Method comparison for molecular typing of French and Tunisian Mycoplasma genitalium-positive specimens

C. Cazanave,1,2,3 A. Charron,1,2 H. Renaudin4 and C. Bébéar1,2,4

1Université de Bordeaux, USC Infections Humaines à Mycoplasmes et Chlamydiae, 33076 Bordeaux, France
2INRA, USC Infections Humaines à Mycoplasmes et Chlamydiae, 33076 Bordeaux, France
3Centre Hospitalier Universitaire de Bordeaux, Service de Maladies Infectieuses B, 33076 Bordeaux, France
4Centre Hospitalier Universitaire de Bordeaux, Laboratoire de Bactériologie, 33076 Bordeaux, France

In this study, 76 French and Tunisian urogenital specimens were subjected to molecular typing by using the two main Mycoplasma genitalium molecular typing methods, the mgpB single nucleotide polymorphism (SNP) typing method and the combination analysis of a variable-number tandem-repeat (VNTR) marker in MG309 and mgpB SNP. Furthermore, we tried to develop a multiple-locus VNTR analysis (MLVA) method. The genome of M. genitalium G37 was analysed for VNTRs and four VNTRs were used for an MLVA. The method, applied directly on clinical specimens, was based on a GENESCAN analysis of VNTR loci labelled with fluorescent dyes by using multiplex PCR and capillary electrophoresis. This method had a 1.00 diversity index (DI) while the mgpB SNP typing and the combination of MG309 and mgpB SNPs had DIs of 0.853 and 0.989, respectively. However, among the sets of two concurrent specimens, taken at the same time from the urogenital tracts of 12 patients, only nine had matching MLVA profiles, while the two other methods gave identical profiles for all specimens amplified, except for one set. Moreover, eight new sequence types were described with the mgpB SNP typing method. The three molecular typing methods revealed a genetic heterogeneity, suggesting that M. genitalium was endemic in France and Tunisia and that the infections were not due to the clonal dissemination of one strain. Comparison of the typing results obtained with the three methods showed that the MLVA assay seemed too discriminatory to be used in future studies of sexual networks of M. genitalium infection. According to the discriminatory power and the feasibility of each mgpB-based method, we recommend that the mgpB analysis be used for general epidemiological studies and that the combination of MG309-STR and mgpB SNP methods should be used for sexual-network studies of M. genitalium infection.

INTRODUCTION

Mycoplasma genitalium is a sexually transmitted pathogen (Hjorth et al., 2006) that is a recognized cause of urethritis in men (Jensen, 2004) and cervicitis (Manhart et al., 2003).

Abbreviations: DI, diversity index; LP, lipoprotein; MLVA, multiple-locus variable-number tandem-repeat analysis; SNP, single nucleotide polymorphisms; ST, sequence type; STI, sexually transmitted infection; STR, short tandem repeat; UPGMA, unweighted pair-group method with arithmetic mean; VNTR, variable-number tandem-repeat.

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A supplementary table is available with the online version of this paper.

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genotyping method was proposed in combination with a short tandem repeat (STR) analysis of lipoprotein (LP) genes and analysis of SNPs in tRNA or mgpB genes (Ma et al., 2008). The majority of molecular typing data for *M. genitalium* clinical specimens is based on the mgpB gene, which encodes the adhesin MgPa (Hjorth et al., 2006; Iverson-Cabral et al., 2006; Musatovova & Baseman, 2009).

Multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) is a new genotyping method (Vergnaud & Pourcel, 2006), based on the variations between isolates in the copy numbers of tandem repeated sequences at different genomic loci. MLVA has been used successfully to subtype some *Mycoplasma* isolates (Dégrange et al., 2009; McAuliffe et al., 2007, 2008; Vranckx et al., 2011).

In the present study, we evaluated the two main molecular typing methods, the mgpB SNP typing method (Hjorth et al., 2006) and the combination analysis of a VNTR marker in MG309 and mgpB SNPs (Ma et al., 2008), for the analysis of a large collection of French and Tunisian *M. genitalium* clinical specimens. We also tried to develop an MLVA scheme, which was compared to the two other methods.

**METHODS**

*M. genitalium* strains isolated in axenic culture and DNA preparation. Thirteen *M. genitalium* strains were used in this study, including the type strain *M. genitalium* G37T (ATCC 33530T) and 12 clinical isolates (Supplementary Table S1, available in JMM Online). These included *M. genitalium* M30 (ATCC 49895) and the seventh passage of the M30 strain (designated M30 early), three isolates obtained from the same French male patient (M6151 on day 1, M6090 on day 41, and M6312 on day 79), six isolates from Denmark and one isolate from Sweden (Jensen et al., 1996, 2004; Bébéar et al., 2008). The growth conditions used for the *M. genitalium* strains have been described previously (Jensen et al., 1996). DNA was isolated with a MagNa Pure LC DNA Isolation kit I (Roche Diagnostics) according to the manufacturer’s instructions.

Clinical specimens. A total of 76 specimens that were collected between 2001 and 2009 from 64 unrelated patients were analysed: 63 specimens from France and 13 specimens from 13 symptomatic Tunisian men. French specimens were obtained in Bordeaux and Paris from 20 symptomatic men, one asymptomatic man, 23 symptomatic women and seven asymptomatic women. Only one specimen was collected per patient, with the exception of 12 patients, from which two specimens were taken from the urogenital tract at the same time. Specimens were first-void urine (23), sperm (1) and cervical (15), vaginal (12) and urethral (25) swabs. Thirteen French specimens from nine different patients were anonymously collected in Bordeaux at the sexually transmitted infections (STI) centre. The remaining 48 specimens from Bordeaux and two specimens from Paris were collected from named hospitalized patients who were non-related. Thirteen specimens from non-related Tunisian men were non-anonymously collected in an STI centre. Characteristics of the specimens are shown in Supplementary Table S1. The study was conducted in accordance with the guidelines of the ethical committees of the participating hospitals. Specimens were collected as part of routine patient management without any additional sampling.

mgpB SNP typing. PCR amplification and sequencing of 281 base pairs (bp) of the MG191 semi-conserved region were performed as previously described using the MgPa-1/MgPa-3 primer set (Jensen et al., 1991). A sequence type (ST) type was assigned to each specimen according to previously described types (Hjorth et al., 2006; Ma et al., 2008; Musatovova & Baseman, 2009). We used the numbering system developed originally in 2006 by Hjorth et al. (2006) with the description of 56 STs, numbered 1–56. In addition to the 56 STs, we successively included the seven new STs described by Ma et al. (2008), numbered 57–63, and the 17 new STs described by Musatovova & Baseman (2009), numbered 64–80. In our study, we then described eight new STs of the mgpB gene, numbered 81–88, and deposited them in GenBank under the accession numbers HM565865–HM565872, respectively.

Identification of TRs. VNTR markers were identified in the *M. genitalium* G37T sequenced genome (Fraser et al., 1995) using the Tandem Repeats Finder program with the Micro-organisms Tandem Repeats database (http://minisatellites.u-psud.fr/GPMS). Loci were chosen if they had >75% matches between DNA sequences of the repeat units.

To screen for variability in the number of TRs, PCR primers targeting the region flanking the TR loci were designed. Forty-seven selected VNTRs were tested with specific primers to amplify DNA from the 13 *M. genitalium* isolates. To confirm that length polymorphisms were the result of repeat copy number variations, products were purified with a Wizard PCR Prep DNA Purification System (Promega) and double-strand sequenced. Preliminary amplification of these 47 loci from isolates showed that only six loci were polymorphic with different allele sizes. After evaluation of the specimens, two of these six VNTR loci were rejected due to lack of adequate discrimination and four VNTR loci were finally chosen for further assessment. Three TR loci, MG307-STR, MG309-STR, and MG338-STR, were named according to a previous study (Ma et al., 2008), and the fourth TR locus, previously identified (Iverson-Cabral et al., 2007) but never used in a typing scheme, was named Mge1.

MLVA typing. The four loci selected for MLVA were subjected to multiplex PCR in two solutions, D1 and D2. The reaction mixtures were composed of 1× Qiagen PCR buffer containing 1.5 mM MgCl₂, 0.2 mM each deoxynucleotide triphosphate, 2.5 mM and 3 mM MgCl₂ for solutions D1 and D2, respectively, 0.625 U HotStar Taq polymerase (Qiagen), 0.5 μM each primer pair (Mge1-F–Mge1-R and MG338-STR-F–MG338-STR-R, for solution D1, or MG307-STR-F–MG307-STR-R and MG309-STR-F–MG309-STR-R, for solution D2), and 5 μl template DNA. Forward or reverse primers were fluorescently labelled depending on the loci to be amplified (Table 1). Both multiplex PCRs were performed under similar conditions: 95 °C for 15 min followed by 40 cycles of 95 °C for 45 s, 58 °C or 54 °C for 1 min for solutions D1 and D2, respectively, and 72 °C for 1 min, with a final elongation step of 72 °C for 10 min. Then, 1 μl each pure or diluted amplicon was mixed with 10 μl Hi-Di formamide (Applied Biosystems) and 0.5 μl GENESCAN ROX 500 size standard (Applied Biosystems). After heat denaturation for 5 min at 95 °C and rapid cooling on ice, the fragments were separated on an ABI 3130 genetic analyser (Applied Biosystems) and electrophoresed. The GENESCAN data were analysed with GeneMapper software (version 4.0; Applied Biosystems) to perform sizing and calculate the numbers of repeats in the PCR fragments.

If no signal was obtained for one or more loci of each duplex PCR, semi-nested or nested PCRs were used. The primers that were used for primary, semi-nested and nested PCRs are listed in Table 1. Amplifications were performed as described above, except that 25 cycles were performed and only 2 μl amplicons from the first PCR were added to the reaction mixtures in the nested PCRs.

To further confirm the presence of heterogeneous repeat populations, some PCR products were sequenced after subcloning into the AT
cloning vector pGEM-T Easy (Promega). Recombinant plasmids were transformed into *E. coli* XL1-Blue competent cells according to standard transformation protocols (Sambrook & Russell, 2001), which permit the cloning of unstable DNA. For specimens containing mixed TR alleles, the proportion of individual alleles was estimated by the cloning results and the heights of the sequence chromatogram peaks. The PCR products were purified and sequenced.

**Data analysis.** The calculated numbers of repeats were imported into the BioNumerics (version 6.0) software package (Applied Maths) to construct a dendrogram Fig. 1(a), which highlighted the diversity among the specimens, with distinct clusters corresponding to the more frequent STs 1, 2, and 4. A new ST (type 81) was identified in 12 French and Tunisian non-related specimens. Interestingly, these specimens were collected between 2002 and 2004. However, this distribution was not identified with the MLVA method Fig. 1(b) or with the combination analysis of the *mgpB* marker and *mgpB* SNP Fig. 1(c). Three consecutive isolates from a male patient from France all belonged to ST 2. All the concurrent specimens from the same patient harboured the same ST, except one set (B14607/B14608) (Supplementary Table S1, Fig. 1a).

**Stability determination.** Five *M. genitalium* isolates (G377 and four clinical isolates, M2282, M2300, M6151 and M2341) were passaged ten times in broth medium to determine the stability of each locus before and after passaging. DNA was double-strand sequenced to identify the number of repeats in each locus.

**RESULTS**

**mgpB SNP typing**

In 73 out of 76 (96%) specimens, a single sequence variant was amplified from each specimen. Sixteen different STs were identified (Supplementary Table S1), and eight new STs (ST 81–88) were described. Sequences were imported to the BioNumerics software package (Applied Maths) to construct a dendrogram Fig. 1(a), which highlighted the diversity among the specimens, with distinct clusters corresponding to the more frequent STs 1, 2, and 4. A new ST (type 81) was identified in 12 French and Tunisian non-related specimens. Interestingly, these specimens were collected between 2002 and 2004. However, this distribution was not identified with the MLVA method Fig. 1(b) or with the combination analysis of the *MG309-STR* marker and *mgpB* SNP Fig. 1(c). Three consecutive isolates from a male patient from France all belonged to ST 2. All the concurrent specimens from the same patient harboured the same ST, except one set (B14607/B14608) (Supplementary Table S1, Fig. 1a).

**MLVA typing**

A set of four 3 nt VNTR markers was used to type the 13 *M. genitalium* isolates and the 76 specimens and their stability was confirmed by sequencing after 10 passages in broth medium. Three of the four VNTRs were located in LP-encoding loci (MG307-STR, MG309-STR and MG338-STR) and had been previously described (Ma et al., 2008). *Mge1*, previously identified by Iverson-Cabral et al. (2007), was located in the *mgpB* locus, which encodes the main adhesin MG191 (Burgos et al., 2006) (Table 2). The MG309-STR and MG338-STR markers were the most discriminatory VNTRs, with 15 and 13 different alleles, respectively. This difference in discriminatory power was also reflected by the diversity...
index (DI) of each VNTR ranging from 0.898 for MG338-STR to 0.797 for Mge1 (Table 2).

The two ATCC strains G37 and M30 had identical genotypes; however, the genotype of the isolate designated M30 early, collected at a different locus, differed from that of the M30 isolate. Two out of three sequential isolates from a male patient from France, M6151 (isolated on day 1) and M6312 (isolated on day 79), had the same MLVA type (Supplementary Table S1), while the third isolate,
M6090 (isolated on day 41), differed at the MG307-STR locus (Supplementary Table S1). Most loci were identified on electropherograms following the first two duplex PCRs (D1 and D2) according to their size ranges and colours. For 25 (32.9%) specimens, one or more VNTRs were not amplified in the duplex PCRs; therefore, nested or semi-nested PCR was applied. The four VNTRs were successfully amplified in all the specimens.

According to the electropherograms, some specimens had multiple alleles at different TR loci, as described previously (Ma et al., 2008). Multiple alleles in at least one TR locus were identified in 28 of the 76 (36.8%) specimens. The proportions of mixed profiles were 22.4, 10.5, 11.8 and 17.1% for Mge1, MG307-STR, MG309-STR and MG338-STR, respectively. To verify the presence of multiple alleles as determined by the Genetic Analyzer, we subcloned PCR products from two selected specimens, B9927 and B9874, and sequenced 10 plasmid clones per specimen. According to the GENESCAN results, specimen B9927 contained a mixture of 5 and 6 repeats at the Mge1 locus and a mixture of 9, 10 and 11 repeats at the MG309-STR locus, and specimen B9874 contained a mixture of 6 and 10 repeats at the MG338-STR locus (Supplementary Table S1).

Specimens were considered to have matching profiles if they had identical alleles at all loci or if they shared at least one common allele at each of the four VNTR loci when mixed TR alleles were present. Sets of two specimens, both obtained from the same patient, had matching genotypes in only 9 out of 12 cases (Supplementary Table S1).

As a complementary analysis, 73 MLVA profiles, corresponding to the specimens typed by both mgpB SNP and MLVA, were imported to the BioNumerics software package (Applied Maths), and the genetic relationships of these specimens were deduced by constructing a hierarchical dendrogram (Fig. 1(b)). This population modelling lacked distinct clusters, highlighting the diversity among the tested specimens. There was no obvious link between the MLVA type and the specimen collection year, patient age, patient gender, clinical syndrome, isolation site or geographical origin.

**Comparison of the three molecular typing methods for M. genitalium**

The MLVA scheme was compared to mgpB SNP typing and to the MG309-STR and mgpB SNP combination analysis recently described (Ma et al., 2008). The diversity index (DI) for each method was calculated using 39 specimens with a single allele at the four loci (Supplementary Table S1). In practice, the DI should be at least 0.90–0.95 for a typing system to be considered more-or-less ideal. The DIs were 1.00 for the MLVA method, 0.853 for the mgpB SNP assay and 0.989 for the MG309-STR and mgpB SNP combination analysis (Table 2). No correlations were detected between the MLVA assay and the two other methods (Fig. 1). Furthermore, a congruence analysis of the MLVA assay with each of the two other methods,
calculated with the BioNumerics software, confirmed the absence of, or a very low, congruence.

**DISCUSSION**

In this study, we applied the two main molecular typing methods described, based on the *mgpB* SNP typing, to our collection of *M. genitalium*-positive specimens and evaluated the applicability of MLVA. We performed these molecular typing methods on a group of 76 geographically diverse and temporally separated specimens from unrelated French and Tunisian patients. Eight new STs were described by the *mgpB* SNP typing method, increasing the number of *mgpB* STs to 88. Both *mgpB* SNP analysis and MG309-STR-*mgpB* SNP combination analysis revealed a genetic heterogeneity suggesting that *M. genitalium* was endemic in France and Tunisia and that infections were not the consequence of the clonal dissemination of a particular strain. These observations have been described for specimens from other geographical areas that were typed by the *mgpB* SNP assay (Hjorth *et al.*, 2006).

We also tried to set up an MLVA assay with four VNTR markers. The design of this set of VNTRs was difficult. Indeed, only six discriminatory markers were identified from the 47 VNTRs initially tested in the 13 screening strains, and only four VNTRs had adequate discriminatory power to be included in the final test for use on specimens. All the specimens were typed, 25 of them after a nested/semi-nested PCR. In comparison, the *mgpB* SNP typing resulted in amplification of DNA from 96% of the specimens, higher than the percentage reported in a recent study using this typing method (38.9%) (Musatovova & Baseman, 2009). Ma *et al.* (2008) had already applied nested PCR to those specimens for which the initial PCR products were not visible or very faint on agarose gels. In our study, the use of nested/semi-nested PCR seemed to be correlated with low amounts of *M. genitalium* DNA in specimens. In fact, it has been shown previously that genital specimens contain low amounts of *M. genitalium* DNA (Jensen *et al.*, 2004).

Among the three typing systems, the MLVA assay was the most discriminatory method, with the highest DI, followed by the combination marker analysis and the *mgpB* SNP method.

One disadvantage of MLVA for *M. genitalium* typing was the presence of mixed repeat patterns that were identified at certain loci in 36.8% of the specimens; this complicates the analysis of the results. However, these mixed patterns could be separated and their copy number quantified by fluorescent fragment sizing without sequencing. The mixed patterns were controlled in our study by subcloning and sequencing two representative specimens. Mixed repeat patterns have been described in *M. genitalium* for the MG307, MG309 and MG338 TRs, and the mixed alleles were identical between couples who were infected simultaneously, confirming the stability of these highly discriminatory genotypes (Ma *et al.*, 2008). Mixed TR alleles are believed to be caused by slipped strand mispairing during DNA replication rather than co-infection with multiple strains (Levinson & Gutman, 1987).

The two ATCC strains G37 and M30 had identical MLVA types that were previously assigned using *mgpB* SNP typing (Hjorth *et al.*, 2006). We confirmed that isolate M30 early was different from the M30 strain, as originally described with *mgpB* SNP typing (Hjorth *et al.*, 2006). No clustering corresponding to geographical location, collection year, specimen origin, patient age, patient gender or clinical syndrome was observed for any of the specimens tested with the MLVA method.

Although MLVA was used successfully for typing all the specimens, when evaluating the reproducibility of MLVA with a group of 24 specimens that was composed of sets of two specimens taken from 12 symptomatic patients at the same time, matching genotype profiles were not observed in three out of the 12 patients. In contrast, although the *mgpB* SNP analysis did not amplify three of the 24 concurrent specimens, all the others, except one pair, (corresponding to eight out of nine patients) harboured identical or matching profiles with the two *mgpB*-based methods.

*In vitro* stability was assessed by looking at five broth-grown *M. genitalium* isolates after 10 passages. Although this is a start, it is not enough to confirm *in vivo* stability. For that purpose, longitudinally obtained clinical specimens would be needed. Only one such series was studied, namely the three sequential isolates M6151, M6090 and M6312. These three isolates, obtained from the same patient on the day of testing as well as 6 and 11 weeks later, differed in MLVA type, one isolate (M6090, taken at 6 weeks) harbouring five TRs on locus MG307 instead of seven, as seen in the two other isolates. In contrast, they had identical profiles by the *mgpB*-based methods (Supplementary Table S1). Although a recontamination could not be excluded, as no information was available about the sexual behaviour of this patient, this suggests instability of the VNTRs *in vivo*. Unfortunately, as sexually transmitted infections are managed anonymously in France, we were not able to evaluate our MLVA scheme for its ability to detect concordant isolates between sexual partners and among sexual networks. Finally, according to correlations between the MLVA results and the *mgpB*-based typing results, as well as the level of variability and the *in vivo* stability problems, the MLVA assay developed here seemed to lack the ability to identify transmission among sexual networks.

In summary, we have described the molecular typing of the first French and Tunisian collection of *M. genitalium*-positive specimens using the well-characterized *mgpB* SNP typing system and a more recently developed system involving a combination of a VNTR and *mgpB* SNP analyses. The MLVA method described here was not tested on samples from known sexual partners. Results gave a
high level of discrimination and this MLVA scheme is considered to be unsuitable for the molecular typing of *M. genitalium* in sexual networks. Therefore, according to the discriminatory power and the feasibility of each *mgpB*-based method, we recommend that *mgpB* analysis be used for general epidemiological studies and the MG309-STR and *mgpB* SNP combination analysis be used for studying *M. genitalium* infection in sexual networks; we do not recommend MLVA for *M. genitalium* discriminatory studies.

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