.99$), fresh beef carcass can be contaminated by several types of pathogenic germs including *L. monocytogenes* during slaughter operations because of its natural presence in soil and water.⁸ Deficiencies in respecting hygienic standards during the slaughter process increase the risk of contamination. In Morocco, the national office of food safety has declared the acute insufficiency of certified red meat's slaughterhouses in the country.⁹ Furthermore, an epidemiological study shows that the majority of listeriosis cases detected in Morocco were related to meat products consumption outside home.⁴ In order to determine the risk of infection with *L. monocytogenes* related to beef consumption, this study aims to determine its contamination level with this germ, virulence genes, serogroups and also its multiresistance to antibiotics.

Methods

Sampling

A total of 140 samples of raw beef meat were collected during four seasons: from 20 December 2016 to 28 February 2017 (winter), from 1 June to 31 August 2017 (summer), from 1 October to 30 November (autumn) and from 1

March to 30 April 2018 (spring). The retail sites were distributed on three types: 40 from supermarkets, 60 from butcheries and 40 from Souk (weekly market). These retail sites were distributed geographically over twelve different regions on the city of Meknes, Morocco, which have a direct relation with the compliance level with hygienic conditions. The samples were kept at 4°C and analyzed after a maximum of 24 hours.

Isolation and detection of *Listeria monocytogenes*

The isolation of *L. monocytogenes* was carried out following the routine method for the Research of *L. monocytogenes* NF V08-055, 1997 (French standard, 1997).¹⁰ This method consists of a pre-enrichment of 25 g of raw beef meat into 225 mL of half Fraser (BIOKAR, Beauvais, France) with an incubation at 30°C during 24 h. Enrichment of 0.1 mL of pre-enrichment into 10 mL of Fraser (BIOKAR) with an incubation at 37°C during 48h. The enrichment cultures were streaked on PALCAM Agar (BIOKAR) and incubated at 37°C during 24 h to 48 h. After incubation, suspect colonies of *L. monocytogenes* (olive green with black center hollow and surrounded by black areas) were cultured on TSYEA agar (BIOKAR). Presumptive strains of *L. monocytogenes* were subjected to the biochemical identification tests: catalase, Gram stain, hemolysis, CAMP test, carbohydrate and API tests.

Detection of virulence genes and serogroups of *Listeria monocytogenes*

Following the conventional polymerase chain reaction (PCR) described by Bouymajane et al.¹¹ The DNA extraction was performed using thermal lysis for a bacterial suspension of isolated strain in 50 µL of H₂O that was subjected three times to 15 min of freezing at -20°C followed by thawing for 3 min at 94°C. The quantification of the extracted DNA was carried out using the Nanodrop 8000 (Thermo Fisher Scientific, Waltham, MA, USA), while the 1% agarose gel was used for its control. The isolates which presented a phenotypic character

Table 1. Specific, virulence and serovars genes of *Listeria monocytogenes*

	Sequences (5'-3')	Length (bp)	Determinant	Amplification condition	Reference
<i>hly</i>	F-TATCCAGGTGCTCTCGTGAAG R-ATTCAGTGAAGCCATTTTCGTC	268	Specific gene of <i>L. monocytogenes</i>	95°C/5min 95°C/80s 60°C/90s 72°C/6min	Meng et al., 2017 ¹⁹
<i>inlA</i>	F-CCTAGCAGGTCTAACCGCAC R-TCGCTAATTTGGTTATGCCC	256			Coroneo et al., 2016 ²⁰
<i>inlC</i>	F-AATTCCCACAGGACACAACC R-CGGGAATGCAATTTTTCCTA	517			Liu et al., 2007 ²¹
<i>inlJ</i>	F-TGTAACCCCGCTTACACAGTT R-AGCGGCTTGGCAGTCTAATA	238		95°C/5min	Liu et al., 2007 ²¹
<i>actA</i>	F-CCAAGCGAGGTAAATACGGGA R-GTCCGAAGCATTTACCTCTTC	650	Virulence genes of <i>L. monocytogenes</i>	94°C/80s 58°C/90s 72°C/6min	Lomonaco et al., 2012 ²²
<i>prfA</i>	F-ACCAATGGGATCCACAAGA R-CAGCTGAGCTATGTGCGAT	467			Bubert et al., 1999 ²³
<i>hlyA</i>	F-ATCATCGACGGCAACCTCGGAGAC R-CACCATTCCCAAGCTAAACCAGTGC	404			Du et al., 2016 ²⁴
<i>plcB</i>	F-AATATTTCAATCAATCGGTGGCTGA R-GGGTAGTCCGCTTTTCGCTCTT	289			Du et al., 2016 ²⁴
<i>Lmo</i> 0737	F-AGGGCTTCAAGGACTTACCC R-ACGATTTCTGCTTGCCATTC	961	<i>L. monocytogenes</i> serovars 1/2a, 1/2c, 3a, 3c.	95°C/5min 94°C/80s	Lotfollahi et al., 2017 ²⁵
ORF 2819	F-AGCAAAATGCCAAACTCGT R-CATCACTAAAGCCTCCCATTG	471	<i>L. monocytogenes</i> serovars 1/2b, 3b, 4b and 4d	55°C/90s 72°C/6min	Lotfollahi et al., 2017 ²⁵

of *L. monocytogenes* were analyzed for the *hly* specific genes for this bacterium. After that, confirmed strains by PCR were tested for the virulence genes *act A*, *hly A*, *inl J*, *inl A*, *inl C*, *plc B* and *prf A*. The different serovars of *L. monocytogenes* strains were determined also by the PCR method using the *Imo* 0737 and ORF 2819 genes, corresponding to eight serotypes: 1/2a, 1/2c, 3a, 3c, 1/2b, 3b, 4b and 4d. In a PCR reaction mixture of 25 µL, the PCR mix of specific genes was composed of 13.9 µL of H₂O, 2.5 µL of buffer (10×), 2 µL of 25 mM MgCl₂, 2.5 µL of 1 µM dNTP mix (KAPA Biosystems, Wilmington, USA), 0.1 µL of Taq DNA polymerase (1 U/µL, KAPA Biosystems), 2 µL of template DNA and 1 µL of each 10 µM primer. Concerning the *act A*, *hly A* and *inl J* virulence genes, the PCR mix was composed of 8.71 µL of H₂O, 2.5 µL of buffer (10×), 3 µL of 25 mM MgCl₂, 2.5 µL of 1 µM dNTP mix (KAPA Biosystems), 0.3 µL of Taq DNA polymerase (1

U/µL, KAPA Biosystems), 2 µL of template DNA and 1 µL of each 10 µM primer. While for the *Inl d*, *Inl C*, *plc B* and *prf A* virulence genes and serotyping genes *Imo* 0737 and ORF 2819, the PCR mix was composed of 11.8 µL of H₂O, 2.5 µL of buffer (10×), 2 µL of 25 mM MgCl₂, 2.5 µL of 1 µM dNTP mix (KAPA Biosystems), 0.2 µL of Taq DNA polymerase (1 U/µL, KAPA Biosystems), 2 µL of template DNA and 1 µL of each 10 µM primer. Temperature profiles differed according to the researched gene (Table 1).

Antimicrobial susceptibility testing

In order to evaluate the level of sensitivity of *L. monocytogenes* isolates to antibiotics, the isolated strains were cultured on TSYE agar medium and tested for 14 different antimicrobials commonly used for human listeriosis therapy (Table 2). After incubation at 37°C during 48 h, the measurement of the

inhibition zone allows to conclude the level of the bacterium's resistance to the antimicrobial according to the CLSI guidelines.¹²

Table 2. Antimicrobial agents used for susceptibility testing of *Listeria monocytogenes*

Antimicrobial agent	Code	Concentration
Ampicillin	AMP10	10 µg
Amoxicillin	AMC30	20/10 µg
Sulfamethoxazole	SMZ200	200 µg
Tetracycline	TE30	30 µg
Chloramphenicol	C30	30 µg
Erythromycin	E15	15 µg
Sulfamethoxazole-trimethoprim	SXT25	25 µg
Penicillin	P10	10 µg
Gentamicin	CN30	30 µg
Imipenem	IPM10	10 µg
Amikacin	AK	30 µg
Streptomycin	S10	10 µg
Vancomycin	VA30	30 µg
Kanamycin	K30	30 µg

Results

Isolation and detection of *L. monocytogenes*

From 140 analyzed beef meat samples, 48 strains had the morphological aspect of *L. monocytogenes*, in which ten have been confirmed by PCR (Figure 1). *L. monocytogenes* was detected in 7.14% of analyzed samples (10/140). The *L. monocytogenes* strain ATCC 19112 was used as a reference.

M – molecular weight marker 100 bp; T+ – positive control; T- – negative control.

Figure 1. PCR amplification of specific *hly* gene

Beef meat retailed in butcheries was the more contaminated with this germ with 6/10 strains, followed by retail meat in supermarkets with 4/10 strains, while no strain of *L. monocytogenes* was isolated from Souk's beef meat. The majority of isolated strains were detected during summer (5 strains) and winter (3 strains), while 2 strains were detected in the autumn season.

Detection of virulence genes and serogroups of *L. monocytogenes*

The results show that the seven researched virulence genes were detected for all isolated strains of *L. monocytogenes* (Figure 2).

M – molecular weight marker 100 bp; T+ – positive control; T- – negative control.

Figure 2. A) PCR amplification of virulence *inl A* and *inl C* genes; B) PCR amplification of virulence *act A*, *hly A* and *inl J* genes. C) PCR amplification of virulence *plc B*, *prf A* genes

Concerning serotyping, eight serotypes were detected in this study (Figure 3). According to results, seven strains were from the Imo 0737 serogroup (3 from supermarkets and 4 from butcheries) while two strains were from the ORF 2819 serogroup (1 from supermarket and 1 from butchery). The variation of retail sites has an effect on the distribution of serotypes of the studied strains.

M – molecular weight marker 100 bp; T+ – positive control; T- – negative control.

Figure 3. PCR amplification of serovars genes of *Listeria monocytogenes*

Antimicrobial susceptibility of isolated strains

The ten isolated strains of *L. monocytogenes* showed a resistance to at least one antimicrobial agent. Four strains (40%) were resistant to at least 2 antimicrobials while two strains (20%) were resistant to 7 antimicrobials; also 30% of *L. monocytogenes* showed a resistance to three or more antimicrobials (Table 3).

Discussion

L. monocytogenes was detected in 7.14% of analyzed beef meat samples (10/140). This prevalence is higher than that found in Egypt (6.67%),¹³ and lower than that found in Poland (19.4%).¹⁴ Our previous study showed that the prevalence of these bacteria was high during summer and winter seasons (8.8%) with an increase in summer (5 strains), which can be explained by the availability of the optimum temperature and humidity conditions for the growth of *L. monocytogenes* in the slaughterhouses and retail sites during this season.¹⁵

Collected beef meat from butcheries and supermarkets were the most contaminated with *L. monocytogenes* (with 10% for each site), however any strain was detected from Souk's beef meat. The high hygienic quality of Souk's beef meat compared to that of the other retail sites was related with the storage duration of meat in butcheries and supermarkets in comparison with

that of Souk which is selling during the same slaughter day.¹⁵ Regarding virulence genes, the seven researched genes were detected in 100% of *L. monocytogenes* strains. *Act A*, *prf A*, *hly A*, *plc B* genes of the *prfA* virulence genes group (pVGC) that represents the most pathogenic genes group of *L. monocytogenes* were detected in this study, as well as the *inl A*, *inl C* and *inl J* virulence genes, which are considered among the most essential tools in the adhesion and invasion of *L. monocytogenes*.¹⁶ In this study, the isolates predominantly belonged to the serogroup (1/2a, 1/2c, 3a and 3c) (70%), which includes one of the most pathogenic serotypes (1/2a), in addition to three others (1/2c, 3a, 3c), while two strains (20%) belonged to the serogroup (1/2b, 3b, 4b and 4d) including two of the most pathogenic serotypes (1/2b and 4b) with two others (3b and 4d). In order to determine the prevalence and different serotypes of *L. monocytogenes* in bovine hides and carcasses, a study performed in Poland indicates that 87% of isolated strains were of the 1/2a serotype.¹⁴ Regarding the resistance of *L. monocytogenes*, all isolated strains were resistant to at least one tested antimicrobial (100%). The isolates showed a very high resistance to amoxicillin (100%) and ampicillin (80%). Moreover, they were moderately resistant to streptomycin (50%), penicillin (50%), erythromycin (50%), vancomycin (50%), tetracycline (50%) and sulfamethoxazole/trimethoprim (50%). Amoxicillin as an amino-penicillin, ampicillin and penicillin are among the beta-lactam antibiotics which have a high inhibitory effect on the Gram-positive bacterial cells in general including *L. monocytogenes*.¹⁷ For this reason, they are frequently used in therapy for the infection with *L. monocytogenes* whereas the standard therapy is formed from amoxicillin or ampicillin in combination with an aminoglycoside like streptomycin.¹⁸

Table 3. Antimicrobial susceptibility of *Listeria monocytogenes* isolated from beef meat

Antimicrobial class	Antimicrobial agent	Code	Resistant, n (%)	Intermediate susceptibility, n (%)	Susceptible, n (%)
Penicillin	Ampicillin	AMP10	8 (80)	1 (10)	1 (10)
	Penicillin	P10	5 (50)	0	5 (50)
	Amoxicillin	AMC30	10 (100)	0	0
Sulfonamide	Sulfamethoxazole	SMZ200	2 (20)	0	8 (80)
Potentiated sulfonamide	Sulfamethoxazole/trimethoprim	SXT25	5 (50)	0	5 (50)
Aminoglycosides	Gentamicin	CN30	1 (10)	0	9 (90)
	Amikacin	AK	0	0	10 (100)
	Streptomycin	S10	5 (50)	1 (10)	4 (40)
	Kanamycin	K30	1 (10)	0	9 (90)
Tetracycline	Tetracycline	TE30	5 (50)	0	5 (50)
Amphenicol	Chloramphenicol	C30	1 (10)	2 (20)	7 (70)
Macrolide	Erythromycin	E15	5 (50)	0	5 (50)
Carbapenem	Imipenem	IPM10	0	0	10 (100)
Glycopeptide	Vancomycin	VA30	5 (50)	0	5 (50)

However, isolated strains were very highly susceptible to imipenem (100%), amikacin (100%), gentamicin (90%), kanamycin (90%), sulfamethoxazole (80%) and chloramphenicol (70%). This result is compatible with a study that was conducted in Romania in which all isolates of *L. monocytogenes* from meat, dairy products and clinical samples were very susceptible for imipenem, amikacin, gentamicin and chloramphenicol.¹⁸ In the present study as in many other recent studies, *Listeria monocytogenes* is becoming increasingly resistant to a wide number of antimicrobials including the beta-lactam antibiotics,¹⁸ which indicates that its danger on the human health is also becoming more and more preoccupying.

Conclusions

In this study, the prevalence of *L. monocytogenes* was 7.14%. This prevalence was moderately high compared to that found in many countries. From forty-eight suspect strains, just ten were confirmed by PCR technique, which indicates the importance of genetic identification tests to identify isolated strains. On the other hand, researched virulence genes in this study play a key role in the multiplication, propagation and pathogenicity of *L. monocytogenes*, while they were totally detected

in all isolated strains. Furthermore, the isolates showed a resistance against the most used antimicrobials for human listeriosis therapy (amoxicillin, ampicillin, penicillin, streptomycin, erythromycin, tetracycline and vancomycin) hence the need to develop more effective antibiotics against this bacterium. These results call for the great necessity to the severe application of the quality systems at the slaughterhouses and retail sites level in Morocco, in order to fight against listeriosis.

Authors' contributions statement: MB prepared the manuscript and conducted the analysis. FRF ensured the necessary equipments and edited the article. NL conducted the PCR amplification. AB prepared the PCR protocol. MS and MM revised and approved the article. All authors read and approved the final version of the manuscript.

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