VERSITA
DOI: 10.2478/rrlm-2013-0025

Research article

Detection and serotyping of *Listeria monocytogenes* in some food products from North-East of Romania

Detecția şi serotipizarea *Listeria monocytogenes* din unele alimente provenite din nord-estul Romaniei

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Abstract

Listeria monocytogenes represents an important hazard to human health because it is capable of causing listeriosis, an atypical foodborne disease with a high fatality rate.The aim of this study was to detect and serotype Listeria monocytogenes in some food products (of animal and vegetable origin), collected during the 2011 period. The detection of Listeria monocytogenes was performed by ISO 11290- 1-A1/2004 standard method and verified by real-time PCR. Numerous subtyping strategies have been developed to characterize Listeria monocytogenes isolates. Serotyping, the traditional method for the subtype characterization of Listeria monocytogenes is not very discriminatory, but it is a universal method which gives important information on prevalence of specific serotypes. The confirmation of Listeria monocytogenes isolated strains was performed by a TaqMan real-time PCR assay, using TaqMan Pathogen Detection Kits (Applied Biosystems). The assay use specific primers and a probe for Listeria monocytogenes. In this study on a total number of 260 samples, 25 (9.61%) gave positive results for Listeria monocytogenes by conventional method and only 22 (8.46%) of them were confirmed by real-time PCR. These results indicate a high efficiency of the real-time PCR method in a short time, compared with classical methods which can provide false positive results. Four different Listeria monocytogenes serotypes were found in nine varieties of food products: 1/2a, 1/2b, 1/2c and 4b.

Keywords: *detection, serotyping, Listeria monocytogenes, food safety*

Rezumat

Listeria monocytogenes reprezintă un pericol important pentru sănătatea omului, producând listerioza, o toxiinfecție alimentară atipică, cu o rată de mortalitate ridicată. Scopul acestui studiu a fost detecția şi serotipizarea tulpinilor de Listeria monocytogenes izolate din unele produse alimentare (de origine animală şi vegetală), în perioada 2011. Detecția tulpinilor de Listeria monocytogenes s-a realizat prin metoda orizontală ISO 11290- 1-A1/2004, iar confirmarea s-a realizat prin tehnica PCR real-time. Pentru caracterizarea serotipurilor de Listeria monocytogenes, s-au dezvoltat numeroase tehnici de subtipizare. Serotipizarea prin

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metoda tradiţională nu permite caracterizarea subtipurilor, însă este metoda universală ce oferă informaţii privind prevalenţa serotipurilor. Confirmarea tulpinilor de Listeria monocytogenes s-a realizat prin tehnica TaqMan real-time PCR, utilizându-se kit-ul TaqMan Pathogen Detection Kits (Applied Biosystems). Această metodă foloseşte primeri specifici şi o sondă nucleotidică pentru Listeria monocytogenes. În urma studiului, din 260 de probe, 25 (9,61%) au fost pozitive pentru Listeria monocytogenes prin metoda clasică şi doar 22 (8,46%) au fost confirmate prin PCR real-time. Rezultatele obţinute indică o eficienţă mai mare a metodei PCR real-time, într-un timp mult mai scurt, comparativ cu metoda clasică, care poate furniza rezultate fals pozitive. Patru serotipuri diferite de Listeria monocytogenes au fost identificate în nouă tipuri de produse alimentare: 1/2a, 1/2b, 1/2c şi 4b.

Cuvinte cheie: *detecţie, serotipizare, Listeria monocytogenes, produse alimentare*

Received: *21st December 2012;* ***Accepted:*** *25th June 2013;* ***Published:*** *9th September 2013.*

Introduction

Listeria monocytogenes is a foodborne pathogen that can cause human listeriosis, a rare but severe illness that may lead to death (1). Numerous sporadic cases and outbreaks of listeriosis have been linked to a wide variety of foods of animal and vegetable origin. *Listeria monocytogenes* infections are particularly dangerous to certain risk groups, including, pregnant women, the elderly, newborns and immunocompromised patients. Manifestations of listeriosis range from self-limited febrile gastroenteritis to severe invasive forms such as meningoencephalitis, septicemia, abortion or perinatal infections with a high fatality rate 30% (2).

Products such as raw milk, soft cheese produced from raw milk, raw meat products and salads are frequently implicated in foodborne with *Listeria monocytogenes*. In this context, hygiene weak points during the slaughtering and milking process are the main critical points for *Listeria* contamination (3).Currently, nosocomial infections with this pathogen are known, but they are rarely, just like direct infections, transmitted due human-animal relationship.

A variety of conventional and rapid methods are available for the detection and identification of *Listeria monocytogenes* in food samples and specimens from animal listeriosis. Traditional culture based methods remain the 'gold standard' with which other methods are compared. They are usually very sensitive. These methods use enrichment procedures with selective agents to reduce the number of contaminating microorganisms and allow multiplication of *Listeria monocytogenes* (4).

The potential of PCR methods for culture confirmation is interesting in light of the possibilities for performing automatic PCR. That PCR method may be at least as sensitive as and often more sensitive than morphological and biochemical identification of isolates have been documented on several occasions (5).

Materials and methods

Food samples

The study was performed on 260 samples, randomly collected from different food manufacturers. The analyzed food categories included unpasteurized milk (n=22), pasteurized milk (n=10), fresh soft bovine cheese (n=15), fresh soft sheep cheese (n=15), fresh cream (n=18), raw sheep meat (n=25), raw poultry meat (n=20), raw pork meat (n=23), raw pork sausages (n=10), raw bovine meat (n=20), snail meat- foot region (n=30), fresh salad (n=15), fresh onion (n=10), raw fish (n=10) and smoked fish (n=17). Each sample was placed in an individual sterile polyethylene bag and transported to laboratory. The samples were first processed by classical microbiological methods, and verified by real-time PCR.

Culture enrichment

The detection of *Listeria monocytogenes* was performed according to the ISO 11290 1-A1/2004. For pre-enrichment step 25 g

or 25 ml of each food sample were added to 225 ml of half-Fraser broth (Oxoid) and homogenized in a sterile stomacher bags for 1 minute. For snail meat (*Helix pomatia*), we used samples of 10 g, collected from the foot region. After 24 hours of incubation at 30ºC, an aliquot of 0.1 ml was used for the enrichment step in 10 ml of Fraser broth (Oxoid) and incubated 24 hours at 37ºC. The content (10 ml) was transferred on selective Palcam medium (Oxoid) and incubated at 37°C for 48 hours.

Isolation and identification on chromogenic media

The presumptive colonies from Palcam medium (five for each plate) were subcultured on ALOA media (Oxoid) for typical *Listeria monocytogenes* morphology. The inoculated plates were incubated at 37° for 24 hours. Characteristic colonies, blue-turquoise surrounded by an opaque halo, were considered as *Listeria monocytogenes*.

DNA extraction

The extraction of bacterial DNA was performed using the PrepMan Ultra kit (Applied Biosystems).

Five presumed colonies from Palcam media were transferred to Fraser enrichment broth and incubated for 24 hours at 37°C. One ml of Fraser broth was transferred into 2 ml microcentrifuge tubes and centrifuged (14000 X *g*) for 3 minutes at room temperature for cell sedimentation. After, the removing of supernatant, the cell pellets were suspended in 100 µl Prepman Ultra reagent and placed in a 100ºC heating block for 10 minutes. The cooling was done at room temperature for 2 minute. The tubes were centrifuged at 14000 X *g* for 3 minutes and the supernatant were transferred in other tubes. For each PCR reaction 2.5 µl of extracted DNA were used.

Real-time PCR reactions

For real-time PCR, we used TaqMan® *Listeria monocytogenes* Detection Kit (Applied Biosystems). The kit contains: specific primers and a TaqMan probe for *Listeria monocytogenes* (TAM) with an IPC detected by a different fluorophore, TaqMan Environmental Master Mix (EMM), which contains the polymerase enzyme that initiates PCR and a RNA/DNA free water as a NC. The specific TaqMan probe for *Listeria monocytogenes* is labelled at the 5' end with the reporter dye 6- FAM and at the 3'with the NFQ-MGB quencher.

Reactions and data analysis were performed in the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). PCR mix (25 µl) contains: 2.5 µl DNA template, 12.5 µl TaqMan Environmental Master Mix 2X, 0.5 µl of each F and R primers (100 µM), 0.25 µl TaqMan probe (50 µM), 8.5 µl RNase/DNase-free water. The amplification conditions were as follow: 10 minutes at 95ºC for DNA denaturation, followed by 40 cycles: 95ºC 15 sec. and 60ºC 1 min.

***Serotyping of Listeria monocytogenes* strains**

From the *Listeria monocytogenes* isolated and confirmed as such by real-time PCR, 22 strains were serotyped.

The isolates were serotyped using commercial *Listeria* antisera (Denka Seiken, Japan), according to manufacturer's instructions. The principle of measurement is based on mixing the reagent with *Listeria monocytogenes* strain and the antigen antibody reaction occurs to produce agglutination.

Results

In our study, we tested a number of 260 samples from various sources, including animal and vegetal food products by classical microbiological method. Five presumptive colonies of *Listeria monocytogenes* of each Palcam plate were tested on chromogenic media, confirmed by real-time PCR and serotyped.

Twenty five samples were positive for *L. monocytogenes* by classical microbiological method, but only 22 were confirmed by real-time PCR (*Table 1*).

On Palcam media we obtained 1 false positive sample in raw sheep meat and 2 false positive samples in snail meat.

Table 1. Detection of *Listeria monocytogenes* in food products by ISO standard method and real-time PCR method

Food type	ISO 11290- 1-A1/2004 standard method		Real-time PCR	
	Positive	Negative	Positive	Negative
Unpasteurized milk	3	19	3	19
Pasteurized milk	0	10	0	10
Fresh soft bovine cheese	0	15	0	15
Fresh soft sheep cheese	2	13	2	13
Fresh cream	1	17	1	17
Raw sheep meat	4	21	3	22
Raw poultry meat	1	19	1	19
Raw pork meat	2	21	2	21
Raw pork sausages	0	10	0	10
Raw bovine meat	2	18	2	18
Snail meat (foot region)	6	24	4	26
Fresh salad	4	11	4	11
Fresh onion	0	10	0	10
Raw fish	0	10	0	10
Smoked fish	0	17	0	17
Total	**25**	**235**	**22**	**238**

Four serotypes were found in food isolates (*Table 2*). Six strains (27.3%) isolated from milk and milk products belonged to serotype 1/2a, eleven strains (50%) isolated from raw meat and fresh salad belonged to serotype 1/2c. Five strains isolated from snail meat and fresh salad belonged to serotype 1/2b (13.6%) and 4b (9.1%).

Discussions

Listeria monocytogenes has an important impact on human health, especially on certain groups such as pregnant women, newborns and immunocompromised individuals. For these groups, unpasteurized milk and milk products, uncooked meat and fresh vegetables contaminated with *Listeria monocytogenes* can be a risk of illness. Even though the bacteria is resistant to freezing and heating treatments, a good sanitation and a proper cooking of the aliments can minimize the risk of contamination with this pathogen (6).

In 2010-2012 period, according to official dates from National Institute of Public Health (Iasi Regional Center), in north-east of Romania, the incidence rate of human confirmed listeriosis was low: 0.02/100.000 in 2010, 0.08/100000 in 2011 and 0.04/100000 in 2012. The incidence by counties of the north-east region was 0.14/100000 (1 case) in Suceava in 2010, 0.33/100000 (2 cases) in Galati in 2011, 0.12/100000 (1 case) in Iasi in 2012. The highest incidence was observed in Bacau county: 0.28/100000 (2 cases) in 2011 and 0.14/100000 (1 case) in 2012.

This low incidence is well below the European average (7) and can be explained by atypical evolution of listeriosis, an insufficient clinical awareness or a lack of a national protocol for laboratory diagnostic so that this disease is eventually unreported.

Table 2. *Listeria monocytogenes* serotypes isolated from food products

Food type	Positive samples (no.)	Serotype			
		1/2a	1/2b	1/2c	4b
Unpasteurized milk	3	3	-	-	-
Fresh soft sheep cheese	2	2	-	-	-
Fresh cream	1	1	-	-	-
Raw sheep meat	3	-	-	3	-
Raw poultry meat	1	-	-	1	-
Raw pork meat	2	-	-	2	-
Raw bovine meat	2	-	-	2	-
Snail meat	4	-	2	1	1
Fresh salad	4	-	1	2	1
Total (%)	22	6 (27.3%)	3 (13.6%)	11 (50%)	2 (9.1%)

Rapid methods are required to detect *Listeria monocytogenes* in food. Still, no single assay can be credited to be sensitive enough to detect *Listeria monocytogenes* from all types of food. We identified 25 samples with presumptive *Listeria monocytogenes* by classical microbiological method, but it is known that all the *Listeria* selective media contains esculin. All *Listeria spp.* hydrolyze esculin and the inclusion of esculin and ferric iron in enrichment or plating media results in the formation of an intense black color. We also know that the pathogenic species *Listeria monocytogenes* and *Listeria ivanovii* appear surrounded with an opaque halo zone on Ottaviani-Agosti media (8). Scotter and al., 2001 (9) showed that using only the standard method, a significant number of false negative results can be obtained when a large number of *Listeria innocua* was present in the samples.

Therefore, it is absolutely necessary to confirm suspect colonies of *Listeria monocytogenes* by biochemical and molecular methods. Recently conventional and real-time PCR assays have been developed for the detection of *Listeria monocytogenes* in foods with very good results (10-14). These methods are rapid and specific compared with classical microbiological method which requires four to five days.

In our study, five presumptive colonies of *Listeria monocytogenes* in each plate was verified by real-time PCR and serotyped. From 25 presumptive *Listeria monocytogenes*, only 22 samples were confirmed by PCR real-time and serotyped. The other 3 false positive samples were identified as other *Listeria spp*. *Listeria monocytogenes* was present in unpasteurized milk and milk products, raw meat and fresh salad.

Listeria spp. is shed in the feces of asymptomatic animal carriers. Therefore, the presence of *Listeria monocytogenes* in milk and meat is normally due to fecal contamination during the milking or slaughtering process (3). The raw milk contamination could be due to the inappropriate sanitary management practices, the feed provided to the cows in which the bacterium can multiply or to the water supply (2).

In other study in 2010 (15), we also isolated 12 strains of *Listeria monocytogenes* in 77 samples of milk, milk products and raw meat. We have observed that the prevalence of *Listeria monocytogenes* in 2010 was higher (12.6% in milk and milk products and 22.7% in raw meat) compared to 2011 (7.5% in milk and milk products and 9.3% in raw meat). We tested a smaller number of samples from other manufacturers.

Presence of *Listeria monocytogenes* in fresh snail samples indicates a primary contamination from the soil, water, or plants, due the fact that this bacterium is ubiquitous and widespread in the environment. Kirkan et al., 2006 (16) found a prevalence of 60% of *Listeria monocytogenes* in fresh and cooked snails meat, higher than we found in our study.

Also, the detection of *Listeria monocytogenes* in fresh salad is probably caused by irrigation with water contaminated with feces. Berrada and al., 2006 (17) detected and quantified *Listeria monocytogenes* in salad by real-time PCR.

Cross-contamination, which can occur within the environment of food-processing equipment, is considered to be a possible source of *Listeria* contamination in processed food. *Listeria monocytogenes* is able to attach to and survive on various working contact surfaces. One reason may be its ability to form biofilms (3).

Serotyping of *Listeria monocytogenes* may be used to determine the prevalence of serogroups, but this technique is less discriminatory than molecular typing methodologies and has limited practical value for investigating the route of spread (18).

Strains of *Listeria* can be assigned to 12 different serotypes, (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7) based on their combination of somatic (O) and flagellar (H) antigens. All of them are considered to be potentially pathogenic, most (>95%) human clinical isolates belong to three serotypes 1/2a, 1/2b and 4b. The 4a and 4c serotypes of lineage are rarely associated with outbreaks of disease despite frequent isolation from a variety of food and environmental samples (4). The majority of food isolates of *L. monocytogenes* belongs to serotype 1/2a or 1/2b, while the mainly clinical isolates belong to serotype 4 (19).

Our results are similar with other researches. Several studies have shown that *Listeria monocytogenes* strains isolated from meat processing environments are frequently of serotypes 1/2a, 1/2/b and 1/2c. This may be associated with enhanced capacity to *Listeria monocytogenes* serotype 1/2c to attach the stainless steel surfaces and form biofilms, in food-processing units (18, 20).

Currently, there are no studies on serotyping *Listeria monocytogenes* isolated from snail meat in Romania. We identified in the North-East of Romania, one strain of *Listeria monocytogenes* from snail meat which belongs to serotype 4b.

We also isolated 4 strains of *Listeria monocytogenes* from fresh salad. Fresh salad is consumed without cooking and *Listeria monocytogenes* is able to survive ingestion because of gastric acid neutralization and subsequently to cause enteritis, bacteremia, and meningitis in susceptible hosts. Lately, several listeriosis outbreaks have been associated to the consumption of raw vegetables, such as e raw celery, tomatoes, lettuce, cabbage or melon.

All AOAC international (Association of Analytical Chemists) approved *Listeria monocytogenes* detection tests, including microbiological, immunological and molecular-based tests, are required to detect 1CFU per 25 g food sample, therefore, all approved tests require culture enrichment (21).

Conclusions

Listeria monocytogenes remains one of the most important hazards for human health and it is very difficult to eradicate this pathogen from food processing unit or environment. The serotype 4b of *Listeria monocytogenes* was identified for the first time in snail meat in North-East of Romania.

Conventional microbiological methods for confirmation of listeria involving chromogenic media are laborious and time consuming and therefore, currently, rapid and highly specific methods are required. PCR real-time provides these requirements and can confirm positive outcomes detected during standard cultural protocols in only 2 hours. However the

high cost of the real-time PCR method constitutes a limiting factor for current utilization in diagnostic laboratories.

Nevertheless, the isolation of *L. monocytogenes* is necessary for epidemiological investigations in case of outbreaks. Molecular biology methods such as DNA macrorestiction analysis by pulsed field gel electrophoresis (PFGE) presently is considered the gold standard for subtyping the food borne pathogens like listeria and for that we need the isolates. Even if there are not cheaper methods their utilization is absolutely necessary in some epidemiological circumstances.

Acknowledgments

This study was financially supported by CNCSIS Romania, grant PD 377/ 2010.

Abbreviations

ALOA-Agar Listeria Ottaviani-Agosti
PCR-Polymerase chain reaction
TAM – Target Assay Mix
IPC – Internal Positive Control
EMM – Environmental Master Mix
NC – Negative Control
FAM - 6-carboxyfluorescein
NFQMGB – Non –Fluorescent Quencher; Dihydrocyclopyrroloindoletripeptide Minor Grove Binding.

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