Isolation of *Mycoplasma genitalium* Strains from the Male Urethra

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Mycoplasma genitalium is a human mycoplasma species which, on the basis of detection by PCR, has been incriminated as a cause of nongonococcal urethritis. Previously, only two strains from the urogenital tract and five strains from extragenital sites have been isolated. We have developed a method for the isolation of this fastidious microbe. *M. genitalium* from PCR-positive urethral specimens was initially propagated in Vero cell cultures grown in serum-free medium supplemented with Ultrosor HY serum substitute. Growth was monitored by PCR. The *M. genitalium* strains grown in cell cultures could subsequently be subcultured in modified Friis's FF broth medium. Several passages in broth medium were required before growth on agar medium was attained. A total of 11 urethral specimens positive for *M. genitalium* by PCR from male patients with urethritis were investigated. Six strains were adapted to growth in broth medium, and four of these strains were cloned. Three specimens were overgrown by other mycoplasmas during propagation in the cell cultures. In only two PCR-positive specimens was propagation of *M. genitalium* unsuccessful. The use of cell culture combined with PCR monitoring of mycoplasmal growth may prove to be more widely applicable for the isolation of other fastidious mollicutes.

In 1980 a new mycoplasma species was isolated from urethral specimens from 2 of 13 men with nongonococcal urethritis (25). It was later named *Mycoplasma genitalium* because of the host tissue location (26). This mycoplasma shared several properties with *Mycoplasma pneumoniae* such as the flask shape and a terminal tip-like structure. Although DNA from *M. genitalium* showed only 1.8% base sequence homology with *M. pneumoniae* (16), the significant antigenic relationship between the two mycoplasma species has hampered diagnostic serology (16).

While *M. genitalium* strains were originally isolated from the urogenital tract (25), no other isolates from this site have since been reported, despite repeated attempts (20, 22).

Five strains of *M. genitalium* have been recovered from extragenital sites, including four strains from the throat of recruits with pneumonia (1) and one strain from a patient with polyarthritis following a primary pneumonia (24). However, these five strains were recovered as mixed cultures with *M. pneumoniae*, and the isolates have raised questions as to the main tissue tropism of *M. genitalium*.

We have developed a method for the isolation of this very fastidious microbe. Clinical specimens were obtained from a study on the prevalence of *M. genitalium* in men with and without urethritis (11). Four of the specimens which were known to contain *M. genitalium* DNA, as determined by PCR, yielded new urogenital strains of this species.

MATERIALS AND METHODS

Patients and specimens. Urethral swabs from men with and without urethritis were collected in SP4 medium (27) as part of a study on the prevalence of *M. genitalium* in men attending the Venereal Disease Clinic of Copenhagen (11). The specimens were divided into aliquots and were stored in liquid nitrogen within 12 h of collection and until use. Eleven specimens with positive reactions

in two different *M. genitalium* PCR assays (11, 12) were selected from patients with symptoms of urethritis.

Selection of cell line for initial propagation. In order to determine the optimal growth conditions for *M. genitalium* in cell cultures, the *M. genitalium* type strain, G-37, was inoculated into cultures of Vero African green monkey kidney, HeLa, McCoy, and HL cells grown in Eagle's minimal essential medium (MEM) containing 3% (of a 2.8% [wt/vol] solution) sodium bicarbonate, 10% inactivated fetal calf serum (FCS; Boehringer Mannheim GmbH, Mannheim, Germany), and 500 IU of penicillin per ml.

Selection of cell culture medium for initial propagation. Vero and HeLa cells were adapted to growth in Eagle's MEM without FCS but supplemented with 2% Ultrosor HY serum substitute (Gibco, BRL, Life Technologies, Roskilde, Denmark) by successive passages in medium with decreasing amounts of FCS and increasing amounts of Ultrosor HY by the procedure recommended by the manufacturer. Vero and HeLa cells were likewise adapted to growth in SP4 medium with 500 IU of penicillin per ml as the sole antibiotic. The adapted cell lines were inoculated with *M. genitalium* G37^T and with two urethral specimens positive for *M. genitalium* by PCR (containing strains M 2288 and M 2304). Growth was monitored by determination of the numbers of CFU and color-changing units (for *M. genitalium* G37^T) and by PCR performed on 10-fold serial dilutions of the pretreated supernatant before PCR.

Propagation of *M. genitalium* from clinical specimens in cell culture. Urethral specimens were collected in 1.8 ml of SP4 broth, and some of those specimens which showed positive reactions in the *M. genitalium* PCR were selected.

Three serial 10-fold dilutions of the clinical specimen were carried out in SP4 medium. Vero cells grown in serum-free medium supplemented with Ultrosor HY were used immediately after trypsinization. Approximately 8×10^5 cells in a volume of 2 ml were mixed with 0.2 ml of each dilution of the clinical specimen; 0.2 ml was stored at -80°C for later use in the PCR. The cultures were incubated in tissue culture tubes with a flat side (Nunc, Roskilde, Denmark) at 37°C for 3 to 4 days. The supernatant was aspirated, and a 0.2-ml aliquot was used for PCR (12) to monitor the growth. Adhering cells were trypsinized or scraped off and were resuspended in 4 ml of fresh medium; 2 ml was frozen in liquid nitrogen and 2 ml was incubated in tissue culture tubes for another 3 to 4 days before repeated passage. When the PCR became strongly positive (usually after three to five passages) the culture was expanded to grow in 10-ml tissue culture flasks (Nunc) by adding 1 ml of the harvested infected cell culture to 9 ml of a suspension of uninfected Vero cells. The resulting 1:10 dilution was necessary to avoid overgrowth of the culture and killing of the cells by the mycoplasmas.

Monitoring of growth by PCR. The growth of *M. genitalium* was monitored by testing the cell culture supernatant by PCR (12) either directly to verify growth or after 10-fold serial dilutions of the pretreated specimen in order to determine the propagation factor. Briefly, 200 μl of the cell culture supernatant was centrifuged and the pellet was resuspended in 100 μl of lysis buffer (10 mM Tris [pH 8.0], 1 mM EDTA, 0.5% Tween 20, 0.5% Nonidet P-40, and 200 μg of proteinase K per ml), and the mixture was incubated at 50°C for 30 min and at 94°C for 15 min. A 20- μl aliquot of the specimen was subjected to PCR for 40 cycles with primers that amplify a 281-bp fragment of the MgPa adhesin gene (12). No attempts were made to estimate the amount of DNA subjected to amplification.

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The quantity of amplified DNA produced from the cell culture-grown *M. genitalium* cells was estimated by comparing the intensities of the bands in an ethidium bromide-stained agarose gel with those of the bands produced by amplification of known amounts of purified *M. genitalium* DNA. This semiquantitative technique was used in order to standardize the assay. The highest 10-fold dilution of the specimen producing a visible band was used as an estimate of the amount of *M. genitalium* cells present in the cell culture supernatant. The concentration at this dilution corresponded to approximately 5 to 10 genome copies when purified DNA was added as the template.

Evaluation of mycoplasma media for optimal growth of *M. genitalium*. Initial studies with *M. genitalium* G-37^T as the test strain titrated in broth medium had shown that SP4 (27) and Friis's modified FF medium (19) were superior to our Hayflick-type medium (16) without thallium acetate, to the soy-peptone medium described by Kenny et al. (13), and to Friis's FF medium without animal serum and yeast extract but supplemented with 10% of a 10% suspension of egg yolk in phosphate-buffered saline, as described by Sasaki et al. (21). Hence, different modifications of the SP4 medium and Friis's FF medium were evaluated.

Both media were prepared with three different brands of FCS purchased from Gibco or Boehringer Mannheim. Horse serum from six different horses (Statens Seruminstitut) and two batches of pooled horse serum (Gibco BRL) were evaluated. The best horse serum was tested in final concentrations of 15, 20, and 25%. The animal serum was substituted with 2% Ultrosor HY. Yeast extract purchased from Difco (Detroit, Mich.) was evaluated against a homemade fresh yeast extract. For preparation of the solid medium, 11 different agaroses purchased from various suppliers and Phytigel agar substitute (Sigma) were tested.

Modified Friis's medium used for isolation of *M. genitalium*. The Friis's broth medium (FB medium) for the isolation of *M. genitalium* consisted of 78% Friis's basal medium (64% Hanks' balanced salt solution, 0.4% [wt/vol] brain heart infusion [Difco], 0.42% [wt/vol] PPLO broth without crystal violet [Difco]) in ultrapure water (Milli Q) supplemented with 5.8% fresh yeast extract prepared from the sterile-filtered (pore size, 0.22 µm; Millipore, Tåstrup, Denmark) centrifuged supernatant of 50% fresh baker's yeast (De Danske Spritfabrikker, Grenå, Denmark) in ultrapure water subjected to acid hydrolysis (pH 4.5) at 80°C for 30 min, 1% (wt/vol) glucose; penicillin G at 500 U/ml, 0.002% (wt/vol) phenol red, and 14.3% heat-treated (56°C for 30 min) horse serum from a single selected horse. The horse serum was stored at 4°C for at least 1 month before use (5a).

Solid medium (FA medium) was prepared by mixing the heat-labile components with 10% of the final volume of Hanks' balanced salt solution containing 1 mg of DEAE dextran per ml and Litex HSA agarose at 56°C to yield a final agarose concentration of 0.5%. The final pH of the medium was adjusted to 7.4.

Preparation of conditioned broth (FBC) and agar (FAC) media. Conditioned cell culture medium was prepared by sterile filtration (pore size, 0.22 µm) of the supernatant of Vero cells grown for 3 days in Eagle's MEM supplemented with 2% of Ultrosor HY serum substitute. Friis's basal medium was partly substituted with conditioned cell culture medium corresponding to 50% of the final volume of the medium. Solid medium was prepared as described above.

Cloning of new strains of *M. genitalium*. Two of the *M. genitalium* strains (strains M 2300 and M 2341) were cloned three times by standard filtration cloning procedures (19). Because of the inability of strains M 2288 and M 2321 to form colonies on solid media after filtration through 0.45-µm-pore-size filters, these two strains were cloned four times by limiting dilution in FB medium after filtration or disruption of microcolonies by forcing the culture through a 25-gauge needle several times (18).

Immunoperoxidase staining of *M. genitalium* colonies. For serological identification of the colonies, a biotinylated rabbit anti-*M. genitalium* antibody (16) and a biotinylated monoclonal antibody (MAb) designated HAK 10 were used (28). HAK 10 reacted specifically with *M. genitalium*, recognizing an epitope with an apparent molecular mass of 140 kDa and exhibiting no cross-reactions with *M. pneumoniae*. A biotinylated MAb designated HAK 3 was used as a negative control. HAK 3 reacts specifically with the P1 adhesin of *M. pneumoniae* (28). Blocks of agar with colonies of *M. genitalium* and *M. pneumoniae* were placed in sterile 24-well tissue culture plates (Costar, Cambridge, Mass.), and the agar blocks were washed three times for 5 min each time in 0.9% NaCl in double-distilled water (DS) containing 0.1% thimerosal; incubated with HAK 10 (1:10,000), HAK 3 (1:1,000), or rabbit anti-*M. genitalium* (1:1,000) for 1 h; washed three times for 5 min each time; incubated for 30 min with horseradish peroxidase-conjugated streptavidin (DAKO, Glostrup, Denmark) diluted 1:1,000; and washed two times for 5 min each time in DS and 5 min in citrate-phosphate buffer (pH 5.5). All dilutions and washes were in DS, and all steps were performed on a shaker at 37°C. Staining was performed at room temperature with 1 ml of staining solution (4 mg of 3,3',5,5'-tetramethylbenzidine [Sigma] per ml, 4 mg of dextran sulfate (Sigma [D-6001; Sigma] per ml, and 32% ethanol in citrate buffer [pH 5.5] with 0.08 µl of 30% H₂O₂ per ml). Reactive colonies were stained purple within 10 to 30 min.

Hemadsorption. Agar blocks with medium-sized colonies were placed on microscope slides in a humidified box, and two drops of a 0.5% suspension of washed human erythrocytes in DS was flooded over the blocks. After a 10-min incubation at 37°C, one drop of 0.1% methylene blue in DS was added (15). After an additional 10 min of incubation, the agar blocks were carefully washed with DS and the colonies were studied microscopically within 5 min.

FIG. 1. Flowchart showing the isolation of *M. genitalium* strains from PCR-positive urethral specimens obtained from male patients with urethritis.

RESULTS

Optimal cell culture conditions. From experiments with the growth of *M. genitalium* G-37^T in different cell lines, Vero and HeLa cells seemed to promote a more rapid growth than McCoy and HL cells, so we adapted only the Vero and HeLa cell lines to other culture media. *M. genitalium* G-37^T could be propagated to some extent in both cell lines and with the three different cell culture media (Eagle's MEM with FCS or Ultrosor HY and SP4). Differences in growth-promoting capabilities were more pronounced when two clinical specimens (M 2288 and M 2304) were used. The *M. genitalium* strains present in the clinical specimens showed no growth in cells adapted to SP4, and *M. genitalium* from one of the specimens (M 2288) grew very poorly in cells with Eagle's medium with FCS. As to cell lines, Vero and HeLa cells did not grow very well in SP4 medium. Vero and HeLa cells grown in serum-free medium seemed to be clearly superior to all other combinations. HeLa cells grew so rapidly that acid was produced in the medium before the growth of *M. genitalium* was optimal. Consequently, Vero cells grown in Eagle's medium supplemented with Ultrosor HY were chosen for the attempts to isolate *M. genitalium* from clinical specimens.

Propagation of new *M. genitalium* strains in cell culture. Of the 11 specimens positive for *M. genitalium* by PCR, 9 could be propagated for several passages in the cell culture (Fig. 1). Two (containing strains M 2231 and M 2282) of the nine were still PCR positive after three and four passages, respectively, corresponding to two passages more than could be expected from simple dilution of the specimen at the time of inoculation, indicating that some multiplication had occurred but that they were then lost. The inoculum of these two specimens contained 10⁴ and 10³ color-changing units of *Ureaplasma urealyticum*, respectively. Another strain (M 2304) was known to contain *Mycoplasma hominis*; it could be propagated with a positive PCR signal at increasing dilution steps for five to six passages, but then the PCR signal gradually disappeared. Large numbers of *M. hominis* organisms were found by culture on cell-free medium. Strains of *M. genitalium* from the remaining six specimens could be grown continuously in the Vero cell culture and reached concentrations in the cell culture supernatant capable of generating a positive PCR result even after a >10⁶-fold dilution.

Generation time of *M. genitalium* in cell culture. We tried to estimate the generation time of *M. genitalium* M 2300 grown in Vero cell culture by monitoring the growth by PCR. After an initial drop in titer by the time of the first passage, the growth rate was nearly exponential if the effect of dilution by passage

FIG. 2. Growth of *M. genitalium* in Vero cell culture determined by titration of cell culture supernatant and expressed as the highest 10-fold dilution of the clinical specimen giving rise to a positive signal in the PCR. The effect of dilution by passage has been taken into account.

was taken into account (Fig. 2). By using the line of best fit, the mean generation time was calculated to be approximately 16 h. Similar experiments with other strains (although with fewer measurements) indicated that this long doubling time was a characteristic feature of primary isolation of *M. genitalium* in this system.

Optimal cell-free culture medium. We investigated several variations of the SP4 medium and of Friis's modified FF medium. In both the SP4 medium and Friis's medium, horse serum was clearly superior to FCS. This was somewhat surprising, since all previously reported isolations were done with SP4 supplemented with FCS. The inferior growth rate in FCS-supplemented medium was not related to a particular brand or lot of FCS and was evident with any kind of horse serum. We found that commercially available pooled horse serum was inferior to the best sera from single horses but was better than other sera from single horses; hence, a few horses were selected to provide serum for Friis's medium. In initial experiments the effect of the concentration of the serum was limited, although 15% seemed to be marginally superior to higher concentrations. Recent duplications of these experiments with the newly isolated strains of *M. genitalium*, however, have indicated that 20% horse serum is optimal. Substitution of the horse serum with 2% Ultrosor HY did not promote the growth of *M. genitalium* well.

Yeast extract from Difco had a poor performance compared with that of our homemade fresh yeast extract. Different concentrations of the Difco yeast extract were not examined.

Since we experienced serious difficulties in growing colonies of our clinical strains of *M. genitalium* on solid media, we investigated the effects of different brands of agar and agarose. An early passage of strain M 2300 was used, and the numbers, sizes, and morphologies of the colonies were determined on each of 12 solid media. The best solidifying compounds were purified Oxoid agar L28 (0.7%), Litex HSA agarose (0.5%), and Phytigel agar substitute (0.2%); these three were equal in their performance.

The conditioned FBC medium with supernatant from the Vero cells was not superior to our standard FB medium when

they were evaluated with *M. genitalium* strains already adapted to growth in cell-free medium. When run in parallel, FBC medium generally performed better than FB medium in the first two to three passages in broth, and the mycoplasmas grown in FBC medium could subsequently be passed into FB medium. The differences between FA and FAC media were more subtle. One strain grew on FAC medium two broth passages earlier than it grew on FA medium. The other three strains grew on both solid media after the same number of broth passages. FAC medium, however, had the obvious disadvantage that it produced false colonies much more rapidly than FA medium did.

Isolation of *M. genitalium* strains. All six *M. genitalium* strains that could be grown continuously in Vero cell cultures could be adapted to grow in FB or FBC medium after 1 to 19 passages in cells, depending on the strain (Table 1). Two strains lost their viability after four and six passages, respectively, in broth. Four strains could be passed repeatedly in broth, but initially they did not form colonies on agar even after prolonged incubation. After various numbers of passages in broth, however, colonies could be observed. The growth characteristics of each strain have been listed in Table 1.

Description of patients from whom *M. genitalium* was isolated. All of the strains were isolated from male patients with urethritis; in none of the patients were gonococci, chlamydiae, ureaplasmas, or *Mycoplasma hominis* detected by conventional culture. Further information about the patients is listed in Table 2.

Immunoperoxidase staining and hemadsorption of *M. genitalium* colonies. Polyclonal antibodies produced in rabbits against *M. genitalium* and *M. pneumoniae* cross-reacted both in disk growth inhibition tests (4) and by immunoperoxidase staining. Hence, we used a biotinylated anti-*M. genitalium* MAb designated HAK 10 which reacted with the MgPa adhesin. This MAb could be used in a dilution of up to 1:50,000 and stained colonies of *M. genitalium* strains M 2300, M 2321, and M 2341 strongly; colonies of the three strains hemadsorbed and caused methylene blue staining of erythrocytes as an indication of hydrogen peroxide production (15). No reaction

TABLE 1. Growth characteristics of *M. genitalium* strains grown in cell-free mycoplasma media

Strain designation	No. of passages in cell culture before growth in broth	No. of passages in broth before visible colonies on agar	Outcome
M 2288	3	7	Cloned four times by limiting dilution; selected clone not hemadsorbing
M 2298	4	>6	Lost viability after six broth passages
M 2300	8	3	Cloned three times by filtration cloning
M 2321	19 ^a	4	Cloned four times by limiting dilution
M 2341	7	2	Cloned three times by filtration cloning
M 2349	1	>4	Lost viability after four broth passages

^a The cloned derivative originated from a broth culture obtained after 27 cell culture passages.

with the MAb was seen with *M. pneumoniae* or *M. genitalium* M 2288. The lack of reactivity of HAK 10 with strain M 2288 coincided with its inability to hemadsorb human erythrocytes and to adhere to plastic surfaces; the ability to produce hydrogen peroxide was retained, as indicated by the appearance of methylene blue-stained erythrocytes detached from the colonies. The ability to hemadsorb was probably lost during the cloning procedures, since we have recently regrown the strain from an early passage and selected hemadsorption-positive colonies. These derivatives are being cloned and show reactivity with HAK 10.

DISCUSSION

Studies of the role of *M. genitalium* in human disease have been hampered by the extreme difficulty in isolating this mycoplasma species and by the lack of specific serological tests. Attempts to repeat the culture procedure used in the original publication (25) have failed to yield new urogenital strains both in published (20, 22) and in several unpublished studies. PCR-based methods have been used with success to determine the

prevalence of *M. genitalium* in different populations (5, 9, 11, 23). The PCR methods have not previously been validated against other methods, nor has the presence of *M. genitalium* DNA in a clinical specimen been correlated to the presence of viable *M. genitalium* organisms. However, good agreement between the prevalence of *M. genitalium* in comparable groups of patients has been found in different studies (9, 11).

Our laboratory performs tests for infection with mycoplasmas in cell cultures, and we were intrigued by the efficiency of cell cultures as habitats for a wide variety of mycoplasma species. In addition to this, as many as 60 to 80% of cell culture strains of *Mycoplasma hyorhinis* grow well in indicator cell lines, but are very fastidious or uncultivable in cell-free media (8, 17). Growth of fastidious spiroplasmas in cell cultures has also been described (6) and as early as 1960, Chanock et al. (2) reported the growth of *M. pneumoniae* in cell culture. Therefore, we decided to attempt the propagation of *M. genitalium* in cell cultures.

Selection of the optimal cell culture system was performed by growing *M. genitalium* G-37^T in various cell lines and cell culture media. Cells adapted to growth in SP4 medium have been used to isolate *M. pneumoniae* from clinical specimens (14), and because the SP4 medium was originally used for the isolation of *M. genitalium*, we also tried this possibility, but without any success. In order to exclude the possibility that antibodies or other constituents in FCS could inhibit the growth of *M. genitalium*, we evaluated the feasibility of using a serum substitute in the cell culture medium. The composition of Ultrosor HY is proprietary, but in the product information it is stated that it is free of immunoglobulins and has a low protein content. It is claimed to provide all growth-promoting properties otherwise supplied by FCS and to stimulate protein secretion from the cells. The successful use of the Ultrosor HY serum substitute in the isolation of new *M. genitalium* strains may have been the outcome of any of the aforementioned properties of the serum substitute or of a combination of these factors. Only two clinical specimens were used to compare the growth of *M. genitalium* on Vero cells in the different cell culture media. Therefore, we cannot exclude the possibility that some of the other strains could have been isolated without the use of serum-free cell culture medium. However, the superiority of the growth rate of the two *M. genitalium* strains inoculated into the Ultrosor HY-grown cell culture was so evident that we refrained from continuing the comparative studies.

The use of a reliable PCR method was found to be essential for the success of the present study. First, it allowed us to select specimens which were known to contain *M. genitalium* (or its DNA). This selection allowed us to concentrate our efforts on a few specimens instead of using an excessive amount of media and time on each of the clinical specimens in case the negative specimens were in excess. Second, the use of PCR allowed us to monitor the growth of the mycoplasmas, which was impor-

TABLE 2. Description of patients from whom *M. genitalium* was isolated

Strain designation	Age of patient (yr)	Duration of urethritis (days)	Previous urethritis	No. of partners in previous month	Homosexual contacts
M 2288	25	>30	No	0	No
M 2298	23	10	Yes	2	No
M 2300	21	4	Yes	2	No
M 2321	47	30	Yes	1	Yes
M 2341	30	3	Yes	2	No
M 2349	39	4	No	1	No

tant. By this means we were able to discard the broth media which were acidified by the continued growth of Vero cells, and the cell culture passage of strains that lost viability because of the overgrowth of other mycoplasmas could be discontinued; furthermore, by the quantitative PCR approach we were able to estimate the generation time of *M. genitalium* in the Vero cell culture system.

An unexpected advantage of PCR monitoring was the use of restriction enzyme analysis of the amplified DNA in the identification of the strains (to be published elsewhere). The restriction enzyme analysis typing enabled us to ensure that the strain of *M. genitalium* which was finally isolated had the same unique restriction enzyme analysis type that was detected directly from the clinical specimen. In the early phase of the study, one of the cell cultures inadvertently became contaminated with *M. genitalium* G-37^T, and because this strain was already adapted to growth in cell-free media, it was initially misinterpreted as our first success in isolating a new strain.

The long generation time of approximately 16 h for *M. genitalium* in the Vero cell culture system might reflect the fact that this system is not fully optimized; nothing is known about the doubling time in vivo. Clyde (3) determined the doubling time for *M. pneumoniae* grown in monkey kidney cell culture (comparable to Vero cell culture) to be approximately 9 h, and the generation time for *Mycobacterium tuberculosis* on artificial media is almost 20 h (1a). Thus, if these generation times can be extrapolated from the in vitro to the in vivo situation, it may have consequences for the therapeutic use of antibiotics calling for a much longer duration of treatment than is usually applied. Furthermore, we and others have shown that *M. genitalium* can be detected intracellularly when it is grown in cell culture (10, 18). The intracellular location and long doubling time might explain why Hooton et al. (7) found *M. genitalium* more frequently in men with recurrent or persistent nongonococcal urethritis than in other groups. These findings were confirmed in another study by the use of PCR (23), which detected *M. genitalium* in 19% of such patients. A lack of detectable *M. genitalium* DNA coincided with a lack of symptoms after a 6-week course of erythromycin treatment.

The optimal growth medium for *M. genitalium* has not yet been defined. It should be noted that conclusions about the optimal growth medium drawn from experiments with laboratory-adapted strains may not be generalized to the primary isolation of more fastidious strains. For example, the growth rate of *M. genitalium* G-37^T was relatively unaffected by changes in the concentration of horse serum of between 15 and 25% in FB medium, whereas the same experiments performed with a fresh clinical isolate indicated an optimal concentration of 20%. In further studies we would therefore recommend this concentration.

We compared a homemade fresh yeast extract with the commercially available preparation from Difco and found that our own preparation was superior. Yeast extract is known to contain substances inhibitory for some mycoplasma strains (19), and some of the effect might have been caused by different concentrations in the two preparations.

Two clinical strains (M 2298 and M 2349) were lost after 4 and 6 passages, respectively, in broth; we suspect minor changes in the growth-promoting capability of our medium to be the reason. These changes in the quality of the medium were not detected by our usual quality control procedures, which consist of recording the number of color-changing units and the rapidity of acidification of the medium after inoculation of a standard inoculum of *M. genitalium* G-37^T. In order to avoid the occurrence of similar situations, we now store an

aliquot of the broth showing acidification at -80°C until the color change of the passage is observed.

The success of the cell culture enrichment procedure in obtaining new isolates of *M. genitalium* could be explained both by the gradual adaptation to growth in an artificial medium and by the increased inoculum size used to seed the mycoplasma broth medium. However, the observation that subtle changes in the broth medium may be detrimental to the survival—and even more so to the growth—of the new clinical strains might also indicate that success or failure of isolation could be a question of the growth-promoting capabilities of the particular lot of medium used and that the cell culture-grown *M. genitalium* simply serves as a continuous source of viable inoculum.

The development of a method for the isolation of *M. genitalium* represents a major breakthrough in the studies of disease possibly related to this microorganism. Clinical trials combining the isolation of *M. genitalium*, antibiotic susceptibility testing of strains, and antibiotic treatment of patients may help to clarify its pathogenetic significance in human disease. In addition, it will allow us to obtain more knowledge about apparent genetic and antigenic variations among strains.

The initial propagation in cell culture with monitoring of growth by PCR may prove to be a general approach in attempts to isolate other fastidious mollicutes from humans and animals.

REFERENCES

1. Baseman, J. B., S. F. Dallo, J. G. Tully, and D. L. Rose. 1988. Isolation and characterization of *Mycoplasma genitalium* strains from the human respiratory tract. *J. Clin. Microbiol.* **26**:2266–2269.
- 1a. Bauer, J. (Mycobacterial Department, Statens Serum Institut). Personal communication.
2. Chanock, R. M., H. H. Fox, W. D. James, H. H. Bloom, and M. A. Mufson. 1960. Growth of laboratory and naturally occurring strains of Eaton agent in monkey kidney tissue culture. *Proc. Soc. Exp. Biol. Med.* **105**:371–375.
3. Clyde, W. A., Jr. 1963. Studies on growth of Eaton's agent in tissue culture. *Proc. Soc. Exp. Biol. Med.* **112**:905–909.
4. Clyde, W. A., Jr. 1964. Mycoplasma species identification based upon growth inhibition by specific antisera. *J. Immunol.* **95**:958–965.
5. de Barbeyrac, B., C. Bernet Poggi, F. Febrer, H. Renaudin, M. Dupon, and C. Bébér. 1993. Detection of *Mycoplasma pneumoniae* and *Mycoplasma genitalium* in clinical samples by polymerase chain reaction. *Clin. Infect. Dis.* **17**(Suppl. 1):S83–S89.
- 5a. Friis, N. Personal communication.
6. Hackett, K. J., and D. E. Lynn. 1985. Cell-assisted growth of a fastidious spiroplasma. *Science* **230**:825–827.
7. Hooton, T. M., M. C. Roberts, P. L. Roberts, K. K. Holmes, W. E. Stamm, and G. E. Kenny. 1988. Prevalence of *Mycoplasma genitalium* determined by DNA probe in men with urethritis. *Lancet* **i**:266–268.
8. Hopps, H. E., and R. A. Del Giudice. 1984. Cell culture models as ancillary tools in the isolation and characterization of mycoplasmas. *Isr. J. Med. Sci.* **20**:927–930.
9. Horner, P. J., C. B. Gilroy, B. J. Thomas, R. O. Naidoo, and D. Taylor-Robinson. 1993. Association of *Mycoplasma genitalium* with acute non-gonococcal urethritis. *Lancet* **342**:582–585.
10. Jensen, J. S., J. Blom, and K. Lind. 1994. Intracellular location of *Mycoplasma genitalium* in cultured Vero cells as demonstrated by electron microscopy. *Int. J. Exp. Pathol.* **75**:91–98.
11. Jensen, J. S., R. Ørsum, B. Dohn, S. Uldum, A.-M. Worm, and K. Lind. 1993. *Mycoplasma genitalium*: a cause of male urethritis? *Genitourin. Med.* **69**:265–269.
12. Jensen, J. S., S. A. Uldum, J. Søndergård-Andersen, J. Vuust, and K. Lind. 1991. Polymerase chain reaction for detection of *Mycoplasma genitalium* in clinical samples. *J. Clin. Microbiol.* **29**:46–50.
13. Kenny, G. E., G. G. Kaiser, M. K. Cooney, and H. M. Foy. 1990. Diagnosis of *Mycoplasma pneumoniae* pneumonia: sensitivities and specificities of serology with lipid antigen and isolation of the organism on soy peptone medium for identification of infections. *J. Clin. Microbiol.* **28**:2087–2093.
14. Kok, T. W., G. Varkanis, B. P. Marmion, J. Martin, and A. Esterman. 1988. Laboratory diagnosis of *Mycoplasma pneumoniae* infection. 1. Direct detection of antigen in respiratory exudates by enzyme immunoassay. *Epidemiol. Infect.* **101**:669–684.
15. Lind, K. 1970. A simple test for peroxide secretion by mycoplasma. *Acta Pathol. Microbiol. Scand. Sect. B Microbiol. Immunol.* **78**:256–257.
16. Lind, K., B. Ø. Lindhardt, H. J. Schütten, J. Blom, and C. Christiansen. 1984. Serological cross-reactions between *Mycoplasma genitalium* and *Myco-*

- plasma pneumoniae*. J. Clin. Microbiol. **20**:1036–1043.
17. McGarrity, G. J., L. Gamon, and L. L. Coriell. 1980. Detection of *Mycoplasma hyorhinis* infection in cell repository cultures. Cytogenet. Cell Genet. **27**:194–196.
 18. Mernaugh, G. R., S. F. Dallo, S. C. Holt, and J. B. Baseman. 1993. Properties of adhering and nonadhering populations of *Mycoplasma genitalium*. Clin. Infect. Dis. **17**:S69–S78.
 19. Razin, S., and J. G. Tully. 1983. Methods in mycoplasmaology, vol. 1. Mycoplasma characterization. Academic Press, Inc., New York.
 20. Samra, Z., M. Borin, Y. Bukowsky, Y. Lipshitz, and D. Sompolinsky. 1988. Non-occurrence of *Mycoplasma genitalium* in clinical specimens. Eur. J. Clin. Microbiol. Infect. Dis. **7**:49–51.
 21. Sasaki, T., M. Shintani, and K. Kihara. 1983. Utility of egg yolk medium for cultivation of *Mycoplasma pneumoniae*. J. Clin. Microbiol. **18**:1167–1173.
 22. Taylor-Robinson, D., P. M. Furr, and N. F. Hanna. 1985. Microbiological and serological study of non-gonococcal urethritis with special reference to *Mycoplasma genitalium*. Genitourin. Med. **61**:319–324.
 23. Taylor-Robinson, D., C. B. Gilroy, and P. E. Hay. 1993. Occurrence of *Mycoplasma genitalium* in different populations and its clinical significance. Clin. Infect. Dis. **17**(Suppl. 1):S66–S68.
 24. Tully, J. G., and J. B. Baseman. 1991. Mycoplasma. Lancet **337**:1296.
 25. Tully, J. G., D. Taylor-Robinson, R. M. Cole, and D. L. Rose. 1981. A newly discovered mycoplasma in the human urogenital tract. Lancet **i**:1288–1291.
 26. Tully, J. G., D. Taylor-Robinson, D. L. Rose, R. M. Cole, and J. M. Bove. 1983. *Mycoplasma genitalium*, a new species from the human urogenital tract. Int. J. Syst. Bacteriol. **33**:387–396.
 27. Tully, J. G., R. F. Whitcomb, H. F. Clark, and D. L. Williamson. 1977. Pathogenic mycoplasmas: cultivation and vertebrate pathogenicity of a new spiroplasma. Science **195**:892–894.
 28. Uldum, S. A. and J. Søndergård-Andersen. 1987. *Mycoplasma pneumoniae*. Immunological characterization of antigens for use in a diagnostic test. M.Sc. thesis. Statens Seruminstitut, Copenhagen, Denmark. (Summary in English.)