Identification of *Listeria* spp. strains isolated from meat products and meat production plants by multiplex polymerase chain reaction

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Abstract

Listeriosis is a foodborne disease caused by *Listeria monocytogenes* and is considered as a serious health problem, due to the severity of symptoms and the high mortality rate. Recently, other *Listeria* species have been associated with disease in human and animals. The aim of this study was to develop a multiplex polymerase chain reaction (PCR) in order to simultaneously detect six *Listeria* species *(L. grayi*, *L. welshimeri*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri*, *L. innocua)* in a single reaction. One hundred eighteen *Listeria* spp. strains, isolated from meat products (sausages) and processing plants (surfaces in contact and not in contact with meat), were included in the study. All the strains were submitted to biochemical identification using the API Listeria system. A multiplex PCR was developed with the aim to identify the six species of *Listeria*. PCR allowed to uniquely identify strains that had expressed a doubtful profile with API Listeria The results suggest that the multiplex PCR could represent a rapid and sensitive screening test, a reliable method for the detection of all *Listeria* species, both in contaminated food and in clinical samples, and also a tool that could be used for epidemiological purposes in food-borne outbreaks. A further application could be the development of a PCR that can be directly applied to the pre-enrichment broth.

Introduction

Human listeriosis is a severe human illness caused by the foodborne pathogen *Listeria monocytogenes.* Human listeriosis has low prevalence but a fatality rate of 16% (EFSA and ECDC, 2015), and primarily affects children, the elderly, pregnant women and people with compromised immune systems (Rocourt *et al.*, 2000). Symptoms are caused primarily by the ability of the pathogen to survive inside the monocytic cells and to cross the blood-brain and the trans-placental barrier (Vazquez-Boland *et al.*, 2001). In pregnant women *L. monocytogenes* is generally asymptomatic in the mother but can lead to abortion, stillbirth or a generalized infection, with sepsis or meningitis in the neonate (Allerberger, 2007). In immune-competent people, listeriosis develops as a typical febrile gastroenteritis, while in immune-compromised adults, such as the elderly and patients receiving immunosuppressive agents, listeriosis can manifest as septicaemia or meningoencephalitis (Allerberger and Wagner, 2010).

Different kinds of foods are implicated in the transmission of the pathogen to humans, primarily ready-to-eat foods, such as fermented sausages, ripened soft cheeses, raw and smoked fish (McLauchlin, 1996a, 1996b; Aureli *et al.*, 2000; Gillespie *et al.*, 2006). Between 2004 and 2011, in sardinian sausage production plants, prevalence of *L. monocytogenes* comprised in a range between 29 and 36.3% were detected in meat samples, of 11% in surfaces not in contact with meat and of 17.4% in surfaces in contact with meat (Meloni *et al.*, 2009; Mureddu *et al.*, 2014).

Although most outbreaks of human listeriosis and 85% of animal cases are caused by *L. monocytogenes*, several authors reported cases of infection by *Listeria seeligeri*, *Listeria ivanovii* and *Listeria innocua* (Rocourt and Grimont, 1983; Bubert *et al.*, 1999; Johnson *et al.*, 2004; Liu *et al.,* 2004).

Human cases of infection with *L. ivanovii* are rare and mainly related to immune-compromised patients (Cummins *et al.*, 1994). Rocourt *et al.* in 1986 reported a case of human listeriosis by *L. seeligeri*, which caused meningitis in an immunocompromised patient. Finally, a case of fatal bacteremia in a 62 years old patient, caused by *L. innocua* was reported in 2003 (Perrin *et al.*, 2003).

Clinical manifestations of listeriosis caused by *L. monocytogenes* and other *Listeria* species are non-specific. Moreover, *L. monocytogenes* shows morphological and biochemical similarity with other *Listeria* species (Vazquez-Boland *et al.*, 2001; Johnson *et al.,* 2004) and the coexistence of various *Listeria* species in the same food has been reported (Ryu *et al.,* 2013). Considering this, many surveys have shown that the presence of *Listeria* species different from *monocytogenes* could conceal the presence of *L. monocytogenes*, resulting in false negative results (Petran and Swanson, 1993; Curiale and Lewus, 1994; Cornu *et al.*, 2002). Therefore, the development of rapid, specific and sensitive diagnostic tests, for the effective control of the disease would be desirable, in order to distinguish *L. monocytogenes* from other *Listeria* species.

The aim of this study was to develop a multiplex polymerase chain reaction (PCR) in order to simultaneously detect 6 *Listeria* species in a single reaction.

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Materials and Methods

Bacterial strains

One hundred eighteen *Listeria* spp. strains, isolated from meat product and processing plants, were included in the study. Moreover, *Listeria* species references strains (*L. monocytogenes*: ATCC 19111; *L. seelingeri*: ATCC 35967; *L. ivanovii*: ATCC 19119; *L. grayi*: ATCC 25401; *L. innocua:* DSMZ 20649; *L. welshimeri:* DSMZ 20650) purchased from the American Type Culture Collection (ATCC) and the Leibniz-Institut DSMZ-Deutsche Sammulung von Mikroorganismen und Zellkulturen GmbH (DSMZ), were used. All *Listeria* strains were cultivated in ALOA (Biolife, Milan, Italy) at 37 °C for 24 h and in Oxford medium (Biolife) at 30°C for 24 h. All isolates with typical *Listeria* spp. morphological characteristics were submitted to confirmatory tests (Gram stain, catalase and oxidase tests).

Biochemical profile determination

All the strains were submitted to biochemical identification using the API Listeria system (BioMérieux, Marcy l'Etoile, France).

Genomic DNA extraction

Genomic DNA of the bacterial strains was extracted using the Wizard Genomic Purification Kit (Promega Corp., Madison, WI, USA) according to the manufacturer's instruction.

Multiplex polymerase chain reaction

A multiplex PCR was developed with the aim to identify the six species of *Listeria* (*L. monocytogenes, L. innocua, L. ivanovii, L. grayi, L. seeligeri and L. welshimeri*) and *Listeria* spp., by using the PCR protocol described by Ryu *et al.* (2013) that has been partially modified in order to obtain the seven differentials bands for each *Listeria* species in a single reaction. Primers concentrations are reported in Table 1. All amplification reactions were performed in a final volume of 25 μL containing 5 μL of DNA, 5 μL of 10X PCR buffer (JumpStart REDTq DNA Polymerase, Sigma-Aldrich, St. Louis, MO, USA), 4 mM of $MgCl_2$, 0.1 mM each of dNTP, and 2 U of JumpStart RED Taq (Sigma-Aldrich). All amplification reactions were performed in a Gene-Amp 2700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) programmed as follows: denaturation at 94°C for 5 min, annealing at 58°C for 30 sec and elongation at 72°C for 30 sec, followed by a final extension period at 72°C for 5 min. The amplified fragments were separated by 3% agarose gel electrophoresis (Roche diagnostics, Milan, Italy) in 1X Tris-acetate EDTA (TAE; Invitrogen, Carlsbad, CA, USA) and stained with ethidium bromide (0.1 mg/mL) for 20 min. The gels were observed and the images acquired by the Gel-Doc UV trans-illuminator (Bio-Rad, Hercules, CA, USA).

Results

Biochemical profile determination

Table 2 shows the results of the biochemical identification with the API Listeria. Seventy three (61.9%) strains belonged to *L. innocua*, 26 (22.03%) to *L. welshimeri* and 9 (7.62%) to *L. monocytogenes.* For the remaining 10 strains (8.47%) the API Listeria test gave a doubtful identification.

Multiplex polymerase chain reaction

Figure 1 shows multiplex PCR results using six *Listeria* species. A gel electrophoresis confirmed that all the primer pairs specifically

Table 1. Primer pairs used for the identification of the six species and *Listeria* spp.

Species	Gene	Primer	Sequences (5'-3')	Product (bp)	Primer concentration (mM)
Listeria species	*prs*	PrsF PrsR	GCTGAAGAGATTGCGAAAGAAG CAAAGAAACCTTGGATTTGCGG	370	0.4
L. grayi	*Oxidoreductasi*	JOgrayiF JOgrayiR	GCGGATAAAGGTGTTCGGGTCAA ATTTGCTATCGTCCGAGGCTAGG	201	0.24
L. innocua	*Lin0464*	Lin0464F Lin0464R	CGCATTTATCGCCAAAACTC TCGTGACATAGACGCGATTG	749	2.5
L. ivanovii	*na*mA	Liv22228F Liv22228R	CGAATTCCTTATTCACTTGAGC GGTGCTGCGAACTTAACTCA	463	0.52
L. monocytogenes	*Lmo1030*	Lmo1030F Lmo1030R	GCTTGTATTCACTTGGATTTGTCTGG ACCATCCGCATATCTCAGCCAACT	509	0.56
L. seelingeri	*Lmo0333*	IseelinF IseelinR	GTACCTGCTGGGAGTACATA CTGTCTCCATATCCGTACAG	673	1.36
L. welshimeri	*scrA*	Lwe1801F Lwe1801R	CGTGGCACAATAGCAATCTG GACATGCCTGCTGAACTAGA	281	2.5

L., Listeria.

Table 2. Results of biochemical and molecular identification.

Matrix isolation	Biochemical identification	Molecular identification
Meat product (61)	*L. innocua*	*L. innocua*
Meat product (15)	L. welshimeri	*L. welshimeri*
Meat product (7)	*L. monocytogenes*	*L. monocytogenes*
Meat product (2)	*L. welshimeri/L. innocua*	*L. welshimeri*
Meat product (1)	*L. welshimeri /L. innocua*	*L. innocua*
Meat product (1)	*L. welshimeri* (doubt)	*L. welshimeri*
Meat product (1)	*L. innocua*	*L. innocua*
Meat product (1)	*L. welshimeri* (unacceptable)	*L. welshimeri*
Meat product (1)	*L. welshimeri* (unacceptable)	*L. welshimeri*
Meat product (1)	*L. welshimeri* (doubt)	*L. welshimeri*
Meat product (1)	*L. welshimeri* (doubt)	*L. welshimeri*
Meat product (1)	*Listeria monocytogenes* (unacceptable)	*L. monocytogens*
Scm (9)	*L. welshimeri*	*L. welshimeri*
Scm (7)	*L. innocua*	*L. innocua*
Scm (1)	*L. welshimeri L. monocytogens* (doubt)	*L. welshimeri*
Swcm (3)	*L. innocua*	*L. innocua*
Swcm (2)	*L. welshimeri*	*L. welshimeri*
Swcm (2)	*L. monocytogens*	*L. monocytogens*
Swcm (1)	*L. innocua*	*L. innocua*

L., listeria; scm, surfaces in contact with meat; swcm, surfaces without contact with meat.

amplified the desired PCR products. This implied that the primers were specific for detection of the *Listeria* species used in this study and would not exhibit false-positives on account of the PCR reaction. All 114 strains were subjected to analysis by the multiplex PCR, confirming the biochemical identification. PCR allowed to uniquely identifying strains that had expressed a doubtful profile with API Listeria (Table 2).

Discussion

Bacteria of genus *Listeria* are major foodborne pathogens. As said, most of the human infections caused to bacteria of genus *Listeria*, are due to *L. monocytogenes* species, but there have been rare cases of infection caused by other species such as *L. seeligeri*, *L. innocua* and *L. ivanovii* (Rocourt *et al.*, 1986; Perrin *et al.*, 2003; Guillet *et al.,* 2010). The coexistence of various species of *Listeria* in the same food matrix is a very common occurrence (Ryu *et al.*, 2013) and there is often a higher prevalence of *Listeria* species other than *monocytogenes*, which further complicates the isolation of *L. monocytogenes*. Among the various detection methods for foodborne pathogens, the most widely used are the selective media that are able to detect live bacterial cells directly from the food matrix (Gracias and McKillip, 2004). These conventional methods are widely used, especially in the food industry, because of the relatively low cost. However, cultural techniques present several limitations due to the time of analysis, as they require a pre-enrichment for the recovery of damaged cells and subsequently, long incubation times for isolation on selective media. Other limiting factors are represented by the detection of false negative results, but especially by the need of confirmation analysis such as biochemical identification realized with the API Listeria test and that has very high costs (Gracias and McKillip, 2004). To overcome these disadvantages, various molecular techniques have been developed. The availability of a PCR assay species-specific for the target genes is a valuable contribution to the identification of all *Listeria* species. Furthermore, this method is a rapid and economic alternative for the detection of the different species, and would be useful for the clinical diagnosis of human patients, and also for the food industry. In this study, we applied a multiplex PCR with high sensitivity for the identification of six species of *Listeria*. The results of the multiplex PCR have shown, in many cases, a higher specificity than biochemical identification. In fact, 8% of the strains analyzed by API Listeria system gave a doubtful result, while the application of the multiplex PCR allowed an unambiguous identification. Furthermore, this molecular assay does not require a colony in purity for the identification of species.

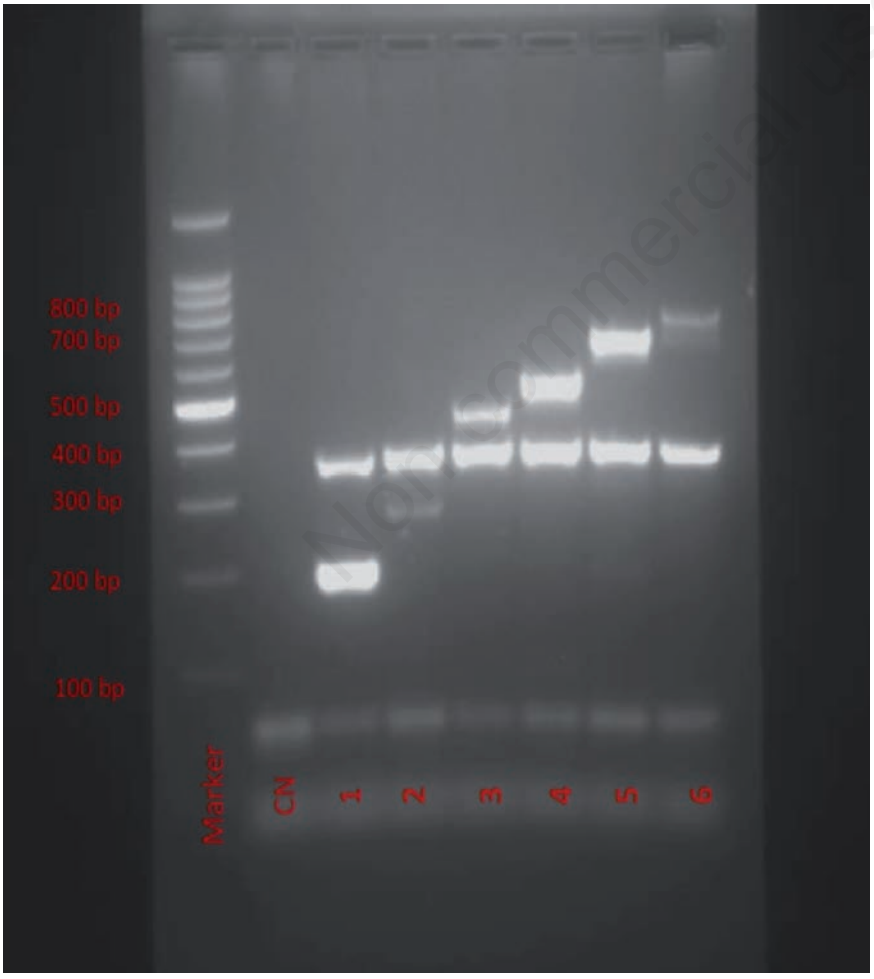

Figure 1. Multiplex polymerase chain reaction results using *Listeria* species type strains. Marker: 100 bp DNA ladder. CN, negative control; 1, *Listeria grayi*; 2, *Listeria welshimeri*; 3, *Listeria ivanovii*; 4, *Listeria monocytogenes*; 5, Listeria seeligeri; 6, *Listeria innocua*.

Conclusions

The results suggest that the multiplex PCR applied in our investigation could represent a rapid and sensitive screening test, a reliable method for the detection of all *Listeria* species, both in contaminated food and in clinical samples, and also a tool that could be used for epidemiological purposes in foodborne outbreaks. A further application could be the development of a PCR that can be directly applied to the pre-enrichment broth.

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