IN SILICO COMPARISON OF DIFFERENT TYPES OF MLVA WITH PFGE BASED ON PSEUDOMONAS *AERUGINOSA GENOMES*

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ABSTRACT

Introduction*: Pseudomonas aeruginosa is an important pathogen in nosocomial infections and developed typing techniques are essential allowing researchers to understanding hospital epidemiology. Monitoring the emergence and transmission of Pseudomonas aeruginosa strains permits the elucidation of the source of infection and routes of bacterial transmission. The aim of the present study was an in silico comparison of Pulsed-field gel electrophoresis with different schemes of Multiple Locus Variable-number Tandem Repeat Analysis in terms of discriminatory power and concordance.*

Materials and methods*: 58 P.aeruginosa whole genomes have been analyzed in silico to determine SpeI-digested PFGE type and subspecies types using different MLVA methods. Resolution power, strength and direction of the concordance between typing methods have been estimated by calculation of the Simpson's index, the adjusted Rand and the adjusted Wallace coefficients.*

Results*: The Simpson's indices of diversity were 1.0 for PFGE and from 0.995 to 0.999 for MLVA schemes with 6-19 markers. The congruence between PFGE and different MLVA methods measured by the adjusted Rand index were from 0.306 to 0.665 on cluster level for PFGE and type level for MLVA. The congruence was slightly higher at the clonal cluster level - from 0.46 to 0.694.*

Conclusion*: Our in silico study for comparing different MLVA schemes with PFGE, based on Pseudomonas aeruginosa genomes showed, on the one hand, the same high level of discriminatory power of PFGE and MLVA even with 6 tandems markers; nonetheless, on the other hand, there was moderate/poor congruence (no more 70%) between PFGE and MLVA schemes on cluster level.*

Key words*: MLVA, PFGE, Pseudomonas aeruginosa, In Silico, Simpson index, Adjusted Rand index, Adjusted Wallace coefficient.*

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Introduction

Identifying different types of very diverse organisms within a species is critical for outbreak investigations and for the control of infectious diseases[(1)]. A large variety of approaches for typing have been developed. These can be divided into two groups - phenotypic and genotypic methods. Importantly, such typing methods must have the ability to distinguish epidemic from endemic or sporadic isolates.

At the same time, methods must be low-cost, rapid, highly reproducible and easy to carry out and to interpret[(1, 2)].

The results obtained with phenotypic techniques have a tendency to vary due to the influence growth conditions and/or growth phase on the products of gene expression. Furthermore, some phenotypic approaches are time consuming, tend to more manual, and require greater skill to run[(1)]. Importantly, as it has been shown in other studies[(3)] the discriminatory power of phenotypic methods is lower in comparison with genotypic variants. Each of these reasons has led to genotypic techniques gradually replacing phenotypic approaches over the past two decades.

There are a large number of genetic typing methods with a high degree of discriminatory

power. One of the most frequently used molecular-genetic methods is pulsed-field gel electrophoresis (PFGE). PFGE is considered as the "gold standard" typing method, due to its excellent discriminatory power and high epidemiological concordance[4]; however, the method remains relatively expensive and time-consuming[5, 6]. Furthermore, this technique tends to suffer from a lack of interlaboratory reproducibility[6].

Another relatively new genetic approach is Multilocus Variable Number Tandem Repeat Analysis (MLVA), which is based on a set of polymorphic tandem repeat loci of bacteria and this method has been successfully used for epidemiological investigations. MLVA is considered as a perspective method with "ideal" criteria for typing, such as being easy to perform, rapid, possessing a high discriminatory power, and having good reproducibility. There are several MLVA schemes for typing of the pathogen *Pseudomonas aeruginosa* currently available. This range from having six to sixteen variable number tandem repeat (VNTR) markers[7-10].

In the present study, we compared different approaches of MLVA with PFGE *in silico* in terms of discriminatory power and coefficients congruence, against the clinical pathogen *Pseudomonas aeruginosa*.

Materials and methods

The study examined 58 *Pseudomonas aeruginosa* genomes (that have been completed and published). These were downloaded from the National Center for Biotechnology Information (NCBI) GenBank[11].

In silico simulation of SpeI enzyme digestion PFGE was performed on *Pseudomonas aeruginosa* genomes transformed into circular structure with Geneious software (Biomatters Lmd.)[12]. PFGE profiles, ranging from 10 to 950 kb, were saved as a TIFF file for further analysis in TotalLab™ 1D software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

Eight different MLVA schemes (reported in table 1) were analyzed by using primer pairs (presented in table 2) and SnapGene (GSL Biotech) and Geneious (Biomatters Lmd.) tools in order to produce amplification products[12, 13]. Based on PCR products, the number of repeats were calculated and rounded up to the nearest integer to obtain the numeric digit code of the isolates. These codes, referred to MLVA profiles, were subsequently used for clustering.

To evaluate genetic variability and relatedness of *Pseudomonas aeruginosa* isolates based on a comparison of bands, the genetic distance was calculated using the Sørensen-Dice coefficient for similarity[16]. From this, a dendrogram was built using unweight-pair group method with arithmetic means via the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. Categorical data (MLVA digit profile) were analyzed using Nei's standard genetic distance[17] and UPGMA algorithm clustering with the "EMA" package for R statistics[18]. Isolates that clustered together, with more than 80% similarity, were considered as genetically related strains[5, 19]. A dendextend R package was used for visualization and dendrogram comparison[20]. Diversity (from Simpson's index) and cluster concordance (Adjusted Rand, Adjusted Wallace) were evaluated via a web source[21].

Results

Fifty-eight genomes of *Pseudomonas aeruginosa* were downloaded and analyzed *in silico*. Prior to converting into circular shape, the genomes were

MLVA19 Tandem [This study]	ms10	ms061	ms77	ms127	ms142	ms172	ms173	ms194	ms207	ms209	ms211	ms212	ms213	ms214	ms215	ms216	ms217	ms222	ms223
MLVA16 Orsay (10)		ms061	ms77	ms127	ms142	ms172			ms207	ms209	ms211	ms212	ms213	ms214	ms215	ms216	ms217	ms222	ms223
MLVA15 Orsay (7)			ms77	ms127	ms142	ms172			ms207	ms209	ms211	ms212	ms213	ms214	ms215	ms216	ms217	ms222	ms223
MLVA12 Tandem		ms061	ms77		ms142	ms172			ms207	ms209	ms211	ms212	ms213	ms214			ms217	ms222	
[this study]																			
MLVA9 Utercht (14)			ms77	ms127	ms142						ms211		ms213		ms215	ms216	ms217		ms223
MLVA9 London (9)		ms061				ms172			ms207	ms209	ms211		ms213	ms214			ms217	ms222	
MLVA7 Tandem (8)	ms10	ms061	ms77	ms127	ms142	ms172	ms173												
MLVA6 Tandem (15)	ms10	ms061	ms77	ms127	ms142	ms172													

\: MLVA schemes with different number of loci (from 6 to 19) for *Pseudomonas aeruginosa* typing.

Marker Name	Forward Primer	Reverse Primer	source
ms10	GCAGGAACGCTTGCAG-CAGGT	CTTCGCCGACCCAGG-GATCA	-8
ms061	CTTGCCGTGCTACCGATCC	CCCCCATGCCAGTTGC	-8
ms77	GCGTCATGGTCTGCATGTC	TATACCCTCTTCGCC-CAGTC	-7
ms127	CTCGGAGTCTCTGCCAACTC	GGCAGGACAGGATCTC-GAC	-7
ms142	AGCAGTGCCAGTTGATGTTG	GTGGGGCGAAGGAGT-GAG	-7
ms172	GGATTCTCTCGCACGAGGT	TACGTGACCT-GACGTTGGTG	-7
ms173	CTGCAGTTCGCGCAAGTC	ATTTCAGCCAGCGTTAC-CAA	-8
ms194	CCTTAGGAGGCGCTGGTC	AGCTGCTGG-CAAGGCTCT	-8
ms211	ACAAGCGCCAGCC-GAACCTGT	CTTCGAACAGGTGCT-GACCGC	-7
ms212	TGCTGGTCGACTACTTCGG-CAA	ACTACGAGAAC-GACCCGGTGTT	-7
ms213	CTGGGCAAGTGTTGGTG-GATC	TGGCGTACTCCGAGCT-GATG	-7
ms214	AAACGCTGTTCGC-CAACCTCTA	CCATCATCCTCC-TACTGGGTT	-7
ms215	GACGAAACCCGTCGCGAA-CA	CTGTACAACGCC-GAGCCGTA	-7
ms216	ACTACTACGTCGAACACGC-CA	GATCGAAGACAA-GAACCTCG	-7
ms217	TTCTGGCTGTCGCGACTGAT	GAA-CAGCGTCTTTTCCTCGC	-7
ms222	AGAGGTGCTTAACGACG-GAT	TGCAGTTCTGCGAG-GAAGGCG	-7
ms223	TTGGCAATATGCCGGTTCGC	TGAGCTGATCGCC-TACTGG	-7
ms207	ACGGCGAACAGCACCAGCA	CTCTTGAGCCTCGGT-CACT	-7
ms209	CAGCCAGGAACTGCGGAGT	CTTCTCGCAACT-GAGCTGGT	-7

Table 2: Primer sequences for VNTR loci of Pseudomonas aeruginosa used in PCR simulation.

used to digest into fragments with a Spe I restriction enzyme in order to simulate PFGE ranges from 10 to 950 kbp. Amplification of MLVA loci were performed with in silico PCR using primer pair set. PFGE with clustering based on band pattern and amplified loci of different MLVA schemes were performed on whole genomes of *Pseudomonas aeruginosa* isolates; these are presented in figure 1.

The characteristics of comparing different methods, such as Simpson's, adj. Rand, adj. Wallace indices and entanglement, are given in Table 3. Discriminant power and agreement between typing methods was calculated based on type and cluster (80% cut-off) levels.

The tanglegram function in R (*Dendextend* package) plots two pairwisely-compared dendrograms (left - PFGE and right - MVLA), side by side, with connections between the same strains and between two trees by lines. This was for visually comparing two hierarchical clustering. Entanglement was used as a quality for the alignment of the two dendrograms (figure 2).

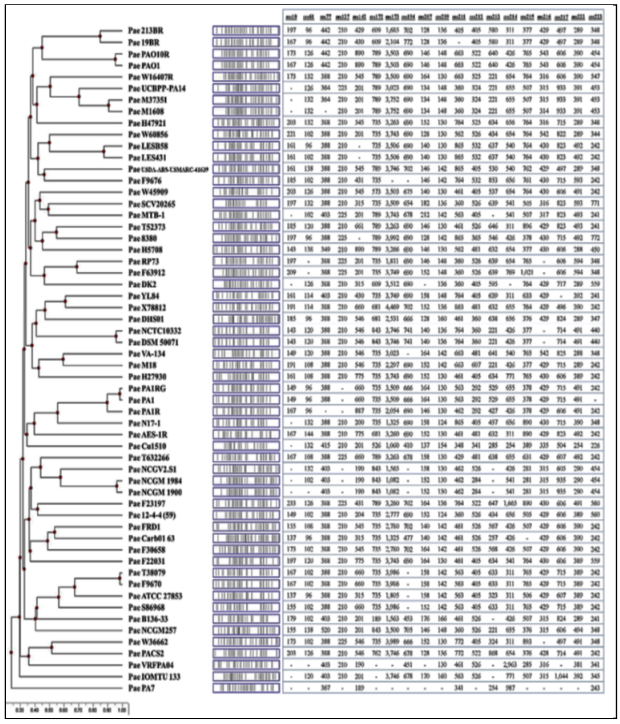

Fig. 1: PFGE patterns and dendrogram for 56 Pseudomonas aeruginosa isolates obtained on whole genome sequences and cut with SpeI enzyme. The dendrogram was constructed by clustering using UPGMA method with TotalLab TL120 1D v2009 (Nonlinear Dynamics Ltd.). There are pcr product of MLVA loci in the table presented in basepair. Dash (-) means nonamplified product.

Discussion

Microbial typing methods are major tools in epidemiological investigation allowing the determination of the clonal relationships between isolates of the same species and clarifying the evolutionary history and population dynamics of microbial pathogens. Many typing approaches, including varieties (as in the case with MLVA), are now available. These approaches are based on ability of the method to identify by phenotypic or genotypic variations within species of microorganism.

PFGE, using the Spe I restriction enzyme (A^CTAGT - a recognition sequence with 1 cut for match), is currently considered to be the gold standard method for subspecies typing of *Pseudomonas aeruginosa*. This is because of the method's high level of typeability, reproducibility, and strong discriminatory power[22]. Spe I digestion with the rare Spe I restriction sites usually generates 14-25 bands per *Pseudomonas aeruginosa* genome[5, 23], although there have been reported band numbers of up to 37[6].

Typing method	group number	Simpson's index [95% CI]	Adjusted Rand coefficient (PFGE(80%)~MLVA)	Adjusted Wallace PFGE (80%) -> MLVA	Adjusted Wallace MLVA -> PFGE (80%)	*Entanglement in comparison with PFGE
PFGE	58	1 [1.000-1.000]	-	0.000	1.000	-
PFGE (80% cut-off)	49	0.994 [0.989-0.999]	-	-	-	-
MLVA19 Tandem	56	0.999 [0.996-1.000]	0.332	0.199	1.000	0.2756
MLVA19 Tandem (80% cut-off)	48	0.993 [0.987-0.999]	0.634	0.698	0.581	-
MLVA16 Orsay	55	0.998 [0.995-1.000]	0.46	0.299	1.000	0.3211
MLVA16 Orsay (80% cut-off)	46	0.992 [0.985-0.998]	0.664	0.798	0.569	-
MLVA15 Orsay	53	0.997 [0.994-1.000]	0.665	0.498	1.000	0.3162
MLVA15 Orsay (80% cut-off)	45	0.991 [0.985-0.997]	0.637	0.798	0.530	-
MLVA12 Tandem	54	0.998 [0.994-1.000]	0.57	0.399	1.000	0.1521
MLVA12 Tandem (80% cut-off)	46	0.992 [0.985-0.998]	0.664	0.798	0.569	-
MLVA9 Utercht	51	0.995 [0.990-1.000]	0.553	0.498	0.623	0.2353
MLVA9 Utercht (80% cut-off)	42	0.988 [0.981-0.995]	0.462	0.696	0.346	-
MLVA9 London	53	0.997 [0.994-1.000]	0.665	0.498	1.000	0.2269
MLVA9 London (80% cut-off)	47	0.992 [0.986-0.998]	0.694	0.798	0.613	-
MLVA7 Tandem	55	0.998 [0.995-1.000]	0.306	0.199	0.665	0.2045
MLVA7 Tandem (80% cut-off)	49	0.994 [0.989-0.999]	0.497	0.497	0.497	-
MLVA6 Tandem	55	0.998 [0.995-1.000]	0.46	0.299	1.000	
MLVA6 Tandem (80% cut-off)	48	0.993 [0.988-0.999]	0.665	0.698	0.634	-

Table 3: Number of *Pseudomonas aeruginosa* types and groups determined with 80% cut-off, Simpson's index of diversity, and adjusted Rand indices, adjusted Wallace coefficients for PFGE and different MLVA schemes and entanglement of PFGE and MVLA tree comparison.

This allows the user to compare PFGE patterns within ranges from around 10 to 800 kbp.[6, 24]. Different studies have shown the discriminant power of the PFGE-Spe I approach, which is between 0.98 and 0.998[25, 26]. Our *in silico* PFGE simulation generated band numbers from 24 to 48 (mean = 34; SD = + 5) in the range 10-950 kbp what allowed to achieve the ultimate (100%) Simpson's index.

MLVA utilizes PCR to amplify the region containing short, repetitive tandem sequences. Amplification products were separated by electrophoresis to determine the size and the number of repeats in the locus. Different MLVA schemes, involving from 6 to 16 loci with repetitive tandem sequences, have been developed for *Pseudomonas aeruginosa* typing[7-10].

In our study, all eight MLVA schemes provided discriminatory power between 0.988 and 0.998, with overlapping 95% confidence intervals. Makaoui Maaˆtallah et al. in their study on typing of *Pseudomonas aeruginosa* (n=85) received the same Simpson's index of diversity (0.980 (0.970-0.991) using MLVA scheme with 15 markers[27]. One of the possible reason why MLVA schemes with 6 or 7 loci produced the same discriminatory ability as MLVA with more loci (e.g. 15 or 16 loci) was because the markers included in each MLVA method had a different discriminatory index (as shown in table 4).

The adjusted Rand indices have a degree of equivalence between the type assignments of two distinct typing methods (PFGE (80% cut-off) and MLVA). These were 0.501 for type level and 0.614 for MLVA cluster level at 80% ($p = 0.0493$) and these ranged from 0.332 to 0.694. This indicates a poor/moderate congruence between PFGE clusters and MLVA clusters/types approaches (as per table 3).

The adjusted Wallace coefficient indicates the probability that pairs of isolates that are assigned to the same type by one typing method are also typed as identical by the other. PFGE with an 80% cut-off were not a particularly good method for predicting MLVA type (the mean of adj. Wallace was 0.361 (from 0.199 to 0.498)).

By contrast, MLVA clusters (more than 80% similarity) were better predicted by PFGE clusters (here the mean of adj. Wallace was 0.911 (from

0.623 to 1.0)). In general, clusters of MLVA typing provided a good prediction for PFGE clusters (the mean of adj. Wallace was 0.723; from 0.497 to 0.798), but due to the higher resolution of PFGE the MLVA method performed poorly in terms of predicting PFGE (adj. Wallace MLVA clusters -> PFGE clusters were from 0.346 to 0.634)).

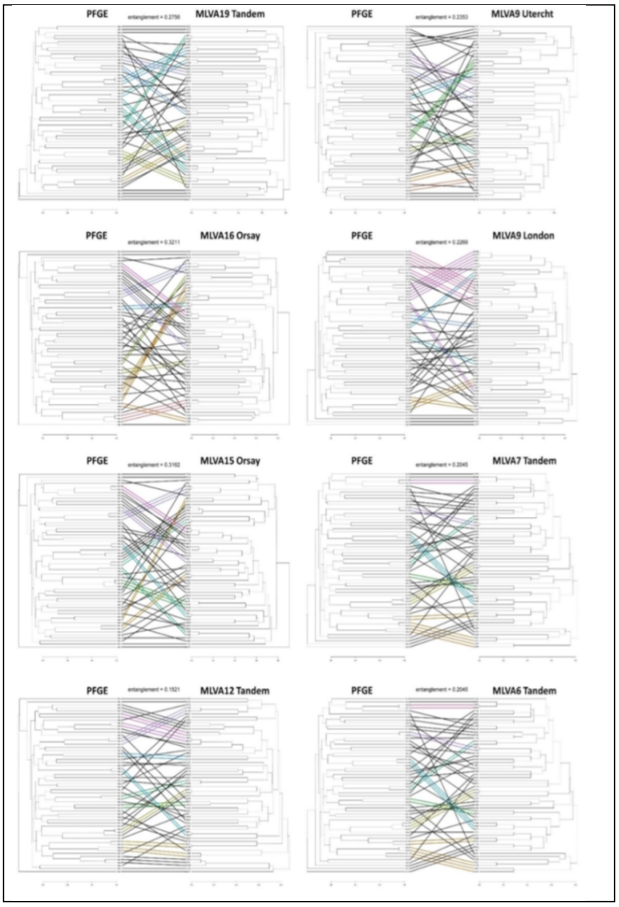

Fig. 2: Tree comparison constructed based on PFGE and different MLVA data of *Pseudomonas aeruginosa* typing with connected lines showing degree of disorder between two dendrograms (entanglement). These pictures and indices were calculated with Dendextend package (R statistics).

In systematic biology, visualization of similarities and differences of phylogenetic trees, in particular in multi-gene analysis, can be provided by drawing two dendrograms side by side with connector lines between species that correspond to each other in the two trees. The resultant tanglegram plot, as presented in this paper, demonstrated discrepancies between the topology structure of MLVA methods and PFGE (figure 2); whereas entanglement outcomes showed approximately the same alignment parameters between different MVLA schemes and PFGE (as shown in table 3).

MLVA marker	# Samples	# Different types	Discriminatory index	Confidence interval (95% CI)
ms10	58	16	0.912	[0.875 - 0.949]
ms061	58	10	0.882	[0.854 - 0.91]
ms77	58	5	0.338	[0.183 - 0.493]
ms127	58	3	0.424	[0.283 - 0.565]
ms142	58	8	0.826	[0.774 - 0.879]
ms172	58	9	0.734	[0.646 - 0.822]
ms173	58	15	0.895	[0.854 - 0.935]
ms194	58	11	0.782	[0.699 - 0.866]
ms207	58	12	0.89	[0.864 - 0.915]
ms209	58	9	0.793	[0.738 - 0.849]
ms211	58	7	0.828	[0.792 - 0.864]
ms212	58	8	0.764	[0.691 - 0.838]
ms213	58	7	0.779	[0.708 - 0.851]
ms214	58	9	0.8	[0.75 - 0.849]
ms215	58	8	0.797	[0.744 - 0.851]
ms216	58	4	0.604	[0.502 - 0.707]
ms217	58	7	0.797	[0.746 - 0.848]
ms222	58	5	0.724	[0.68 - 0.767]
ms223	58	6	0.724	[0.653 - 0.794]

Table 4: Resolution power and 95% CI of each VNTR marker included in MLVA scheme for *Pseudomonas aeruginosa* typing.

In summary, our in *silico* study for comparing different MLVA schemes with PFGE, based on *Pseudomonas aeruginosa* genomes showed, on the one hand, the same high level of discriminatory power of PFGE and MLVA even with 6 tandems markers; nonetheless, on the other hand, there was poor congruence between PFGE and some MLVA schemes, and moderate agreement in cases with other MLVA approaches. In general, concordance between PFGE clusters and MLVA clusters was higher than between PFGE clusters and MLVA types, although this was never more than 69.4%. Based on different principles of PFGE and MLVA typing methods, there were understandable different outcomes in terms of the topology of hierarchical clustering.

Interestingly, PFGE and MLVA methods could independently determine the *Pseudomonas aeruginosa* PA7 strains as taxonomic outliers[28].

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