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J Antimicrob Chemother 2022; **77**: 2901–2907
https://doi.org/10.1093/jac/dkac281 Advance Access publication 18 August 2022

Journal of Antimicrobial Chemotherapy

Challenges of *in vitro* propagation and antimicrobial susceptibility testing of *Mycoplasma genitalium*

Rachel Pitt [1]*, Dolcibella Boampong[1], Michaela Day [1], Jorgen Skov Jensen [2] and Michelle Cole [1]

[1]*UK Health Security Agency, 61 Colindale Avenue, London, NW9 5EQ, UK;* [2]*Statens Serum Institut, Copenhagen, Denmark*

*Corresponding author: Rachel.pitt@ukhsa.gov.uk

The sexually transmitted bacterial pathogen *Mycoplasma genitalium* has proved a complex organism to work with in the laboratory setting. Exhibiting an extremely fastidious nature, successful *in vitro* propagation of *M. genitalium* has remained elusive for many researchers. Antimicrobial resistance to both first- and second-line recommended therapies (macrolides and fluoroquinolones, respectively) is commonly reported. However, phenotypic susceptibility testing is not routinely performed, due to the difficulties of *in vitro* growth. Instead, molecular detection of known resistance determinants is used to infer susceptibility/resistance. However, associations between determinant detection and clinical treatment failure are not always clear. Furthermore, molecular assays have limited use for detection of emerging resistance mechanisms. The present review collates and discusses the development of successful culture systems for initial isolation of this organism and current methodologies employed for phenotypic susceptibility testing to aid researchers in this field. As with *Neisseria gonorrhoeae*, future treatment options are extremely limited for *M. genitalium* and, if this sexually transmitted infection is to remain treatable, phenotypic susceptibility testing will play an invaluable role in evaluation of potential therapeutics. As such, retainment of these techniques is imperative.

Introduction

First isolated in 1981 from urethral specimens from men with non-gonococcal urethritis (NGU),[1] *Mycoplasma genitalium* is a recognized cause of NGU in men and cervicitis in women.[2] In addition, infection with *M. genitalium* has been associated with pelvic inflammatory disease, spontaneous abortion and preterm birth.[3] However, data suggest that the majority of infections are asymptomatic.[4,5] True prevalence data are difficult to ascertain, due to disparities in diagnostic testing and reporting for this organism, but studies have demonstrated prevalence rates of up to 17% in patients seeking sexual health care[6,7] and 1%–3% in the general population in high-income countries.[8,9] Similarly to *Chlamydia trachomatis*, risk factors for infection with *M. genitalium* include increased number of sexual partners, non-white ethnicity and younger age (≤24 years old).[10] However, the peak age for infection is later than that for *C. trachomatis*.[8,11] After its initial isolation in the 1980s syndromic management was used until the advent of molecular assays. This is because whilst it is possible to culture *M. genitalium in vitro* it is not a suitable method for primary diagnostic testing as it is laborious, lacks sensitivity and is not timely as the organism grows exceptionally slowly. This is converse to other, less fastidious, *Mycoplasma* species, such as *Mycoplasma hominis*, which are readily isolated in the primary diagnostic laboratory. As such, in the modern laboratory, detection of *M. genitalium* is performed via sensitive and specific nucleic acid amplification tests (NAATs) only.[12]

By nature, *M. genitalium* is susceptible to few classes of antimicrobial and, thus, treatment options are limited. Current guidelines recommend use of the macrolide azithromycin as first-line therapy and the fluoroquinolone moxifloxacin as second-line therapy.[12] Antimicrobial resistance to these treatment options has been increasingly reported. Phenotypic investigation of antimicrobial resistance has been hampered by the lack of clinical isolates, due to the extremely fastidious nature of this organism, the lack of standardized methodology for performing susceptibility assays and the absence of clinical breakpoint criteria for interpretation of results. For practical purposes, antimicrobial resistance has been inferred by detection of mutations in genes associated with resistance. Macrolide resistance mutations (MRMs) are common with 30%–100%[13] of infections reported as resistant and, as such, it is recommended that a molecular macrolide-susceptibility assay is carried out as part of the diagnostic process, prior to treatment.[12,14] Resistance to fluoroquinolone antimicrobials caused by quinolone resistance-associated mutations (QRAMs) is less often reported; however, a recent review estimated prevalence in European countries of 5%,[15] with higher rates of 8% detected at the national reference laboratory in England[10] and one study from China reported 89% of specimens with QRAMs and 88% with dual resistance.[16]

The following report aims to discuss the different methods available for culture and susceptibility testing of *M. genitalium* and the challenges that working with this organism pose.

Biology

M. genitalium is a curious organism with a distinct flask-shaped morphology belonging to the *Mollicutes* (meaning soft skin) class.

It lacks a cell wall and, as such, cannot be visualized using traditional staining methods, such as Gram staining. *M. genitalium* is exceptionally fastidious and has evolved through genomic reduction from a Gram-positive ancestor common to *Clostridia* to its current state.[2] The genome consists of just 580 kb, encoding approximately 480 genes.[17] Measuring 0.6–0.7 μM in length, it is one of the smallest known self-replicating organisms and is considered the best example of a natural minimal cell.[2,17] *M. genitalium* replicates by binary fission in both intracellular and extracellular environments after adhesion to many cell types, including the mucosal epithelia and spermatozoa.[2,18] In fact, studies have shown that *M. genitalium* become intracellular in only 10% of Vero cells *in vitro*.[19] Even compared with other fastidious *Mycoplasma* species, *M. genitalium* grows exceptionally slowly, requiring weeks to months for primary isolation. This is thought, in part, related to the complete reliance of the organism on its host as a source of amino acids, as *M. genitalium* has no genes encoding amino acids.[20]

In vitro propagation of *M. genitalium*

The original strains of *M. genitalium* isolated in 1981 [named G37, which became the type-strain (G37^T), and M30] were cultured after direct inoculation into the complex SP4 mycoplasma medium with incubation for approximately 50 days;[1] relying on observed colour change of a phenol-red indicator as confirmation of growth. This technique, however, proved unsuccessful for other researchers using urogenital specimens[21,22] and, whilst the exact reason behind this is unclear, it has been suggested that the composition of the media used in those initial experiments was somehow unique and subsequently unreproducible.[2] As such, there have been limited reports of *in vitro* isolation of clinical strains of *M. genitalium* from urethral,[23,24] cervical/endocervical[24] and urine[25–27] specimens since, none using the original method/media. However, four *M. genitalium* isolates were described as being recovered from respiratory tract isolates of *Mycoplasma pneumoniae*[28] and one from an *M. pneumoniae* isolate from synovial fluid.[29] These isolates have been deposited in the ATCC strain collection together with the initial *M. genitalium* G37^T and M30 strains from the original isolations. By WGS, however, these strains have been shown to be identical to the G37 strain,[30] whereas an earlier M30 isolate obtained from the Mollicutes Collection (Gainesville, FL, USA) is genetically distinct.[30] Thus, researchers should be aware that the ATCC strains do not reflect any genetic diversity. It also stresses the need for laboratories attempting isolations to do strain typing from the primary specimen as well as from the resulting isolate to document lack of cross-contamination with the laboratory-adapted and rapidly growing G37^T strain.

With the failure of direct inoculation in mycoplasma broth media for *in vitro* isolation, researchers[23] turned to cell culture methods based on the recognized ability of other *Mycoplasma* species (e.g. *Mycoplasma hyorhinis*, *Mycoplasma orale* and *Mycoplasma fermentans*) to infect almost any cell line[31] and evidence that *M. genitalium* could attach to and invade Vero African green monkey kidney cells.[19,32] Co-culture in cells proved an important development for initial propagation of *M. genitalium* and is now the accepted standard method.[33] The specifics of cell lines and media used vary between laboratories, however, and isolation remains labour intensive and slow, with very long incubation periods.[26] No attempts have been made to systematically compare different cell lines and culture media, but probably there will be strains preferring one method, whereas others will grow better using an alternative method.

Vero African green monkey kidney cells adapted to grow in serum-free media [supplemented with a serum substitute, such as Ultroser™ G (Sartorious)][23] and Hep-2 human heteroploidy cells supplemented with FCS[25] have produced good results for isolation of clinical strains of *M. genitalium*. Different base media, including Eagle's modified essential medium (MEM)[23,25,26] and 199 medium,[24] have all been used with success in cell culture models. Irrespective of the base media, supplements are included, such as sodium bicarbonate[26] and yeast extract,[25] to enrich and balance pH and antimicrobials are included to limit contamination of cultures from commensal bacteria (e.g. penicillin G or vancomycin and polymyxin B) and to suppress yeast in the clinical specimens (e.g. amphotericin B or nystatin). Although, high concentrations of antimicrobials in the culture media can inhibit the isolation of *M. genitalium* even when the organism itself is impervious to that antimicrobial.[26] As such, maximal concentrations should be 200 IU/mL penicillin G and 500 μg/mL polymyxin B.[26,33]

Depending on the clinical specimen type, pre-processing prior to infection of the cell line may be required. For swab specimens (e.g. urethral or cervical) an aliquot of swab transport medium may be directly inoculated into the cell culture system. However, urine specimens have been found to be cytotoxic to cell lines when added directly.[26,33] As such, a series of washing steps was developed by researchers, whereby the urine specimen was centrifuged and resuspended in tissue culture media prior to inoculation of the cell lines.[25,26] This process not only removed cytotoxic components of the urine but also concentrated any *M. genitalium* present. Low bacterial load in clinical specimens, characteristic of the slow-growing nature of *M. genitalium*, is a further hindrance of *in vitro* isolation. It follows that the lower the initial load the longer the incubation period would be until isolation is detectable and the more likely for loss during passage or due to contaminants would be. It has been reported that approximately 60% of isolates could grow in Vero cells in a month when bacterial load >1000 copies per 100 μL clinical specimen (swab or urine).[27] In addition, storage of clinical specimens, particularly urine, prior to culture negatively impacts on the success of isolation of *M. genitalium*, with reported viability loss after 2 days of storage of urine specimens at room temperature.[33] As such, specimens should be inoculated into tissue culture as soon as is practicable.

Detection of growth of *M. genitalium* in SP4 relied on observation of a colour change instigated by acidification of a phenol-red pH indicator in the medium, as mentioned previously. However, this method is subjective and not specific to *M. genitalium* and can only be used in axenic culture (i.e. culture systems free of all other living cells) because cultured cells acidify the medium.[33] With the advent of widespread use of first PCR and then real-time PCR, observational interpretation has been superseded by quantitative *M. genitalium*-specific molecular assays.[34–36]

Axenic culture and cloning

Following initial isolation in cells lines, adaptation to axenic growth can be attempted. There is an enormous variation in

the ability of different isolates to grow in mycoplasma broth medium. Some will adapt to axenic growth after high-titre growth has been obtained in cell culture, whereas other isolates appear to be unable to adapt to broth culture. Plating on mycoplasma agar usually requires several passages in broth and some isolates do not grow on solid medium at all. Growth on solid medium is, however, a requirement in order to ensure clonality of the isolate and to obtain strain status. Classical methods require three rounds of filtration-cloning, where 0.45 μM filters are used to ensure single cells,[32] but the filtration step has proven impossible to perform for a high proportion of isolates, so limiting dilution or colony picks after disruption of clumps by passing the culture though a narrow gauge cannula has been used.[23] Initially, frequent loss of isolates during the cloning process was described,[23,26] so keeping a back-up for each step in liquid nitrogen or at −80°C is strongly advised. These additional steps further lengthen the culture timeline of *M. genitalium*, with one group reporting that from initial inoculation of the cell lines to completion of cloning took about one calendar year.[26] Adaptation of the isolates to axenic culture produces strains that require less complex culture methods, but comparability of laboratory-adapted strains that have been in culture for long periods of time with recent clinical isolates must be considered.

Antimicrobial susceptibility, testing and resistance

As stated previously, *M. genitalium* is susceptible to few classes of antimicrobial, namely tetracyclines, macrolides and fluoroquinolones; however, the degree of susceptibility varies.[2] As with other members of the *Mollicutes* class, β-lactam antimicrobials are ineffective, due to the lack of a cell wall,[37] hence their inclusion in cell culture media to limit contamination. *In vitro* data suggest that most *M. genitalium* isolates are susceptible to doxycycline[38] when MICs are interpreted using the closely related *M. pneumoniae* breakpoints[39] as no such breakpoints currently exist for *M. genitalium* specifically. In reality, these breakpoints may not be appropriate as clinical trial data indicate poor efficacy at eradicating infections, with only a 30%–40% cure rate.[40] As such, monotherapy with doxycycline is not recommended; however, there is evidence that pretreating with doxycycline prior to use of a recommended first-line (macrolide) or second-line (fluoroquinolone) therapy increases the chance of treatment success.[4,41] This is thought likely because although doxycycline may not cure the infection it reduces the bacterial load, in turn reducing the likelihood of the presence of resistance-associated mutations.[12] The mechanism by which *M. genitalium* 'resists' doxycycline therapy is at present unknown and currently no correlation between doxycycline MIC *in vitro* and treatment failure or success has been reported.[40] Pretreatment with doxycycline is recommended as standard of care in the UK national guidelines for treatment of *M. genitalium* infection.[12]

Due to the inherent issues with performing culture of *M. genitalium*, phenotypic antimicrobial susceptibility testing (AST) is performed rarely and by only a few specialist laboratories. Consequently, molecular assays (both commercial[42–44] and/or in-house) are widely used to infer susceptibility of isolates in clinical specimens, through the presence or absence of the detection of mutations known to be associated with antimicrobial resistance in the target genes. Macrolide resistance is well characterized in *M. genitalium* and is caused by mutations in region V of the 23S rRNA gene, which forms a component of the 50S ribosomal subunit. Mutations are primarily reported at loci 2058 and 2059 (*Escherichia coli* numbering) and isolates harbouring these mutations have demonstrated high MICs of azithromycin in *in vitro* susceptibility assays.[45] These mutations are also commonly reported in patients who have failed treatment with azithromycin.[46–59]

Fluoroquinolone resistance is less well understood, but is mediated by mutations in the QRDR of the *parC* gene, which encodes topoisomerase IV, an enzyme essential in DNA replication. Whilst many mutations have been reported in this gene[46,48,50,51,53,54,56,60–75] those at amino acid positions S83 and D87 (*M. genitalium* numbering) are most frequently detected and associated with resistance. Indeed, even at these positions there is evidence to suggest certain mutations (S83I, S83R, D87N and D87Y)[76] are more often associated with treatment failure and/or increased MICs than others (e.g. S83N). Whilst mutations have been reported in the *gyrA* QRDR, the association between these and antimicrobial resistance is even less clear than for the *parC* mutations. Where mutations are apparent in *gyrA*, those that contribute to treatment failure seem to occur concurrently with *parC* mutations (e.g. G248T/S83I).[76] It has been reported that treatment failure (and decreased phenotypic susceptibility) with moxifloxacin was more likely to occur if *gyrA* mutation M95I or D99N was present as well as the *parC* mutation S83I, suggesting an additive effect of these mutations.[76,77]

Phenotypic AST

Common to *C. trachomatis*, no standardized methodology exists for phenotypic AST of *M. genitalium*. Whilst published methods are available, all are reliant on successful isolation of the organism in the first instance and, as already discussed, culture of *M. genitalium* is not straightforward and this is itself the limiting factor. As such, any methods used are for research purposes only and cannot be used to guide patient therapy.

Methods employed for AST of *M. genitalium* vary based on the ability of the isolates to grow in axenic culture. Where axenic strains are used, methods follow those recommended for other *Mycoplasma* species, i.e. broth dilution or agar dilution,[78,79] and should follow the CLSI guideline for human mycoplasmas.[80] For *M. genitalium* the broth dilution method employs serial 2-fold dilutions of the antimicrobials titrated in SP4 medium in microtitre plates containing known concentrations of *M. genitalium* isolates.[81–83] Plates are incubated at 37°C and MICs assigned to the lowest concentration of antimicrobial that inhibits colour change of the medium at the time where the control wells without antimicrobial compound change colour. As clinical isolates grow at different rates each susceptibility assay should be analysed independently and only in relation to itself. The agar dilution method is not commonly used for *M. genitalium*[84] as it is better suited to larger series of strains and involves inoculation of dilutions of *M. genitalium* isolates onto agar plates containing serial dilutions of antimicrobial, which are then incubated at 37°C. There has been some debate over the interpretation of these

plates;[78] however, to standardize criteria, MICs should be assigned to the lowest antimicrobial concentration that inhibits colony formation at the time where colonies are formed on the growth control plate and for the dilution that produces a colony count of 30–300 colonies.[80]

As the majority of *M. genitalium* isolates have not been adapted for axenic culture, a method was developed,[27] whereby serial 2-fold dilutions of antimicrobials were titrated into Vero cell cultures containing known concentrations of *M. genitalium* isolates.[64,81,85,86] Culture plates are sealed and incubated at 37°C/5% CO_2 for 3 weeks and supernatant is harvested from each well for DNA extraction and qPCR.[35] MICs are defined as the lowest antibiotic concentration resulting in >99% inhibition of growth in comparison with the antibiotic-free control. Researchers have demonstrated comparability of results obtained using this method with those from the broth dilution method.[27,87]

Future directions and research needs

With increasing reports of antimicrobial resistance to both first- and second-line antimicrobials worldwide, the importance of successful culture and phenotypic AST of *M. genitalium* cannot be overstated. Only through phenotypic testing can novel antimicrobials be assayed to establish their efficacy in inhibiting this clinically important pathogen. Establishment of an *M. genitalium* bio-bank, or further submission to a bio-bank repository already established (e.g. ATCC or NCTC), of cultured clinical isolates with differing genotypic susceptibilities would enable researchers to access representative strains. These strains would be essential in determining the wild-type distribution of *M. genitalium* for different antimicrobials, which in turn would allow establishment of breakpoints for interpretation of AST results.

Culture of this organism is recommended primarily to enhance our understanding and it is not the suggestion of this review that it should be used as a primary diagnostic technique. Provision of universal access to sensitive and specific primary diagnostic NAATs is crucial and should be a priority where these assays are not currently available. In the research laboratory, improvements to the success of primary isolation methodologies are urgently required. Establishing the most appropriate specimen type and additives (e.g. transport buffer) for longer-term preservation of viability of the organism in clinical specimens is required if wider expansion of culture is to be achieved. Due to the length of incubation, complexities of culture methods and associated costs, any *in vitro* isolation should be attempted on NAAT-confirmed *M. genitalium*-positive clinical specimens only. Further to this, as has already been discussed, isolation success is greatly dependent on the initial bacterial load in the clinical specimen. Therefore, it is recommended that bacterial load is quantified prior to inoculation of any cell culture system, but even surprisingly low-load specimens may occasionally lead to an isolate.

Surveillance of antimicrobial resistance in *M. genitalium* is of great importance to facilitate appropriate interventions, promote antimicrobial stewardship and inform treatment guidelines. Currently, surveillance of this organism is in its infancy. Two successful pilot surveillance programmes have been performed in England.[10,88] However, these utilized genotypic methods to infer antimicrobial resistance only. Where resistance mechanisms are well characterized, e.g. for macrolides, using genotypic detection of mutations may be appropriate. However, where mechanisms are less clear, e.g. for fluoroquinolones, application of phenotypic testing may play an important role. However, in reality, culture is likely to play a limited role in surveillance programmes, due to the issues described in this report.

Conclusions

M. genitalium is a clinically relevant pathogen and infection with this organism can result in significant clinical sequelae. The unusual biology and exceptional fastidiousness of this organism present unique challenges to scientists in the laboratory where traditional methods of culture and susceptibility testing are not appropriate.

Whilst cell culture methods have proven successful for isolating *M. genitalium*, the length of incubation time required for this means that it has no practical use in the diagnostic laboratory. The advent of sensitive and specific NAATs for the detection of *M. genitalium*-specific material has revolutionized diagnosis of infection with this organism.

AST, an increasingly important aspect of the diagnostic pathway for *M. genitalium*, is reliant on molecular detection of mutations known to be associated with antimicrobial resistance in other organisms because of the difficulties faced when attempting *in vitro* isolation. Whilst molecular methods are useful for antimicrobials where resistance mechanisms are well characterized, e.g. macrolides, there is limited use where the significance of mutations is less well understood, e.g. *parC* and *gyrA*. In addition, molecular techniques do not allow for understanding clinical treatment failures, for monitoring of emerging resistance or for investigation of susceptibility to new antimicrobial agents. As such, it is imperative that methods for performing phenotypic AST of *M. genitalium* are not lost, albeit in the confines of specialist and research laboratories only. Currently there are less than 10 specialist laboratories worldwide performing *M. genitalium* culture. Ideally, it would be possible to improve and standardize both culture and AST methodologies to allow wider application outside of these specialist laboratories. However, due to the inherent complexities of working with this organism and associated financial burden, application may be limited. This is of particular concern in low-income countries. Of greater importance is providing universal access to primary diagnostic NAATs and molecular AST methodologies.

What is clear is that working with *M. genitalium in vitro* is not straightforward. Cell culture methods are laborious, time-consuming and expensive, with variable success rates; however, if we are to maintain *M. genitalium* as a treatable infection, these hurdles must be overcome.

Acknowledgements

We would like to acknowledge Dr Helen Fifer, Dr John Saunders and Dr Paddy Horner for their contribution to this work.

Funding

This work was supported by funding from the National Institute for Health Research, HPRU in Blood Borne and Sexually Transmitted Infections at University College London, HPRU Competition 2019.

Transparency declarations

J.S.J. serves on scientific advisory boards for Roche, Abbott, Cepheid, Nabriva and GSK, and has received speaker's honoraria from Hologic, Cepheid and SpeeDx, all outside the submitted work. All other authors: none to declare.

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