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Isolation of *Mycoplasma genitalium* from patients with urogenital infections: first report from the Latin-American region

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Abstract

Isolation of *Mycoplasma genitalium* from clinical specimens remains difficult. We describe a modified culture system based on Vero cells grown in medium 199 with 2% foetal bovine serum (FBS). The culture system was evaluated using early passage *M. genitalium* strains M6271 and M6311 with growth monitoring by quantitative TaqMan PCR. Eleven endocervical swabs and one male urethral swab positive by 16S rRNA and MgPa1–3 PCRs were quantified and inoculated into Vero cell suspensions in medium 199 supplemented with 2% FBS and antibiotics. Cultures were incubated for 14 days. Cell passages and growth monitoring by TaqMan PCR were performed until the growth of *M. genitalium* reached $\geq 10^6$ geq/mL. Confirmation of the new *M. genitalium* strains was performed by sequencing a 281 bp fragment of *mgpB*. The growth of *Mycoplasma genitalium* strains was recorded for all urogenital swab specimens in the modified cell-culture system. Growth of *M. genitalium* was obtained within 2 months and yielded 12 *M. genitalium* strains with all 11 isolates from females of an identical, but unique genotype. To our knowledge, this is the first successful isolation of *M. genitalium* in the Latin-American region. The use of Vero cell culture in 199 medium with 2% FBS is a method comparable to the Ultroser G culture system for isolation of *M. genitalium*. Genotyping of clinical samples and isolates should be performed to document the absence of cross-contamination.

Keywords: Co-culture, isolation, *Mycoplasma genitalium*, qPCR, urogenital
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Introduction

Mycoplasma genitalium was first isolated from urethral swabs from patients with non-gonococcal urethritis in 1980 [1]. This microorganism is now considered an established cause of male non-gonococcal urethritis and female cervicitis [2, 3]. An important improvement in the primary isolation procedure of *M. genitalium* was the use of co-culture in Vero cells [4–6]. This methodology allows growth from clinical samples in approximately 2 months, but is still extremely slow and labour intensive [5, 6].

Vero and Hep-2 cells have been used for co-cultivation and the best results were obtained using a serum-free medium [4]. Nevertheless, foetal bovine serum has shown good growth-promoting capabilities for *M. genitalium* cell-assisted growth and some strains were recovered from urine samples using it [7]. At present, *M. genitalium* strains have been isolated from samples in the Unites States of America, Europe, Japan and Australia [1, 4–6], but no strains have been obtained from the Latin-American region.

In Cuba, a previous study carried out by Rodríguez *et al.* [8] showed a high frequency of *M. genitalium* in men with urethritis but, so far, no strains have been obtained from Cuban patients, and genetic characteristics of the circulating strains remain unknown. The isolation and genetic characterization of *M. genitalium* strains from Cuban patients is important for studies of antimicrobial susceptibilities that permit a better management of urogenital infection in men and women. With the objective of isolating *M. genitalium* strains from urogenital samples of Cuban patients, a modified co-culture method was developed.

Part of this work was presented at the 19th congress of the International Organization for Mycoplasmology (IOM), Toulouse, France, 15–20 July 2012 (abstract no. 109).

Methods

All procedures below were performed in the National Reference Laboratory for Mycoplasma Research at "Pedro Kourí" Tropical Medicine Institute (IPK), Havana, Cuba. This study was approved by the IPK Ethics Committee, and all participants provided informed consent.

Cell culture and *M. genitalium* strains

Vero cells (ATCC CCL-81) were cultured in 199 medium (Gibco, Paisley, Scotland, UK) with 5% foetal bovine serum (FBS; Hyclone, Logan, UT, USA) for 7 days. *M. genitalium* strains M6271 and M6311 only adapted to growth in cell culture (obtained from the Mycoplasma Laboratory at Statens Serum Institut, Denmark) were used as control strains for the evaluation of the culture systems.

Real-time PCR for *M. genitalium* growth monitoring and quantification

LightCycler® (Roche, Mannheim, Germany) TaqMan PCR (qPCR) of a 78 bp fragment of the *mgpB* gene [9] was used. Briefly, an 'in house' PCR mix was prepared, containing per PCR-reaction (20 μL): 1× of 10× PCR reaction buffer (25 mM $MgCl_2$; 2 mM deoxyribonucleotides, 2 mg/mL BSA, 100 mM Tris HCl pH 8 and 500 mM KCl), 75 nM of MgPa-380 probe, 1 μM of MgPa-355F and MgPa-432R primers; and 1 U of *Taq*-polymerase (Qiagen, Hilden, Germany). Five microlitres of DNA extractions obtained by the osmotic and thermic shock method [10] was used as template. In each run, 5 μL of *M. genitalium*-DNA standards with concentrations of 1000, 100, 10 and 1 genome equivalent per μL (geq/μL) were used for construction of the standard curve. The PCR set-up and analysis were performed using a LightCycler® 1.5 equipment (Roche). A two-step program described by Jensen *et al.* in 2004 [9] was used.

Cell culture system for *M. genitalium* isolation and propagation

Vero cells in 199 medium with 2% of Hyclone–FBS, 1× of vancomycin–colistin–nystatin–trimethoprim (VCNT) antimicrobial supplement (Biolife, Milano, Italy) and 500 U/mL of penicillin G (Sigma, St. Louis, MO, USA), was selected for attempting the isolation of *M. genitalium*. The culture systems was evaluated using an early passage of *M. genitalium* strains M6271 and M6311, with growth monitoring by qPCR as described above. Briefly, cell culture supernatants of M6271 and M6311 strains were quantified and adjusted to contain 8×10^5 geq/mL. One millilitre of this standardized inoculum was mixed with 3 mL of Vero-cell suspension (2.5×10^5 cells/mL) in a cell-culture tube (Nunc, Roskilde, Denmark). Cell-culture tubes were incubated at 37°C for 21 days. At days 0, 7, 14 and 21; 0.1 mL of the cell culture supernatant was harvested for monitoring the growth by qPCR.

Isolation of *M. genitalium* from clinical specimens

A modification of the protocols described by Jensen *et al.* [4] and Hamasuna *et al.* [5] was followed for the initial isolation of *M. genitalium* from clinical specimens. Briefly, a total of 12 *M. genitalium* PCR-positive urogenital specimens (11 endocervical and one male-urethral swab) from 11 women and one man with urogenital infections attending the "Pedro Kourí" Tropical Medicine Institute's Hospital, between February and July of 2011 for sexually transmitted diseases, were used. The samples were screened positive by a 16S rRNA PCR [11] for *M. genitalium* and confirmed as positive using the MgPa 1–3 PCR [12]. Quantification of *Mycoplasma genitalium* was performed by qPCR and aliquots of the *M. genitalium*-positive specimens in Friis medium (FB medium) [4] were maintained at −80°C for attempts at isolation.

One millilitre of the frozen aliquot of positive sample was thawed and diluted in 199 medium to obtain a final *M. genitalium* concentration of 2×10^4 geq/mL. Vero-culture tubes containing 3 mL of Vero-cell suspension (2.5×10^4 cells/mL) in 199 medium supplemented with 2% FBS, 1× of VCNT and 500 U/mL of penicillin G, were inoculated with 1 mL of the samples. The cells were observed daily for the first week and then at 2- to 3-day intervals. If cells detached, fresh Vero cells were added (about 2.5×10^4 cells/mL). The medium was not changed during the first 14 days even if it was acidified.

Propagation of *M. genitalium* strains

Attached Vero cells were carefully scraped off 14 days after the initial inoculation, and 2 mL of the cell-containing supernatant was added to a new tube with 2 mL of a fresh Vero cell suspension. At the same time, 0.1 mL was taken for growth monitoring by qPCR. The remaining cell-containing medium was stored at −80°C as back-up. When *M. genitalium* growth was confirmed by qPCR, and the concentration reached 10^6–10^7 geq/mL, cell-containing medium was stored at −80°C for future attempts of axenic isolation.

Sequencing of MgPa-1/MgPa-3 amplicons

Primary confirmation of the new *M. genitalium* strains was performed at the IPK by sequencing the 281 bp fragment of the *mgpB* gene obtained by the confirmatory PCR from genomic DNA of early passage of the strains, using the

methodology described by Hjorth *et al.* [13] PCR products were purified using the QIAquick PCR Purification kit (Qiagen) and sequencing was performed using the Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) the same primers as used for the confirmatory PCR. The sequencing fragments were resolved on a genetic analysis system CEQ 8800 (Beckman Coulter). Finally, the sequences were edited and assembled using Sequencher, version 4.10 (Gene Codes Corporation, Ann Arbor, MI, USA). The genotype was confirmed at Statens Serum Institute (SSI), Denmark, by sequencing the same amplicon described above from the original clinical samples and genomic DNA of early passage of the strains. Phylogenetic analysis was conducted using MEGA software version 5.

Results

Evaluation of the cell culture system for *M. genitalium* isolation and propagation

M. genitalium strains M6271 and M6311 successfully grew in the selected culture system (Vero cells in 199 medium w/2% FBS), showing a similar growth patterns as previously reported for *M. genitalium* strains in cell culture [14]. The growth curve of *M. genitalium* strains obtained by qPCR is shown in Fig. 1. Growth of *Mycoplasma genitalium* was achieved 3 weeks post-inoculation.

Isolation of *M. genitalium* from clinical specimens

The 12 *M. genitalium*-positive samples were quantified by qPCR. All specimens showed *M. genitalium* concentrations greater than 60 geq/μL (Table 1). Growth of the *M. genitalium* strains was recorded from all 11 female urogenital swab specimens and the single male urethral-swab specimen in the modified cell-culture system. Growth in all positive samples was obtained within a period of 2 months and yielded 12 new *M. genitalium* isolates (Table 1). All the isolates had the expected growth patterns as previously described by Hamasuna *et al.* in 2005 [14].

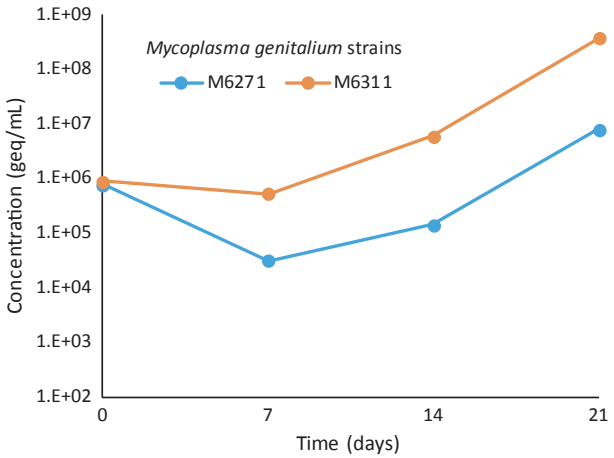

FIG. 1. Growth curves of early passage of *Mycoplasma genitalium* strains M6271 and M6311 in Vero cell culture in 199 medium with 2% foetal bovine serum.

TABLE 1. Urogenital specimens cultured for *Mycoplasma genitalium*

Sample	Specimen type	Concentration of *M. genitalium* by qPCR (geq/μL)	Strain	First day of confirmed *M. genitalium* growth in Vero cells
MICH 1	Endocervical	67	B1	70
RAPH 2	Endocervical	5.4×10^2	B2	42
GAB 3	Endocervical	6.7×10^2	B3	56
JOF 4	Endocervical	6.1×10^2	B4	28
CHAM 5	Endocervical	5.9×10^2	B5	28
URI 6	Endocervical	9.1×10^2	B6	28
ZAQ 7	Endocervical	7.5×10^2	B7	42
JC 8	Endocervical	1.6×10^2	B8	42
PHE 9	Endocervical	2.3×10^2	B9	42
JVH 10	Endocervical	1.5×10^2	B10	42
BAM 11	Endocervical	1.9×10^2	B11	14
347-U	Urethral	5.8×10^4	B12	14

Sequencing of MgPa-1/MgPa-3 amplicons

When the MgPa-typing method was applied on the isolates, the same genotype was found in all the 11 female isolates while the male isolate showed a different genotype (GenBank accession nos. KF017612 and KF017613). All the strains were different to $G37^T$ and to the other *M. genitalium* reference strains that are maintained in our laboratory (Fig. 2). Sequencing results of the clinical samples showed that the male isolate had the same genotype in clinical samples and in cell culture. Unfortunately, none of the female clinical samples yielded any amplicons when sequencing was attempted at SSI. The two genotypes are new and have not been previously reported in the literature.

Discussion

The use of Vero cells for isolation of *M. genitalium* has been shown to be the best method for isolating new strains. The original procedure was published by Jensen *et al.* [4] and Hamasuna *et al.* [5], and described the use of a serum-free medium: minimal essential medium supplemented with Ultroser G serum substitute (Ciphergen, Cergy-Saint-Christophe, France). However, in the present investigation, some modifications were introduced and evaluated with early cell-culture

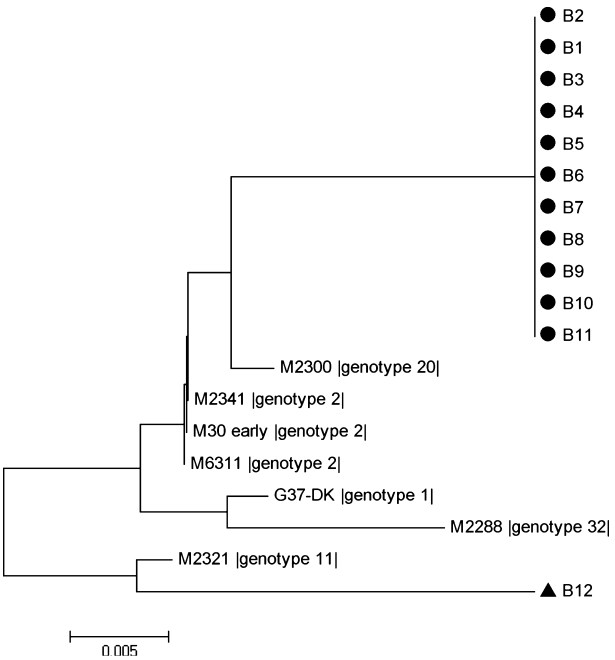

FIG. 2. Dendrogram showing phylogenetic relationship between Cuban isolates (B-strains: black circles identify the female isolates and black triangles identify the male isolate) and *Mycoplasma genitalium* reference strains (G37 and M-strains) maintained at "Pedro Kourí" Tropical Medicine Institute (IPK).

passages of *M. genitalium* clinical strains. The modification of the culture system (cell media and supplements) showed that 199 medium with 2% FCS supports the growth similarly to that reported for minimal essential medium with Ultroser serum substitute [4]. Unfortunately, due to lack of availability of Ultroser G, no direct comparisons could be performed. The good results with the 199 medium make good sense as the CMRL-1069 medium, which is used in the SP-4 culture medium for fastidious mycoplasmas used to isolate *M. genitalium* for the first time [1], is a modification of the 199 medium. At present, only two reports on the use of FBS-containing medium have been published, but none of these used the 199 basal medium [4,7]. The use of a low-serum concentration may permit enough growth-promoting components of the serum but with less *M. genitalium* growth-suppressing activity of antibodies and other compounds in the serum. Although later results with 199 medium with 10% of FBS has shown better grown-promoting properties for a few strains others have shown a slower grown rate than in 199 medium with 2% FBS (data not shown). Consequently, we recommend the use of 2% FBS as the optimal concentration.

Due to the heavy bacterial contamination of female samples, an additional antimicrobial supplement was needed. The VCNT supplement was chosen because it is used routinely in the selective media for *Neisseria gonorrhoeae* [15]. Additionally, penicillin was included as this antibiotic has been shown not to interfere with the growth of *M. genitalium*, although it could be argued that the spectrum of this antibiotic was covered already. The antibiotic mixture proved to be highly efficient as only the B1 isolate was contaminated with bacterial over-growth at 7 days post-inoculation, but after a 0.45 μm filtration, the bacterial contamination was eliminated and the *M. genitalium* strain was recovered.

The MgPa-1/3 genotyping clearly showed that all the strains were different to $G37^T$ and the other *M. genitalium* reference strains that are maintained in our laboratory (Fig. 2). However, the high genetic homogeneity found in the female isolates could probably only be explained by some unfortunate cross-contamination between the cultures during the prolonged handling of the cell cultures. Although the risk of cross-contamination is well known, and although all possible care was taken during the propagation of the cell lines, it seems unlikely that the genotypes of all specimens should be identical. Unfortunately, confirmation of the genotype directly from the clinical specimens failed for technical reasons, so it is impossible to determine if more than one of the female samples were of the same genotype and leading to new, unique strains. However, the two genotypes found in the male and female specimens were unique when compared to previous entries in the public databases, and thus, represent new strains. Future sequencing studies will be performed to assess the genotype distribution in the Cuban population.

To our knowledge, this is the first successful isolation of *M. genitalium* in the Latin-American region. Some previous attempts using direct culture of clinical samples in SP-4 and Friis media have been undertaken by our group but without any successful results.

In conclusion, we successfully implemented a modification of the co-culture method allowing us to isolate *M. genitalium* strains from at least two urogenital specimens from Cuban patients. Although 12 specimens from PCR positive specimens yielded cultivable strains we could only prove that two of them represented genuine new strains stressing the need for genotyping of both the clinical sample and the isolated strain. Furthermore, the procedure is still slow and labour intensive and other improvements in the culture methodology would be advantageous. Future work will focus on adaptation of the strains to growth in axenic culture and on determining the antibiotic susceptibility profile which is completely unknown in Cuba at present. This will guide the optimal treatment of *M. genitalium* infections in Cuba and possibly also in other Latin-American countries.

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