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Comparison of two AFLP methods and PFGE using strains of *Listeria monocytogenes* isolated from environmental and food samples obtained from Piedmont, Italy

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Highlights

► We typed 103 well characterized *L. monocytogenes* food and environmental strains.

► We compared two AFLP methods and PFGE in characterizing the same set of samples.

► We evaluated discriminatory power, typeability and concordance of the three methods.

► We evidenced a low typeability for one of the AFLP method considered.

► We observed similar discriminatory power and a good agreement between methods.

Keywords AFLP; PFGE; *Listeria monocytogenes* subtyping

Abstract

Listeria monocytogenes ranks among the most frequent causes of death due to foodborne illness (20–30% case fatality rate).

Discriminative subtyping methods are important to detect the relatedness of isolates and verify epidemiologic associations. AFLP analysis is a DNA fingerprinting technique based on the selective amplification of genomic restriction fragments. In this study, two AFLP methods and PFGE were compared in regard to discriminatory power, typeability and concordance.

A total of 103 unrelated *L. monocytogenes* strains isolated from different environmental and food sources were analyzed. Strains were isolated from samples obtained from food-production plants, supermarkets and small food markets in Piedmont, Italy.

All methods clustered *L. monocytogenes* strains into two genetic lineages, Lineage I and II. The three methods were compared using the 82 isolates which were typeable with all techniques. The calculated pair-wise Pearson's correlation coefficients (r) showed close agreement between all three methods.

Our findings suggest that the AFLP II method can be successfully used to subtype *L. monocytogenes* strains isolated from foods and food processing facilities.

1. Introduction

Listeria monocytogenes is a foodborne pathogen causing listeriosis mainly in certain well-defined high-risk groups. In 2008, the reported incidence of listeriosis in Europe was 0.3/100,000 population. In Italy, it has mostly caused sporadic cases with a reported incidence of 0.1/100,000 in 2007 and similar to incidences reported in 2005 (EFSA, 2007). More specifically, there were 45 notified cases reported in the last 10 years (1998–2008) in the Piedmont region, area where this study was carried out (Ministero del Lavoro, 2006). Because of its high case-fatality rate (20–30%), listeriosis ranks among the most frequent causes of death due to foodborne illness (EFSA, 2010).

L. monocytogenes has been recovered from raw or processed foods including dairy products, meat products, vegetables and seafood and can be found in the environment, mostly in soil and silage.

In order to reduce *L. monocytogenes* contamination, to confirm outbreaks sources, establish transmission patterns, and eliminate reservoirs of epidemic strains, there is a need for subtyping methods capable of discerning epidemiologically related strains.

Phenotypic methods, such as serotyping and phage typing, often yield low discriminatory power. However, serology is still largely being used to determine the prevalence of specific serotypes of bacterial species.

Since the 1980s, the number of genotypic methods was extended by the development of restriction-fragment-based methods such as pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). PFGE typing was shown to be a very accurate and reproducible method for fine structure comparison and molecular subtyping of *L. monocytogenes* (Graves and Swaminathan, 2001 and Hyytiä-Trees et al., 2007).

AFLP analysis is a DNA fingerprinting technique based on the selective amplification of genomic restriction fragments. AFLP has been reported to yield more complex patterns when compared to other available DNA fingerprinting methods, thus likely increasing strain discrimination (Razin, 2006). The strategy of using two different restriction enzymes and subsequent selective amplification results in the generation on highly discriminatory banding patterns (Aarts et al., 1999 and Vogel et al., 2001). In addition, the use of fluorescent labels allows AFLP patterns to be viewed by automated laser fluorescence analysis, permitting inter-laboratory data comparison (Aarts et al., 1999). These features, combined with the possibility for

automation and high-throughput analysis, make AFLP an interesting alternative to currently used whole-genome fingerprinting techniques (Aarts et al., 1999 and Vogel et al., 2001).

Recently, a number of new AFLP protocols using different restriction enzymes, were shown to have high typeability, discriminatory power and reproducibility (Keto-Timonen et al., 2007, Keto-Timonen et al., 2003, Parisi et al., 2010 and Wulff et al., 2006). However, these studies were difficult to compare as different numbers of strains from different sources were analyzed.
Therefore, the purpose of the present study was to compare, in terms of typeability and discriminatory power, two available AFLP methods with PFGE using a set of 103 well-characterized *L. monocytogenes* strains isolated from environmental and food samples collected in Italy.

2. Materials and methods

2.1. Test strains

A total of 103 unrelated and well-characterized *L. monocytogenes* strains isolated from different environmental and food sources were analyzed (Fig. 1). Food samples were collected from food-production plants, supermarkets and small food markets from Piedmont, Italy. The environmental samples consisted of swabs collected from the environment in food production plants and work surfaces in contact with meat or dairy products. Strains were isolated from: meat and meat processing plants (n = 64), dairy products and dairy plants (n = 30), prepared foods (stuffed fresh pasta n = 2, seafood salads n = 4) and fish (n = 3).
Identification of *L. monocytogenes* was performed using the AFNOR V08/055 procedure (1997), biochemical confirmation, API-Listeria (BioMerieux), and serotype identification (Denka Seiken, Tokyo, Japan) with minor modifications (Fig. 1). Further confirmation was based on a *L. monocytogenes* specific *prfA* PCR assay (D'Agostino et al., 2004).

2.2. Subtyping

2.2.1. PFGE

Genomic DNA from *L. monocytogenes* strains was prepared for PFGE analysis in agarose plugs as described by Graves and Swaminathan (2001). Agarose-immobilized DNA was restricted using AscI and ApaI (New England Biolabs, Beverly, MA). Restricted DNA was electrophoresed in 1% SeaKem Gold agarose in 0.5X TBE at 6 V/cm in a Chef DR III system (Bio-Rad, Hercules, CA, USA). A linear ramping factor with pulse times from 4.0 to 40.0 s at 14 °C and an angle of 120° were applied for 21 h (Graves and Swaminathan, 2001).

2.2.2. AFLP

AFLP I and AFLP II procedure(s) were performed as described by Vogel et al. (2001) and by Parisi et al. (2010), respectively. AFLP protocols were based on different restriction enzymes: *BamHI*/*EcoRI* for AFLP I and *HindIII*/*HhaI* for AFLP II. Genomic DNA was extracted using DNeasy Tissue kit (Qiagen) according to the manufacturer's instructions. Amplified products were analyzed on an ABI 310 automated sequencer (Applied Biosystems, Foster City, CA, USA).

2.3. PFGE and AFLP pattern analysis

Similarity between PFGE patterns was determined with Bionumerics software (Applied Maths, Kortrijk, Belgium) using the Dice coefficient and an optimization value of 1.2% for AscI and 1.4% for ApaI. Band position tolerance was 1.4% for both the enzymes. Results were then combined using the same software by averaging the two generated distance matrices and generating a single dendrogram using the Complete Linkage algorithm. Two cut-off similarity values were fixed: isolates showing a PFGE Similarity Level (S.L.) > 90% were grouped in the same “PFGE group”, while an S.L. of 100% grouped isolates in the same “PFGE

type”. A PFGE type was shared between one or more strain while unique types were comprised of a single strain.

Concerning AFLP data, after electrophoresis patterns were collected with the GeneScan software (Applied Biosystems, Foster City, CA, USA). Densitometric values were then transferred to BioNumerics software (Applied Maths, Kortrijk, Belgium). Normalization was performed by using the reference positions of the DNA size standard GeneScan-500 (Applied Biosystems, Foster City, CA, USA). A similarity matrix was created by determining the Pearson product–moment correlation coefficient (r). Cluster analysis was performed by the Complete Linkage algorithm. As with PFGE data analysis, isolates showing a S.L. > 90% were grouped in the same “AFLP group”, while an S.L. > 95% grouped the isolates in the same “AFLP type”.

The cut-off point for the AFLP type definition was chosen considering 5 randomly selected strains analyzed each in triplicates. For each strains the similarity level (S.L.) of the three replicates was calculated. In order to calculate the cut-off for identifying indistinguishable strains the lowest S.L. was chosen for each strains (n=5) and then averaged.

2.4. Statistical analysis

To evaluate the discriminatory power of the three assays Simpson's index of diversity (DI) was calculated as described by Hunter and Gaston (1988). The related 95% confidence interval was calculated using the formula of Grundmann et al. (2001). The concordance between methods was determined using BioNumerics software for comparing similarity matrices, applying Pearson's correlation coefficient (r).

3. Results

3.1. Identification

All 103 test strains that previously had been biochemically identified as *L. monocytogenes* were also confirmed as *L. monocytogenes* by *prfA* PCR.

3.2. PFGE

All 103 test strains were typeable with both *AscI* and *ApaI* macrorestriction analysis. The generated patterns divided the strains into 56 types grouped into two major clusters (I and II) diverging at a S.L. of 36.6% (Fig. 1). These clusters reflect the genetic division of *L. monocytogenes* into Lineage I and II, respectively. Among the 23 strains comprising Cluster I (S.L. = 48.4%) 6 PFGE groups, 6 PFGE types defined previously and 8 unique types could be identified. Among the 80 strains belonging to Cluster II (S.L. = 47.4%) 12 PFGE groups, 17 PFGE types and 25 unique types were identified (Fig. 1). The discriminatory power of PFGE was 0.980 (95% C.I.:0.970–0.990).

3.3. AFLP I

This method allowed the characterization of only 82 strains. The analyses of the remaining 21 strains were repeated twice before classifying them as untypeable. Of those, 20 were isolated from dairies and related production environments, and belonged to serotype 1/2a (n = 11) and serotype 4b/4e (n = 10) (Fig. 1). In order to confirm these results, DNA extraction and AFLP analyses were performed twice on these strains but in all cases no bands were detected.

AFLP I divided the 82 strains into 42 types, grouped into two major clusters (I and II) that diverged at a S.L. of 18.3%. Among the 69 strains belonging to Cluster I (S.L. = 34.5%) 10 AFLP groups, 14 AFLP types and 18 unique types could be identified. Among the 13 strains belonging to Cluster II (S.L. = 64.4%) 3 AFLP groups, 3 AFLP types and 7 unique types were observed (Fig. 1). The discriminatory power of AFLP I was 0.972 (95% C.I.:0.959–0.985).

3.4. AFLP II

All 103 *L. monocytogenes* strains were typeable with this method. AFLP II divided the *L. monocytogenes* strains into 38 types, grouped into two clusters (I and II) divergent at 16.38% S.L. Among the 23 strains comprising Cluster I (S.L. = 74.1%) 5 AFLP groups, 5 AFLP types and 5 unique types were identified. Among the 80 strains belonging to Cluster II (S.L. = 45.6%) 14 AFLP groups, 17 AFLP types and 11 unique types could be observed (Fig. 1). The discriminatory power of AFLP II was 0.971 (95% C.I.:0.963–0.978).

3.5. Comparison of typing methods

The comparison among the different methods was performed on the 82 isolates typed with all three techniques (Fig. 1). In fact, the calculated pair-wise Pearson's correlation coefficients (r) showed good concordance between the techniques: AFLP I and AFLP II: $r = 0.87$, ($p < 0.001$); AFLP I and PFGE: $r = 0.78$, ($p < 0.001$); AFLP II and PFGE: $r = 0.82$, ($p < 0.001$).

4. Discussion

In this research, two AFLP methods and PFGE were compared in regard to discriminatory power, typeability and concordance. All methods clustered *L. monocytogenes* strains into two major clusters. These two clusters may reflect the division of *L. monocytogenes* species into three genetic lineages: Lineage I (serotypes 1/2b, 4b, 3b), Lineage II (1/2a, 1/2c, 3a) and Lineage III (4a, 4c, some 4b) (Wiedmann et al., 1997, Ward et al., 2004 and Neves et al., 2008). The clustering of isolates allowed the identification of two outliers (ID: 29 and 43, not included in the earlier mentioned 103 strains) serotyped as *L. monocytogenes* serotype 4a and 4c. However, 16S rDNA sequencing was carried out to confirm these findings. Interestingly, the results classified these two strains as *L. seeligeri* and *L. welshimeri*, therefore they were removed from the presented dendrogram (Fig. 1). This study supports the use of PFGE and AFLP as an useful tool for the assessment and verification of PCR identification.

Overall, this study showed a very similar discriminatory power between the two AFLP methods and PFGE, when the 82 strains typed with all 3 techniques were considered. On the other hand, typeability of AFLP I and AFLP II were respectively 80% and 100%, thus suggesting the latter as the most suitable AFLP typing method. Interestingly, the AFLP I protocol was previously applied to *L. monocytogenes* strains isolated from fish and related environment and a 100% typeability was reported (Vogel et al., 2001). The same protocol showed an 80% typeability in the present study; however 20 of the 21 untypeable strains were isolated from dairy samples. Typing results obtained with the other two techniques (Fig. 1), showed untypeable strains cluster together in two major groups. This may suggest that these strains have peculiar genetic characteristics, such as modification or methylation of restriction sites, making them untypeable with AFLP I. In particular, it has already been shown that genomic DNA of some *L. monocytogenes* strains isolated from dairy is able to resist digestion with Sau3AI, thus suggesting methylation of cytosine at GATC sites effectively inhibiting cleavage (Zheng and Kathariou, 1997). Moreover, according to the manufacturer's website, *EcoRI* is sensitive to CpG methylation, being sensitive to blockage by some combinations of overlapping, while *BamHI* does not appear to have any methylation sensitivity.

Therefore, considering these findings, complementation experiments were carried out in order to determine whether the *EcoRI* enzyme was able to digest genomic DNA of the untypeable strains. However, results showed that the enzyme was able to digest the tested DNA, resulting in smearing for both positive control and untypeable strains (data not shown). Further testing will be needed to elucidate the reason of such results.

When considering only the 82 strains typeable with both AFLP methods, the results were in high agreement and in accordance with PFGE results, confirming AFLP as a valuable alternative tool for subtyping *L. monocytogenes* strains. In addition, the Simpson's discriminatory indexes and the related confidence intervals (95%) confirmed the similar discriminatory power of the three methods. PFGE showed higher

agreement with AFLP II: 50% of PFGE types grouped strains that were identical with AFLP II. In 11 cases, strains belonging to the same PFGE-types were separated both by AFLP I and AFLP II, thus evidencing the high correlation between these two AFLP methods. These data revealed that the three methods are similar in differentiating strains but they do not produce the exact same clustering results. Therefore, the combination of AFLP and PFGE results could be combined to achieve a better differentiation of the strains analyzed.

The major advantages of AFLP over PFGE are its high throughput, possibilities for automation, and its applicability not only for strain characterization, but also for determining molecular evolution of bacteria (Keto-Timonen et al., 2003). Overall, our findings suggest that AFLP typing can be successfully applied to subtype strains of transmission of *L. monocytogenes* from foods and food environments.

Despite the advantages of AFLP II, it should be noted that PFGE has greatly facilitated the epidemiologic investigation of listeriosis outbreaks. Moreover, PFGE has been used to subtype thousands of isolates worldwide with the creation of large databases containing food, and environmental isolates that will greatly aid future epidemiologic investigations. However, AFLP can be successfully applied to the subtyping of *L. monocytogenes* strains in laboratories where PFGE is not available. In addition, AFLP may be used as part of routine surveillance in production plants in order to reveal sources and routes of transmission. This information can then be used to design more effective intervention strategies to prevent contamination of foods.

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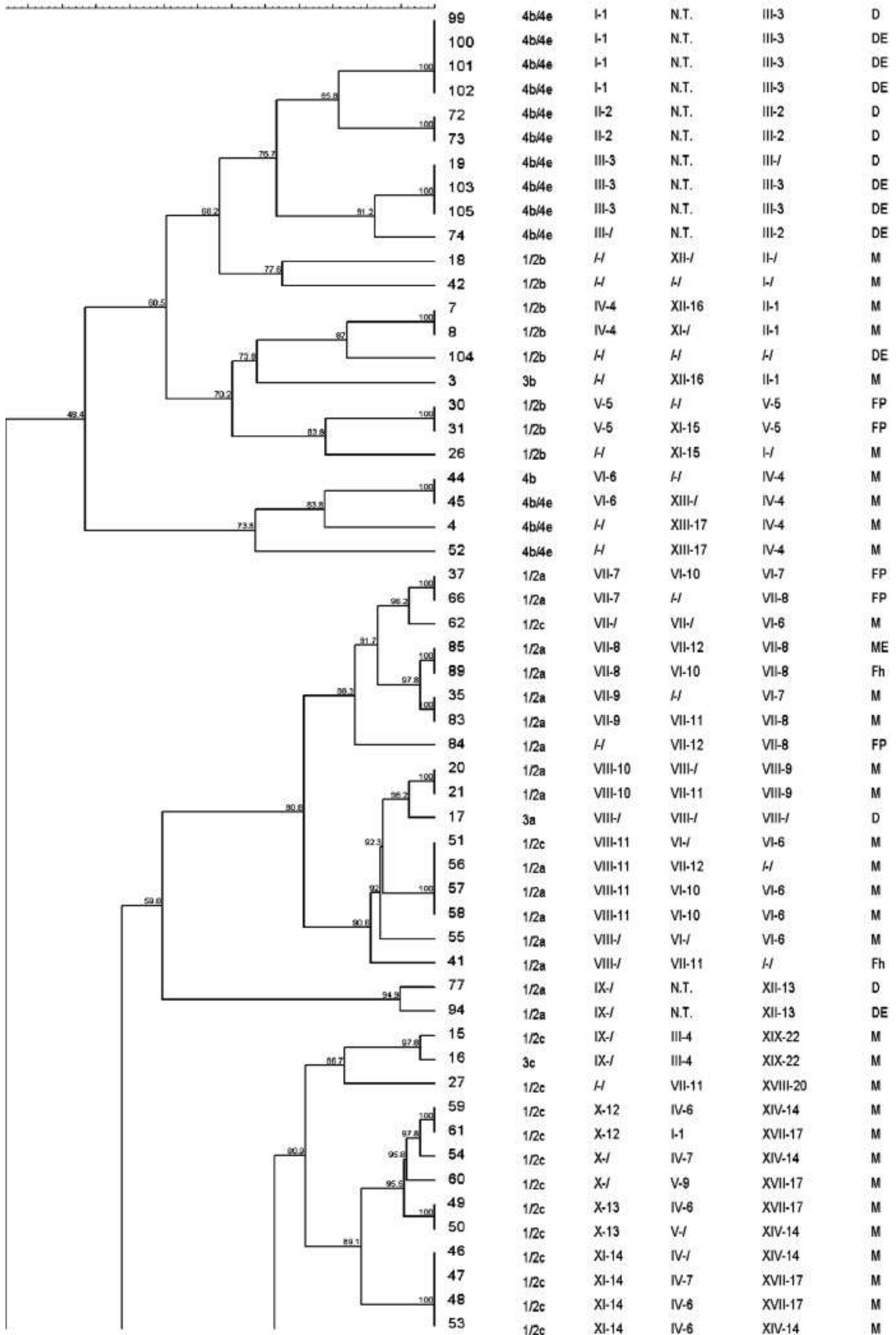
Fig. 1. Dendrogram showing the PFGE clustering of strains with corresponding ID, serotype, AFLP I + II profiles and source information for each strain. In the AFLP profile columns the roman numbers indicate the group the strains belong to, the Arabic numbers, the type of each strain. The symbol “/” replaces the roman or the Arabic numbers or both, when the isolate does not belong to a group or to a type or either. N.T. in AFLP I column stands for Not Typeable. The source column indicates: D (Dairy), DE (Dairy Environment), M (Meat), ME (Meat Environment), PF (Prepared Food) and Fh (Fish).



asc + apa
99 4b/4e I-1 N.T. III-3 D
100 4b/4e I-1 N.T. III-3 DE
101 4b/4e I-1 N.T. III-3 DE
102 4b/4e I-1 N.T. III-3 DE
72 4b/4e II-2 N.T. III-2 D
73 4b/4e II-2 N.T. III-2 D
19 4b/4e III-3 N.T. III-/ D
103 4b/4e III-3 N.T. III-3 DE
105 4b/4e III-3 N.T. III-3 DE
74 4b/4e III-/ N.T. III-2 DE
18 1/2b -/ XII-/ II-/ M
42 1/2b -/ -/ I-/ M
7 1/2b IV-4 XII-16 II-1 M
8 1/2b IV-4 XI-/ II-1 M
104 1/2b -/ -/ -/ DE
3 3b -/ XII-16 II-1 M
30 1/2b V-5 -/ V-5 FP
31 1/2b V-5 XI-15 V-5 FP
26 1/2b -/ XI-15 I-/ M
44 4b VI-6 -/ IV-4 M
45 4b/4e VI-6 XIII-/ IV-4 M
4 4b/4e -/ XIII-17 IV-4 M
52 4b/4e -/ XIII-17 IV-4 M
37 1/2a VII-7 VI-10 VI-7 FP
66 1/2a VII-7 -/ VII-8 FP
62 1/2c VII-/ VII-/ VI-6 M
85 1/2a VII-8 VII-12 VII-8 ME
89 1/2a VII-8 VI-10 VII-8 Fh
35 1/2a VII-9 -/ VI-7 M
83 1/2a VII-9 VII-11 VII-8 M
84 1/2a -/ VII-12 VII-8 FP
20 1/2a VIII-10 VIII-/ VIII-9 M
21 1/2a VIII-10 VII-11 VIII-9 M
17 3a VIII-/ VIII-/ VIII-/ D
51 1/2c VIII-11 VI-/ VI-6 M
56 1/2a VIII-11 VII-12 -/ M
57 1/2a VIII-11 VI-10 VI-6 M
58 1/2a VIII-11 VI-10 VI-6 M
55 1/2a VIII-/ VI-/ VI-6 M
41 1/2a VIII-/ VII-11 -/ Fh
77 1/2a IX-/ N.T. XII-13 D
94 1/2a IX-/ N.T. XII-13 DE
15 1/2c IX-/ III-4 XIX-22 M
16 3c IX-/ III-4 XIX-22 M
27 1/2c -/ VII-11 XVIII-20 M
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54 1/2c X-/ IV-7 XIV-14 M
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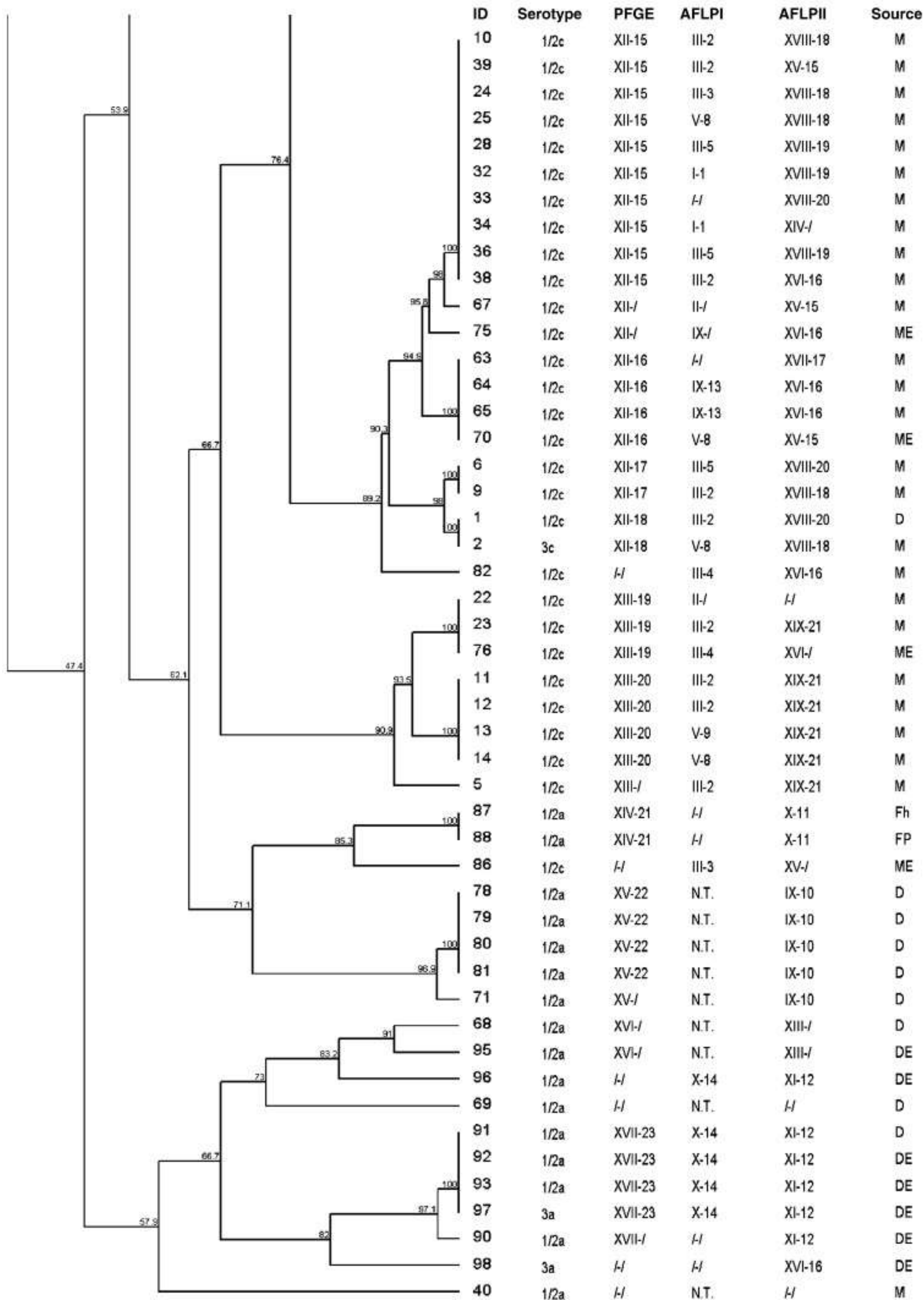
ID Serotype PFGE AFLPI AFLPII Source
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25 1/2c XII-15 V-8 XVIII-18 M
28 1/2c XII-15 III-5 XVIII-19 M
32 1/2c XII-15 I-1 XVIII-19 M
33 1/2c XII-15 /-/ XVIII-20 M
34 1/2c XII-15 I-1 XIV-/ M
36 1/2c XII-15 III-5 XVIII-19 M
38 1/2c XII-15 III-2 XVI-16 M
67 1/2c XII-/ II-/ XV-15 M
75 1/2c XII-/ IX-/ XVI-16 ME
63 1/2c XII-16 /-/ XVII-17 M
64 1/2c XII-16 IX-13 XVI-16 M
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70 1/2c XII-16 V-8 XV-15 ME
6 1/2c XII-17 III-5 XVIII-20 M
9 1/2c XII-17 III-2 XVIII-18 M
1 1/2c XII-18 III-2 XVIII-20 D
2 3c XII-18 V-8 XVIII-18 M
82 1/2c /-/ III-4 XVI-16 M
22 1/2c XIII-19 II-/ /-/ M
23 1/2c XIII-19 III-2 XIX-21 M
76 1/2c XIII-19 III-4 XVI-/ ME
11 1/2c XIII-20 III-2 XIX-21 M
12 1/2c XIII-20 III-2 XIX-21 M
13 1/2c XIII-20 V-9 XIX-21 M
14 1/2c XIII-20 V-8 XIX-21 M
5 1/2c XIII-/ III-2 XIX-21 M
87 1/2a XIV-21 /-/ X-11 Fh
88 1/2a XIV-21 /-/ X-11 FP
86 1/2c /-/ III-3 XV-/ ME
78 1/2a XV-22 N.T. IX-10 D
79 1/2a XV-22 N.T. IX-10 D
80 1/2a XV-22 N.T. IX-10 D
81 1/2a XV-22 N.T. IX-10 D
71 1/2a XV-/ N.T. IX-10 D
68 1/2a XVI-/ N.T. XIII-/ D
95 1/2a XVI-/ N.T. XIII-/ DE
96 1/2a /-/ X-14 XI-12 DE
69 1/2a /-/ N.T. /-/ D
91 1/2a XVII-23 X-14 XI-12 D
92 1/2a XVII-23 X-14 XI-12 DE
93 1/2a XVII-23 X-14 XI-12 DE
97 3a XVII-23 X-14 XI-12 DE
90 1/2a XVII-/ /-/ XI-12 DE
98 3a /-/ /-/ XVI-16 DE
40 1/2a /-/ N.T. /-/ M

Appendix A. Supplementary data

32	XII-15	I-1	XVIII-19	1/2c	M
34	XII-15	I-1	XIV-/	1/2c	M
61	X-12	I-1	XVII-17	1/2c	M
33	XII-15	/-/	XVIII-20	1/2c	M
22	XIII-19	II-/	/-/	1/2c	M
67	XII-/	II-/	XV-15	1/2c	M
1	XII-18	III-2	XVIII-20	1/2c	D
23	XIII-19	III-2	XIX-21	1/2c	M
38	XII-15	III-2	XVI-18	1/2c	M
39	XII-15	III-2	XV-15	1/2c	M
11	XIII-20	III-2	XIX-21	1/2c	M
12	XIII-20	III-2	XIX-21	1/2c	M
5	XIII-/	III-2	XIX-21	1/2c	M
9	XII-17	III-2	XVIII-18	1/2c	M
10	XII-15	III-2	XVIII-18	1/2c	M
24	XII-15	III-3	XVIII-18	1/2c	M
86	/-/	III-3	XV-/	1/2c	ME
78	XIII-19	III-4	XVI-/	1/2c	ME
82	/-/	III-4	XVI-18	1/2c	M
15	IX-/	III-4	XIX-22	1/2c	M
16	IX-/	III-4	XIX-22	3c	M
28	XII-15	III-5	XVIII-19	1/2c	M
36	XII-15	III-5	XVIII-19	1/2c	M
6	XII-17	III-5	XVIII-20	1/2c	M
63	XII-18	/-/	XVII-17	1/2c	M
49	X-13	IV-6	XVII-17	1/2c	M
53	XI-14	IV-6	XIV-14	1/2c	M
48	XI-14	IV-6	XVII-17	1/2c	M
59	X-12	IV-6	XIV-14	1/2c	M
46	XI-14	IV-/	XIV-14	1/2c	M
47	XI-14	IV-7	XVII-17	1/2c	M
54	X-/	IV-7	XIV-14	1/2c	M
2	XII-18	V-8	XVIII-18	3c	M
70	XII-16	V-8	XV-15	1/2c	ME
14	XIII-20	V-8	XIX-21	1/2c	M
25	XII-15	V-8	XVIII-18	1/2c	M
50	X-13	V-/	XIV-14	1/2c	M
13	XIII-20	V-9	XIX-21	1/2c	M
60	X-/	V-9	XVII-17	1/2c	M
57	VIII-11	VI-10	VI-6	1/2a	M
58	VIII-11	VI-10	VI-6	1/2a	M
37	VII-7	VI-10	VI-7	1/2a	FP
89	VII-8	VI-10	VII-8	1/2a	Fh
55	VIII-/	VI-/	VI-6	1/2a	M
51	VIII-11	VI-/	VI-6	1/2c	M
41	VIII-/	VII-11	/-/	1/2a	Fh
83	VII-9	VII-11	VII-8	1/2a	M
21	VIII-10	VII-11	VIII-9	1/2a	M
27	/-/	VII-11	XVIII-20	1/2c	M
56	VIII-11	VII-12	/-/	1/2a	M
84	/-/	VII-12	VII-8	1/2a	FP
85	VII-8	VII-12	VII-8	1/2a	ME
62	VII-/	VII-/	VI-6	1/2c	M
17	VIII-/	VIII-/	VIII-/	3a	D
20	VIII-10	VIII-/	VIII-9	1/2a	M
35	VII-9	/-/	VI-7	1/2a	M
66	VII-7	/-/	VII-8	1/2a	FP
64	XII-16	IX-13	XVI-16	1/2c	M
65	XII-16	IX-13	XVI-16	1/2c	M
75	XII-/	IX-/	XVI-16	1/2c	ME
96	/-/	X-14	XI-12	1/2a	DE
97	XVII-23	X-14	XI-12	3a	DE
92	XVII-23	X-14	XI-12	1/2a	DE
91	XVII-23	X-14	XI-12	1/2a	D
93	XVII-23	X-14	XI-12	1/2a	DE
98	/-/	/-/	XVI-16	3a	DE
87	XIV-21	/-/	X-11	1/2a	Fh
88	XIV-21	/-/	X-11	1/2a	FP
90	XVII-/	/-/	XI-12	1/2a	DE
26	/-/	XI-15	I-/	1/2b	M
31	V-5	XI-15	V-5	1/2b	FP
8	IV-4	XI-/	II-1	1/2b	M
30	V-5	/-/	V-5	1/2b	FP
3	/-/	XII-16	II-1	3b	M
7	IV-4	XII-16	II-1	1/2b	M
18	/-/	XII-/	II-/	1/2b	M
104	/-/	/-/	/-/	1/2b	DE
4	/-/	XIII-17	IV-4	4b/4e	M
52	/-/	XIII-17	IV-4	4b/4e	M
45	VI-6	XIII-/	IV-4	4b/4e	M
44	VI-6	/-/	IV-4	4b	M
42	/-/	/-/	I-/	1/2b	M