International *Mycoplasma pneumoniae* typing study: interpretation of *M. pneumoniae* multilocus variable-number tandem-repeat analysis

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Abstract

Typing of *Mycoplasma pneumoniae* by multiple-locus variable-number tandem repeat analysis (MLVA) is increasingly in use. However, no specific internationally agreed guidance is available. Thirty *M. pneumoniae* DNA samples including serial dilutions of a type strain were sent to six international laboratories to perform MLVA and results were compared. Good correlation was observed, indicating that this methodology can be robustly performed in multiple sites. However, differences due to interpretation of fragment size, repeat sequence identification and repeat numbering led to inconsistency in the final profiles assigned by laboratories. We propose guidelines for interpreting *M. pneumoniae* MLVA typing and assigning the number of repeats.

Keywords: Interpretation guidelines, molecular typing, multiple-locus variable-number tandem repeat analysis (MLVA), *Mycoplasma pneumoniae*, *standardisation*
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Introduction

Mycoplasma pneumoniae causes human respiratory tract infections [1]. Typing of isolates and positive clinical samples is necessary to support epidemiologic data for the detection of outbreaks and understand the transmission of infection. In contrast to typing based on sequence differences in the *P1* gene of *M. pneumoniae* [2], multiple-locus variable-number tandem repeat analysis (MLVA) is reportedly highly discriminatory [3] and is now increasingly in use for strain characterization internationally [4–11]. Investigating the five loci selected (MPN1, MPN13–16), it has been reported that the MPN1 locus is not stable, thus calling into question the reliability of the marker [12]. Therefore, several authors have proposed an alteration to the naming system to reflect this [12,13]. Despite the availability of general guidelines for the MLVA procedure [14,15], specific internationally agreed guidelines for the execution and interpretation of MLVA of *M. pneumoniae* are not yet available.

In this study, 24 *M. pneumoniae* clinical isolates were included, as well as the reference strain M129 (ATCC 29342). The clinical isolates, all derived from sputum specimens, were obtained from the Public Health England Respiratory and Vaccine Preventable Bacteria Reference Unit culture collection. DNA was extracted from bacterial cultures in *Mycoplasma* liquid medium (Mycoplasma Experience Ltd., UK) using the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche). The commercial quantitative type strain NCTC10119 FH (Minerva Biolabs) was used to determine sensitivity of MLVA at four dilutions (1000, 100, 10 and 1 copy/μL). MLVA was performed in a blinded manner using a previously described method [3] in six international laboratories (China, England, France, Germany, Netherlands, United States of America). Results were collated, including fragment size, calculated MLVA repeat number and MLVA profile. No guidelines were given to the participating laboratories other than that already available in the literature. Naming of profiles was based on the method in which naming is

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based on a string of allele numbers in the order MPN1, MPN13, MPN14, MPN15, MPN16 showing the actual number of repeats at each locus [5]. 'No amplification' was assigned to loci that failed to amplify [16].

Results

Regarding fragment sizes, excellent parity was seen among laboratories, with <7 bp difference in fragment sizes between all DNA samples and all laboratories. Table 1 includes expected fragment size and repeat numbers in order to clarify predicted fragment size and repeat number. Regarding the fragment repeat numbers, a total of three errors on assigning and collating the MLVA repeat number from the accurate fragment size (transcription errors) were noted from two separate laboratories (Table 2). In addition, there was an inconsistency in the results reported for two of the loci, MPN13 and MPN15. This was due to a different interpretation of fragment repeat number when encountering a point number. Specifically, four of the laboratories rounded ≥3.2 copies up to 4 repeats for MPN13, whereas two other laboratories rounded <3.5 down to 3 repeats. This highlights the need for an internationally agreed protocol regarding the interpretation of MLVA repeat numbers. In addition, one laboratory made calculating errors linked to the determination of the sequence of MPN15. The MPN15 sequence was manually determined as TGTCCATTTTTACTTCCATCAT, in contrast to the accurate TTGTCCATTTTTTCTTCCATC sequence calculated using tandem repeat finder software with settings match, mismatch, indel of (2,3,5). It should be noted that the use of settings other than (2,3,5) can give alternative repeat sequence and length for some loci. For example, using settings (2,7,7) for *M. pneumoniae* M129, the MPN15 repeat would be only 20 bp with a different sequence (TTGTCCATTTTTTTTCCATC instead of TTGTCCATTTTTTCTTCCATC).

Excluding the three transcription errors, and after correcting for interpretation differences by rounding up partial repeat numbers to the next integer value, all laboratories determined identical fragment repeat numbers for the M129 strain, and 20 of the 24 clinical isolates gave consistent fragment repeat numbers (Table 2). Actual technical differences were seen in only four samples: samples 8, 10, 21 and 30. Interestingly, these samples had lower-than-average DNA concentration on initial DNA extraction (less than 3 μg/mL DNA, compared to 7 μg/mL for the other samples). To compare the sensitivity, a serial dilution of the NCTC10119 strain was included. All laboratories determined the MLVA profile in the presence of 1000 copies/μL. However, only three laboratories obtained a full profile, while the other three reported partial profiles for

TABLE 1. Target sequences, repeat size and fragment size of *Mycoplasma pneumoniae* MLVA loci MPN1, MPN13, MPN14, MPN15 and MPN16 according to number of repeats

Characteristic	MPN1	MPN13	MPN14	MPN15	MPN16
Repeat sequence based on M129 TRF (2,3,5)[a]	CCGAGCTAAGCG	TATTAATAACTATTCT	TGGACAAAATGGAAGTAAAAA	TTGTCCATTTTTTCTTCCATC	ATTTTTTAAAAGTTTTTATTTATCCGTTTTTGACAACTGCTTTTTGTT
Repeat size (bp)	12	16	21	21	47
Repeat number					
0	287	364	294	108	259
1	299	380	315	129	306
2	311	396	336	150	353
3	323	412	357	171	400
4	335	428	378	192	447
5	347	444	399	213	494
6	359	460	420	234	541
7	371	476	441	255	588
8	383	492	462	276	635
9	395	508	483	297	682
M129 fragment size (bp)	333	415	399	241	353
M129 repeat number	3.8→4	3.2→4	5	6.3→7	2

MLVA, multiple-locus variable-number tandem repeat analysis.
The *M. pneumoniae* M129 MLVA type is 44572 with 3.8 repeats in MPN1 (rounded up to 4), 3.2 repeats in MPN13 (rounded up to 4), 5 repeats in MPN14, 6.3 repeats in MPN15 (rounded up to 7) and 2 repeats in MPN16.
[a]Tandem repeat finder software. The settings used are (2,3,5) (match, mismatch, indel) [17]. The use of other settings can give alternative repeat sequence and length for some loci—for example, using settings (2,7,7) for *M. pneumoniae* M129: MPN13 CTTATTAATAACTATT 2.3 repeats of 16 bp, and MPN15 TTGTCCATTTTTTTTTCCATC 6.3 repeats of 20 bp.

TABLE 2. MLVA profiles collated from six international laboratories after investigation of 30 *Mycoplasma pneumoniae* DNA samples

Sample	Expected profile[a]	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6
1 (M129)	44572	44572	43562	44572	44572	44572	43562
2 (1000 copies/μL)[b]	43662	43662	42652	43662	43662	43662	42652
3 (100 copies/μL)	43662	43662	42652	4–662	4356 -[c]	43662	- 255[c] -
4 (10 copies/μL)	43662	43662	- - 652	4–6-2	- - - - -	43662	- - - - -
5 (1 copy/μL)	43662	- - - - -	- - - - -	- - 6 - -	- - - - -	- - - - -	- - - - -
6	43662	43662	42652	43662	43662	43662	42652
7	43662	43662[d]	42652	43662	43662	43662	42652
8	53662	63662[c]	52652	53662	53662	53662	52652
9	54572	54572	53562	54572	54572	54572	53562
10	63662	63662	62652[d]	63662	63662	63662	62542[c]
11	34572	34572	33562	34572	34572	34572	33562
12 (M129)	44572	44572	43562	44572	44572	44572	43562
13	63562	63562	62552[d]	63562	63562	63562	62552
14	33562	33562	32552	33562	33562	33562	32552
15	63562	63562	62552	63562	63562	63562	62552
16	53662	53662	52652	53662	53662	53662	52652
17	23662	23662	22652	23662	23662	23662	22652
18	44572	44572	43562	44572	44572	44572	43562
19	23562	23562	22552	23562	23562	23562	22552
20	43572	43572	42562	43572	43572	43572	42562
21	54572	54572	53562	54572	54572	54572	53542[c]
22	54572	54572	53562	54572	54572	54572	53562
23	43662	43662	42652	43662	43662	43662	42652
24	34572	34572	33562	34572	34572	34572	33562
25	63562	63562	62552	63562	63562	63562	62552
26	34572	34572	33562	34572	34572	34572	33562
27	33662	33662	32652	33662	33662	33662	32652
28	23662	23662	22652	23662	23662	23662	22652
29	33662	33662	32652	33662	33662	33662	32652
30	54572	- - - - -	- - - - -	54572	5–572	54572	- - 562[c]

Repeat number different from the expected result are underlined. A hyphen indicates no amplification.
MLVA, multiple-locus variable-number tandem repeat analysis.
[a]Naming of profiles was based on the method in which naming is based on a string of allele numbers in order of MPN1, MPN13, MPN14, MPN15 and MPN16 showing the actual number of repeats at each locus.
[b]Samples 2, 3, 4 and 5 are dilutions of the NTCT10119 FH commercial standard strain with 1000, 100, 10 and 1 copies/μL, respectively.
[c]MLVA profile remains different from the expected MLVA profile after correction for transcription errors and interpretation differences.
[d]MLVA profiles with initial transcriptional errors.

the 100 copies/μL standard; two of these partial profiles differed in the repeat number for MPN14. A full profile was obtained with the 10 copies/μL standard by only two laboratories. None of the laboratories obtained a full profile for the lowest dilution tested (1 copy/μL). When examining both the serial dilution and the low-loaded sample results, it was apparent that in laboratory 5, which increased the number of amplification cycles to 45 for samples that gave poor results with 25 cycles of amplification, the typing method showed a greater sensitivity.

Discussion

This study highlights the need for standardization of interpretive criteria for data analysis internationally. It indicates that comparison of existing published MLVA data between laboratories may be flawed in some cases, diminishing reliability of strain investigation involving more than one laboratory. The following recommendations are considered pertinent by our collaborative group in enabling standardization of interpretive data.

First, predicted fragment sizes and repeat numbers should be assigned using the information provided in Table 1. Sequence of repeat fragments listed in Table 1 should be considered as the sequence of interest.

Second, If tandem repeat finder software is used (http://tandem.bu.edu/trf/trf.html) [17] to determine repeat numbers, the following settings should be used: match, mismatch, indel (2,3,5).

Third, the repeat number should be expressed as whole integers, and partial sequences should be rounded up to the next integer number. The rounding up or down convention is matter of debate [14,15]. However, as previously reported [15], rounding the partial number of repeats up and not down will avoid rounding down to zero a repeat number such as 0.7, which is ambiguous, as it may be understood as 'lack of repeat.' Thus, using a 'rounding up' convention, zero will unambiguously be defined as 'lack of repeat.' Moreover, by retaining a rounding-up approach, future data will correspond with historical data in previous publications related to *M. pneumoniae* MLVA typing.

Fourth, the MPN1 target should be removed from future analyses due to its instability [12,13]. The identification of additional MLVA targets that have greater stability than target MPN1

and enable greater discrimination power than MPN16 should be advanced. With the removal of the MPN1 allele, adoption of the following naming system is recommended: MLVA-1, -2, -3 and -4, where each digit corresponds to repeat numbers at loci MPN13, MPN14, MPN15 and MPN16, respectively.

In conclusion, although whole genome sequencing has become rapid and affordable to replace older typing methods in the future for either clinical strains or clinical specimens [18], MLVA typing using the method by Dégrange *et al.* [3] is widely in use for *M. pneumoniae*. MLVA typing was performed with good correlation in six international laboratories, indicating that this methodology can be correctly performed on *M. pneumoniae* at different locations. Differences due to interpretation of fragment size, repeat sequence identification and repeat numbering led to inconsistencies in the final profiles assigned by laboratories. With users following the interpretation guidelines we provide, full interlaboratory strain comparison should be achieved.

Conflict of Interest

None declared.

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