JOURNAL OF CLINICAL MICROBIOLOGY, June 2006, p. 2078–2083 Vol. 44, No. 6
0095-1137/06/$08.00+0 doi:10.1128/JCM.00003-06
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Sequence-Based Typing of *Mycoplasma genitalium* Reveals Sexual Transmission†

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Received 2 January 2006/Returned for modification 9 February 2006/Accepted 22 March 2006

***Mycoplasma genitalium* causes male nonchlamydial, nongonococcal urethritis and is associated with cervicitis and pelvic inflammatory disease in women. Epidemiological studies indicate that *M. genitalium* is sexually transmitted, and the aim of the present study was to further substantiate this by means of a DNA typing system. A typing assay based on a diagnostic *mgpB* gene PCR was developed, evaluated, and applied directly to urogenital specimens. The assay had a low limit of detection and hence a high typeability. Sequences of isolates from 52 unrelated patients were divided into 29 different sequence types, giving a discriminatory index of 0.95. Two to six *M. genitalium*-positive specimens were collected from each of 44 patients over a median interval of 56 days (range, 11 to 1,395). Forty had the same sequence type in consecutive specimens. Specimens collected from two men were repeatedly positive at intervals of 472 and 1,395 days, respectively, but the sequence types had changed. A new strain was introduced in one sexual dyad, and the sequence types changed subsequently. Seventy-nine *M. genitalium*-positive specimens from 19 couples were investigated, and all partners initially had concordant sequence types, but one couple had discordant types at one time point before a newly introduced strain took over. The present typing system is simple and reproducible and has an excellent discriminatory capacity which might prove useful in studies of sexual networks and for evaluation of treatment failures. In the laboratory, this system may document the uniqueness of newly isolated *M. genitalium* strains.**

Mycoplasma genitalium is a cause of nonchlamydial, nongonococcal urethritis in men (11, 21) and is associated with cervicitis and endometritis in women (2, 5, 19, 23). *M. genitalium* has also been associated with salpingitis and tubal factor infertility, although few studies have addressed these complications (4, 20). The organism is extremely difficult to isolate from clinical specimens; thus, it has not been possible to compare strains using classical microbiological techniques.

At present, the detection of *M. genitalium* is dependent mainly on PCR methods. The PCR can be based on the MgPa-1/MgPa-3 primer set located in conserved regions of the *mgpB* gene and producing an amplified fragment of 281 bp, as previously described (14, 15). This primer set has a very low limit of detection in clinical specimens and is consequently suited for diagnostic use. Furthermore, based on restriction enzyme analysis, it was found in 1991 that the region amplified with these primers was somewhat heterogeneous (15). Since then, sequencing has shown the presence of several mutations in the few available clinical strains compared to the sequence of the type strain *M. genitalium* G37 (13).

The aim of the present study was to document, by DNA-based typing, that *M. genitalium* is transmissible through sexual contact. We determined the discriminatory index (DI), i.e., the likelihood that unrelated strains sampled from the test population would be placed into different typing groups (10), by sequencing specimens from unrelated patients. The stability of the typing system was determined by analyzing patients with consecutive positive specimens.

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MATERIALS AND METHODS

Specimens. *M. genitalium*-positive specimens were obtained from patients recruited in Sweden, Norway, Denmark, Japan, the United States, the United Kingdom, Germany, Russia, and Australia as part of other studies. All studies were approved by the relevant ethical committees. Specimens were first-void urine (FVU) and cervical and urethral swab specimens.

Genetic diversity as expressed by the number of established sequence types was determined by comparing all 267 specimens sequenced, regardless of the availability of information regarding possible contact between the patients.

Reproducibility of the typing system was not evaluated in the classical sense with repeated typing of the same specimen but by selecting 2 male patients with positive *M. genitalium* PCR results for both urethral swab and FVU specimens at 7 time points and 12 female patients with positive specimens from more than one anatomical site, i.e., urethra, cervix, or FVU, at 17 time points.

Stability was analyzed by comparing 156 consecutive *M. genitalium*-positive specimens collected from 36 patients seen in Norway and Sweden as well as 7 specimens collected from patients seen in New Orleans, La., and three consecutive *M. genitalium* isolates (M6090, M6151, and M6312) obtained by culture from a French male patient.

The DI (10) was determined by comparing the number of different sequence types in specimens obtained from 52 unrelated patients from whom information about partners was available. The analysis was extended to include patients from whom information about partners was not available but excluded repeated samples and samples from reported partners. In this extended analysis, 144 patients were included.

Concordance of sequence types between couples was determined by comparing 79 *M. genitalium*-positive specimens from 19 patients with reported sexual

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relationships. Patients having repeatedly positive specimens were included in the stability study, and those with more than one positive specimen from any anatomical site were included in the reproducibility study. The first male FVU specimen from each couple with a sexual relationship was included in the determination of the discriminatory index.

DNA sequencing. PCR products were obtained with the MgPa-1/MgPa-3 primer set (15) using sample preparation and optimized reaction conditions as previously described (12, 14). The PCR products were purified using a QiaQuick PCR purification kit (QIAGEN, Hilden, Germany). Sequencing was performed with the ABI Prism Big Dye terminator reaction kit v. 2.0 (Applied Biosystems, Foster City, CA) and read on an ABI 377 or ABI 3100 genetic analyzer. Both strands of the amplified fragments were sequenced.

The sequences were aligned and compared with the 281-bp sequence located between MgPa-1 and MgPa-3 in the genome of *M. genitalium* strain G37^{T} (GenBank accession number NC 000908) using BioNumerics 4.0 (Applied Maths, Sint-Martens-Latem, Belgium).

RESULTS

Genetic diversity. A comparison of all 267 specimens resulted in 56 different sequence types and 1 mixed sequence. A file containing all 56 sequence types in FASTA format is available for download in the supplemental material. The sequence name includes the name of the prototype sequence, i.e., the specimen number as well as the proposed sequence type number. For strains isolated by culture, the first available strain of the particular type was selected as the prototype sequence, and for the remaining types the name refers to the specimen number included in the calculation of the discriminatory index.

An alignment of the 56 sequences with the *M. genitalium* G37 sequence showing the differences is also available for download in the supplemental material.

Discriminatory index. The sequences from the 52 unrelated patients were divided into 29 different sequence types, and the resulting DI was 0.95 (Fig. 1). Expanding the analysis to include 144 patients from whom partner information was unavailable but excluding repeat specimens and specimens from known partners revealed 54 different sequence types and resulted in a DI of 0.93.

Stability. Two to six *M. genitalium*-positive specimens were obtained from a total of 36 men over a median observation time of 56 days (range, 11 to 1,395). The same sequence type was found in consecutive specimens from 33 of the men. In positive specimens collected from two men at intervals of 472 and 1,395 days, respectively, the sequence types had changed. Both had received treatment and provided several negative specimens during the time interval. They also reported several new partners during the observation period. Thus, they had most likely been infected with new strains. One man was part of a sexual dyad (couple A), and the same sequence type was found in specimens obtained from him at five visits over a 127-day period. On day 87, his female partner provided *M. genitalium*-positive specimens from urethra and cervix with a sequence type identical to that of the index patient. On day 127, this couple attended the clinic together, and a specimen from the index patient had the original sequence type whereas the cervical and urethral swab specimens from his partner had a new sequence type and a mixed type, respectively. No new partners were mentioned in the charts. On day 280, the index patient reattended the clinic, and both the FVU and the urethral swab specimens showed the sequence type found in the female partner's cervical specimen at day 127. The index patient reported that he had not had any partners since the visit at day 127, and it therefore seems likely that the female partner introduced a new strain.

M. genitalium-positive specimens were collected from eight women on two to four visits over a median observation period of 75 days (range, 37 to 201), and seven of the specimens had stable sequence types. The specimens from the female partner of couple A described above, collected over a 40-day interval, had different sequences.

Reproducibility. The 24 specimen sets obtained from two anatomical sites at the same visit yielded the same sequence types except for the urethral and cervical swabs from the female patient in couple A described above; the urethral swab specimen had a mixed sequence, with dual peaks in the chromatogram at the variant positions clearly reflecting the sequences of the old and the newly introduced strains.

Sexual transmission. A total of 79 *M. genitalium*-positive specimens from 19 couples were investigated. For 18 of the couples, nine different sequence types were identified, and the partners of each of the 18 couples had the same sequence type. Five sequence types included only one couple, three sequence types three couples, and one sequence type four couples (Fig. 2). Couple A described above had a new sequence type introduced apart from their initial unique sequence type but had initially had the same sequence types.

DISCUSSION

The present study further substantiates the idea that *M. genitalium* is a sexually transmitted pathogen by investigating 19 couples. For all couples, the sequence type found in specimens from the male partner was identical to that found in the female partner. In one of the couples (couple A), however, a new sequence type appeared to be introduced by the female partner, since cervical specimens obtained at an interval of 40 days differed in sequence type, whereas the urethral specimen obtained at the second visit contained a mixture of the two sequence types. In the male partner, the new sequence type appeared in both the urethral specimen and the first-void urine specimen at the subsequent visit 5 months later.

Sexual transmission of *M. genitalium* has previously been strongly suggested by epidemiological studies which have found a concordance rate of infection between sexual partners ranging from 46 to 63% for female partners of *M. genitalium*-infected male index patients (1, 2, 7, 16).

The present typing system was developed on the basis of a diagnostic PCR assay (15) which allowed for a very low limit of detection and hence a high typeability. The observation that the 281-bp amplicon produced by the MgPa-1/MgPa-3 primer set contained a certain degree of variability was made during the initial validation of the assay. It was noted that only the isolates available from ATCC contained the EcoRI restriction enzyme cleavage site expected from the published sequence. Furthermore, some of the amplicons from clinical specimens also lacked an SspI site found in the type strain (15). In a later study, during the development of a TaqMan quantitative PCR assay, sequencing revealed a much higher heterogeneity than expected, and it was suggested that the MgPa-1/MgPa-3 assay could be applied for molecular typing purposes (13).

Typeability was not systematically evaluated, since specimens producing only faint bands in the MgPa-1/MgPa-3 PCR

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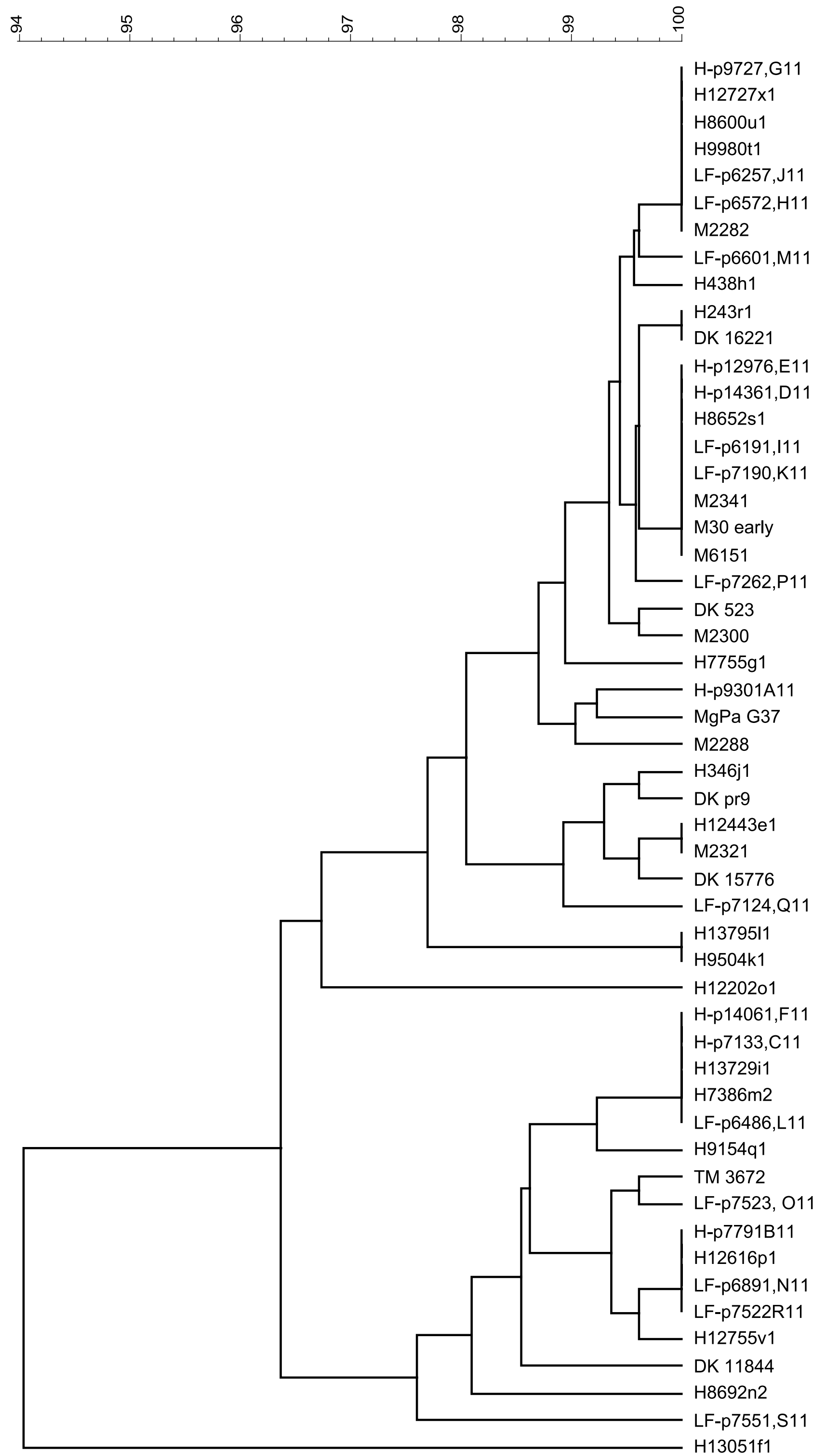

FIG. 1. Dendrogram showing clustering of 52 DNA sequences acquired from urogenital specimens and *M. genitalium* strains obtained from patients with no known sexual relationship.

were not included in the study. It should be possible, however, to reamplify faint bands and produce enough amplicon for sequencing. Except for the urethral specimen from the female partner of couple A which produced ambiguous sequences due to an infection with two different strains, all amplicons produced unambiguous sequences.

The typing system described here was shown to have the excellent discriminatory index of 0.95. In other words, if the *M.*

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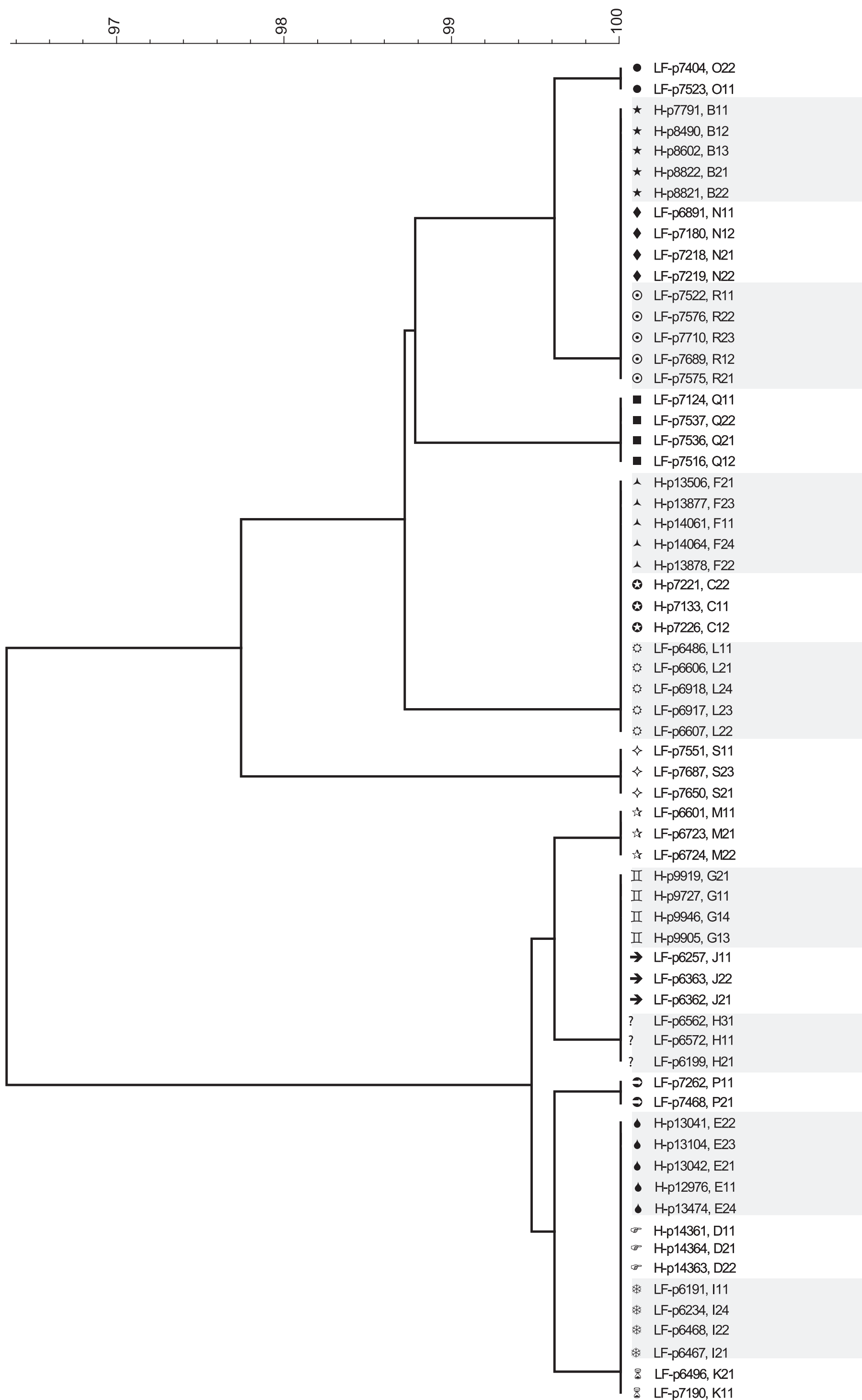

FIG. 2. Dendrogram showing clustering of 65 specimens from 18 *M. genitalium*-positive couples. Some patients provided specimens from several anatomical sites and/or on more than one occasion. The presence of the same sequence at several time points in the cluster demonstrates the stability of the typing system. The gray shading and the symbols to the left of the specimen numbers show specimens belonging to the same couple in sequence type clusters comprising more than one couple. Note that all sequences within couples are the same. Each specimen is listed with its laboratory specimen number, with couples identified by the letters A through S and each letter followed by a two-digit number, with the first digit indicating sex (1, male; 2, female) and the second digit indicating the sequence of the specimen. Thus, 11 denotes the first specimen from the male partner and 23 the third specimen from the female partner.

genitalium strains from two patients were unrelated, they would fall within different sequence types in 95% of the cases. This is well over the 0.90 level suggested by Hunter and Gaston (10) for an efficient typing system. The discriminatory index was determined for a collection of 52 specimens from mostly Scandinavian patients where information about sexual partners

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was available. However, when the panel was expanded to include 144 patients who were expected not to have sexual relations, but where this information was not directly available, the discriminatory index remained as high as 0.93. This group of specimens represented a huge geographical diversity as well as a considerable temporal separation.

Two important aspects of a typing system are reproducibility and stability, i.e., the ability to assign a strain to the same cluster when the typing is repeated or when it is performed on samples taken at various time points. The reproducibility was not investigated by repeating the sequencing of individual specimens but was estimated by examining specimens from different anatomical sites from the same patient and collected at the same time. Only for the female patient from couple A, where a new strain was introduced, was a mixed sequence found in the urethral swab specimen, showing the high reproducibility of the method. Due to the poor treatment efficacy of doxycycline (6), a relatively high number of consecutive *M. genitalium*-positive specimens could be collected. Sequence conservation was documented even up to 895 days after collection of the first specimen, demonstrating the ability of *M. genitalium* to cause persistent infections and the apparent lack of spontaneous clearance of the infection. Only two patients apart from couple A presented with different sequence types, at intervals of 472 and 1,395 days, respectively. These patients, however, had received treatment and submitted negative specimens after treatment before they were reinfected with new strains. These reinfections demonstrate that the protective immunity after a natural infection appears to be limited.

The genetic heterogeneity suggests that *M. genitalium* is endemic in the areas studied here and that infections are not due to the dissemination of a single strain. On the other hand, a few big clusters containing a relatively high number of strains may suggest either a clonal spread of certain, more-virulent strains or a particularly common sequence type, reflecting the limitations of the typing system. One such cluster contained sequence types from specimens collected in Denmark, Norway, Russia, Germany, the United Kingdom, France, Japan, and several sites in Sweden, illustrating the widespread geographic representation of this sequence type. Further discrimination could probably be achieved by combining the MgPa-1/MgPa-3 typing system with elements of the variable-number tandem repeat typing system or of the rRNA sequence typing system, both described by Ma and Martin (18).

With a discriminatory index of 0.95, it was very surprising to find that all seven strains available from the ATCC shared the same sequence type. Although the two original ATCC isolates from the urogenital tract, $G37^T$ and M30, had the same sequence type, this could be explained by a common source yielding strains that were cultivable. However, when an early passage (passage 7) of the M30 strain was acquired from the Mollicutes Collection of Cultures and Antisera, Gainesville, Florida, it lacked the characteristic EcoRI site and clustered together with other strains having another relatively common sequence type. Moreover, the four respiratory tract isolates (3) and a strain isolated from synovial fluid (22) also had sequence types identical to that of the G37 strain. These five strains had all been isolated from cultures of *M. pneumoniae*. Among the other 260 sequences we studied, none had the EcoRI site found in the ATCC strains, and it therefore appears likely that the ATCC strains were contaminated and overgrown with the G37 strain or that the isolates from the extragenital sites were contaminated at some point. These findings are in good agreement with previous studies (17, 18) and may have serious implications in many respects. Except for two studies (8, 9), all antimicrobial susceptibility studies have relied on the ATCC strains and thus underestimate the variability in susceptibility among isolates. Studies of antigen variation and validations of diagnostic PCR assays may also be in danger of misinterpretation due to the lack of strains representing different sequence types.

The present typing system is simple and reproducible and has an excellent discriminatory capacity which might prove useful in studies of sexual networks and for evaluation of treatment failures in terms of whether they are related to reinfection with new strains or to persistence. In the laboratory, this system may also help to document the uniqueness of newly isolated *M. genitalium* strains.

ACKNOWLEDGMENTS

We are grateful for specimens received for *M. genitalium* PCR testing as part of other studies by Harald Moi, Olafiakliniken, Oslo, Norway; David Taylor-Robinson, London, United Kingdom; Ryoichi Hamasuna, Miyazaki, Japan; Ruxandra Boit, Heidelberg, Germany; and Jens Boman, Umeå, Sweden.

This work was supported in part by NIAID grant 1 U19 AI061972-01.

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