Serotyping and antibiotic sensitivity of *Listeria monocytogenes* isolated from cheeses produced in the region of Algiers (Algeria)

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ABSTRACT

Listeria monocytogenes causes invasive syndromes with the high mortality rates in specific population groups. Cheeses have been frequently involved in epidemics around the world. The objective of this study was to assess the prevalence of *L. monocytogenes*, study its serotyping and antibiotic resistance in the samples collected at different stages of cow milk cheese production in three production units located in the Algiers region. A total of 385 samples of dairy products were analyzed using the standard procedure EN ISO 11290-1, and the *L. monocytogenes* isolates were serotyped by polymerase chain reaction. The overall prevalence was 5.2% (20/385). The highest prevalence was in the hard cheese processing unit (3.12%) followed by the pressed cheese production unit (1.82%) and the soft cheese production unit (0.26%). Among these isolates, four serotypes identified, serotypes 4b (50%) and 1/2b (35%) are the most dominant followed successively by serotypes 1/2a (10%) and 4c (5%). Depending on the step of production, 11 strains of *L. monocytogenes* are isolated from packaged grated cheese, seven strains from the raw milk, one strain during refining and 1/2 b strain has been isolated bya surface swabbing. The study of the antimicrobial sensitivity of the isolates of *L. monocytogenes* showed significant sensitivity to antibiotics commonly used in animal and human listeriosis. In conclusion, the presence of serotypes 4b, 1/2b and 1/2a of *L. monocytogenes* in the samples is of great concern to public health as these serotypes can cause listeriosis in humans.

Key words: cow, listeriosis, milk, prevalence, serotypes, antimicrobial resistance

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Introduction

Listeria species include Gram-positive, facultatively anaerobic, psychrotrophic, rod-shaped, non-spore-forming bacteria. Currently, the genus *Listeria* contains nineteen different species (Doijad et al., 2018), of which *L. monocytogenes* and *L. ivanovii* are pathogenic, and the former is the major causative agent of listeriosis in human and other mammals (Garedew et al., 2015). *L. monocytogenes* is an opportunistic bacterial pathogen that poses a significant risk to human health because it is capable of causing disease, mainly in certain groups of newborns, the elderly, immunocompromised and high-risk pregnant women (Vazquez-Boland etal., 2001). In this high-risk group, listeriosis has a high lethality rate of 20-30%. Human listeriosis may occur in the form of mild febrile gastroenteritis or severe invasive disease such as meningoencephalitis or septicemia (Torres et al., 2005).

Ingesting food contaminated with *L. monocytogenes* is the main route of transmission of the pathogen to humans (Dussurget et al., 2008). Omnipresent in nature, *L. monocytogenes* can contaminate foods from a wide variety of environmental reservoirs during food processing, distribution and storage, making this opportunistic pathogen extremely difficult to control and manage in the food industry (Kathariou, 2002). *L. monocytogenes* has been isolated from various food products including raw and cooked meats, milk products, seafood and vegetables (Luo et al., 2017). Most cases of foodborne listeriosis are related to contaminated ready-to-eat products during and after treatment (Jemmi et al., 2006). This highlights the importance of monitoring the environment of food processing plants, identifying potential sources of contamination and transmission pathways in the food production chain, particularly with regard to persistent strains of *L. monocytogenes*. The presence of persistent strains could result from a deficiency in the cleaning and disinfection procedures, which allow the survival and adaptation of *L. monocytogenes* strains (Thévenot et al.,2006).

Although more than 13 serotypes of *L. monocytogenes* have been described, three serotypes (1/ 2a, 1/2b and 4b) are responsible for the vast majority of clinical cases (Tappero et al., 1995). Interestingly, although serotype 1/2a is the most frequently isolated from food, serotype 4b causes the majority of human epidemics (Gilot et al.,

1996). Therefore, it is likely that the serotype designation is associated with the virulence potential.

The conventional bacteriological methods used to identify *L. monocytogenes* are not always reliable and often take time and are laborious. Thus, more reliable, fast and cost-effective molecular techniques such as polymerase chain reaction (PCR) methods have been developed for the detection of these pathogens in foods (Rodriguez-Lazaro et al., 2004).

Increased use of antibiotics for therapeutic purposes in animals and humans has led to the development of antibiotic resistance, a major public health problem (Poyart-Salmeronet al., 1990). Studies have shown the existence of *L. monocytogenes* strains resistant to one or more antibiotics, such as nalidixic acid, oxacillin, tetracycline, gentamicin, penicillin, ampicillin, streptomycin, erythromycin, kanamycin, sulfonamide, trimethoprim and rifampicin. Therefore, it is important to demonstrate the existence of antibiotic resistance in *L. monocytogenes* strains in food (Poyart-Salmeronet al., 1990). The transmission of the resistant strains to humans via contaminated food products may have a serious impact on public health (Wang et al., 2015). Processed ready-to-eat products, including raw milk cheeses, are particularly affected by the Listeria danger. Thus, the objective of the present study was first to determine the prevalence and level of contamination with *L. monocytogenes* in three types of raw milk cheese throughout the process of their manufacture, in a second step to determine serotypes of isolates of *L. monocytogenes* and thirdly the evaluation of their antimicrobial resistance.

Material and methods

Sample collection

Sampling was carried out in three cheese production and processing units located in the Algiers region. The first unit produces a soft cheese (camembert type) and the second produces a hard cheese (Edam type). Both types of cheese are made from raw milk. The third unit processes an imported hard cheese (Maasdam type). During the period from 2014 to 2016, five visits were made for each unit. A total of 385 samples were taken at different points in the production chain. The number and distribution of samples are shown in Table 1. Quantities of 25 g (semi-products, finished products) or 25 ml of milk were collected. At each visit, surface swabbings are made. The swabbed surfaces are those that come in contact with the product (vat, molds, knife, cutting machine...). All samples were immediately transported to the laboratory in a refrigerated box at +4 ° C and analyzes were performed within two hours.

Table 1. Distribution of samples on the stages of production

Type of production Stage of production	Pressed cheese (Edam)	Soft cheese (Camembert)	Hard cheese (Lady mass)
Raw milk	75	60	-
Pasteurized milk	5	5	-
Curd	10	10	-
Refinement	10	10	-
Final product	25	25	-
Swab surface	25	25	25
Loose cheese	-	-	25
Cheese slice	-	-	25
Grated Cheese	-	-	25
Total	150	135	100

Isolation and identification of *L. monocytogenes*

To detect the presence of *L. monocytogenes*, all samples are analyzed using the standard procedure EN ISO 11290-1 (ISO, 1997). In summary, 25 g (25 ml) of each sample are pre-enriched in 225 ml half-Fraser broth (Merck, Germany) and homogenized in a stomacher (Stomacher Lab-Blender 400, UK) for two minutes. The homogenates are incubated at 30 ° C for 24 h. After incubation, 0.1 ml of this medium was transferred to 10 ml of Fraser broth (secondary enrichment medium) (Merck, Germany) and incubated at 37 ° C for 48 h. The enrichment culture streaked onto Oxford and Palcam Listeria Selective Agar (Merck) and the inoculated plates incubated at 37 °C for 48 h. Up to five suspected colonies were picked from the plate and streaked onto a trypticase soya agar (Merck) plate with 0.6% yeast extract and incubated at 37 °C for 48 h. Suspected colonies were verified by Gram staining, catalase reactions, oxidase tests, CAMP tests, motility at 20–25 °C, Methyl Red-Voges-Proskauer (MR-VP) reactions and nitrate reduction. Isolates have been confirmed by using the API Listeria strips according to the manufacturer's recommendations (BioMérieux, France).

Serotype identification by multiplex PCR

Genomic DNA was extracted from isolates by Chelex resin method (Amills et al., 1997). Serogroup and serovar determinations were performed by multiplex PCR according to the method described by Doumith et al. (2004) using the primers lmo0737 (906 bp), lmo1118 (691 bp), ORF2819 (471 bp), ORF2110 (597 bp) and prs (370bp) for all Listeria species (Sigma-Aldrich) (Table 2). Initially, PCR conditions were optimized using varying concentrations of the reagents. PCR was performed in a 50-μL reaction mixture containing 1X PCR buffer (Sigma); 2 mM MgCl2 (Sigma); 0.2 mM of each dNTP (Sigma); 2 U of Taq polymerase (Sigma); 1 μM each of lmo0737, ORF2819, and ORF2110 primers; 1.5 μM of

lmo1118 primer; and 5 μL of template DNA. PCR cycling conditions were as follows: initial denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 40 s, annealing at 53°C for 75 s, and extension at 72° C for 75 s with a final extension at 72 °C for 7 min. After electrophoresis, strains identified that produced PCR products of the following sizes were: 691 bp identified as serotype 1/2a (or 3a), 471 bp identified as serotype 1/2b (or 3b), 691 to 906 bp identified as serotype 1/2c (or 3c), 471 to 597 bp identified as serotype 4b (or 4d or 4e), and 370bp for all *Listeria* species.

Antimicrobial susceptibility

Susceptibility tests were performed by standard disk diffusion method on Mueller Hinton Agar (MHA) (Oxoid) following the procedures recommended by the Clinical and Laboratory Standards Institute (CLSI, 2010). *Staphylococcus aureus* ATCC 29213 was used as a control strain. Fresh bacterial colonies cultured at 37 ° C for 24 h in a tryptic soybean broth (Merck) were taken to prepare a suspension adjusted to 0.5 McFarland. 1ml of this suspension was inoculated on Mueller-Hinton agar (Oxoid) supplemented with 0.5% defibrinated sheep blood plates and spread uniformly. The plates were incubated at 35°C for 18–24 h. Sixteen antibiotics were chosen: amoxicillin (10 μg), amoxicillin + clavulanic acid (30 μg), ampicillin (10 μg), chloramphenicol (30 μg), colistin (10 μg), doxycycline (30 μg), erythromycin (15 mg), gentamicin (10 μg), kanamycin (30 μg), nitrofurantoine (30 μg), penicillin G (10 units), rifampicin (5 μg), spiramicin (100 μg), streptomycin (10 μg), tetracyclin (30 μg) and trimethoprim-sulfamethoxazol (1.25/23.75 mg).

Results

A total of 20 (5.19%) isolates of *L. monocytogenes* were obtained from 385 samples analyzed. Twelve (60%) positive samples came from the cheese processing unit. Seven (35%) isolates were from the pressed cheese production unit (6 isolates from raw milk and 1 isolate during ripening). Finally, one (5%) raw milk sample from the manufacture of camembert was also positive for *L. monocytogenes*. Isolated strains belonged to serotypes 4b (n = 10; 50%), 1/2b (n = 7; 35%), 1/2a (n = 2; 10%), and 4a/4c (n = 1; 5 %) (Figure 1). The distribution of serotypes obtained at different stages of production is shown in Table 3.

Table 4 summarizes the antibiotic susceptibility patterns of the isolates. A 10% of the isolates were resistant to amoxicillin, spiramycin, doxycyclin and nitrofurantoin, 5% were resistant to amoxicillin-clavulanic acid and rifampicin and 50% of the isolates were resistant to streptomycin and colistin. All isolates were sensitive to other antibiotics.

Figure 1. Serotype identification of *L. monocytogenes* isolates by multiplex PCR. M: 100-bp DNA ladder, Lane 1: *L. monocytogenes* reference strain serotype 1/2c, 691–906 bp (*L. monocytogenes* ATCC 7644); Lane 2: *L. monocytogenes* reference strain serotype 4b, 471–597 bp (*L. monocytogenes* RSKK 475); Lanes 4 and 10: serotype 1/2b isolate; Lanes 5 and 6: serotype 1/2a isolate; Lanes 3, 7, 8 and 9: serotype 4b isolate; Lane 11: negative control.

Table 2. List of *L. monocytogenes* primers used in the PCR assay (Doumith etal., 2004)

Gene Primer sequence (5'-3')	Product size (bp)	Serovar specificity
Lmo 0737 F-AGGG-CTTCAAGGACT-TACCC R-ACGATTTCTGC-TTGCCATTC	691	Serovars 1/2a, 1/2c, 3a, and 3c
Lmo1118 F-AGGG-GTCTTAAATCCTG-GAA R-CGGCTTGTTCGG-CATACTTA	906	Serovars 1/2c and 3c
ORF2819 F-AG-CAAAATGC-CAAAACTCGT R-CATCACTAAAGC-CTCCCATTG	471	Serovars 1/2b, 3b, 4b, 4d, and 4e
Prs F- GCTGAA-GAGATTGC-GAAAGAAG R- CAAAGAAACC-TTGGATTTGCGG	370	All Listeria species

Table 3. Prevalence of *L. monocytogenes* serotypes in different manufacturing steps of the analyzed cheeses

Type and number of products	Samples	*L. monocytogenes* serotypes				
		1/2a	1/2b	4a / 4C	4b	Total
Pressed cheese (edam) 150	Raw Milk	2	2	0	2	6
	Refinement	0	0	0	1	1
Soft cheese (Camembert) 135	Raw Milk	0	0	0	1	1
Hard cheese (Lady mass) 100	Grated Cheese	0	4	1	6	11
	Swab Surface	0	1	0	0	1
Total 385		2	7	1	10	20 (5.19%)

Table 4. Antimicrobial resistance profiles of *L. monocytogenes* isolates from the cheeses

Antibiotic disks	Serotypes (n=20)				Total number of resistant isolates
	1/2a (n = 2)	1/2b (n = 7)	4a/4C (n=1)	4b (n=10)	
Amoxicillin (25 µg)	-	2	-	-	2
Amoxicillin +acide clavulanic (30 µg)	-	1	-	-	1
Colistin (10 µg)	1	5	-	4	10
Doxycyclin (30 µg)	-	1	-	1	2
Nitrofurantoin (30 µg)	-	-	-	2	2
Rifampicin (30 µg)	-	-	-	1	1
Spirammycin (10 µg)	1	1	-	-	2
Streptomycin (10 µg)	2	2	1	5	10

Discussion and conclusions

Several studies are conducted on the presence of *L. monocytogenes* in dairy products. In Algeria, the prevalence of *L. monocytogenes* is 1.9% to 3.2% (Lebres et Mouffok 2000; Hamdi et al., 2007). In India, consideration of other types of foods involved in the spread of *L. monocytogenes* such as milk, ice cream, fruit salads, meat and fish, Nayak at al. (2015) found an overall prevalence of 1.5%**.**

The prevalence recorded for the three units visited are variable. Thus, the highest prevalence was obtained at the processing unit of hard cheese (12%) followed by the pressed cheese production unit (4.6%) and finally the one that produces the soft cheese (0.7%). In fact, the Camembert production unit differs from the other two by the application of the HACCP system, a very important concept for controlling foodborne pathogens, in particular *L. monocytogenes*. Listeria has traits that allow it to survive and grow in the environments that are not habitable by other pathogens. This makes *L. monocytogenes* a potential indicator of careless quality assurance standards, inadequate cleaning procedures, or poor employee hygiene (Jemmi etal., 2006). Depending on the prod uction stage, *L. monocytogenes* was isolated mainly from grated cheese (10 isolates) with no contamination of the raw material. This result suggests that the environment is at the origin of this contamination. This is particularly important because the organism is easily detected in the outside environment and can also form biofilms resistant to normal cleaning procedures (Moretro et al., 2004). *L. monocytogenes* was secondarily isolated from raw milk collected from the collector tanks (8 isolates) with a higher incidence at the pressed cheese plant (7 isolates). This contamination can have several origins, among which the contamination at the farms and the lack of hygiene of the tanks with existence of the persistent strains (Hamdi etal., 2007). This result also demonstrates the importance of foodborne listeriosis prevention by developing and implementing effective HACCP programs to reduce the presence of *L. monocytogenes* at all critical points in the production and distribution chain (from fork to fork). Food safety is ensured by the establishment of a food safety master plan. For raw milk products, control of processing cannot be ensured without absolute control over the hygienic quality of raw milk (Belleflamme etal., 2006). A *L. monocytogenes* strain was isolated during ripening of pressed cheese. During ripening, enzymes of microbial origin play a very active role in texture changes and flavor development, which gives the cheese its organoleptic characteristics. This phenomenon also causes a rise in the pH value at the crust. This increase in pH and aerobic conditions at the surface are favorable for the development of *L. monocytogenes* (Belleflamme et al., 2006).

In our study, serotypes 4b and 1/2b were most frequently isolated followed by serotypes 1/2a and 4c. These results are in agreement with those obtained in a cheese factory in São Paolo, Brazil (85 strains of *L. monocytogenes* isolates belonged to serotypes 1/2b, 1/2c and 4b with a predominance of serotype 4b) (Barancelli et al., 2014). Similarly, in Colombia, Munoz (2012) found that the most prevalent serotypes among 1424 food samples collected between 2000 and 2009 were 4b, 1/2b, 1/2a, with an obvious predominance of 4b serotypes. In Chile, Montero at al. (2015) found a slightly different distribution. The serotype 4b (46%) was the most widespread followed by 1/2a (32%), and then 1/2b (13%). The variations observed might be due to the differences in the types of foods included in each study. In Japan, the most common serotypes isolated from ready-to-eat foods (including natural cheese, meat products, seafood and vegetables to pick) were 1/2a (47.6%), 1/2b (20.6%) and 4b (14.3%) (Shimojima, 2016). Similar results have been reported in other countries in the northern hemisphere. In Poland, Korsak et al. (2012) analyzed 471 strains of *L. monocytogenes* isolates from different foods and found the following serotype distribution: 1/2a (54.4%), 1/2 c (25.5%), 1/2b (12.5%) and 4b (7.6%). In Ireland, O'Connor et al.(2010) found that the most common serotype present in different food categories was 1/2a followed by 4b. Finally, in China, Chen at al. (2015) found that the most common raw food isolates were serotypes 1/2a and 1/2b.

In the present study, 75% of *L. monocytogenes* isolates belong to the I lineage (1/2b and 4b); the remaining isolates belong to lineage II (1/2a). Both lineages include serotypes most commonly found among the human listeriosis cases. Our results contrast with other reports that showed that strains belonging to lineage II were the most widespread in food (Blatter et al., 2010, O'Connor et al., 2010). The authors suggest that the greatest genetic variation seen in lineage II strains is consisting o adaptation to more diverse

environments. In Algeria, the *L. monocytogenes* isolates of clinical cases by Naim (1987) and Ramdani (1992) belong to serovars 1/2b and 1/2a (respectively), all other isolates from the clinical cases or foods belong to serovars 4b (Lebres, 2002; Hamdi et al., 2007). In Tunisia, only serovar 4b is implicated in the studies of human listeriosis (Boukadida et al., 1994). In Morocco, El Marrakchi et al. (1998) report food isolates belonging to serovar 4b and 1/2b. As is the case in other countries, the serotypes found in this study are responsible for human listeriosis (Montero et al., 2015; O'Connor et al., 2010; Shimojima et al, 2016). Serotypes 4b, 1/2a and 1/2b are incriminated in most human listeriosis outbreaks (Wiedmann et al., 2011). Among the 13 serotypes of *L. monocytogenes* in the literature, serovar 4b is mainly responsible for most epidemics in humans (Gasanov et al., 2005). Isolates 4b and 1/2b account for more than 70% of the analyzed samples (raw milk and cheese). This result suggests the role of these food categories in the propagation of *L. monocytogenes* strains belonging to serotypes frequently found in the cases of human listeriosis.

An increasing number of reports have been accumulated concerning the isolation of *L. monocytogenes* strains resistant to one or more antibiotics from food products (Gomez et al., 2014). Ampicillin, oxacillin and penicillin are the most active β-lactams that inhibit the synthesis of bacterial cell wall peptidoglycan (Miller et al.,2014). *L.monocytogenes* is naturally susceptible to β-lactams, and the standard antibiotic therapy for human listeriosis includes penicillin/ ampicillin alone or combined with an aminoglycoside (gentamicin) (Al-Nabulsi et al., 2015). In the present study, all the strains tested show a sensitivity of 100% with respect to several antibiotics tested: ampicillin, penicillin, erythromycin, chloramphenicol, tetracycline, gentamicin, kanamycin and cotrimoxazole. The same result is reported by Hibert at al. (2004) who do not record any cases of resistance to *L. monocytogenes* strains of food origin to the following antibiotics: tetracycline, penicillin, gentamicin, cotrimoxazole, erythromycin and chloramphenicol. It is important to note that all strains tested were sensitive to ampicillin, which is the main drug of choice for the treatment of listeriosis. Its association with gentamicin has also been reported and successfully used in the treatment of listeriosis, a situation supported by this study since all strains were susceptible to gentamicin. No resistance to the combination of trimethoprim-sulfamethoxazole have been observed, which is important considering its appointment as an alternative in the treatment of listeriosis, mainly in patients with penicillin intolerance (Wiedmann et al., 2011). In general, most strains of *L. monocytogenes* isolates in this study are sensitive to antibiotics commonly used in animal and human listeriosis. Several authors also report 100% sensitivity to the majority of antibiotics used as choice or substitution therapy in case of human listeriosis (Korak et al., 2012, Cosansu et al., 2012). The results obtained in this study are in contrast with those reported by other authors who proved resistance to several antibiotics tested (Wang et al., 2015; Obaidat et al., 2015).

This variability emphasizes several points: the absence of listeria in heat-treated milk indicates that pasteurization is sufficient to kill this bacteria. The low prevalence (3% in raw milk, only) recorded at the level of the soft cheese production unit shows the interest of setting up a food safety control system (Type HACCP) as a means of effective control against this contaminant.

The detection of serotypes 4b, 1/2b and 1/2a and the predominance of serotype 4b (serotype most often incriminated in cases of human listeriosis) shows that the Algerian consumer is not immune to possible outbreak of listeriosis. For the control of this public health problem efforts will have to be applied at three levels: the authorities concerned by the revision of the current regulations in this area, and the rigor in its application; the agri-food industry by the control of food safety through the application of legal requirements (HACCP, BPH / GMP) and consumers, who must participate in a concerted effort taking a minimum of precautions.

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Serotipizacija i antibiotska osjetljivost bakterije *Listeria monocytogenes* izolirane iz sireva proizvedenih u regiji Algiers (Alžir)

SAŽETAK

Listeria monocytogenes uzrokuje invazivne sindrome sa visokom stopom mortaliteta kod pojedinih grupa stanovništva. Sirevi su česti izvori epidemija u svijetu. Cilj ovog istraživanja jeste odrediti prevalencu *L. monocytogenes,* identificirati serotipove i ispitati antibiotsku rezistenciju na uzorcima prikupljenim u različitim fazama proizvodnje sira od kravljeg mlijeka na tri lokacije u regiji Algiers. Ukupno je analizirano 385 uzoraka mliječnih proizvoda koristeći standardnu proceduru EN ISO 11290-1, a izolati *L. monocytogenes* su serotipizirani PCR metodom. Ukupna prevalenca iznosi 5.2% (20/385). Najviša prevalenca je u jedinicama za proizvodnju tvrdog sira (3.12%), potom presovanog (1.82%) i mekog sira (0.26%). Među ovim izolatima su identificirana četiri serotipa od kojih su dominantni serotipovi 4b (50%) i 1/2b (35%), a slijede serotipovi 1/2a (10%) i 4c (5%). Istraživanje antimikrobne osjetljivosti izolata *L. monocytogenes* je pokazalo signifikantnu senzitivnost na antibiotike koji se obično koriste kod listerioze životinja i ljudi. U zaključku, prisustvo serotipova 4b, 1/2b i 1/2a *L. monocytogenes* u uzorcima je od velikog značaja za javno zdravstvo obzirom da su ovi serotipovi uzročnici listerioze ljudi.

Ključne riječi: krava, listerioza, mlijeko, prevalenca, serotipovi, antimikrobna rezistencija