

Original Article

LPS-PCR typing of ovine *Pasteurella multocida* isolates from Iran based on (L1 to L8) outer core biosynthesis loci

Mirhaghgoye Jalali ¹, F., Jabbari ^{1, *}, A.R., Esmaelizad ², M.

1. *Pasteurella* National Research Laboratory, Razi Vaccine and Serum Research Institute, Agricultural Research, Education, and Extension Organization, Karaj, Iran

2. Department of Central Laboratory, Razi Vaccine and Serum Research Institute, Agricultural Research, Education, and Extension Organization, Karaj, Iran

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Corresponding Author: a.jabbari@rvsri.ac.ir

ABSTRACT

Pasteurella multocida is a gram-negative bacterial pathogen that is causative agent of a wide range of diseases in many animal species and humans. Lipopolysaccharides (LPS) are an important virulence factor, minor changes to structure of which can exert dramatic effects on pathogenicity of *P. multocida* in its host. LPS can be used for the identification and classification of strains with somatic typing systems. The aim of this study was to identify the LPS genotypes of the ovine *P. multocida* isolates obtained from pneumonia cases in Iran. The LPS genotype of the isolates was determined using eight specific primers for LPS outer core biosynthesis loci. The LPS genes were amplified by polymerase chain reaction (PCR), then they were sequenced and compared to the sequences registered in the GenBank. Of the 32 ovine *P. multocida* isolates tested, 21 (65.62%) isolates belonged to genotype L6, 9 (28.12%) isolates contained genotype L3, 1 (3.12%) isolate had both L3 and L6 loci, and 1 (3.12%) isolate remained untypeable. The LPS-PCR was able to type 31 of 32 field ovine isolates from Iran. According to the phylogenetic analysis, L3 genotype isolates were grouped into two distinct lineages. LPS gene sequences among L6 genotypes of ovine *P. multocida* isolates from Iran and the related sequences in the GenBank were highly similar (>99.5%). LPS-PCR is an accurate genotyping method that was able to classify *P. multocida* strains into one of the eight distinct LPS genotypes.

Keywords: *P. multocida*, Sheep, LPS outer core, PCR-typing

LPS-PCR typage des isolats iraniens de *Pasteurella multocida* de mouton sur la base des gènes externes (L1 à L8) LPS

Résumé: *Pasteurella multocida* est une bactérie Gram négative pathogène chez de nombreuses espèces animales ainsi que pour l'homme. De toute évidence, les lipopolysaccharides (LPS) représentent un facteur pathogène important et le moindre changement dans leur structure peut fondamentalement affecter le pouvoir pathogène de *Pasteurella multocida*. De plus, les LPS peuvent être utilisés comme un facteur important dans le système de typage somatique pour l'identification et la classification des bactéries utilisées. Les génotypes des LPS des isolats de moutons ont été déterminés avec 8 paires d'amorces spécifiques à certains gènes exprimés par le noyau externe des LPS. Ensuite, les séquençages des gènes de LPS amplifiés par PCR ont été déterminés et ont été comparés aux séquences déjà existantes dans la banque de gènes. Parmi les 32 isolats de *Pasteurella multocida* de mouton testés, 21 isolats (62.65%) avaient le génotype L6, 9 isolats (28.12%) le génotype L3, 1 isolat (3.12%) montraient les deux génotypes L3 et L6; et un isolat (3.12%) était inclassable. La méthode LPS-PCR a été en mesure de déterminer le génotype de 31 des 32 isolats étudiés. Sur la base de l'analyse phylogénétique, les isolats montrant le génotype L3 ont été regroupés en deux sous-catégories. Le pourcentage de similitude des séquences des gènes LPS entre les différents isolats génotypiques de mouton répertoriés dans

la banque de gènes était considérablement élevé (>99.5%). Cette étude montre que la LPS-PCR est une méthode de géotypage précise qui peut classer les isolats de *Pasteurella multocida* dans l'un des huit géotypes des LPS.

Mots clés: *Pasteurella multocida*, mouton, noyau externe de LPS, le typage PCR

INTRODUCTION

Pasteurella multocida is a gram-negative coccobacillus belonging to the Pasteurellaceae family that can cause a wide range of diseases in animals and humans (Christensen and Bisgaard, 2000). This bacterium has been identified as the causative agent of many economically important diseases in a wide range of hosts with diverse clinical signs. It causes important diseases, including hemorrhagic septicemia in cattle and atrophic rhinitis in pigs, fowl cholera in birds, sniffles in rabbits, as well as enzootic pneumonia and shipping fever in cattle, sheep, and pigs. *P. multocida* also causes opportunistic infections in humans often following cat or dog bites (Wilkie et al., 2012). *P. multocida* virulence factors comprise of endotoxin, outer membrane proteins, toxin, iron binding system, heat shock proteins, neuraminidase production, capsule, and LPS, with the last two known as the key factors. Capsule plays a significant role in resistance to phagocytosis and complement while complete LPS is critical for pathogenicity in the host (Harper et al., 2006). LPS stimulate humoral immunity and are considered protective antigens (Brogden and Rebers, 1978). LPS produced by *P. multocida* consist of a hydrophobic lipid A molecule; core-oligosaccharide is divided into two parts of inner and outer core, both consisting of a series of sugars linked in a specific way. The LPS of *P. multocida* do not contain O-polysaccharid, thereby they are termed rough LPS (Raetz and Whitfield, 2002). LPS produced by all strains consist of a highly conserved inner core and a variable outer core. The outer core region of LPS is a highly variable component. Each of the strains expresses structurally distinct LPS (St Michael et al., 2009; Harper et al., 2011a; Harper et al., 2012; Harper

et al., 2013a; Harper et al., 2013b; Harper et al., 2014). The ability to distinguish LPS structures is important for diagnosis and control of outbreaks (Harper et al., 2014). The genes required for the biosynthesis of the outer core region of the LPS in all *P. multocida* strains are located within a single locus between the conserved genes *priA* and *fpg*. With knowledge of the genetics of LPS biosynthesis in *P. multocida*, strains can be differentiated based on the genetic organization of the LPS outer core biosynthesis loci (Harper et al., 2011b). *P. multocida* strains have classically been differentiated using serological techniques. Strains can be classified into five capsular serogroups (i.e., A, B, D, E, and F) using an indirect hemagglutination assay. Capsular genotyping method was first implemented by Townsend et al. (2001) using multiplex polymerase chain reaction (PCR). Heddleston serotyping is currently the only method used to differentiate *P. multocida* strains on the basis of LPS type. Heddleston somatic typing strains can be classified into 16 somatic or LPS serotypes using the Heddleston gel diffusion precipitin test (Heddleston et al., 1972). Harper showed that only eight unique LPS outer core biosynthesis loci were found in the 16 Heddleston type strains. Molecular methods such as PCR test can be used for identifying and typing this bacterium (Harper et al., 2014). The aim of this study was to identify the LPS genotypes of the ovine *P. multocida* isolates obtained from pneumonia cases in Iran.

MATERIALS AND METHODS

Specimens. A total of 32 field isolates of *P. multocida* from sheep with respiratory diseases from endemic areas in Isfahan (4), Kerman (2), Ghom (7), Tehran (5), and Fars (14) provinces were included in the study. The reference strains of *P. multocida* used in

the study were PMI33 (capsular serogroup A) and PMI34 (capsular serogroup B) acquired from National Pasteurella Research Laboratory, Razi Vaccine and Serum Research Institute (RVSRI), Karaj, Iran. The codes and the regions of the isolates are presented in Table 1. They were identified as *P. multocida* using standard biochemical procedures, including catalase, oxidase, indol production, urease activity, ornithine decarboxylase production, and carbohydrate fermentation associated with *Pasteurella multocida* (PM)-PCR, for the detection of *kmtI* species specific gene fragment (Townsend et al., 1998; Ewers et al., 2006).

Table 1. The codes and regions of *Pasteurella multocida* isolates used in this study

Code of the isolates	Region
PM01-PM05-PM06-PM07-PM08-PM15-PM17-PM21-PM22-PM23-PM24-PM25-PM26-PM31	Shiraz
PM02-PM03-PM04-PM13	Esfahan
PM09-PM28-PM29-PM30-PM32	Tehran
PM10-PM11	Kerman
PM12-PM14-PM16-PM18-PM19-PM20-PM27	Ghom

performance liquid chromatography (HPLC) grade water. After boiling for 20 min and centrifugation (13000 x g, 5 min), 3 µl of supernatant was used as DNA template for PCR assay. The concentration of the DNA template was determined using a spectrophotometer at optical density of 260/280 nm (Eppendorf, Germany).

PCR method. Prior to the study of LPS outer core biosynthesis genes, we performed a PM-PCR protocol for species-specific amplification of the *kmtI* gene, as described by Townsend et al. (1998). The capsular type of the isolates was determined by Cap-PCR as described previously (Townsend et al., 2001). Thereafter, the isolates were examined for the presence of unique LPS outer core biosynthesis loci including *Pcq*, *nctA*, *gatF*, *latB*, *nctB*, *rmlA*, *ppgB*, and *natG* genes. The related specific primers were as published in previous study (Harper et al., 2014). The details of the LPS biosynthesis loci, sequences of the oligonucleotide primers, and the expected size of each amplicon are described in Table 2.

Table 2. DNA sequences and genetic location of the primers used in the lipopolysaccharide genotyping of ovine *P. multocida* isolates

Locus	Primer	Sequence (5'-3')	Location	Product size (bp)
L1	BAP6119-F	ACATTCAGATAATACACCCG	<i>pcgD</i>	1107
	BAP6120-R	ATGGAGCACCTAGTAACCC	<i>pcgB</i>	
L2	BAP6121-F	CTTAAAGTAACTCGCTATTGC	<i>nctA</i>	810
	BAP6122-R	TTTGATTCCCTTGGGATAGC	<i>nctA</i>	
L3	BAP7213-F	TGCAGGCGAGAGTTGATAAACCATC	<i>gatF</i>	474
	BAP7214-R	CAAAGATTGGTTCCAAATCTGAATGGA	<i>gatF</i>	
L4	BAP6125-F	TTTCCATAGATTAGCAATGCCG	<i>latB</i>	550
	BAP6126-R	CTTTATTTGGTCTTTATATATACC	<i>latB</i>	
L5	BAP6129-F	AGATTGCATGGCGAAATGGC	<i>rmlA</i>	1175
	BAP6130-R	CAATCCTCGTAAGACCCCC	<i>rmlC</i>	
L6	BAP7292-F	TCTTTATAATTACTCTCCCAAGG	<i>nctB</i>	668
	BAP7293-R	AATGAAGGTTTAAAAGAGATAGCTGGAG	<i>nctB</i>	
L7	BAP6127-F	CCTATATTTATATCTCTCCCC	<i>ppgB</i>	931
	BAP6128-R	CTAATATATAAACCATCCAACGC	<i>ppgB</i>	
L8	BAP6133-F	GAGAGTTACAAAAATGATCGGC	<i>natG</i>	255
	BAP6134-R	TCCTGGTTCATATATAGGTAGG	<i>natG</i>	

DNA extraction method. All the *P. multocida* strains were grown at 37 °C in Brain Heart Infusion (BHI) broth. Overnight culture of each sample in BHI was used for the extraction of DNA by heat treatment. Then, 1 ml aliquot of each culture was centrifuged (13000 x g, 15 min) and the resultant pellets were washed twice and resuspended in 200 µl of high-

The PCR reactions were performed in a final volume of 20 µl at the following reagent concentrations: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 µM d NTP, 0.5 µM of each primer, 1.5 mM MgCl₂, 2.5 U Taq polymerase enzyme, and 1 µl of template DNA. Template DNA was replaced by water for the negative control reaction. DNA of the reference strains (mentioned above) was

used as positive control. Amplification was performed for 35 cycles. The PCR conditions were initial denaturation at 95 °C for 1 min, annealing at 54 °C for 50 s, and extension at 72 °C for 1 min. The final cycle was followed by an extension at 72 °C for 5 min.

Electrophoresis. The amplified products were separated by agarose gel electrophoresis (1% agarose) at 5 v/cm for 2 h. The agarose gel was stained by ethidium bromide. DNA fragments were observed by UV trans-illuminator and photographed. The size of the amplified fragments was determined by comparison with the standard DNA marker. The PCR products of selective isolates were purified and sequenced.

Sequencing and phylogenetic analysis. PCR products for sequencing were purified using the PCR Product Purification Kit (Roche, Germany). All the purified PCR products were sequenced by MacroGen Laboratory, South Korea. Sequences in the GenBank database were searched by BLAST. The comparison of the sequence alignments and phylogenetic tree was performed by Megalign software. The alignments of Iranian isolates were compared to each other and the LPS sequences of other countries registered in GenBank (Table 3).

RESULTS

All of the isolates were confirmed as *P. multocida* by PM-PCR and belonged to capsular type A as showed by Cap-PCR. The results of LPS-PCR analysis for the presence and frequency of LPS genes are outlined in Table 4. The LPS-PCR with specific primers for genotypes L3 and L6 produced PCR fragments with the expected molecular weight and size (Figure 1). Sequence analysis of the representative of the L3 (6 fragments) and L6 (15 fragments) genotypes confirmed the PCR results. Of the 32 ovine *P. multocida* isolates tested, 21 (65.62%) isolates belonged to genotype L6, 9 (28.12%) isolates contained genotype L3, 1 (3.12%) isolate had both L3 and L6 loci, and 1 (3.12%) isolate remained untypeable (Table 4). The LPS-PCR was able to type 31 of 32 field ovine isolates from Iran. According to the phylogenetic tree, all the isolates containing L3 genotype were grouped into two distinct

lineages (Figure 2). L6 LPS genes of ovine *P. multocida* from Iran and other related sequences in GenBank were considerably similar (>99.5%; Table 5).

Table 3. *Pasteurella multocida* isolates available in GenBank and their related accession numbers

Isolate	Origin	Accession number
L3 – ATCC	S wine	CP008918.1
L3 – HBO3	S wine	CP003328.1
L3 – P1662	Turkey	KF314826.1
L3 – P1059	Turkey	KF314825.1
L3 – 36950	Bovine	CP003022.1
L3 – KOBC5	Swine	HQ162680.1
L3 – CU	Turkey	HQ162671.1
L3 – P1662	Turkey	HQ162670.1
L3 – P1059	Turkey	HQ162669.1
L3 – PM70	Avian	AE004439.1
L6 – P1573	Human	KJ689443.1
L6 – 3480	Swine	CP001409.1
L6 – HNO6	Swine	CP003313.1
L6 – P2723	Turkey	HQ162679.1
L6 – P2237	Turkey	HQ162678.1
L6 – P1573	Human	HQ162677.1
L6 – ATCC	Swine	HQ162676.1
L6 – P903	Swine	HQ162675.1
L6 – P2100	Turkey	HQ162674.1
L6 – P61	Swine	HQ162673.1
L6 – P470	Swine	HQ162672.1

Table 4. The number and frequency of lipopolysaccharide genotypes in the studied ovine *P. multocida* isolates

Genotype	Strains	Number	Frequency
L3	SPM06-SPM07-SPM08-SPM09-SPM10-SPM16-SPM26-SPM27-SPM32-SPM01-SPM02-SPM03-SPM04-SPM05-SPM11-SPM12-SPM13-SPM14-	9	28.12%
L6	SPM15-SPM17-SPM18-SPM19-SPM20-SPM21-SPM23-SPM24-SPM25-SPM29-SPM30-SPM31	21	65.62%
L3-L6	SPM22	1	3.12%
Untypeable	SPM28	1	3.12%

DISCUSSION

Heddlestone serotyping is the only method used to differentiate *P. multocida* strains on the basis of LPS type. Data indicate that there were clear disagreements between Heddlestone serotyping and LPS genotypes as determined by the LPS-PCR. Importantly, PCR assigned strains to a single genotype, whereas serotyping frequently assigned strains to multiple Heddlestone serovars. Heddlestone method cannot

accurately type some isolates; thus, reliability of Heddlestone typing is questionable (Wilson et al., 1993; Singh et al., 2013).

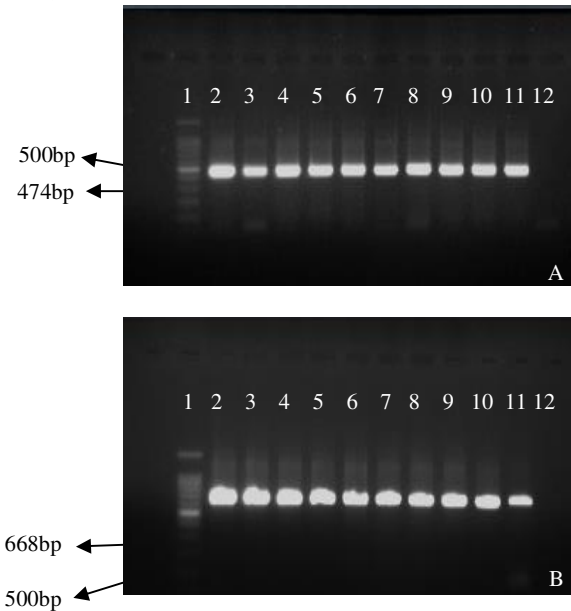


Figure 1. PCR product of L6, L3 genes on 1% agarose gel in ovine isolates, (A) L3 genotype with amplicon size (474bp), (B) L6 genotype with amplicon size (668bp) in ovine isolates and a 100 bp ladder marker was loaded in lane 1 of each gel. (1) Ladder, (2-11) isolates contain L3, L6 genotype, (12) negative control.

Compared to the Heddlestone serotyping techniques, PCR-based typing methods have several advantages such as general applicability and high discriminatory power for assessing genome variation in isolates, but they are remarkably susceptible to minor variations. Harper et al. (2014) illustrated that only eight unique LPS outer core biosynthesis loci were found in the 16 Heddlestone serotype strains, and Heddlestone serovar type strains were classified into eight genotypes using multiplex PCR (Harper et al., 2014). Each LPS genotype contains variation or truncation of the LPS structure that can arise from random point mutations or deletions in the LPS outer core biosynthesis genes resulting in the inactivation of LPS assembly biosynthesis genes; these mutations can cause change

of function or a total loss of function (Harper et al., 2014). In this study, capsular type of the isolates was determined, and all the isolates contained capsular type A. Previous studies showed that the majority of Iranian ovine samples contained capsular type A; thus, it is recognized as the most prevalent type in Iran (Shayegh et al., 2009). Molecular LPS genotyping demonstrated that ovine isolates of *P. multocida* mainly belonged to the two LPS types of L3 and L6. Our findings showed that genotype L6 with 65.62% frequency was dominant among the isolates in Iran. However, one isolate (3.12%) was positive for both L3 and L6 genotypes and one isolate (3.12%) remained untypeable. Phylogenetic analysis showed a high level of similarity (>99.5%) among L6 *P. multocida* isolates and few variations among L3 isolates. In a previous study, multiplex PCR was used for LPS genotyping of 58 avian field isolates. Genotypes L1, L3, L4, and L6 were found among the isolates. However, 10 strains were untypeable. Nucleotide sequence analysis of the LPS outer core biosynthesis locus revealed that nine of the untypeable strains contained an L3 LPS locus with significant nucleotide differences, where the L3 primers were located and strain PM135 contained an L7 LPS locus with a major deletion in the L7 primers area (Harper et al., 2014). LPS-PCR was able to type 31 of 32 field isolates; however, strain SPM28 was untypeable. This can be due to a large deletion in the annealing region of the LPS gene. Large deletions are rare; analyses indicated most mutations within the *P. multocida* LPS outer core biosynthesis loci involved single-point mutations. Similarly, in a study by Harper et al. (2014), strain PM135 remained untypeable (Harper et al., 2011a; Harper et al., 2013a; Harper et al., 2013b; Harper et al., 2014). A specific vaccine against ovine pasteurellosis is not available in Iran. However, some farmers use bovine pasteurellosis vaccine for this purpose in the sheep. The LPS typing rendered a part of the rudimentary information needed for preparation and evaluation of a specific *Pasteurella* vaccine by using local isolates.

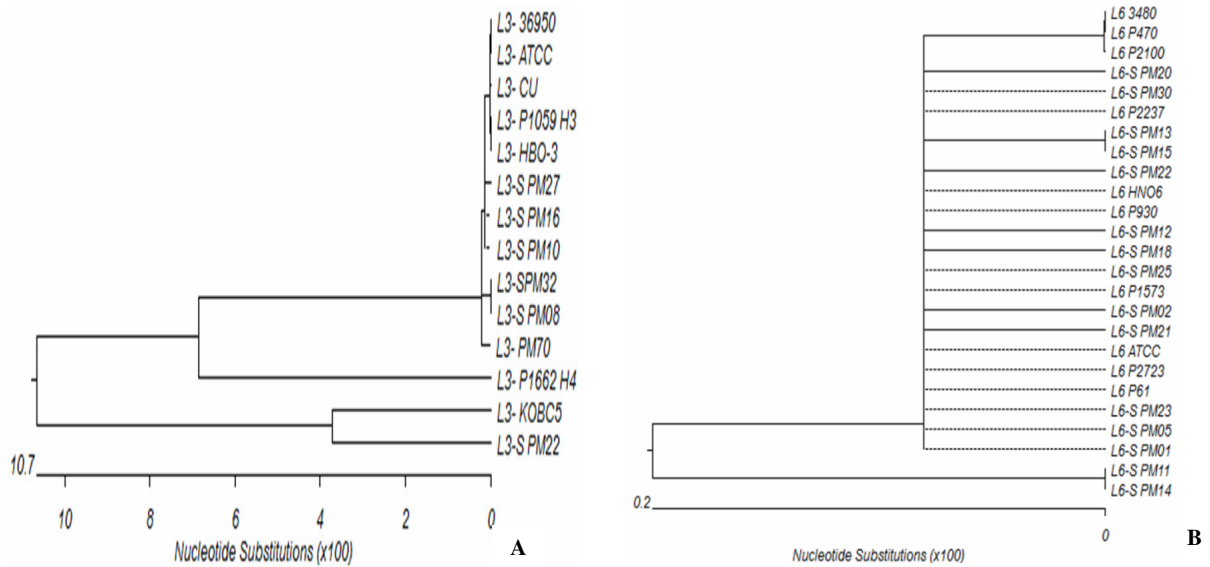


Figure 2. Phylogenetic tree based on nucleotide sequences of L3 (A) and L6 (B) genotypes of Iranian ovine *P. multocida* isolates and GenBank sequences.

Table 5. Percent of identity and divergence sequences of L3, L6 genotypes of Iranian ovine *P. multocida* isolates and GenBank sequences

		Percent Identity															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14		
Divergence	1	100.0	100.0	86.9	100.0	87.4	99.5	99.7	99.7	99.7	99.5	99.5	81.1	100.0	1	L3- 36950	
	2	0.0	100.0	86.9	100.0	87.4	99.5	99.7	99.7	99.7	99.5	99.5	81.1	100.0	2	L3- ATCC	
	3	0.0	0.0	100.0	86.9	87.4	99.5	99.7	99.7	99.7	99.5	99.5	81.1	100.0	3	L3- CU	
	4	14.6	14.6	14.6	100.0	87.4	81.9	87.4	87.1	87.1	87.1	87.1	87.1	92.7	86.9	4	L3- KOBCC5
	5	0.0	0.0	0.0	14.6	100.0	87.4	99.5	99.7	99.7	99.7	99.5	99.5	81.1	100.0	5	L3- P1059 H3
	6	13.9	13.9	13.9	21.0	13.9	100.0	87.4	87.7	87.7	87.7	87.4	87.4	76.4	87.4	6	L3- P1662 H4
	7	0.5	0.5	0.5	14.0	0.5	13.9	0.0	100.0	99.7	99.7	99.5	99.5	81.6	99.5	7	L3- PM70
	8	0.3	0.3	0.3	14.3	0.3	13.6	0.3	0.0	100.0	99.7	99.7	81.4	99.7	8	L3- SPM10	
	9	0.3	0.3	0.3	14.3	0.3	13.6	0.3	0.0	0.0	100.0	99.7	99.7	81.4	99.7	9	L3- SPM16
	10	0.3	0.3	0.3	14.3	0.3	13.6	0.3	0.0	0.0	0.0	99.7	99.7	81.4	99.7	10	L3- SPM27
	11	0.5	0.5	0.5	14.3	0.5	13.9	0.5	0.3	0.3	0.0	100.0	81.4	99.5	11	L3- SPM32	
	12	0.5	0.5	0.5	14.3	0.5	13.9	0.5	0.3	0.3	0.0	0.0	100.0	81.4	99.5	12	L3- SPM08
	13	21.6	21.6	21.6	7.4	21.6	28.7	20.9	21.2	21.2	21.2	21.3	21.3	81.1	13	L3- SPM22	
	14	0.0	0.0	0.0	14.6	0.0	13.9	0.5	0.3	0.3	0.3	0.5	0.5	21.6	81.1	14	L3- HBO-3
		1	2	3	4	5	6	7	8	9	10	11	12	13	14		

		Percent Identity																														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25						
Divergence	1	100.0	100.0	99.7	99.8	99.8	99.7	99.8	100.0	100.0	100.0	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	99.8	100.0	100.0	99.8	100.0	100.0	1	L6- SPM01			
	2	0.2	100.0	99.7	99.7	99.5	99.7	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	2	L6- SPM02		
	3	0.0	0.2	100.0	99.7	99.8	99.7	99.8	100.0	100.0	100.0	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	99.8	100.0	100.0	99.8	100.0	100.0	3	L6- SPM05			
	4	0.3	0.3	0.3	100.0	99.5	99.7	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	4	L6- SPM11	
	5	0.2	0.3	0.2	0.5	100.0	99.5	99.7	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	5	L6- SPM12	
	6	0.2	0.3	0.2	0.5	0.3	100.0	99.5	99.7	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	6	L6- SPM13
	7	0.3	0.3	0.3	0.0	0.5	0.5	100.0	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	99.7	7	L6- SPM14
	8	0.2	0.3	0.2	0.5	0.3	0.0	0.5	100.0	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	8	L6- SPM15
	9	0.0	0.2	0.0	0.3	0.2	0.2	0.3	0.2	100.0	100.0	100.0	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	100.0	9	L6- SPM18	
	10	0.0	0.2	0.0	0.3	0.2	0.2	0.3	0.2	0.0	100.0	100.0	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	100.0	10	L6- SPM20	
	11	0.0	0.2	0.0	0.3	0.2	0.2	0.3	0.2	0.0	0.0	100.0	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	100.0	11	L6- SPM14	
	12	0.0	0.2	0.0	0.3	0.2	0.2	0.3	0.2	0.0	0.0	0.0	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	12	L6- SPM22		
	13	0.0	0.2	0.0	0.3	0.2	0.2	0.3	0.2	0.0	0.0	0.0	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	13	L6- SPM23		
	14	0.0	0.2	0.0	0.3	0.2	0.2	0.3	0.2	0.0	0.0	0.0	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	14	L6- SPM25		
	15	0.0	0.2	0.0	0.3	0.2	0.2	0.3	0.2	0.0	0.0	0.0	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	15	L6- SPM30		
	16	0.2	0.3	0.2	0.5	0.3	0.3	0.5	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	16	L6- 3480	
	17	0.0	0.2	0.0	0.3	0.2	0.2	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	100.0	99.8	100.0	100.0	99.8	100.0	100.0	17	L6- ATCC			
	18	0.0	0.2	0.0	0.3	0.2	0.2	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	100.0	99.8	100.0	100.0	99.8	100.0	100.0	18	L6- HNO6			
	19	0.0	0.2	0.0	0.3	0.2	0.2	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	100.0	99.8	100.0	100.0	99.8	100.0	100.0	19	L6- P61		
	20	0.2	0.3	0.2	0.5	0.3	0.3	0.5	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	20	L6- P930	
	21	0.0	0.2	0.0	0.3	0.2	0.2	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	100.0	99.8	100.0	100.0	99.8	100.0	100.0	21	L6- P930	
	22	0.0	0.2	0.0	0.3	0.2	0.2	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	100.0	99.8	100.0	100.0	99.8	100.0	22	L6- P1573	
	23	0.2	0.3	0.2	0.5	0.3	0.3	0.5	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	23	L6- P2100	
	24	0.0	0.2	0.0	0.3	0.2	0.2	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24	L6- P2237	
	25	0.0	0.2	0.0	0.3	0.2	0.2	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25	L6- P272	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25						

Ethics

I hereby declare all ethical standards have been respected in the preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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