Original Article

Identification of Variable-Number Tandem-Repeat (VNTR) Sequences in *Acinetobacter pittii* and Development of an Optimized Multiple-Locus VNTR Analysis Typing Scheme*

HU Yuan[1], LI Bo Qing[2], JIN Da Zhi[3], HE Li Hua[1], TAO Xiao Xia[1], and ZHANG Jian Zhong[1,#]

1. State Key Laboratory of Infectious Disease Prevention and Control, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China; 2. Binzhou Medical College, Yantai 256603, Shandong, China; 3. Zhejiang Provincial Center for Disease Control and Prevention, Hangzhou 310009, Zhejiang, China

Abstract

Objective To develop a multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) assay for *Acinetobacter pittii* typing.

Methods Polymorphic VNTRs were searched by Tandem Repeats Finder. The distribution and polymorphism of each VNTR locus were analyzed in all the *A. pittii* genomes deposited in the NCBI genome database by BLAST and were evaluated with a collection of 20 well-characterized clinical *A. pittii* strains and one reference strain. The MLVA assay was compared with pulsed-field gel electrophoresis (PFGE) for discriminating *A. pittii* isolates.

Results Ten VNTR loci were identified upon bioinformatic screening of *A. pittii* genomes, but only five of them showed full amplifiability and good polymorphism. Therefore, an MLVA assay composed of five VNTR loci was developed. The typeability, reproducibility, stability, discriminatory power, and epidemiological concordance were excellent. Compared with PFGE, the new optimized MLVA typing scheme provided the same and even greater discrimination.

Conclusion Compared with PFGE, MLVA typing is a faster and more standardized alternative for studying the genetic relatedness of *A. pittii* isolates in disease surveillance and outbreak investigation.

Key words: *A. pittii; MLVA typing; VNTR*

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INTRODUCTION

Acinetobacter species are receiving increasing attention as significant opportunistic pathogens. Among them, members of the phenotypically undifferentiated *A. calcoaceticus-A. baumannii* (Acb) complex, namely *A. baumannii*, *A. pittii* (genomospecies 3), and *A. nosocomialis* (genomospecies 13TU), are the three most common *Acinetobacter* species isolated in clinical settings[1-2]. *A. baumannii* has long been considered the most clinically important species with the greatest number of healthcare-related outbreaks and reports of multidrug resistance. More recently, as a consequence of improved laboratory speciation, it is becoming apparent that non-*baumannii* species of *Acinetobacter*, particularly *A. pittii* and *A. nosocomialis*, are clinically significant

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#Correspondence should be addressed to Professor ZHANG Jian Zhong, Tel: 86-10-58900707, E-mail: zhangjianzhong@icdc.cn

Biographical note of the first author: HU Yuan, female, PhD, major in pathogenic biology.

human pathogens with increasing reports of hospital outbreaks and antibiotic resistance. Especially, the emergence of New Delhi metallo-b-lactamase-1 (blaNDM-1)-producing *A. pittii* constantly appears in several countries[2,3-8], and the cross-genus transfer of blaNDM-1 to *E. coli* was achieved, which underlines the potential of *A. pittii* as a resistance reservoir for the dissemination of blaNDM-1[9]. However, because of the difficulties in identification, epidemiological and clinical studies of *Acinetobacter* spp. often investigate the Acb complex as a single entity, which is practical but limits our full understanding of *A. pittii*. Therefore, there is an urgent need to strengthen the epidemiological surveillance of *A. pittii*.

A. pittii is increasingly being identified as a causative agent of nosocomial infections[1,10]. Recent studies have determined that *A. pittii* accounted for 19% of Acb complex bacteremia in the USA[11], 5.3% of blood cultures in Korea[12], 8% of clinical isolates in Taiwan[13], 19.9% of Acb complex isolates in France[14], and 19.5% of *Acinetobacter* spp. blood culture isolates in Norway[15]. An investigation on the distribution of *Acinetobacter* over a 5-year period in Germany also found that *A. pittii* was the most prevalent species within the Acb complex[16]. This surprising result revealed the potential of *A. pittii* to widely spread and cause outbreaks.

Several typing methods have been developed for epidemiological typing and population genetic studies of *A. baumannii*, including macrorestriction analysis with pulsed-field gel electrophoresis (PFGE)[17], multilocus sequence typing (MLST)[18-19], and multiple-locus variable-number tandem repeats (VNTRs) analysis (MLVA)[20-21]. Using these methods, the clonal spread of *A. baumannii* was revealed, and the evolutionary success of the international clonal lineages I, II, and III was identified. However, the ecology and epidemiology of most non-*baumannii* Acb species are not well understood, primarily because present specific phenotypic techniques have, to date, been insufficient and short of rapid, easy-to-perform, cost-effective typing methods. MLVA, based on the copy numbers of tandem-repeat units (TRs) of VNTR loci, is easy to perform and can be easily shared and has thus become the reference typing method for several bacterial species[22]. The aim of this study was to establish an MLVA scheme for *A. pittii*. For this purpose, we searched for the presence of shared VNTR markers in sequenced *A. pittii* strains and set up a standard MLVA protocol based on simple PCR and capillary gel electrophoresis. The overall performance of the MLVA method was assessed in a study of a selected collection of clinical *A. pittii* isolates and compared with the reference typing method of PFGE.

MATERIALS AND METHODS

Bacterial Strains

A collection of 20 well-characterized *A. pittii* strains, one reference *A. pittii* strain, and 28 non-*A. pittii* strains were used (Table 1). Except seven ATCC strains, all the tested strains were isolated from patients in seven Chinese cities (one hospital per city and one isolate per patient). Two pairs of the strains were epidemiologically related. Strains HBXH135 and HBXH83 were isolated from two patients in the same ward, and strains R18 and R28 were isolated from different patients during the same outbreak. All isolates were identified as *A. pittii* by MALDI TOF Mass Spectrometry[23] and verified using sequence analysis of the 16S-23S ribosomal DNA intergenic space[13].

Detection and Description of VNTRs

The available draft genome of ANC 3678 was selected to search for VNTR loci using Tandem Repeats Finder available at http://tandem.bu.edu/trf/trf.html. The polymorphism of the target tandem repeats and the conservation of the flanking sequence were analyzed by *A. pittii* GENOME BLAST. A total of 22 draft genomes deposited in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sites/genome) were screened for the presence of potential VNTR loci. The expected amplicon size, repeat length, number of repetitions, and deduced size of flanking regions were analyzed. VNTR loci that showed polymorphisms in the repeat region but were conserved in the flanking regions and distributed in more than half of the available genomes were selected. The occurrences of the selected VNTR loci in a collection of 21 *A. pittii* strains were assessed by PCR and verified by PCR sequencing. PCR primers are listed in Table 2. For the five loci included in the MLVA scheme, the forward primer of each primer set was fluorescently labeled with FAM or HEX. PCRs were done in monoplex, and then, the multiplexed PCR products were sized on 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA). The bands were sized relative to the DNA marker (GeneScan LIZ®1200 size marker, Applied Biosystems) by using the

Table 1. Characteristics of Strains Used in This Study

Study Code	Strain Designation	Species	Yr of Isolation	Origin
A. pittii strains				
1	ATCC17922	*A. pittii*		
2	LBQ1	*A. pittii*	2010	Yantai
3	LBQ6	*A. pittii*	2010	Yantai
4	LBQ14	*A. pittii*	2010	Yantai
5	LBQ22	*A. pittii*	2010	Yantai
6	LBQ23	*A. pittii*	2010	Yantai
7	S12159	*A. pittii*	2005	Beijing
8	S12192	*A. pittii*	2005	Beijing
9	S12383	*A. pittii*	2005	Beijing
10	S12482	*A. pittii*	2005	Beijing
11	S13041	*A. pittii*	2005	Beijing
12	S13299	*A. pittii*	2005	Beijing
13	HBXH135[a]	*A. pittii*	2010	Wuhan
14	HN252	*A. pittii*	2010	Haining
15	R18[a]	*A. pittii*	2005	Beijing
16	R28[a]	*A. pittii*	2005	Beijing
17	14	*A. pittii*	2005	Beijing
18	H015	*A. pittii*	2010	Taizhou
19	BJ73	*A. pittii*	2007	Beijing
20	HBXH83[a]	*A. pittii*	2010	Wuhan
21	HN162	*A. pittii*	2010	Haining
Non-*pittii Acinetobacter* strains				
1	ATCC17903	*A. nosocomialis*		
2	W053	*A. nosocomialis*	2010	Wenzhou
3	HN095	*A. nosocomialis*	2010	Haining
4	FZ1	*A. nosocomialis*	2012	Fuzhou
5	BJR1	*A. nosocomialis*	2005	Beijing
6	BJR3	*A. nosocomialis*	2005	Beijing
7	BJR6	*A. nosocomialis*	2005	Beijing
8	HN128	*A. nosocomialis*	2010	Haining
9	HN145	*A. nosocomialis*	2010	Haining
10	BJRM11	*A. nosocomialis*	2004	Beijing
11	ATCC19003	*A. baumannii*		
12	HN086	*A. baumannii*	2010	Haining
13	LBQ13	*A. baumannii*	2010	Yantai
14	BJ23	*A. baumannii*	2007	Beijing
15	BJRM30	*A. baumannii*	2005	Beijing
16	BJ27	*A. baumannii*	2007	Beijing
17	FZ3	*A. baumannii*	2012	Fuzhou
18	HN001	*A. baumannii*	2010	Haining
19	LBQ20	*A. baumannii*	2010	Yantai
20	S11013	*A. baumannii*	2005	Beijing
21	ATCC17924	*A. bereziniae*		
22	R12	*A. bereziniae*	2005	Beijing
23	R11	*A. bereziniae*	2005	Beijing
24	13	*A. bereziniae*	2004	Beijing
25	HBXH110	*A. junii*	2010	Wuhan
26	ATCC15309	*A. lwoffii*		
27	ATCC17906	*A. haemolyticus*		
28	ATCC17909	*A. johnsonii*		

Note. [a]Epidemiological concordance panel. Strains HBXH135 and HBXH83 were isolated from two patients in same ward. Strains R18 and R28 were isolated from different patients during the same outbreak.

GeneMapper® software version 4.0 (Applied Biosystems). Amplification was performed with an initial denaturation for 5 min at 94 °C, followed by 30 cycles of denaturation for 10 s at 94 °C, annealing for 30 s at 55 °C, and elongation for 30 s at 72 °C, with a final elongation for 1 min at 72 °C. The number of repeats in the VNTR alleles was estimated by subtracting the flanking region size from the amplicon size and then dividing by the repeat unit length (Table 2). The number of repeats was rounded to the nearest integer number.

PFGE Typing

PFGE analysis of ApaI-digested genomic DNA was performed for the whole collection of 21 unique *A. pittii* isolates as previously described for *A. baumannii*[17]. A dendrogram was created with the BioNumerics software (Applied Maths) by using the Dice coefficient and the unweighted-pair group method using average linkages (UPGMA), with a 1.5% tolerance limit and 1.5% optimization[17]. A similarity threshold of 95% was used to define the isolates belonging to the same PFGE type.

Evaluation of A. pittii Typing Methods

The polymorphism index for individual or combined VNTR loci was calculated using the Hunter-Gaston diversity index (HGDI)[24], an application of Simpson's index of diversity. HGDI was also determined for PFGE typing. Confidence intervals (CIs) were calculated as previously described[25]. All calculations were performed using the free online tool Comparing Partitions (available at www.comparingpartitions.info).

Evaluation of the MLVA typing assay was performed according to the guidelines previously described[26]. The reproducibility of the method was assessed at intra-laboratory levels. Two sets of two epidemiologically related isolates each were used to determine the epidemiological concordance (Table 1). Stability was determined for all the 21 *A. pittii* strains by 30 serial passages on MH agar. DNA was extracted at each passage for MLVA analysis. Typeability was estimated by analysis of all the 21 *A. pittii* isolates.

Table 2. Oligonucleotide Primers, and Characteristics of VNTRs Analyzed in This Study

VNTR Marker	ORF	Predicted Function of the Translated VNTR Sequence	Expected Amplicon Size [bp (no. of repeats)]	Repeat Size (bp)	Flanking Regions Size (bp)	Primer Designation	Primer Sequence
Pittii_V	F930_00029	cation transporter (Co/Zn/Cd efflux system component)	196(5)	6	166	V-F	FAM-CAGTTGAAATTTTTTATAATGAAGC
						V-R	GCATCTGCAACTACATGTAAAAA
Pittii_L	F930_01763	DNA polymerase III subunit gamma/tau	485(18)	6	383	L-F	HEX-CTCATGATGATTTGGGTCAAAT
						L-R	ATTTGAGTTTGAAATATAGCGATG
Pittii_M	F930_00926	hypothetical protein	539(2)	33	348	M-F	CCATGAGCTGGTTGAGCTG
						M-R	GGTGGTCTTGTTAGTGGCGT
Pittii_P	F930_01019	hypothetical protein	631(4)	57	376	P-F	GATAATTGTATCTGCAACTGTATCAA
						P-R	TGAATATCCTGTTTCAGACCTAGC
Pittii_Q	F930_01019	hypothetical protein	468(3)	57	283	Q-F	GCTAGGTCTGAAACAGGATATTCA
						Q-R	CGCAACTGAGTCTGCTACTGGT
Pittii_J	F930_01691	protein hfq	555(8)	18	406	J-F	AGAAGACTTGCGCTTGCATA
						J-R1	GTACAGGCTTCCCAGCACA
Pittii_G	F930_03156	hypothetical protein	467(10)	9	377	G-F2	ATTCATCATTAGATGCCGTCTG
						G-R2	CCGCCCTAAACTTGCTATCTC
Pittii_R	F930_00471	hypothetical protein	710(3)	24	651	R-F1	ATAATCACCAGTTTTTTGTTCATC
						R-R1	ATATTCACCGTCTGTTCAGCA
Pittii_S	APICBIBUN_07861	copper resistance protein A	735(4)	30	623	ST-F1	CATTAATTGTCCAAATATAGCGC
						ST-R1	GGAATCAGATGCGAATGCTT
Pittii_I	F930_01081	hypothetical protein	583(3)	30	493	I-F1	TATATTGTTGAGCCGTATGCAA
						I-R	TTAGATGAGCAGAACCAACCA

RESULTS

Detection of VNTRs in A. pittii Genomes

A total of 116 VNTR loci were revealed in the draft genome of *A. pittii* ANC 3678, but only 21 of them were found to be polymorphic by the NCBI GENOME BLAST. Of the 21 VNTR loci, 11 were finally eliminated because of either their distribution in less than half of the available genomes or the flanking sequence variation, and the remaining 10 VNTRs were considered suitable candidates for the development of an MLVA scheme.

Selection and Typeability of VNTR Loci

The occurrences of the 10 selected VNTR loci in a collection of 21 *A. pittii* strains were assessed. Six VNTR markers obtained amplification products for all the tested strains, but only five of them (Pittii_V, Pittii_L, Pittii_P, Pittii_Q, Pittii_M) showed polymorphism, and the variation of the repeat numbers was confirmed by direct sequencing of the PCR products (Table 3). Therefore, these five VNTR loci showed full typeability (*T*=1.00). The remaining four markers, namely, Pittii_G, Pittii_R, Pittii_S, and Pittii_I, yielded lower typeability values (0.52≤T ≤0.76). This was due to failures of some PCR amplifications (Table 4). Locus Pittii_J showed full amplifiability but no variation in TRs with the present diversity panel of *A. pittii* strains (Table 4). To determine whether the amplification failures were due to mismatches in the primer target regions, PCR primers were redesigned, however, PCR products were still not obtained for all the amplification failure cases, raising the possibility that these loci are not widespread in the *A. pittii* population. As a result, Pittii_G, Pittii_R, Pittii_S, Pittii_I, and Pittii_J were not included in a first-line MLVA scheme, although these VNTRs could be reconsidered under circumst- ances in which additional information is needed.

Based on the above results, an MLVA scheme was developed to include five VNTR markers, in the order Pittii_V, Pittii_L, Pittii_M, Pittii_P, Pittii_Q. To test the species specificity of the five loci, 28 non-*pittii Acinetobacter* strains were selected (Table 1). No VNTR amplification was observed for Pittii_L, Pittii_P, and Pittii_Q, but all the tested *A. nosocomialis*, *A. baumannii*, and *A. bereziniae* strains obtained amplicons for Pittii_V and Pittii_M, suggesting that the two loci were widespread in the *Acinetobacter* strains.

Reproducibility of the MLVA Assay

Reproducibility was assessed at intra-laboratory levels. The MLVA profiles of the whole collection of strains were tested separately in two independent assays performed by different people. The MLVA profiles were in complete agreement for all the tested strains.

Discriminatory Power of the MLVA Assay

The 21 tested strains showed high genetic diversity as revealed by PFGE. Except the epidemiologically related isolates, no more than two isolates clustered together by a threshold of 85%. A total of 20 MLVA types and 19 PFGE types were obtained (Figure 1, Table 3). The dendrogram generated by PFGE analysis was then matched with the results of MLVA for direct comparison (Figure 1). Each strain showing a unique PFGE profile was also assigned to a unique MLVA type. Thus, MLVA (0.995; 95% CI, 0.983-1.000) demonstrated the same excellent discriminatory power as that of PFGE typing (0.9; 95% CI, 0.974-1.000).

To fully evaluate the discriminatory value of MLVA assay, we ran an in silico analysis on all the *A. pittii* genomes available by BLAST, and the results are shown in Table 3 marked by '#'. The tandem-repeat regions of some VNTR loci were located at the end of the related contig of some draft genome, and hence the repeat numbers were not confirmed (marked by '*' in Table 3) and were not included in the HGDI calculation. Using the 21 tested strains and 22 in silico-generated MLVA dataset, a high discriminatory power of MLVA was obtained (HGDI=0.991, Table 5). Individual HGDI values for the five VNTR markers ranged between 0.318 and 0.925 (Table 5). Greater diversity was observed for the short repeat markers (0.845 for pittii-V, 0.925 for pittii-L).

Epidemiological Concordance of the MLVA Assay

A panel consisting of two sets of epidemiologically related *A. pittii* isolates was used to assess the epidemiological concordance (Table 1), which was found to be excellent. One set of epidemiologically related isolates, R18 and R28, was characterized by identical MLVA types (type M5), and the other set (HBXH135 and HBXH83) showed two MLVA profiles differing in only one unit at one short repeat locus Pittii_L (Figure1).

Stability of the MLVA Assay

To assess the stability of our MLVA scheme, 30

Table 3. MLVA Allelic Profile of 43 *A. pittii* Strains

Strain Designation	pittii-					MLVA Type
	V	L	M	P	Q	
S12192	5	19	5	3	3	M2
R18	5	27	5	3	3	M5
R28	5	27	5	3	3	M5
S12383	8	15	5	3	3	M12
14	11	17	5	3	3	M8
LBQ1	5	16	5	3	4	M13
LBQ23	5	20	5	4	2	M1
HN162	10	10	5	4	2	M16
S13041	4	10	5	4	2	M17
HN252	5	18	5	4	3	M6
LBQ14	6	18	5	4	3	M7
H015	6	25	5	4	3	M10
S12482	6	8	5	4	3	M20
S13299	7	15	3	4	3	M19
HBXH135	10	17	5	4	3	M3
HBXH83	10	18	5	4	3	M4
LBQ22	10	21	5	4	3	M18
BJ73	11	17	5	4	3	M14
LBQ6	4	17	5	4	3	M9
ATCC17922	5	15	5	4	3	M15
S12159	5	18	5	4	4	M11
CIP 70.29[#]	6	10	3	3	3	M21
DSM 21653[#]	6	10	3	3	3	M21
DSM 25618[#]	6	10	3	3	3	M21
NBRC 110506[#]	6	11	5	3	3	M22
ANC 3678[#]	4	10	5	3	3	M23
TG6411[#]	4	10	5	3	3	M24
D499[#]	5	5	5	4	2	M25
DSM 9306[#]	5	18	5	4	2	M26
42F[#]	4	12	5	4	2	M27
LC510[#]	4	14	3	4	2	M28
SH024[#]	10	23	5	4	3	M29
ANC 4052[#]	9	11	5	5	3	M30
NBRC 110508[#]	4	11[*]	5	4	3	
ANC 4050[#]	4	11[*]	6	6	3	
NBRC 110510[#]	6	11[*]	3	3	3	
NBRC 110505[#]	6	11[*]	5	3	3	
TE2[#]	6	13	5	*	*	
CR12-42[#]	8	3[*]	5	4	3	
NBRC 110504[#]	9	11[*]	3	4	3	
NBRC 110507[#]	9	11[*]	5	4	3	
NBRC 110509[#]	9	11[*]	5	4	3	
T167[#]	9	3[*]	5	1	3	

Note. [#]Whole genome sequences (from which all VNTR coli sequence were extracted in silico). [*]Partial sequence, the tandem repeat region was located at the end of the related contig in the draft genome, so the repeat number was not confirmed.

Table 4. HGDI and Characters for Individual VNTR Locus not Included in the MLVA Scheme in 21 *A. pittii* strains

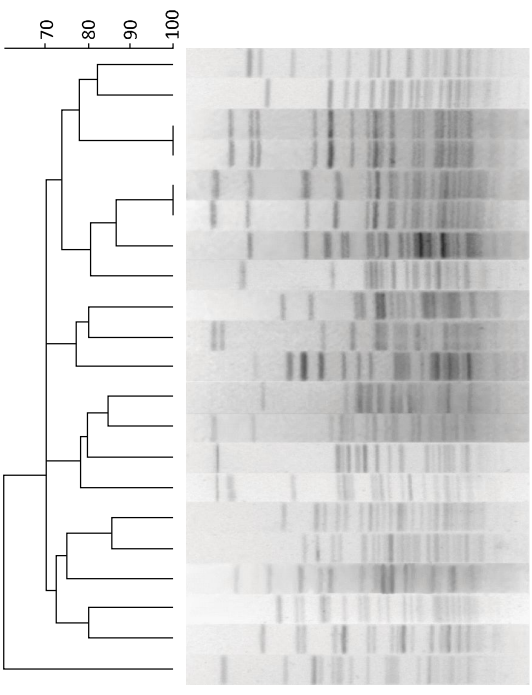
VNTR marker	Repeat Size (bp)	Allele Size Range [bp (no. of repeats)]	HGDI	CI[a]	K[b]	No. of Amplification Failures (%)
Pittii_G	9	419(5)-584(23)	0.871	0.770-0.973	10	7(33.3)
Pittii_R	24	710(3)-782(6)	0.79	0.698-0.883	5	8(38.1)
Pittii_S	30	735(4)-1016(13)	0.733	0.581-0.886	7	10(47.6)
Pittii_I	30	583(3)-643(5)	0.729	0.632-0.826	4	5(23.8)
Pittii_J	18	555(8)	0	0.000-0.270	1	0

Note. [a]Precision of the diversity index, expressed as 95% upper and lower boundaries. [b]Number of different repeats present at this locus in this sample set.

Table 5. HGDI for Individual or Combined VNTR Loci Included in the MLVA Scheme in 43 *A. pittii* Strains

VNTR marker	Repeat Size (bp)	Allele Size Range [bp (no. of repeats)]	HGDI	CI[a]	K[b]	Most Frequent no. of Repeats (%)
Pittii_V	6	190(4)-232(11)	0.845	0.805-0.885	8	5/6(23.3)
Pittii_L*	6	431(8)-545(27)	0.925	0.880-0.970	18	10(16.3)
Pittii_M	33	473(3)-572(6)	0.318	0.162-0.474	3	5(81.4)
Pittii_P*	57	574(3)-631(4)	0.546	0.442-0.650	6	4(58.1)
Pittii_Q*	57	411(2)-525(4)	0.361	0.200-0.522	4	3(76.7)
MLVA*			0.991	0.978-1.000	38	

Note. [a]Precision of the diversity index, expressed as 95% upper and lower boundaries. [b]Number of different repeats present at this locus in this sample set. *Strains with unconfirmed repeat numbers were not included in the HGDI calculation.



Strain	PFGE type	MLVA type	MLVA allelic profile pittii-V	pittii-L	pittii-M	pittii-P	pittii-Q
LBQ23	F1	M1	5	20	5	4	2
S12192	F2	M2	5	19	5	3	3
HBXH135	F3	M3	10	17	5	4	3
HBXH83	F3	M4	10	18	5	4	3
R18	F4	M5	5	27	5	3	3
R28	F4	M5	5	27	5	3	3
HN252	F5	M6	5	18	5	4	3
LBQ14	F6	M7	6	18	5	4	3
14	F7	M8	11	17	5	3	3
LBQ6	F8	M9	4	17	5	4	3
H015	F9	M10	6	25	5	4	3
S12159	F10	M11	5	18	5	4	4
S12383	F11	M12	8	15	5	3	3
LBQ1	F12	M13	5	16	5	3	4
BJ73	F13	M14	11	17	5	4	3
ATCC17922	F14	M15	5	15	5	4	3
HN162	F15	M16	10	10	5	4	2
S13041	F16	M17	4	10	5	4	2
LBQ22	F17	M18	10	21	5	4	3
S13299	F18	M19	7	15	3	4	3
S12482	F19	M20	6	8	5	4	3

Figure 1. Typing concordance between MLVA and PFGE typing. Cluster analysis of 21 *A. pittii* strains analyzed by PFGE is shown on the left and the MLVA allelic profiles are provided on the right for direct comparison.

in vitro passages with all the 21 *A. pittii* strains were performed. Before the 25th passage, no change in the MLVA profiles was observed. Subsequently, an increase of one TR copy number at locus Pittii_L occurred in two isolates. No TR copy number changes were observed in the remaining four loci. The rapid TR alteration at this locus is consistent with its high discriminatory power. Therefore, it should be considered as a rapidly evolving marker with a less phylogenetic value.

DISCUSSION

The primary aim of this study was to develop an MLVA scheme and evaluate its utility as a genotyping method for *A. pittii.* The MLVA scheme we propose included three moderately diverse long repeat VNTR markers (repeat units of 33-57 bp) and two highly variable short repeat VNTR markers (repeat units of 6) (Table 5). The long repeat loci, characterized by a small number of alleles with low variability, presumably reflecting a low mutation rate, are important to define the phylogenetic relationships among strains evolving over a long period. Conversely, the short repeat loci showed high levels of polymorphism and provided variability inside the clonal lineages, which is crucial in the case of disease surveillance and/or investigations of outbreaks. By combining three long repeat and two short repeat VNTR markers, an excellent discriminatory power of the MLVA assay was achieved (HGDI=0.991) (Table 5), at least with the present diversity panel of *A. pittii* strains.

The epidemiological concordance was also excellent when the epidemiologically related isolates R18 and R28, from two patients during the same outbreak, were analyzed. The MLVA results for the epidemiologically related isolates HBXH83 and HBXH135 showed difference in only one unit at short repeat locus Pittii_L. Our *in vitro* 30-passage stability test confirmed that Pittii_L was capable of being altered in a relatively small number of generations (one TR copy number increase occurred in two of the 21 tested isolates at the 25th passage). Thus, the rapid evolution of Pittii_L should be taken into account when comparing between epidemiologically related isolates during an outbreak.

At present, identification of *A. pittii* still depends on molecular biology methods, which are difficult to perform routinely in the clinical setting and often lead to high rates of misidentification. Hence, if the MLVA typing scheme is specific for *A. pittii*, it could provide additional clues to determine whether the strain identification is correct or not. With the present panel of non-*A.pittii* species, primers for Pittii_L, Pittii_P, and Pittii_Q are specific for *A. pittii.* This should be attention in the future works.

To our knowledge, this is the first MLVA typing scheme developed for *A. pittii*, and excellent reproducibility and discriminatory power were obtained. Because all the genomes deposited in the database were draft genomes, it is possible that some VNTR candidates that were initially discarded due to their apparent low degree of polymorphism or low distribution could be reconsidered. In the future, with improvement of the number of isolates typed by MLVA, the applicability of this MLVA typing scheme for disease surveillance and outbreak tracing will be further evaluated. Compared with PFGE, MLVA typing is a faster, more standardized alternative for studying the genetic relatedness of *A. pittii* isolates.

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