Comparison of Multiple-Locus Variable number tandem repeat Analysis and Pulsed Field Gel Electrophoresis in molecular subtyping of Listeria monocytogenes *isolates from Italian cheese*

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Keywords
Cheese,
Discriminatory power,
Listeria monocytogenes,
MLVA,
PFGE.

Summary
Subtyping of *Listeria monocytogenes* strains is an essential epidemiological tool to trace contamination and determine evolutionary relationships among different strains. Pulsed field gel electrophoresis (PFGE) is the current gold standard method for *Listeria* characterization. Multiple-Locus Variable-number tandem repeat Analysis (MLVA) is a rapid subtyping method based on Polymerase Chain Reaction (PCR) amplification that has been successfully developed for subtyping bacterial genera. The purpose of this study was to evaluate MLVA for subtyping *L. monocytogenes* strains isolated from Italian cheese and to compare it with PFGE. The type ability and discriminatory power of MLVA was determined on a collection of 90 isolates corresponding to 5 serotypes and 29 pulsotypes with enzymes *Asc*I and *Apa*I. A panel of 5 variable-number tandem repeat *loci* was used. MLVA and PFGE showed very similar discriminatory power (Simpson's Index of diversity 0.840 with 95% Confidence Interval [CI] 0.780/0.899 and 0.837 with 95% CI 0.768/0.906, respectively). MLVA is an easy test to perform. It is relatively fast, reproducible and could be implemented in any molecular laboratory but, according to the performed protocol, it is not sufficient for discriminating the *L. monocytogenes* strains isolated from cheese. This method could be combined with PFGE to increase the discrimination in molecular subtyping of these strains.

Confronto delle tecniche Multiple-Locus Variable number tandem repeat Analysis *(MLVA) e* Pulsed Field Gel Electrophoresis *(PFGE) per la tipizzazione molecolare di ceppi di* Listeria monocytogenes *isolati da formaggi italiani*

Parole chiave
Formaggio,
Listeria monocytogenes,
MLVA,
PFGE,
Potere discriminante.

Riassunto
La tipizzazione dei ceppi di *Listeria monocytogenes* è uno strumento epidemiologico fondamentale per la tracciabilità della contaminazione e per lo studio delle relazioni evolutive tra ceppi diversi del microrganismo. La *Pulsed Field Gel Electrophoresis* (PFGE) è, attualmente, il metodo più usato per la caratterizzazione dei ceppi di *Listeria*. La *Multiple-Locus Variable number tandem repeat Analysis* (MLVA) è un metodo di tipizzazione rapido basato su PCR, sviluppato con successo per diversi generi batterici. L'obiettivo dello studio è stato di valutare la MLVA come metodo di tipizzazione dei ceppi di *Listeria monocytogenes* isolati da formaggi italiani e compararla con la PFGE. Le capacità di tipizzazione e differenziazione della MLVA sono state determinate su 90 ceppi corrispondenti a 5 differenti sierotipi e 29 profili di PFGE ottenuti con gli enzimi di restrizione *Asc*I e *Apa*I. Per la MLVA è stato utilizzato un protocollo basato su 5 *loci* di sequenze ripetute in tandem. MLVA e PFGE hanno mostrato potere discriminante molto simile (Indice di diversità di Simpson rispettivamente di 0,840 con 95% CI 0,780/0,899 e di 0,837 con 95% CI 0,768/0,906). La MLVA è un metodo facile da eseguire, relativamente veloce, riproducibile e facilmente sviluppabile in qualsiasi laboratorio che effettui analisi molecolari ma, in accordo con il protocollo eseguito, non

è risultato sufficientemente discriminante per i ceppi di *Listeria monocytogenes* isolati da formaggio. Il metodo potrebbe essere vantaggiosamente combinato con la PFGE per aumentare la differenziazione nella tipizzazione molecolare di questi ceppi.

Introduction

Listeriosis is the third leading cause of death among major pathogens commonly transmitted by food. Despite it being a rare disease, hospitalization due to Listeriosis is much more common than hospitalization due to other foodborne infections (CDC 2013). *Listeria* species are ubiquitous organisms that are widely distributed in the environment, especially in plant matter and soil. The principal reservoirs of *Listeria* are soil, forage, and surface water. The main route of transmission to humans is believed to be through consumption of contaminated food. In the European Union (EU) ready to eat (RTE) foods with a relatively long shelf-life, such as fishery and heat-treated meat products, and ready-to-eat cheese are considered an important food-borne source of human *Listeria monocytogenes* infections. In 2009 and 2011, cheese was the main implicated vehicle reported by Member States (MSs) in the EU annual report on food-borne outbreaks caused by *L. monocytogenes* (EFSA 2013). Strains of *L. monocytogenes* need to be identified and characterized in a timely manner in order to diagnose infection, identify clusters, outbreaks, and address environmental persistence, so to mitigate the risks of contamination through the food chain (Saleh-Lakha *et al.* 2013). Molecular subtyping is an essential epidemiological tool to trace contamination from food processing plants and to determine evolutionary relationships among different strains. In the last 3 decades, a multitude of such methods have been developed and a number of them have become firmly established in the toolkit of epidemiologists, while others have become obsolete with the appearance of newer techniques. Pulsed field gel electrophoresis (PFGE), for example, has played a major role in the investigation of food-borne outbreaks for more than 20 years. To date, PFGE is the 'gold standard' method for *L. monocytogenes* characterization and it is still widely used for listeriosis surveillance and outbreak investigation. Recently, PFGE has been increasingly complemented with multiple-locus variable-number of tandem repeats analysis (MLVA) (Eurosurveillance editorial team 2013, Gerner-Smidt *et al.* 2006). This is a rapid, relatively easy and cheap subtyping technique that has been successfully developed for subtyping various bacterial genera (Chenal-Francisque *et al.* 2013). Multiple-locus variable-number of tandem repeat analysis is a PCR-based method that can be used to discriminate among different strains of a bacterium and can, therefore, infer genetic relationships amongst them. This approach is based on the detection of the number of tandem repeats (TRs) at a specific *locus* in the genome of a microorganism. These can vary as a consequence of DNA polymerase enzyme slippage, during replication and these differences can be detected using PCR primers designed to anneal the flanking regions (Murphy *et al.* 2007). Typically, multiplex PCR amplification of the repeat and flanking regions is followed by amplicon sizing using capillary electrophoresis. The number of repeat copy units, or allele number, at each location is inferred from the measured amplicon size. The string of alleles from multiple *loci* forms the MLVA profile (Nadon *et al.* 2013).

The purpose of this study was to evaluate the MLVA protocol for subtyping *L. monocytogenes* strains isolated from Italian cheese and to assess the performance of MLVA and PFGE so to compare the discriminatory power of the former with the current 'gold standard' constituted by the latter.

Materials and methods

Bacterial isolates

The strain typing capability and discriminatory power of MLVA were determined on a collection of *L. monocytogenes* strains isolated at the Italian National Reference Laboratory for *L. monocytogenes*, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale' between 2005 and 2012. Ninety isolates were analysed. Forty seven *L. monocytogenes* strains had already been typed through serotyping and PFGE with PulseNet protocol[1] (Acciari *et al.* 2011), in this study, the remaining 43 strains were also typed using the same procedures. In addition, all the 90 isolates were processed using MLVA. The strain collection included 4 isolates from Brie, 1 isolate from Crescenza, 37 isolates from Gorgonzola, 2 isolates from Grana Padano, 1 isolate from Pecorino, 19 isolates from Ricotta, and 26 isolates from Taleggio. Strains were preserved using Microbank vials at -80°C.

[1] Pulsenet USA. 2009. One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Listeria monocytogenes* by Pulsed Field Gel Electrophoresis (PFGE).

Serotyping

Strains were serotyped according to the method described in the United State Food and Drug Administration *Bacteriological analytical manual* (Bennett and Weaver 2001), using commercial sera for somatic (O) and flagellar (H) antigens (Denkan Seiken Co. Ltd, Tokyo, Japan).

Pulsed-field gel electrophoresis

Listeria monocytogenes strains were characterised by PFGE according to PulseNet protocol (Pulsenet USA 2009) involving the restriction enzymes *Asc*I and *Apa*I, and *Salmonella* serotyped *Braenderup* (H9812) as the standard. Bacterial suspensions were included in agarose, lysed, washed, and digested with the restriction enzymes. The digested samples underwent electrophoresis in SeaKem Gold agarose 1% (Lonza, Rockland, USA) in the Chef Mapper XA (BioRad Inc, Hercules, California, USA) at 6 V/cm with a pulse time between 4 and 40 seconds for 19 hours.

Analysis of pulsed-field gel electrophoresis profiles

The PFGE profiles were analyzsed using BioNumerics software version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). The similarities between the *Asc*I and *Apa*I macrorestriction profiles (MRPs) were calculated using the Dice coefficient applying an optimization coefficient and band tolerance of 1.0% for both enzymes. Starting from the single similarity matrixes for each enzyme, the software created a single combined matrix, obtained from the average of the values of the single tests; each matrix was considered of equal importance, regardless of restriction enzyme. Pulsotypes were obtained combining *Asc*I and *Apa*I MRPs. Clustering was performed and dendrograms generated using the unweight pair group method with arithmetic mean (UPGMA).

MLVA protocol

A panel of 5 variable-number tandem repeat (VNTRs) *loci* was subjected to analysis. MLVA was conducted according to Lindstedt protocol (Lindstedt *et al.* 2008) with exception of a few changes regarding: primers labeling, primers concentration, temperature of annealing of 1 of the multiplex reaction, and the ratio of the 2 multiplexed reactions products in the final pooled mix. In particular, LMV7 forward primer was labeled with NED instead of HEX, different primer concentration was used instead of 10pmol (Table I). An annealing temperature of 62°C instead of 63°C was used for R2 and the ratio of 3:1 instead 5:1 was used for the final R1:R2 pooled mix.

Each amplification run included 1 *L. monocytogenes* strain, chosen to test the stability of the method, and a no-DNA template control.

DNA extraction

Each isolate was streaked for isolation and grown on 5% sheep blood agar at 37±2°C for 24-48 hours. After incubation, bacterial cells were suspended in 500 µl TE solution (50mM Tris- HCl pH 8.0, EDTA pH 8.0) and heated at 100°C for 15 minutes to obtain cell lysis. After centrifugation (Eppendorf 5402 rotor F-45-18-11, Milan, Italy) at 15000 x g for 6 minutes at 4°C, the supernatant containing DNA was transferred to a clean test tube.

Multiplex PCR reaction

A reaction mix of the total volume of 25 µl containing 3 µl of extracted DNA (final concentration 50-60 ng/µl), 12.5 µl Multiplex PCR Master Mix 2X (Quiagen®, Hilden, Germany) was used for PCR. The concentrations and labeling of each primer are shown in Table I.

The primers were multiplexed in 2 reactions, R1 and R2: R1 containing primers LMV2 and LMV6 and R2 containing primers LMV1, LMV7 and LMV9.

The PCR reactions were performed in a Thermal Cycler GeneAmp 9700 (AppliedBiosystem®, Carlsbad, CA, USA). PCR reactions were run under the following conditions: initial step at 95°C for 15 minutes, 30 cycles at 94°C for 30 seconds for denaturation, R1 at 60°C and R2 at 62°C for 90 seconds for annealing, and at 72°C for 90 seconds for extension, with a final extension step at 72°C for 10 minutes.

Capillary electrophoresis

PCR amplification products of R1 and R2 reactions were pooled in a 3:1 ratio and PCR pooled products were diluted 1:20 before loading. From R1-R2 pooled solution, 1 µl was added to 9 µl of

Table I. *Primer labeling and final concentrations used in the PCR reactions R1- R2 conducted on* L. monocytogenes *strains isolated at the Italian National Reference Laboratory for* L. monocytogenes*, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', between 2005 and 2012.*

Primer	Final concentration (µM)	Primer labeling
LMV1	0.05	HEX
LMV2	0.15	6-FAM
LMV6	0.05	6-FAM
LMV7	0.10	NED
LMV9	0.15	HEX

formamide and 0.05 µl of GeneFloTM 625 DNA Ladder ROX Labeled (EURX Ltd., Gdansk, Poland) internal size standard.

Capillary electrophoresis was performed on an ABI 3500 Genetic Analyzer (Applied Biosystem, Carlsbad, CA, USA). The protocol used was "Long Fragment Analysis 50-POP7". Before the beginning of the run, a denaturation at 94°C for 5 minutes was provided.

Data analysis

Each fragment was identified by peak size (bp) using GeneMapper® Software Version 4.0, allele numbers were assigned according to Pasteur Institute MLVA database[2].

A typical electropherogram is shown in Figure 1. Each peak represents a PCR product. *Loci* LMV2 and LMV6 were depicted as blue peaks, *loci* LMV1 and LMV9 were depicted as green peaks, and *locus* LMV7 was depicted as a black peak. The DNA size standard labelled with the dye ROX was depicted in red which presented the internal size standard.

The MLVA profile was presented in the order of the allele string as follows: LMV1-LMV2-LMV6-LMV7-LMV9. This allele string of 5 numbers identified the strain MLVA type (MT).

Statistical analysis

To evaluate the ability of MLVA and PFGE methods to discriminate between strains and the discriminatory power of each *locus*, Simpson's index of diversity was endorsed (Hunter *et al.* 1988):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} nj(nj-1)$$

Where

N= total number of strains tested

S= total number of different types identified

nj= total number of j type identical strains

Results

Serotypes

Serotyping performed by serological agglutination revealed that the 90 isolates could be grouped into 5 serotypes (1/2a, 1/2b, 1/2c, 3a and 4b). No auto agglutination was observed.

The main serotypes detected were 1/2a (75.6%) followed by serotype 1/2c (11.1%), and serotype 4b (10.0%). Serotype 3a was detected in 1 strain only.

Pulsed-field gel electrophoresis

The 90 isolates corresponded to 29 PFGE patterns obtained with PulseNet protocol using restriction enzymes *Asc*I and *Apa*I. The most frequent pulsotypes were pulsotype B (37.8%), pulsotype I (11.1%), pulsotype L (7.8%), pulsotype J and X (5.6%); the remaining pulsotypes were associated with 1 or 2 strains.

The analysis of PFGE patterns, according to similarity of macrorestriction profiles, pointed out 5 main clusters. Cluster I, mainly represented by pulsotype B, included 51 strains with similarity values of 74.1%. Cluster II included 7 strains with a similarity of 100% (pulsotype L). Cluster III consisted of 4 strains with a similarity of 85.3% and with pulsotypes different from each other. Cluster IV, mainly represented by pulsotypes I and J, included 17 strains showing a similarity of 79.2%. Cluster V included 9 strains belonging to 4 pulsotypes (X-Z-Y-AB) with similarity values of 85.5%. All the isolates associated to

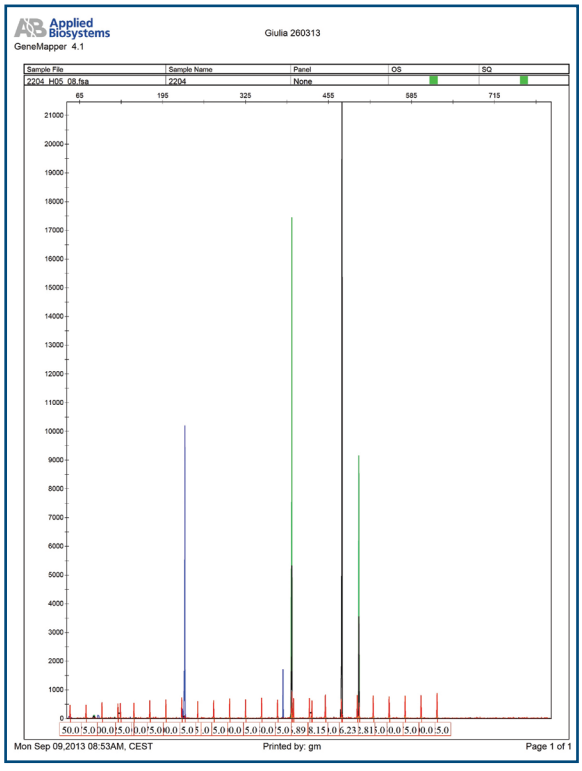
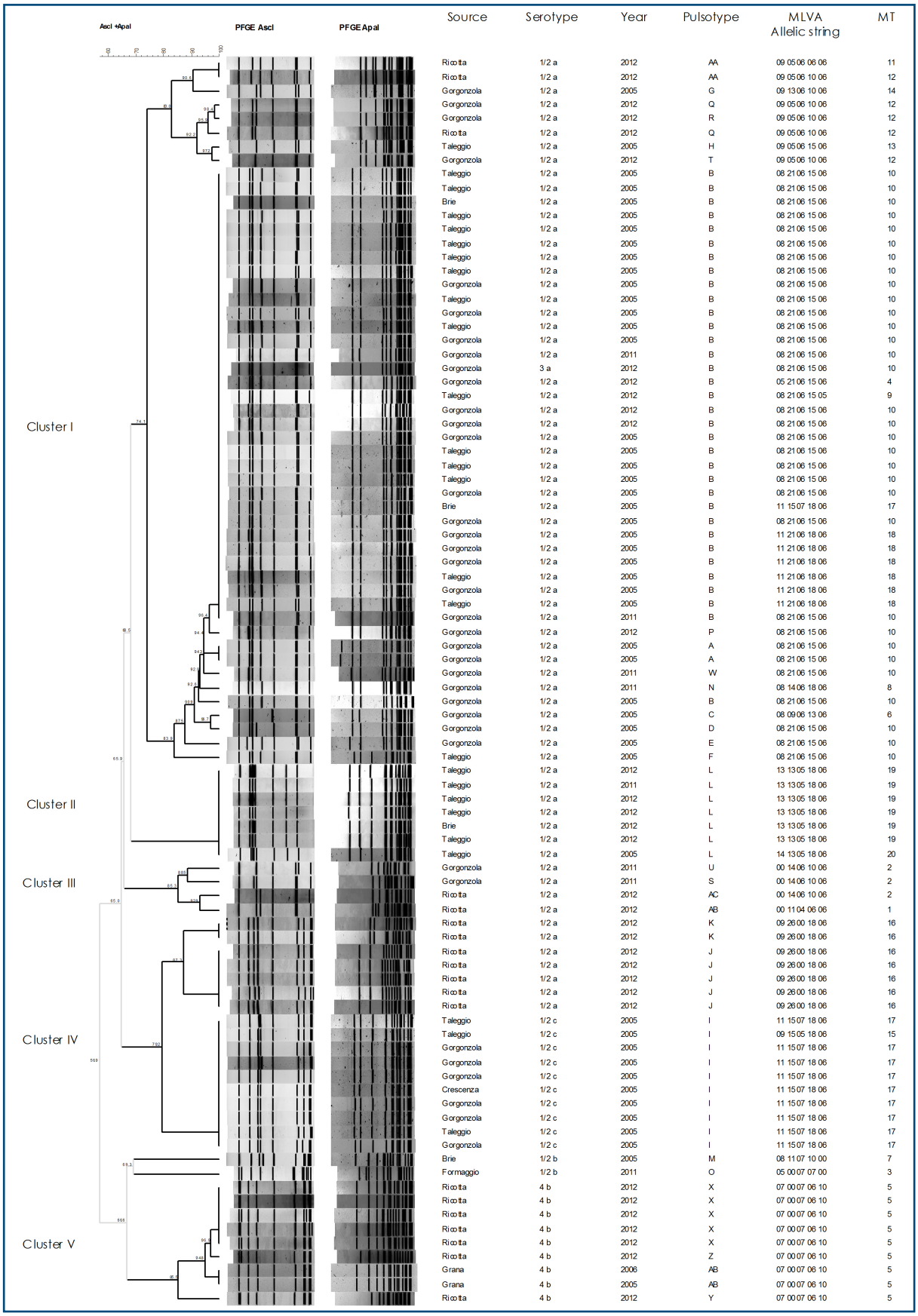


Figure 1. *A typical MLVA electropherogram of* Listeria monocytogenes *strains isolated at the Italian National Reference Laboratory for* L. monocytogenes*, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', between 2005 and 2012. Loci* LMV2 and LMV6 were depicted as blue peaks, *loci* LMV1 and LMV9 were depicted as green peaks, and *locus* LMV7 was depicted by black. The DNA size standard labeled with the dye ROX, which presented the internal size standard, was depicted in red.

[2] http://www.pasteur.fr/cgi-bin/genopole/PF8/mlva/mlvadb.pl.



Source	Serotype	Year	Pulsotype	MLVA Allelic string	MT
Ricotta	1/2 a	2012	AA	09 05 06 06 06	11
Ricotta	1/2 a	2012	AA	09 05 06 10 06	12
Gorgonzola	1/2 a	2005	G	09 13 06 10 06	14
Gorgonzola	1/2 a	2012	Q	09 05 06 10 06	12
Gorgonzola	1/2 a	2012	R	09 05 06 10 06	12
Ricotta	1/2 a	2012	Q	09 05 06 10 06	12
Taleggio	1/2 a	2005	H	09 05 06 15 06	13
Gorgonzola	1/2 a	2012	T	09 05 06 10 06	12
Taleggio	1/2 a	2005	B	08 21 06 15 06	10
Taleggio	1/2 a	2005	B	08 21 06 15 06	10
Brie	1/2 a	2005	B	08 21 06 15 06	10
Taleggio	1/2 a	2005	B	08 21 06 15 06	10
Taleggio	1/2 a	2005	B	08 21 06 15 06	10
Taleggio	1/2 a	2005	B	08 21 06 15 06	10
Taleggio	1/2 a	2005	B	08 21 06 15 06	10
Taleggio	1/2 a	2005	B	08 21 06 15 06	10
Gorgonzola	1/2 a	2005	B	08 21 06 15 06	10
Taleggio	1/2 a	2005	B	08 21 06 15 06	10
Gorgonzola	1/2 a	2005	B	08 21 06 15 06	10
Taleggio	1/2 a	2005	B	08 21 06 15 06	10
Gorgonzola	1/2 a	2005	B	08 21 06 15 06	10
Gorgonzola	1/2 a	2011	B	08 21 06 15 06	10
Gorgonzola	3 a	2012	B	08 21 06 15 06	10
Gorgonzola	1/2 a	2012	B	05 21 06 15 06	4
Taleggio	1/2 a	2012	B	08 21 06 15 05	9
Gorgonzola	1/2 a	2012	B	08 21 06 15 06	10
Gorgonzola	1/2 a	2012	B	08 21 06 15 06	10
Gorgonzola	1/2 a	2005	B	08 21 06 15 06	10
Taleggio	1/2 a	2005	B	08 21 06 15 06	10
Taleggio	1/2 a	2005	B	08 21 06 15 06	10
Taleggio	1/2 a	2005	B	08 21 06 15 06	10
Gorgonzola	1/2 a	2005	B	08 21 06 15 06	10
Brie	1/2 a	2005	B	11 15 07 18 06	17
Gorgonzola	1/2 a	2005	B	08 21 06 15 06	10
Gorgonzola	1/2 a	2005	B	11 21 06 18 06	18
Gorgonzola	1/2 a	2005	B	11 21 06 18 06	18
Gorgonzola	1/2 a	2005	B	11 21 06 18 06	18
Taleggio	1/2 a	2005	B	11 21 06 18 06	18
Gorgonzola	1/2 a	2005	B	11 21 06 18 06	18
Taleggio	1/2 a	2005	B	11 21 06 18 06	18
Gorgonzola	1/2 a	2011	B	08 21 06 15 06	10
Gorgonzola	1/2 a	2012	P	08 21 06 15 06	10
Gorgonzola	1/2 a	2005	A	08 21 06 15 06	10
Gorgonzola	1/2 a	2005	A	08 21 06 15 06	10
Gorgonzola	1/2 a	2011	W	08 21 06 15 06	10
Gorgonzola	1/2 a	2011	N	08 14 06 18 06	8
Gorgonzola	1/2 a	2005	B	08 21 06 15 06	10
Gorgonzola	1/2 a	2005	C	08 09 06 13 06	6
Gorgonzola	1/2 a	2005	D	08 21 06 15 06	10
Gorgonzola	1/2 a	2005	E	08 21 06 15 06	10
Taleggio	1/2 a	2005	F	08 21 06 15 06	10
Taleggio	1/2 a	2012	L	13 13 05 18 06	19
Taleggio	1/2 a	2011	L	13 13 05 18 06	19
Taleggio	1/2 a	2012	L	13 13 05 18 06	19
Taleggio	1/2 a	2012	L	13 13 05 18 06	19
Brie	1/2 a	2012	L	13 13 05 18 06	19
Taleggio	1/2 a	2012	L	13 13 05 18 06	19
Taleggio	1/2 a	2005	L	14 13 05 18 06	20
Gorgonzola	1/2 a	2011	U	00 14 06 10 06	2
Gorgonzola	1/2 a	2011	S	00 14 06 10 06	2
Ricotta	1/2 a	2012	AC	00 14 06 10 06	2
Ricotta	1/2 a	2012	AB	00 11 04 06 06	1
Ricotta	1/2 a	2012	K	09 26 00 18 06	16
Ricotta	1/2 a	2012	K	09 26 00 18 06	16
Ricotta	1/2 a	2012	J	09 26 00 18 06	16
Ricotta	1/2 a	2012	J	09 26 00 18 06	16
Ricotta	1/2 a	2012	J	09 26 00 18 06	16
Ricotta	1/2 a	2012	J	09 26 00 18 06	16
Ricotta	1/2 a	2012	J	09 26 00 18 06	16
Taleggio	1/2 c	2005	I	11 15 07 18 06	17
Taleggio	1/2 c	2005	I	09 15 05 18 06	15
Gorgonzola	1/2 c	2005	I	11 15 07 18 06	17
Gorgonzola	1/2 c	2005	I	11 15 07 18 06	17
Gorgonzola	1/2 c	2005	I	11 15 07 18 06	17
Crescenza	1/2 c	2005	I	11 15 07 18 06	17
Gorgonzola	1/2 c	2005	I	11 15 07 18 06	17
Gorgonzola	1/2 c	2005	I	11 15 07 18 06	17
Taleggio	1/2 c	2005	I	11 15 07 18 06	17
Gorgonzola	1/2 c	2005	I	11 15 07 18 06	17
Brie	1/2 b	2005	M	08 11 07 10 00	7
Formaggio	1/2 b	2011	O	05 00 07 07 00	3
Ricotta	4 b	2012	X	07 00 07 06 10	5
Ricotta	4 b	2012	X	07 00 07 06 10	5
Ricotta	4 b	2012	X	07 00 07 06 10	5
Ricotta	4 b	2012	X	07 00 07 06 10	5
Ricotta	4 b	2012	X	07 00 07 06 10	5
Ricotta	4 b	2012	Z	07 00 07 06 10	5
Grana	4 b	2006	AB	07 00 07 06 10	5
Grana	4 b	2005	AB	07 00 07 06 10	5
Ricotta	4 b	2012	Y	07 00 07 06 10	5

Figure 2. *Cluster analysis of 90* Listeria monocytogenes *strains isolated from Italian cheese samples at the Italian National Reference Laboratory for* L. monocytogenes, *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', between 2005 and 2012 based on combined* AscI *and* ApaI *macrorestriction profiles.* Similarity analysis was performed using Dice coefficients, with a 1.0% band position tolerance and 1.0% optimization. Clustering and dendrogram construction were performed using the Unweighted Pair Group Method Analysis with averages (UPGMA). MT= MLVA Type.

serotype 4b clustered in this last group. The only 2 strains with serotype 1/2b, corresponding to pulsotype M and O, did not result associated to any cluster (Figure 2).

Multiple-Locus Variable number tandem repeat Analysis

The MLVA protocol was implemented on 5 VNTR *loci*. MLVA of the all isolates resulted in 20 different MLVA types (MT), the 8 main MT accounting for 86.7% of the isolates. The most common profiles were 08-21-06-15-06 (MT 10) accounting for 35.6%, 11-15-07-18-06 (MT 17) accounting for 11.1%, and 07-00-07-06-10 (MT 5) accounting for 10.0 % (Figure 3).

The majority of isolates displayed all amplification products, although some of these gave no peaks for 1 of Variable Number of Tandem Repeat (VNTR). Only 1 isolate gave no peaks for 2 of VNTR. Numbers of alleles, percentages of null alleles, and discriminatory power obtained for each VNTR are described in Table II.

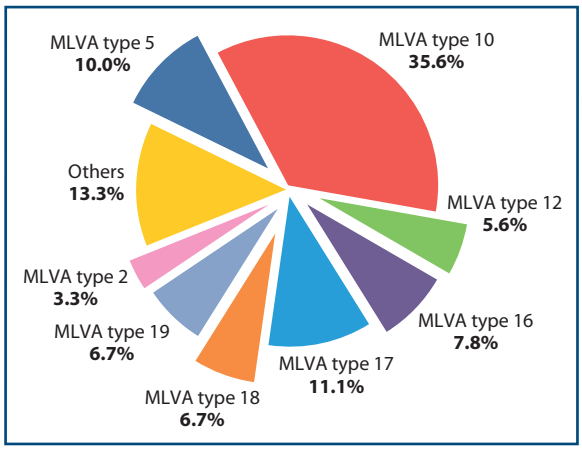


Figure 3. *MLVA type percentages of* Listeria monocytogenes *strains isolated from cheese samples.*

Table II. *Numbers of alleles, percentages of null alleles and discriminatory power obtained for each VNTR conducted on* Listeria monocytogenes *strains isolated at the Italian National Reference Laboratory for* L. monocytogenes*, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', between 2005 and 2012.*

Locus name	Repeat size (bp)	No. of alleles	No. of isolates with null allele[a]	Simpson index	CI 95%
LMV1	6	8	4 (4.4%)	0.768	0.710 - 0.826
LMV2	9	9	10 (11.1%)	0.754	0.676 - 0.831
LMV6	15	5	7 (7.8%)	0.583	0.490 - 0.676
LMV7	9	6	0	0.693	0.635 - 0.750
LMV9	9	4	2 (2.2%)	0.241	0.128 - 0.354

[a]The frequency of null alleles among isolates tested is shown in parentheses.
bp = base pair; CI = confidence interval.

The 8 main MT included strains predominantly linked to a specific pulsotype except by strains belonging to MT 2 and 12 (Figure 4).

Figure 2, showing MLVA allelic string and the corresponding MLVA type, revealed the presence of strains with identical PFGE patterns corresponding to different MT and vice versa. It is worth stressing that strains belonging to pulsotype B showed 5 MT (cluster I), while MT 5 showed 4 pulsotypes (cluster V).

The discriminatory power of MLVA and PFGE methods in molecular subtyping of *L. monocytogenes* strains from Italian cheese was very similar. MLVA showed a Simpson's Index of diversity (SID) of 0.840 (95% CI; range 0.780/0.899). On the other hand, PFGE differentiated these isolates into 29 pulsotypes showing a SID of 0.837 (95% CI; range 0.768/0.906). Combining these techniques allows for obtaining a higher discriminatory power (0.902, 95% CI; range 0.856/0.948) than the one of each technique alone.

Discussion/Conclusion

This study aimed at evaluating a 5-*loci* MLVA protocol in order to characterize *L. monocytogenes* strains isolated from Italian cheese and to compare the discriminatory power of MLVA versus the current 'gold standard' PFGE. The strains were analysed by serotyping, PFGE, and MLVA.

According to previous studies, serotype 1/2a was confirmed as the main serotype in food isolates (Gianfranceschi *et al.*, 2009), accounting for 75.6% of all the *L. monocytogenes* isolates. Previous studies (Acciari *et al.* 2011, Lunestad *et al.* 2013) highlighted the predominance of this serotype in isolates from cheese and from cases of listeriosis associated to cheese consumption and showed that the predominance of serotype 1/2a could be due to a greater resistance to bacteriocins and to conditions prevalent in the production processes.

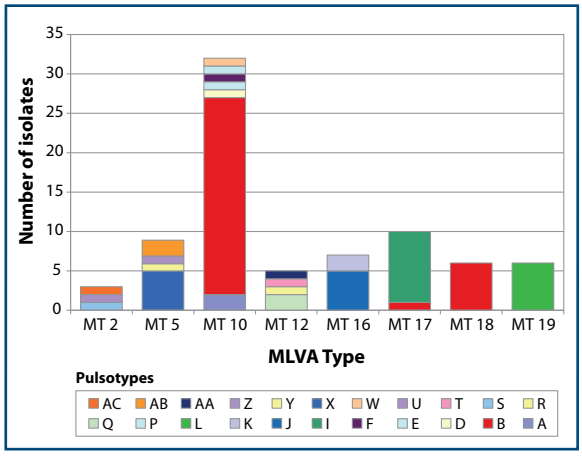


Figure 4. *Distribution of pulsotypes among the main MLVA types.*

Several studies have compared and correlated PFGE and MLVA as typing methods used for *L. monocytogenes* strains. MLVA has attracted intense interest as it has practical advantages that are largely recognized.

The MLVA method is one of several molecular techniques used for genotyping bacterial pathogens to investigate the epidemiology of outbreaks, as well as to trace the contamination routes in food-processing factories (Lunestad *et al.* 2013).

MLVA is easy to perform, relatively fast, and reproducible technique and could be deployed in any molecular laboratory. From a technical point of view, MLVA has a potential high-throughput as 96 samples can be potentially loaded in a single run and involves simple and rapid procedures resulting in a total time, for each sample, of approximately 6 hours from a pure culture. PFGE is much more laborious and time-intensive, as it needs more than 48 hours to obtain a 2 enzymes complete profile of a sample. Moreover, data analysis for MLVA is simple and objective, with established tables for copy-number calling for each *locus*, resulting in an easy to understand numerical barcode that facilitates inter-laboratory data exchange. In comparison, PFGE is much more subjective in data interpretation and requires a trained specialist for pulsed field pattern designation, leading to more variability in inter-laboratory results (Saleh-Lakha *et al.* 2013).

However, PFGE is still considered the gold standard method used in listeriosis surveillance systems of European Union, United States, and Canada (Chenal-Francisque *et al.* 2013, Eurosurveillance editorial team 2013) .

Jadhav and colleagues reviewed the literature on relevant methods for *L. monocytogenes* subtyping (Jadhav *et al.* 2012) and reported the following order in terms of decreasing discriminatory power: MLVA>PFGE>MLST>ribotyping. However, other authors (Sperry *et al.* 2008) demonstrate a lower discrimination of MLVA than PFGE.

Lindstedt and colleagues showed that MLVA gave overall the same resolution as PFGE even if it appeared more discriminatory in a sample set and less discriminatory in another one. This was probably due to a sample selection bias (Lindstedt *et al.* 2008). Our study indicates that MLVA had the same discriminatory power of PFGE based on both *Asc*I and *Apa*I enzymes and that it was generally in good agreement with PFGE clusters and serotyping data.

The strain typing capability obtained with PFGE was higher than the one obtained with MLVA. This event could be due to the relatively high percentages of null alleles detected. This is in agreement with previous studies in which MLVA panels have been associated with PCR failures for some VNTR *loci* leading to an incomplete characterization of some isolates (Chenal-Francisque *et al.* 2013, Li *et al.* 2013)

Regarding single *locus* discriminatory power, according to a previous work conducted with the same protocol (Li *et al.* 2013), LMV9 showed the lowest SID. Furthermore, LMV2 *locus* showed the highest numbers of alleles confirming a higher mutation rate than the rest of the considered *loci*. The lower SID obtained for LMV2, LMV6, and LMV9 *loci* could be due to a higher percentage of null alleles observed or to a lower number of strains tested.

The overall low discriminatory powers obtained for the single *locus* could be attributable to a potential correlation of some isolates. Some authors stress that the sMLVA method is highly dependent on the clone and high discrimination could be achieved only including in the study unrelated strains or performing a more-*loci* protocol (Chenal-Francisque *et al.* 2013).

In conclusion, according to the protocol performed, MLVA is not sufficiently discriminating for strains of *L. monocytogenes* isolated from cheese and it cannot replace PFGE in outbreak investigation. A higher number of selected VNTR markers should be used to achieve a better discriminatory power.

However MLVA method showed advantages over PFGE (high reproducibility, unambiguous results). Standardization would provide benefits to international surveillance. Given the high reproducibility, unambiguous results and advantages of MLVA method, the Pasteur institute is making efforts to implement an 11-*loci* protocol and to gain an inter-laboratory standardization through calibration of fragment sizing, reproducibility and MLVA reference set of strains (Chenal-Francisque *et al.* 2013).

At the moment this assay could be a useful screening method or could be combined with PFGE to increase the discrimination in molecular subtyping in a 2-steps MLVA-PFGE strategy in listeriosis surveillance.

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