Experimental Infection of Pig-Tailed Macaques (*Macaca nemestrina*) with *Mycoplasma genitalium*

Gwendolyn E. Wood,^a Dorothy L. Patton,^b Peter K. Cummings,^b
Stefanie L. Iverson-Cabral,^a Patricia A. Totten^{a,c}

Department of Medicine, Division of Allergy and Infectious Diseases,^a Department of Obstetrics and Gynecology,^b and Department of Global Health, Pathobiology Interdisciplinary Program,^c University of Washington, Seattle, Washington, USA

ABSTRACT *Mycoplasma genitalium* is an underappreciated cause of human reproductive tract disease, characterized by persistent, often asymptomatic, infection. Building on our previous experiments using a single female pig-tailed macaque as a model for *M. genitalium* infection (G. E. Wood, S. L. Iverson-Cabral, D. L. Patton, P. K. Cummings, Y. T. Cosgrove Sweeney, and P. A. Totten, Infect Immun 81:2938–2951, 2013, <https://doi.org/10.1128/IAI.01322-12>), we cervically inoculated eight additional animals, two of which were simultaneously inoculated in salpingeal tissue autotransplanted into abdominal pockets. Viable *M. genitalium* persisted in the lower genital tract for 8 weeks in three animals, 4 weeks in two, and 1 week in one; two primates resisted infection. In both animals inoculated in salpingeal pockets, viable *M. genitalium* was recovered for 2 weeks. Recovery of viable *M. genitalium* from lower genital tract specimens was improved by diluting the specimen in broth and by Vero cell coculture. Ascension to upper reproductive tract tissues was not detected, even among three persistently infected animals. *M. genitalium*-specific serum antibodies targeting the immunodominant MgpB and MgpC proteins appeared within 1 week in three animals inoculated both cervically and in salpingeal pockets and in one of three persistently infected animals inoculated only in the cervix. *M. genitalium*-specific IgG, but not IgA, was detected in cervical secretions of serum antibody-positive animals, predominantly against MgpB and MgpC, but was insufficient to clear *M. genitalium* lower tract infection. Our findings further support female pig-tailed macaques as a model of *M. genitalium* infection, persistence, and immune evasion.

KEYWORDS *Mycoplasma genitalium*, animal model, antibody response, persistent infection, primate

Mycoplasma genitalium is a fastidious, cell wall-less bacterium notable for its small size (0.1 μ m), reduced genome (580 kbp), parasitic lifestyle, and apparent specificity for the human host. In men, *M. genitalium* is a frequent cause of acute and chronic nonchlamydial nongonococcal urethritis (1–4), and in women, *M. genitalium* is increasingly recognized for its role in cervicitis, pelvic inflammatory disease, preterm birth, and spontaneous abortion (reviewed in reference 5). Additional studies have implicated *M. genitalium* in tubal factor infertility (6, 7) and endometritis (8). Significantly, *M. genitalium* infection increases cervical shedding (9) and the risk of acquiring and transmitting HIV (10, 11), further highlighting the potential adverse outcomes resulting from this underappreciated bacterial pathogen.

M. genitalium infection can persist for months to years in infected patients (12–19), despite the presence of antibodies to *M. genitalium* in genital exudates of infected women (20) and in the sera of infected men (21). These data suggest that *M. genitalium*

Received 22 August 2016 Returned for modification 19 September 2016 Accepted 7 November 2016

Accepted manuscript posted online 21 November 2016

Citation Wood GE, Patton DL, Cummings PK, Iverson-Cabral SL, Totten PA. 2017. Experimental infection of pig-tailed macaques (*Macaca nemestrina*) with *Mycoplasma genitalium*. Infect Immun 85:e00738-16. <https://doi.org/10.1128/IAI.00738-16>.

Editor Guy H. Palmer, Washington State University

Copyright © 2017 American Society for Microbiology. All Rights Reserved.

Address correspondence to Patricia A. Totten, patotten@uw.edu.

evades the local and systemic immune response, potentially increasing the likelihood of sexual transmission and ascension to the upper reproductive tract. Recent studies suggest that many patients are unaware of their *M. genitalium* infection. For example, Manhart et al. (22) found that none of the *M. genitalium*-positive patients enrolled in the U.S. general population-based AddHealth study reported symptoms of vaginal or urethral discharge, and in a British national survey study, Sonnenberg et al. (23) determined that 94% of men and 56% of women infected with *M. genitalium* were asymptomatic.

The asymptomatic nature of *M. genitalium* infection predicts that patients are unlikely to seek treatment for this pathogen. In addition, accurate and reliable detection of current *M. genitalium* infection is challenging, requiring nucleic acid amplification techniques not yet commercially available in the United States, further hampering appropriate treatment. Growth of this fastidious pathogen from infected patients is impractical, as its recovery from clinical specimens requires lengthy coculture with mammalian cell lines, a technique available in only a few specialized laboratories (24, 25; P. A. Totten, unpublished). Once diagnosed, treatment is complicated by the inherent resistance of *M. genitalium* to cell wall synthesis inhibitors, incomplete effectiveness of doxycycline, and development of azithromycin resistance in many strains (26). Moxifloxacin resistance has been reported (27), and worrisome multidrug resistance is increasing (28).

Despite its clinical importance, the virulence strategies of *M. genitalium* are largely unknown. Among its best-characterized proteins are the immunodominant MgpB and MgpC adhesins located in its complex tip organelle, which is required for attachment to host cells and motility. Sequences within the *mgpB* and *mgpC* genes vary through a process of segmental recombination during which segments of the *mgpBC* expression site exchange DNA with archived partial copies of these genes, known as MgPar sites, scattered throughout the genome (19, 29, 30). Furthermore, *in vitro* experiments demonstrated that adherent *M. genitalium* cultures decrease the integrity of endocervical cells, increasing the passage of HIV through epithelial barriers (31), which provides a molecular explanation for the increased risk of HIV acquisition in *M. genitalium*-infected women (10, 11). *In vitro* epithelial cell models revealed that *M. genitalium* infection induces host defense and inflammation pathways (32, 33), as does a purified fragment of the immunogenic lipoprotein MG309 (34). A serine/threonine phosphatase (MG207) has been implicated in cytotoxicity to culture cells (35), and a peptide methionine sulfoxide reductase (MsrA) promotes survival *in vivo* in the lungs of hamsters (36). The effect of these potential virulence factors on *M. genitalium* pathogenesis could best be assessed in relevant animal models.

In the current study, we explored the ability of pig-tailed macaques (*Macaca nemestrina*), to serve as an animal model to explore the molecular pathogenesis and immunopathogenesis of *M. genitalium*. We previously described experimental infection of a single female pig-tailed macaque (37), simultaneously utilizing cervical and salpingeal pocket models to assess lower and upper genital tract infection, respectively. These experiments demonstrated that (i) chronic infection of the lower genital tract could be established following cervical inoculation with the type strain of *M. genitalium* (G37), (ii) humoral and cervical antibodies specific for *M. genitalium* (including the immunodominant MgpB and MgpC proteins) were produced, and (iii) antigenic variation of MgpB coincided with the production of antibodies to the original infecting sequence that failed to react with an MgpB antigenic variant that predominated at 8 weeks postinoculation (37). The ability of *M. genitalium* to infect upper reproductive tract tissue was demonstrated by the persistence of *M. genitalium* within autotransplanted fallopian tubes for 2 weeks. In light of these promising results, we expanded our preliminary experiments to include eight additional primates inoculated cervically, two of which also underwent the salpingeal pocket model of upper reproductive tract infection, the results of which are reported herein. Additionally, methods for the recovery of viable bacteria from infected sites were optimized, and the production of *M. genitalium*-specific antibodies in serum and cervical exudates was analyzed. Our

FIG 1 Duration of lower genital tract infection in primates inoculated cervically with *M. genitalium* and timing of biopsy specimen collection. All primates were inoculated cervically with viable *M. genitalium* at week 0. Black bars indicate time points at which viable *M. genitalium* was recovered from the lower genital tract by Vero cell coculture, and gray bars indicate times at which viable *M. genitalium* was not detected. Primates indicated within brackets were infected at the same time with the same inoculum. AF, ampulla and fimbria biopsy specimens collected; Cx, cervical biopsy specimens collected. Primates A07071, A07080, and A01220 (described previously [37], shown here for comparison) were also inoculated in multiple salpingeal pockets.

results demonstrate the suitability of the *M. nemestrina* primate model for the study of *M. genitalium* persistence, pathogenesis, and immune response to infection.

RESULTS

In 2013 we published our findings exploring the utility of pig-tailed macaques as an animal model to study the immunopathogenesis of *M. genitalium* infection by inoculating the cervix and multiple salpingeal pockets in a single animal (37). In the current study, we expanded this work by cervically inoculating and following eight additional animals, two of which were also inoculated in autologously transplanted salpingeal pockets. In these experiments we examined (i) methods for detection and recovery of viable organisms during the course of infection, (ii) persistence of lower reproductive tract infection, (iii) ascension to the upper reproductive tract, (iv) infection of autologously transplanted salpingeal tissue, and (v) the antibody response to infection.

Cervical infection of pig-tailed macaques. Analysis of preinoculation specimens (vaginal swab, cervical swab, and cervical cytobrush) indicated that *M. genitalium* DNA and viable bacteria were absent in these primates (data not shown). Subsequently, primates were infected in pairs with the same inoculum as indicated in Fig. 1, and the presence of *M. genitalium* DNA and viable organisms in the lower genital tract was assessed at intervals for 8 weeks by PCR and culture. Including the previously reported pilot primate (37), cervical *M. genitalium* infection was established in seven of nine animals, with bacteria persisting for four to 8 weeks in six animals and for 1 week in one (Fig. 1). Interestingly, two primates resisted infection (Fig. 1). A single primate (A07145) underwent mock cervical infection with phosphate-buffered saline (PBS), and importantly, no *M. genitalium* was recovered from this animal at any time point during this phase of the experiment (data not shown). Following this control experiment, primate A07145 was cervically inoculated with *M. genitalium* and followed for 8 weeks (Fig. 1 and Table 1).

Recovery of viable *M. genitalium* from the lower genital tracts of infected primates. To quantitate viable organisms recovered from the lower genital tract, serial 10-fold dilutions of each specimen were cultured in H broth supplemented with 100 U/ml penicillin, 50 µg/ml vancomycin, and 50 µg/ml colistin (HPVC broth) and observed for color change (from red to yellow), indicating growth of *M. genitalium*. In our

TABLE 1 Recovery of *M. genitalium* from experimentally infected primates^a

		Detection of <i>M. genitalium</i> at wk:																			
Inoculation	Primate	Specimen type	1			2			3			4			6			8			
			qPCR ^b	Broth ^c	Vero ^d	qPCR	Broth	Vero	qPCR	Broth	Vero	qPCR	Broth	Vero	qPCR	Broth	Vero	qPCR	Broth	Vero	
Cervical only	J00106	Vaginal swab	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		Cervical swab	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
	K99329	Cervical cytobrush	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		Vaginal swab	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
	T00039	Cervical swab	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		Cervical cytobrush	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
	A07145	Vaginal swab	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		Cervical swab	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
	Cervical and salpingeal pocket	A07071	Cervical cytobrush	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			Vaginal swab	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A07080		Cervical swab	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		Cervical cytobrush	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
A01220 ^e		Vaginal swab	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		Cervical swab	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		Cervical cytobrush	ND	ND	ND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

^aSpecimens collected prior to inoculation (week -3 and week 0) and 3 weeks after completion of azithromycin treatment (week 12) were negative for *M. genitalium* by qPCR and culture, as were all specimens from primates K02241 and 05073 (data not shown).

^b*M. genitalium* genomes detected in 6 μ l of primate specimen as determined by quantitative PCR. Samples were considered positive (+) if the number of genomes was more than twice that of background levels in preinoculation specimens. -, *M. genitalium* DNA not detected; ND, not determined.

^cGrowth of *M. genitalium* in HPV broth was determined by color change (red to orange or yellow) in any of five tubes inoculated with 10-fold serial dilutions (10^{-1} to 10^{-5}) of primate specimen after 3 to 4 weeks of incubation. +, growth; -, no growth; ND, not determined.

^dGrowth in Vero cell cocultures, determined by detecting *M. genitalium* genomes in 6 μ l of culture supernatant by qPCR after 3 to 4 weeks of incubation. Positive cultures (+) contained 6×10^5 to 4×10^6 genomes per ml of culture supernatant.

^eResults for primate A01220, previously described (37), are shown here for comparison. Specimens were collected at week 5 instead of week 6.

TABLE 2 Growth inhibition of *M. genitalium* recovered from lower genital tract specimens of primate J00106^a

–Log dilution	Vaginal swab		Cervical swab		Cervical cytobrush	
	qPCR ^b	Color ^c	qPCR	Color	qPCR	Color
1	1.50×10^3	Pink	1.13×10^5	Red	3.22×10^4	Red
2	4.84×10^3	Pink	5.58×10^8	Yellow	3.86×10^7	Red
3	1.29×10^6	Red	4.89×10^8	Yellow	2.12×10^8	Yellow
4	$<1 \times 10^3$	Red	3.57×10^4	Red	7.73×10^4	Red
5	$<1 \times 10^3$	Red	1.14×10^4	Red	1.07×10^4	Red

^aTenfold serial dilutions of 0.3 ml of each specimen were made in 2.7 ml HPVC broth and then incubated at 37°C for 24 days. Colors were recorded, and the number of genomes was determined by qPCR. Specimens shown were collected 1 week after cervical inoculation.

^bGenomes per ml of HPVC broth.

^cPink, alkaline; red, neutral; yellow, acidic.

first iteration of the experiment (Fig. 1), cultures of the 10^{-2} and 10^{-3} dilutions of specimen from primate J00106 unexpectedly turned yellow before the 10^{-1} dilution even though they contained 10- and 100-fold less specimen (Table 2). These results are consistent with the lower number of genomes detected by quantitative PCR (qPCR) in the specimens diluted 10^{-1} and grown for 24 days and with the finding that the H broth turned yellow only when the cultures obtained a density of $>2 \times 10^8$ genomes per ml. Conversely, the medium turned pink (indicating an increase in pH) in lower dilutions of the vaginal specimens, suggesting the growth of other bacteria (such as *Mycoplasma hominis*) that produce alkaline end products in this medium. As an alternative strategy to enumerate viable *M. genitalium*, HPVC agar plates were inoculated with fresh primate specimen (or with HPVC broth cultures of specimens); however, the specific identification of *M. genitalium* was masked by the growth of numerous microscopic colonies that lacked the typical *M. genitalium* colony morphology (data not shown). Together, these results suggest that components of the primate specimens or unidentified normal flora impeded our ability to detect, isolate, and enumerate viable *M. genitalium* from the cervical and vaginal primate specimens in low dilutions of the primate specimens.

Although the *M. genitalium* strain inoculated in primates (strain G37-C) grows axenically, our observation that primate genital tract secretions inhibit *in vitro* growth prompted us to consider alternate culture methods to optimize detection of viable *M. genitalium*. Growth of clinical strains of *M. genitalium* directly from human patients is enhanced by, and is usually dependent on, coculture with mammalian cells (24, 25). To determine if such techniques could improve our recovery of viable *M. genitalium* from primate genital tissues, we inoculated primate genital tract specimens into Vero cell cocultures, monitored *M. genitalium* growth by qPCR, and compared these results to those for growth detected by color change in HPVC broth. Among the 144 total lower genital tract specimens collected from the six primates infected cervically in this study (including two primates that resisted infection, which are not shown in Table 1), 57 (39.6%) were positive for growth by color change in HPVC broth (at any dilution), while 74 specimens (51.4%) were positive in Vero cell coculture by qPCR (Table 1) ($P < 0.0001$ for difference in recovery between HPVC broth and Vero cell coculture). All 57 lower tract specimens that were HPVC broth positive were also positive for growth in Vero cell coculture. These results were unexpected given that the HPVC broth cultures were inoculated with more specimen (0.3 ml) than Vero cell cocultures (0.2 ml), but they confirmed that growth in HPVC broth was inhibited in these primate specimens. Among the 17 HPVC broth-negative specimens that grew in Vero cell cocultures, eight were vaginal swabs, five were cervical swabs, and four were cervical cytobrush specimens, indicating that growth inhibition in HPVC broth was not specific to sample collection site. Additionally, this inhibition of *M. genitalium* growth in HPVC broth was common to all seven primates with cervical infections (Table 1 and data not shown). Interestingly, *M. genitalium* proliferated in Vero cells at dilutions of primate specimen (1:43) that were inhibited in HPVC broth, perhaps because Vero cells provide a more

robust culture environment or because the polymyxin B added to Vero cell cocultures inhibited growth of competitor/inhibitor bacteria. These results confirm that measuring *M. genitalium* strain G37 growth by color change in HPVC broth culture is inadequate for detecting viable bacteria in lower genital tract specimens. These results also emphasize the importance of using Vero cell cocultures to recover viable *M. genitalium* from pig-tailed macaques, and possibly other animal models, even when using strains that grow reliably in axenic cultures.

Detection of *M. genitalium* in infected primates by qPCR. In addition to detecting *M. genitalium* growth in HPVC broth and Vero cell cocultures, we also assessed the persistence of bacteria in the primates by qPCR of DNA purified directly from lower genital tract specimens. Of the 144 total lower genital tract samples collected from all eight primates (including primates K02241 and 05073, which resisted infection) during the 8 weeks of the experiment, 73 (50.7%) were positive for *M. genitalium* DNA by qPCR, 93% of which were positive for *M. genitalium* growth in Vero cell cocultures (Table 1). Only six specimens (5.6%) were positive for growth in Vero cell coculture but negative by qPCR, likely due to the differences in the volume of specimen analyzed (6 μ l for qPCR versus 0.2 ml for Vero cell cultures). Conversely, a positive qPCR result in the absence of *M. genitalium* growth was observed for only five (3.5%) lower tract specimens. Three of these specimens (the cervical swab and cervical cytobrush collected at week 4 from K99329 and the cervical swab collected at week 2 from A07145) may have contained only DNA from nonviable bacteria, as at subsequent time points these primates were negative for *M. genitalium* by all measures (Table 1). The fact that *M. genitalium* DNA was not detected even at week 1 in the two primates that resisted infection (primates 05073 and K02241) suggests that this organism (and its DNA) was rapidly cleared from the genital tracts of these two primates.

Because qPCR has the limitations of small specimen volume analyzed and the inability to differentiate live from dead bacteria, we conclude that Vero cell coculture is the optimal method for tracking active *M. genitalium* infection in the lower genital tracts of pig-tailed macaques. Using this measure, we found that four primates (J00106, T00039, A07080, and A01220) were persistently infected for the entire 8 weeks of the experiment, two (K99329 and A07071) cleared their infections after 4 weeks, one (A07145) was infected for 1 to 2 weeks, and two (K02241 and 05073) resisted infection or were infected for less than 1 week (Fig. 1 and Table 1).

Evaluation of ascension to the upper reproductive tract. As *M. genitalium* is associated with diseases of the upper reproductive tract in women, we analyzed fallopian tube tissues at 4 and 8 weeks after cervical inoculation, as indicated in Fig. 1, to determine if ascension from the lower to the upper reproductive tract occurred. Salpingeal tissues (ampulla and fimbriae) from the left or right side were collected by laparotomy, homogenized, and then analyzed by qPCR and culture (HPVC broth and Vero cell cocultures), similar to our experiments with the lower genital tract specimens. As expected, no *M. genitalium* DNA or viable bacteria were detected in the control primate inoculated with PBS or from four biopsy specimens obtained from primates that had resisted lower genital tract infection. However, six biopsy specimens obtained from three animals with persistent lower genital tract infection were also negative for viable *M. genitalium* and DNA. These results suggest that *M. genitalium* did not ascend to the upper reproductive tract after cervical inoculation or that our methods lacked the sensitivity to detect low numbers of bacteria.

Salpingeal pocket inoculations. In addition to evaluating the ascension to the upper reproductive tract in cervically infected primates, we investigated the ability of *M. genitalium* to infect fallopian tube tissue in two primates (A07071 and A07080) using the salpingeal pocket model (37, 38). In this model, autologous salpingeal tissue is transplanted into multiple abdominal pockets, and 7 weeks later, *M. genitalium* is injected into the fluid surrounding the salpingeal tissue. This model potentially has the advantage of allowing the analysis of multiple inoculations of a single animal over time. Pockets were inoculated with *M. genitalium* (or PBS as a control) at week 0, and then

FIG 2 Maps of abdominal salpingeal pockets for primates A07071 and A07080. Circles indicate pockets inoculated with PBS control or *M. genitalium* (Mg), and boxes indicate pockets excised at each time point. The presence (+) or absence (-) of viable *M. genitalium* was determined by growth in HPVC broth or Vero cell coculture. [^], this pocket was positive by qPCR for *M. genitalium* DNA but negative by HPVC broth and Vero cell coculture.

individual pockets were removed at intervals, homogenized, and tested for *M. genitalium* DNA by qPCR and for viable *M. genitalium* by growth in HPVC broth and Vero cell coculture (Fig. 2). As expected, no *M. genitalium* DNA or viable bacteria were detected in any of the 12 pockets excised at 1 to 8 weeks after injection with PBS (data not shown). Of the 20 salpingeal pockets in primate A07071 inoculated with *M. genitalium* at week 0, viable bacteria were recovered from only one pocket that was excised at week 2. Similarly, of the 14 *M. genitalium*-inoculated pockets of primate A07080, two contained viable *M. genitalium*: one collected at week 1 and the other at week 2. A single pocket collected at week 1 from primate A07080 was positive by PCR but failed to produce any *M. genitalium* growth, suggesting that DNA derived from dead bacteria had persisted in the pocket. Unlike the case for the lower genital tract specimens, growth of *M. genitalium* in HPVC broth was not inhibited by homogenized tissue (data not shown). These results are similar to our previous findings in which viable *M. genitalium* also persisted in salpingeal pockets for 2 weeks postinoculation in our pilot primate, A01220 (37).

Humoral antibody response in *M. genitalium*-infected primates. Serum specimens collected from each primate prior to and throughout the experiment were diluted 1:100 and analyzed for antibody reactivity to *M. genitalium* whole-cell lysates by immunoblotting (Fig. 3). Preexisting *M. genitalium* reactivity was low in all primates. Not unexpectedly, animals in which *M. genitalium* infection was not established (primates K02241 and 05073) or in which infection was quickly resolved after only 1 week (primate A07145) also failed to produce *M. genitalium*-reactive IgG (Fig. 3 and data not shown). However, of the three primates (J00106, K99329, and T00039) (Fig. 1) inoculated only in the cervix in which bacteria persisted for 4 weeks or longer, only one (primate J00106, infected for 8 weeks) mounted an *M. genitalium*-specific antibody response that increased over the course of infection, peaking at around 6 to 8 weeks postinoculation. In contrast, serum diluted 1:100 from primates K99329 (infected for 4

FIG 3 Immunoblot reactivity of primate serum IgG to *M. genitalium* whole-cell lysates. Each panel shows the antibody reactivity of serum (diluted 1:100) from a single primate at the indicated week postinoculation (0 to 12). Immunoblots using a 1:20 dilution of pre- and postinoculation sera from primate T00039 showed background reactivity that did not change over time (data not shown). Specific rabbit antiserum (Rb) was used to locate MgpB (black arrowhead) and MgpC (open arrowhead) on immunoblots. Molecular size markers (in kDa) are indicated on the left and apply to both immunoblots in the row.

weeks) and T00039 (infected for 8 weeks) failed to demonstrate an increase in antibody reactivity to *M. genitalium*. The primates inoculated both cervically and in multiple salpingeal pockets (A07071 and A07080) exhibited a robust antibody response to *M. genitalium* whole-cell lysates with reactivity detected by week 1 (Fig. 3), consistent with

FIG 4 Immunoblot reactivity of primate cervical IgG to *M. genitalium* whole-cell lysates. Primate numbers are indicated above the panels; the numbers above each lane indicate the week after inoculation that the cervical exudate was obtained. Black and open arrowheads indicate migration of MgpB and MgpC, respectively, identified with specific rabbit antiserum (not shown). Molecular size markers (in kDa) are indicated on the left.

the higher inoculum in these two primates and the immune response in our original pilot primate, A01220 (37). All three animals that produced an *M. genitalium*-specific immune response demonstrated an increase in reactivity to the antigens MgpB and MgpC (Fig. 3), consistent with the immunodominance of these proteins among humans (6, 7, 20, 21) and our pilot primate A01220 (37). Two animals (A07071 and A07080) developed antibodies to low-molecular-mass proteins coincident with *M. genitalium* inoculation; the identity of these proteins was not pursued. The *M. genitalium*-specific humoral IgG response persisted for 12 weeks in these three animals (J00106 and A07071 [Fig. 3] and A07080 [data not shown]), which was 4 weeks after azithromycin treatment cured the infection. These animals were returned to the colony and not studied further.

Presence of *M. genitalium*-reactive antibodies in the primate genital tract.

Cervical swab specimens were analyzed for IgG and IgA reactivity to *M. genitalium* whole-cell lysates by immunoblotting (Fig. 4). For primates that resisted infection, or cleared the infection after 1 week, we compared cervical antibody reactivities of week 0 and week 2 specimens. For persistently infected primates, we compared cervical antibodies collected at week 0 to those collected at the last *M. genitalium*-positive time point. *M. genitalium*-specific cervical antibodies were detected in primates J00106, A07071, and A07080, consistent with the presence of antibodies in serum. Cervical IgG reactivity was similar to serum IgG in that strong reactivity to MgpB and MgpC was observed (Fig. 4). Primates that lacked *M. genitalium* antibodies in serum were also negative for an *M. genitalium*-specific IgG response in cervical specimens (Fig. 4). For all primates, even those exhibiting a humoral IgG response, *M. genitalium*-reactive IgA was undetectable in cervical swab specimens (data not shown).

Local inflammatory response to infection. Cervical specimens (swabs and cytobrush) were collected throughout the experiment and analyzed for the presence of polymorphonuclear leukocytes (PMNs) by differential staining and light microscopy. PMNs were rarely found except when blood was present due to menses (data not shown), suggesting the absence of a local inflammatory response.

DISCUSSION

In this study, we demonstrated that the female pig-tailed macaque (*Macaca nemestrina*) is a valuable animal model for the study of *M. genitalium* infection, an increasingly recognized cause of reproductive tract infection in men and women. In this model, macaques were inoculated with *M. genitalium* (strain G37) in the cervix and in autologously transplanted salpingeal tissue and followed for 8 weeks, with assessment of the establishment and persistence of infection and the specific antibody response. Including the single primate described previously (37), cervical infection persisted throughout the study (8 weeks) in four primates, for 4 weeks in two primates, and for

1 week in one primate, whereas two animals resisted infection. In the three animals inoculated concurrently in salpingeal pockets and the cervix (37), viable bacteria were recovered from the pockets for 2 weeks postinoculation and specific antibodies were produced within 1 week. In contrast, among the three animals with persistent (≥ 4 weeks) infection after cervical inoculation alone, antibodies were induced in only one primate, detected at 4 weeks postinoculation. In all antibody-positive primates, IgG isotype antibodies to the immunodominant MgpB and MgpC proteins were detected in sera and the cervix, paralleling the immune response in humans (6, 7, 20, 21).

An important finding of this study is that growth of *M. genitalium* from primate genital tract specimens was inhibited in HPVC broth, necessitating coculture with Vero cells for optimal recovery. Coculture with Vero cells, a method developed for the propagation of clinical strains from patient specimens (24, 25), enabled the recovery of *M. genitalium* from 74 specimens, compared to 57 specimens cultured in HPVC broth ($P < 0.0001$), a surprising finding given that strain G37 grows reliably in HPVC broth. Growth inhibition could be overcome by dilution, suggesting that these isolates were able to grow in HPVC broth rather than having undergone an adaptation *in vivo* that resulted in an *in vitro* growth deficit. In addition, Vero cell-cocultured primate isolates subsequently grew normally in HPVC broth (data not shown). While the nature of the inhibiting factor in lower genital tract secretions is not known, it was common to all of the *M. genitalium*-infected primates, in both vaginal and cervical specimens. Despite using a cocktail of three antibiotics (penicillin, vancomycin, and colistin), contaminating microscopic bacterial colonies grew on H agar plates (data not shown), indicating the presence of unknown bacteria that may inhibit growth of *M. genitalium* or compete for nutrients *in vitro*. Improved growth in Vero cultures may be explained by the differences between Vero cell medium and HPVC broth (e.g., fetal bovine serum [FBS] versus horse serum, H broth versus Eagle minimal essential medium [EMEM], and different antibiotics) that may favor *M. genitalium* growth *in vitro* or, alternatively, because Vero cells themselves provide a more robust culture environment enabling *M. genitalium* to overcome growth inhibition. Conversely, Vero cells may provide a suboptimal growth condition for inhibitory bacteria present in the specimen. Further exploration of this phenomenon is under way in our laboratory.

Persistence of *M. genitalium* cervical infection for >8 weeks in four (44%) primates mimics untreated infection in men and women (12, 14, 16, 39–41). For example, *M. genitalium* persisted in the lower genital tract for >2 to 3 months in 50% of enrolled Kenyan and Ugandan sex workers (12, 14) and for >12 to 21 months in 26% of students studied in London (41). Persistent *M. genitalium* infection has been established in other animal models, including vaginally infected chimpanzees (42), urethrally inoculated male chimpanzees (43), and the upper reproductive tracts of vaginally infected mice (44). Each of these other animal models has its limitations; for example, chimpanzees are no longer available for scientific research, and mice require hormone treatment to establish infection (44). Pig-tailed macaques, although expensive, may provide a more suitable system in which to study this human pathogen.

Two (22%) of nine primates in our study resisted cervical infection, a finding that requires further investigation. The presence of preexisting antibodies does not explain this resistance, because the preinoculation antibody reactivity was low in all primates. However, differences in vaginal pH, measured throughout the experiment for six of the nine cervically inoculated primates (data not shown), could affect the growth of *M. genitalium* *in vivo*. At the time of inoculation, the vaginal pH of one primate (K02241) that resisted infection was 5.5, much lower than that of the infected animals (pH 6.25 to 8.5, consistent with previous observations for pig-tailed macaques [45]). Although vaginal pH readings are not available for the other primate (05073) that resisted infection, it is tempting to speculate that low pH prevents the establishment of lower genital tract infection, as *M. genitalium* is rapidly killed at a pH of less than 6.5 (46). While vaginal pH in humans is normally between 3.8 and 4.5, the increased pH (>4.5) characteristic of bacterial vaginosis may in part explain the association of this syndrome with *M. genitalium* infection (47, 48). In addition, semen rapidly increases vaginal pH to

6.1 to 7.2 (49, 50) during intercourse, which may enhance heterosexual transmission. Future studies using contemporary, low-passage strains and assessing the effect of vaginal pH, the microbiome, and other host factors are needed to evaluate the differences in susceptibility to *M. genitalium* infection and persistence.

M. genitalium was not detected in the upper reproductive tract of any primate inoculated cervically, suggesting that ascension (i) requires longer than 8 weeks, (ii) is an infrequent event, or (iii) does not occur in pig-tailed macaques. Strategies similar to those employed to study *Chlamydia trachomatis*-induced salpingitis, including multiple cervical inoculations and/or direct tubal inoculation (51–54), or the use of contemporary *M. genitalium* strains may be required to model upper tract infection. Efforts to infect the upper reproductive tract in this animal model should be continued, especially in light of the results of McGowin et al. (44), in which vaginal inoculation with either of two strains of *M. genitalium* resulted in hydrosalpinx at 3 to 10 weeks after infection in estradiol-treated mice. In that mouse study, bacteria were rapidly cleared from the vagina (within 3 days), yet viable *M. genitalium* was recovered from the oviduct tissue of 14% of the animals with hydrosalpinx (44).

As an alternative strategy to study upper reproductive tract infection, we utilized the salpingeal pocket model (37, 38), in which autologous salpingeal tissue is transplanted into abdominal pockets, inoculated directly, and then excised at various times to assess the presence of viable *M. genitalium*. This model has the advantages of allowing multiple inoculations and analysis at various time points in a single animal, thereby limiting animal-to-animal variation and reducing the high costs of primate experiments. Furthermore, as the transplanted tissue is enclosed within a vascularized dermal cyst, the inoculum does not disperse into surrounding tissue and instead is maintained in close proximity with the salpingeal tissue. Including our pilot primate (37), viable *M. genitalium* was recovered from two (of eight) pockets after 1 week and from five (of nine) pockets after 2 weeks. The low rate of persistence in these pockets may indicate failure to establish an infection or rapid clearance by specific antibodies arising by week 1 in these heavily inoculated animals. The persistence of infection in the genital tracts of these same animals may indicate that *M. genitalium* can avoid clearance by the lower concentration of antibodies present in cervicovaginal secretions than in serum (55).

Cervical infection alone induced specific antibodies in only one of three persistently infected animals, and these arose after 4 weeks of infection, perhaps unsurprising given that the lower genital tract lacks well-developed inductive immune sites (55). Similarly, in previous experiments with male chimpanzees, serum antibodies were detectable at 5 to 11 weeks after intraurethral infection (42, 43) and 3 to 4 weeks after vaginal inoculation of female chimpanzees (42). In humans, antibody reactivity to *M. genitalium* is associated with tubal factor infertility in women (6, 7) and with urethritis in men (21, 56). Our previous study (20) of women attending a sexually transmitted disease (STD) clinic in Seattle, WA, showed that 70% of PCR-positive women produced *M. genitalium*-reactive cervicovaginal antibodies, 92% of which reacted with MgpB and 69% of which reacted with MgpC. We and others have proposed that antigenic variation of the immunodominant surface proteins, MgpB and MgpC, contributes to immune evasion of *M. genitalium* leading to persistence of infection, and we have shown that the appearance of antibodies to specific variant sequences of MgpB correlates with the clearance of those sequences from the genital tract of our pilot primate (37). Together these findings may suggest multiple strategies to avoid antibody clearance, including (i) poor/delayed induction of antibodies, (ii) low abundance at the site of infection, and (iii) antigenic variation.

Our finding that cervical infection failed to induce an infiltration of PMNs was surprising given the association of *M. genitalium* infection with cervicitis in women (5) and the production of inflammatory cytokines by cells infected *in vitro* (32–34, 57, 58). The absence of PMNs may indicate that a longer duration of infection, higher infectious load, or inoculation with a strain not passaged *in vitro* is required to induce inflammation. However, these results are consistent with the lack of signs or symptoms of infection and the lack of increased cervical PMNs in a subset of infected patients. For

example, Manhart et al. (59) detected elevated PMNs in only 48% of *M. genitalium*-infected (and in 29% of *M. genitalium*-negative) women, and Pepin et al. (60) detected cervical pus in 16.5% of infected women compared to 7.2% of uninfected women. The induction of cervical cytokines and inflammatory cells, and their effects on *M. genitalium*, needs to be studied further in this model.

We conclude that the pig-tailed macaque is a valuable model for the study of the pathogenesis and immunobiology of *M. genitalium* infection. The utility of this model is enhanced by the similarity of this primate to humans, including reproductive tract anatomy, menstrual cycle, and vaginal flora (61), but is limited by its high cost and availability in only a few research centers. The variety of outcomes (resistance to infection, persistence, and clearance) after cervical inoculation suggests that this model may be useful for exploring host and bacterial factors involved in resistance to infection, spontaneous clearance, and persistence *in vivo*. The persistence of lower genital tract infection provides a valuable opportunity to study the interplay of antibody production and antigenic variation in MgpB and MgpC. Clearly there is much to be learned about the molecular pathogenesis of *M. genitalium* infection that could be addressed in this model.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Mycoplasma genitalium* strain G37-C, our single-colony filter-cloned isolate of the type strain G37, which contains the *mgpB/mgpC* expression site and MgPar sequences identical to those of the fully sequenced genome (29), was used in all experiments. This strain has undergone numerous *in vitro* passages since its isolation and deposition into ATCC in 1983 (66). *M. genitalium* was grown in H broth (62) containing 100 U/ml penicillin at 37°C in ambient air to prepare the primate inoculum and in H broth supplemented with 100 U/ml penicillin, 50 µg/ml vancomycin, and 50 µg/ml colistin (HPVC broth), or on HPVC agar plates, and Vero cell coculture (with 100 U/ml penicillin, 50 µg/ml vancomycin, and 5 µg/ml polymyxin B) to recover *M. genitalium* from primate specimens (see below). Because *M. genitalium* cultures do not increase in turbidity over time, growth is monitored by a color change (red to orange or yellow) due to the production of acid from glucose or by qPCR to measure an increase in genomes. To prepare the primate inoculum, *M. genitalium* was grown in H broth in 75-cm² tissue culture flasks until cultures turned orange. Adherent bacteria were scraped into the existing culture medium in the flasks, washed twice with PBS by centrifugation (20,000 × g, 10 min), quantitated by qPCR, and then adjusted to a final concentration of 1×10^9 genomes per ml. A fresh inoculum was prepared for each pair of primates.

Experimental animals. Prior approval for these experiments was obtained from the University of Washington Institutional Animal Care and Use Committee and the Washington National Primate Research Center, where the animals were housed. Eight healthy, female pig-tailed macaques (*Macaca nemestrina*), ranging in age from 7.5 to 11 years old and weighing 5.5 to 11 kg, were used in our cervical infection model, two of which also were used in the salpingeal pocket model (see below). Each primate was sexually mature and a proven breeder. Primate 05073 was obtained from a feral colony in Indonesia; the other primates were captive bred in the United States. The macaques were housed separately throughout the protocol. For ease of analysis, two primates were infected at a time with the same inoculum and then followed, treated, and tested for cure before repeating the protocol on additional pairs of primates as indicated in Fig. 1. Primates were treated with azithromycin after specimen collection at week 8; *M. genitalium*-negative status was confirmed at week 12 by qPCR and culture of lower reproductive tract specimens, at which point the animals were returned to the Washington National Primate Research Center colony.

Cervical infection model. Lower reproductive tract specimens (vaginal swab, cervical swab, and cervical cytobrush) were collected 3 weeks prior to inoculation to verify the *M. genitalium*-negative status of each primate by culture and qPCR (see below) and to confirm that the antibiotic cocktail included in HPVC broth, HPVC agar plates, and Vero cell cocultures inhibited the growth of normal flora (data not shown). Specimens were collected by rolling Dacron swabs on the surface of the vagina or cervix or by inserting and rotating a cytobrush in the cervical os to collect cells. Week 0 vaginal and cervical swabs were collected just prior to inoculation; cervical cytobrush specimens were not collected at this time to avoid abrasions that might influence the establishment of *M. genitalium* infection. Primates were inoculated in pairs with an identical inoculum: 1.0 ml PBS containing $\sim 1 \times 10^9$ genomes ($\sim 1 \times 10^8$ color change units [CCU]/ml) of *M. genitalium* applied at the cervix. Immediately following primate inoculation, serial 10-fold dilutions of the inoculum were cultured in H broth to confirm its viability.

Beginning 1 week after inoculation, lower tract specimens were collected at the time points indicated (Table 1). First, two vaginal swabs were collected; the first swab was suspended in 2 ml mycoplasma transport medium (MTM) (63) for culture and qPCR, and the second swab was suspended in PBS for future studies. Next, two cervical swabs and two cervical cytobrush specimens were collected, the first of each was suspended in 2 ml MTM for culture and qPCR, while the second was rolled onto a glass slide for detection of PMNs and then suspended in PBS for analysis of cervical antibodies.

Culture of viable *M. genitalium* from infected primates. *M. genitalium* was cultured from lower genital tract specimens by inoculating 2.7 ml of HPVC with 0.3 ml of specimen in MTM and then making

four additional 10-fold serial dilutions in the same medium. Our preliminary experiments indicated that this antibiotic cocktail was the most effective at inhibiting the overgrowth of normal flora, without inhibiting *M. genitalium* (data not shown). Growth in HPVC broth was considered positive if any of the five dilutions changed from red to orange or yellow, indicating the production of acid, during 24 days of culture. Cultures that became turbid, indicating overgrowth of normal flora, were always *M. genitalium* negative by qPCR. HPVC agar plates were inoculated with dilutions of primate specimens or with aliquots of HPVC broth cultures and then examined for microscopic colonies after 24 to 30 days of incubation.

To complement the recovery of *M. genitalium* in HPVC broth, primate specimens were also inoculated into Vero cell cultures, prepared by seeding 25-cm² flasks with 1×10^5 Vero cells in 5 ml EMEM supplemented with 10% FBS and 100 U/ml penicillin and incubating overnight at 37°C in 5% CO₂. The adherent Vero cells were washed once with PBS and then inoculated with 0.2 ml of primate specimen (or MTM alone as a negative control) in 8.5 ml of EMEM supplemented with 10% FBS, 6% yeast dialysate, 100 U/ml penicillin, 50 µg/ml vancomycin, and 5 µg/ml polymyxin B and incubated at 37°C in 5% CO₂. Growth of *M. genitalium* in Vero cell cocultures was monitored by qPCR of culture supernatants collected after 1, 8, 15, 22, and 29 days incubation. No *M. genitalium* DNA was detected in Vero cell cultures inoculated with sterile MTM. Similarly, primate specimens were considered negative if *M. genitalium* DNA was undetectable by qPCR. On the other hand, *M. genitalium*-positive Vero cell cultures were easily identified, as they contained $>10^5$ genomes per ml of culture supernatant. The statistical comparison of the recovery of *M. genitalium* in HPVC broth versus Vero cell cocultures was performed by McNemar's test.

Biopsies of upper reproductive tract. Biopsy specimens of intact fallopian tubes were collected via laparotomy from primates inoculated cervically at week 4 (right side) and week 8 (left side) to determine if *M. genitalium* had ascended from the lower genital tract to upper reproductive tract tissues (Fig. 1). Ampulla and fimbria tissues were separately homogenized in 2 ml MTM and tested for viable *M. genitalium* by culture in HPVC broth (0.3 ml of homogenate and four serial 10-fold dilutions) and Vero cell cocultures (one flask inoculated with 0.2 ml and a second flask inoculated with 1 ml of homogenate) and for DNA by qPCR using the methods outlined below.

Control primate. One primate, A07145, was mock inoculated with PBS and followed for 8 weeks by collecting lower genital tract and serum specimens, at which time fallopian tube biopsy specimens (ampulla and fimbriae) were collected from the right side. All specimens collected were negative for *M. genitalium* DNA by qPCR, growth in HPVC broth, and Vero cell coculture and for antibody reactivity by immunoblot analysis (data not shown). Six weeks later, this same primate was inoculated cervically with *M. genitalium* and again followed for 8 weeks to assess *M. genitalium* infection. However, fallopian tube biopsy specimens were not collected during this time because lower tract specimens indicated that the infection cleared spontaneously after only 1 week.

Cervical biopsies. Cervical biopsy specimens were collected from six *M. genitalium*-inoculated primates at week 8 (Fig. 1), just prior to azithromycin treatment, for future studies. Two primates, A07071 and A07080, were biopsied 7 weeks prior to inoculation, when salpingeal tissue was autotransplanted, and then again at week 2 or week 4, respectively (Fig. 1). EuroMed minitipped biopsy forceps (1 mm by 3 mm) were used to remove cervical tissue, and silver nitrate styptic was used to halt bleeding. This procedure did not terminate the persistent cervical infection in these animals, as subsequent samples contained viable *M. genitalium* (Fig. 1 and Table 1).

Salpingeal pocket model. Similar to the case for our previous experiment with primate A01220 (37), two additional primates (A07071 and A07080) underwent the salpingeal pocket model procedure (37, 38) at the same time as cervical inoculation. In this model, salpingeal tissues were surgically collected by minilaparotomy, dissected into 3- to 5-mm segments, and then autotransplanted into subcutaneous pockets in the anterior abdominal wall, where a dermal cyst forms, enclosing the transplanted tissue (38). Seven weeks later, these primates were inoculated cervically as described above and into each of multiple (16 to 20) salpingeal pockets (0.2 ml of 1×10^9 genomes/ml injected into the fluid-filled space surrounding the transplanted tissue). Five or six pockets in each primate were mock inoculated with PBS alone. At the times indicated in Fig. 2, the cysts containing salpingeal tissue from *M. genitalium*-inoculated and control pockets was removed, homogenized in 2 ml MTM using a disposable tissue grinder, and assessed for the presence of *M. genitalium* DNA by qPCR and for viable *M. genitalium* by growth in HPVC broth and Vero cell cocultures, as described above.

M. genitalium qPCR assays. *M. genitalium* genomes were quantitated by isolating total DNA from 150 µl of sample (inoculum, primate specimens [including lower genital tract and homogenized salpingeal tissue], HPVC broth cultures, and Vero cell culture supernatants) using the MasterPure DNA purification kit (Epicentre, Madison, WI). The 7900 HT Fast real-time PCR system and reagents (Applied Biosystems, Grand Island, NY) were used to quantitate *M. genitalium* genomes using a modification of the procedure of Jensen et al. (64). Reaction mixtures consisted of 2 µl purified DNA (corresponding to 6 µl of specimen), 0.6 µM primers (MgPa-355F and MgPa-432R), 125 nM 6-carboxyfluorescein (FAM)-labeled probe (MgPa-380), and TaqMan Fast Universal PCR Mastermix No AmpErase UNG in a total volume of 10 µl, with the following cycling conditions: 95°C for 10 min and then 40 cycles of 95°C for 1 s and 60°C for 15 s. Each sample was quantitated in triplicate compared to a standard curve prepared with serial 10-fold dilutions of *M. genitalium* whole-cell DNA. The concentration of DNA used to generate the standard curve was determined using the Quant-iT PicoGreen double-stranded DNA (dsDNA) kit (Invitrogen, Grand Island, NY) according to the manufacturer's instructions.

Immunoblot assays. Serum specimens collected from each primate prior to inoculation, throughout each experiment, and after antibiotic treatment were frozen at -20°C for later detection of *M. genitalium*-reactive antibodies by immunoblotting. *M. genitalium* G37-C lysates for immunoblots were prepared

from bacteria grown in SP-4 broth (65) to avoid detecting antibodies inadvertently elicited against components of H broth (e.g., horse serum) used to grow the inoculum. Lysates (10 µg total protein, determined using the bicinchoninic acid [BCA] protein assay kit [Thermo Scientific, Rockford, IL]) were electrophoresed through a 4 to 20% SDS-polyacrylamide gel (Mini-Protein TGX; Bio-Rad, Hercules, CA) with a prestained protein size marker (HiMark Pre-Stained HMW protein standard; Invitrogen, Grand Island, NY), transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen), and then reacted with primate serum (diluted 1:100, unless otherwise noted) or cervical swab specimens (diluted 1:10) and peroxidase-conjugated secondary antibodies (1:1,000 dilution of goat anti-human IgG whole molecule or goat anti-human IgA alpha chain; Sigma-Aldrich, St. Louis, MO) with colorimetric detection.

MgpB- and MgpC-specific rabbit antibodies. MgpB-specific antibodies were produced by immunizing rabbits with a keyhole limpet hemocyanin (KLH)-conjugated peptide corresponding to amino acids 1123 to 1136 (KLIEPVDATSAATNC, with a terminal cysteine added for conjugation to KLH) (Sigma Genosys, The Woodlands, TX). MgpC was detected with serum from a rabbit immunized with His-tagged recombinant MgpC amino acids 36 to 308 (37). For immunoblots, these antisera were diluted 1:1,000 (anti-MgpB) or 1:5,000 (anti-MgpC) and detected with peroxidase-conjugated goat anti-rabbit IgG (whole molecule; Sigma-Aldrich) and colorimetric detection.

Cytology. Slides prepared from cervical swabs or cervical cytobrush specimens were air dried, stained using the Harleco Hemacolor stain kit (EMD Millipore, Billerica, MA), and examined by light microscopy for the presence of PMNs, an indicator of local inflammation.

ACKNOWLEDGMENTS

This work was supported by grants AI082316 and AI074898 from the National Institutes of Health and P51 OD010425 from the NIH Office of Research Infrastructure Programs (ORIP) to the Washington National Primate Research Center and the Department of Medicine, University of Washington. University of Washington STD/AIDS Research Training Grants 124 NIH/NIAID T32 AI7140-33 and -34 supported S.L.I.-C.

REFERENCES

- Deguchi T, Yoshida T, Yokoi S, Ito M, Tamaki M, Ishiko H, Maeda S. 2002. Longitudinal quantitative detection by real-time PCR of *Mycoplasma genitalium* in first-pass urine of men with recurrent nongonococcal urethritis. *J Clin Microbiol* 40:3854–3856. <https://doi.org/10.1128/JCM.40.10.3854-3856.2002>.
- Manhart LE, Broad JM, Golden MR. 2011. *Mycoplasma genitalium*: should we treat and how? *Clin Infect Dis* 53(Suppl 3):S129–S142. <https://doi.org/10.1093/cid/cir702>.
- Jensen JS, Orsum R, Dohn B, Uldum S, Worm AM, Lind K. 1993. *Mycoplasma genitalium*: a cause of male urethritis? *Genitourin Med* 69:265–269.
- Totten PA, Schwartz MA, Sjostrom KE, Kenny GE, Handsfield HH, Weiss JB, Whittington WL. 2001. Association of *Mycoplasma genitalium* with nongonococcal urethritis in heterosexual men. *J Infect Dis* 183:269–276. <https://doi.org/10.1086/317942>.
- Lis R, Rowhani-Rahbar A, Manhart LE. 2015. *Mycoplasma genitalium* infection and female reproductive tract disease: a meta-analysis. *Clin Infect Dis* 61:418–426. <https://doi.org/10.1093/cid/civ312>.
- Clausen HF, Fedder J, Drasbek M, Nielsen PK, Toft B, Ingerslev HJ, Birkelund S, Christiansen G. 2001. Serological investigation of *Mycoplasma genitalium* in infertile women. *Hum Reprod* 16:1866–1874. <https://doi.org/10.1093/humrep/16.9.1866>.
- Svenstrup HF, Fedder J, Kristoffersen SE, Trolle B, Birkelund S, Christiansen G. 2008. *Mycoplasma genitalium*, *Chlamydia trachomatis*, and tubal factor infertility—a prospective study. *Fertil Steril* 90:513–520. <https://doi.org/10.1016/j.fertnstert.2006.12.056>.
- Cohen CR, Manhart LE, Bukusi EA, Astete S, Brunham RC, Holmes KK, Sinei SK, Bwayo JJ, Totten PA. 2002. Association between *Mycoplasma genitalium* and acute endometritis. *Lancet* 359:765–766. [https://doi.org/10.1016/S0140-6736\(02\)07848-0](https://doi.org/10.1016/S0140-6736(02)07848-0).
- Manhart LE, Mostad SB, Baeten JM, Astete SG, Mandaliya K, Totten PA. 2008. High *Mycoplasma genitalium* organism burden is associated with shedding of HIV-1 DNA from the cervix. *J Infect Dis* 197:733–736. <https://doi.org/10.1086/526501>.
- Mavedzenge SN, Van Der Pol B, Weiss HA, Kwok C, Mambo F, Chipato T, Van der Straten A, Salata R, Morrison C. 2012. The association between *Mycoplasma genitalium* and HIV-1 acquisition in African women. *AIDS* 26:617–624. <https://doi.org/10.1097/QAD.0b013e32834ff690>.
- Vandepitte J, Weiss HA, Bukuya J, Nakubulwa S, Mayanja Y, Matovu G, Kyakuwa N, Hughes P, Hayes R, Grosskurth H. 2013. Alcohol use, *Mycoplasma genitalium*, and other STIs associated with HIV incidence among women at high risk in Kampala, Uganda. *J Acquir Immune Defic Syndr* 62:119–126. <https://doi.org/10.1097/QAI.0b013e3282777167>.
- Cohen CR, Nosek M, Meier A, Astete SG, Iverson-Cabral S, Mugo NR, Totten PA. 2007. *Mycoplasma genitalium* infection and persistence in a cohort of female sex workers in Nairobi, Kenya. *Sex Transm Dis* 34:274–279.
- Oakeshott P, Hay P, Taylor-Robinson D, Hay S, Dohn B, Kerry S, Jensen JS. 2004. Prevalence of *Mycoplasma genitalium* in early pregnancy and relationship between its presence and pregnancy outcome. *BJOG* 111:1464–1467. <https://doi.org/10.1111/j.1471-0528.2004.00276.x>.
- Vandepitte J, Weiss HA, Kyakuwa N, Nakubulwa S, Muller E, Buve A, Van der Stuyt P, Hayes R, Grosskurth H. 2013. Natural history of *Mycoplasma genitalium* infection in a cohort of female sex workers in Kampala, Uganda. *Sex Transm Dis* 40:422–427. <https://doi.org/10.1097/OLQ.0b013e32828bfcfc>.
- Horner P, Thomas B, Gilroy CB, Egger M, Taylor-Robinson D. 2001. Role of *Mycoplasma genitalium* and *Ureaplasma urealyticum* in acute and chronic nongonococcal urethritis. *Clin Infect Dis* 32:995–1003. <https://doi.org/10.1086/319594>.
- Taylor-Robinson D, Gilroy CB, Thomas BJ, Hay PE. 2004. *Mycoplasma genitalium* in chronic non-gonococcal urethritis. *Int J STD AIDS* 15:21–25. <https://doi.org/10.1258/095646204322637209>.
- Taylor-Robinson D, Horner PJ. 2001. The role of *Mycoplasma genitalium* in non-gonococcal urethritis. *Sex Transm Dis* 77:229–231. <https://doi.org/10.1136/sti.77.4.229>.
- Sena AC, Lensing S, Rompalo A, Taylor SN, Martin DH, Lopez LM, Lee JY, Schwabke JR. 2012. *Chlamydia trachomatis*, *Mycoplasma genitalium*, and *Trichomonas vaginalis* infections in men with nongonococcal urethritis: predictors and persistence after therapy. *J Infect Dis* 206:357–365. <https://doi.org/10.1093/infdis/jis356>.
- Iverson-Cabral SL, Astete SG, Cohen CR, Rocha EP, Totten PA. 2006. Intrastrain heterogeneity of the *mgpB* gene in *Mycoplasma genitalium* is extensive in vitro and in vivo and suggests that variation is generated via recombination with repetitive chromosomal sequences. *Infect Immun* 74:3715–3726. <https://doi.org/10.1128/IAI.00239-06>.
- Iverson-Cabral SL, Manhart LE, Totten PA. 2011. Detection of *Mycoplasma genitalium*-reactive cervicovaginal antibodies among infected women. *Clin Vaccine Immunol* 18:1783–1786. <https://doi.org/10.1128/CVI.05174-11>.
- Svenstrup HF, Jensen JS, Gevaert K, Birkelund S, Christiansen G. 2006. Identification and characterization of immunogenic proteins of *Myco-*

- plasma genitalium*. Clin Vaccine Immunol 13:913–922. <https://doi.org/10.1128/CVI.00048-06>.
22. Manhart LE, Holmes KK, Hughes JP, Houston LS, Totten PA. 2007. *Mycoplasma genitalium* among young adults in the United States: an emerging sexually transmitted infection. Am J Public Health 97: 1118–1125. <https://doi.org/10.2105/AJPH.2005.074062>.
 23. Sonnenberg P, Ison CA, Clifton S, Field N, Tanton C, Soldan K, Beddows S, Alexander S, Khanom R, Saunders P, Copas AJ, Wellings K, Mercer CH, Johnson AM. 2015. Epidemiology of *Mycoplasma genitalium* in British men and women aged 16–44 years: evidence from the third National Survey of Sexual Attitudes and Lifestyles (Natsal-3). Int J Epidemiol 44:1982–1994. <https://doi.org/10.1093/ije/dyv194>.
 24. Hamasuna R, Osada Y, Jensen JS. 2007. Isolation of *Mycoplasma genitalium* from first-void urine specimens by coculture with Vero cells. J Clin Microbiol 45:847–850. <https://doi.org/10.1128/JCM.02056-06>.
 25. Jensen JS, Hansen HT, Lind K. 1996. Isolation of *Mycoplasma genitalium* strains from the male urethra. J Clin Microbiol 34:286–291.
 26. Manhart LE, Jensen JS, Bradshaw CS, Golden MR, Martin DH. 2015. Efficacy of antimicrobial therapy for *Mycoplasma genitalium* infections. Clin Infect Dis 61(Suppl 8):S802–S817. <https://doi.org/10.1093/cid/civ785>.
 27. Couldwell DL, Tagg KA, Jeoffreys NJ, Gilbert GL. 2013. Failure of moxifloxacin treatment in *Mycoplasma genitalium* infections due to macrolide and fluoroquinolone resistance. Int J STD AIDS 24:822–828. <https://doi.org/10.1177/0956462413502008>.
 28. Lau A, Bradshaw CS, Lewis D, Fairley CK, Chen MY, Kong FY, Hocking JS. 2015. The efficacy of azithromycin for the treatment of genital *Mycoplasma genitalium*: a systematic review and meta-analysis. Clin Infect Dis 61:1389–1399. <https://doi.org/10.1093/cid/civ644>.
 29. Iverson-Cabral SL, Astete SG, Cohen CR, Totten PA. 2007. *mgpB* and *mgpC* sequence diversity in *Mycoplasma genitalium* is generated by segmental reciprocal recombination with repetitive chromosomal sequences. Mol Microbiol 66:55–73. <https://doi.org/10.1111/j.1365-2958.2007.05898.x>.
 30. Ma L, Jensen JS, Myers L, Burnett J, Welch M, Jia Q, Martin DH. 2007. *Mycoplasma genitalium*: an efficient strategy to generate genetic variation from a minimal genome. Mol Microbiol 66:220–236. <https://doi.org/10.1111/j.1365-2958.2007.05911.x>.
 31. Das K, De la Garza G, Siwak EB, Scofield VL, Dhandayuthapani S. 2014. *Mycoplasma genitalium* promotes epithelial crossing and peripheral blood mononuclear cell infection by HIV-1. Int J Infect Dis 23:31–38. <https://doi.org/10.1016/j.ijid.2013.11.022>.
 32. McGowin CL, Radtke AL, Abraham K, Martin DH, Herbst-Kralovetz M. 2013. *Mycoplasma genitalium* infection activates cellular host defense and inflammation pathways in a 3-dimensional human endocervical epithelial cell model. J Infect Dis 207:1857–1868. <https://doi.org/10.1093/infdis/jit101>.
 33. McGowin CL, Annan RS, Quayle AJ, Greene SJ, Ma L, Mancuso MM, Adegboye D, Martin DH. 2012. Persistent *Mycoplasma genitalium* infection of human endocervical epithelial cells elicits chronic inflammatory cytokine secretion. Infect Immun 80:3842–3849. <https://doi.org/10.1128/IAI.00819-12>.
 34. McGowin CL, Ma L, Martin DH, Pyles RB. 2009. *Mycoplasma genitalium*-encoded MG309 activates NF- κ B via Toll-like receptors 2 and 6 to elicit proinflammatory cytokine secretion from human genital epithelial cells. Infect Immun 77:1175–1181. <https://doi.org/10.1128/IAI.00845-08>.
 35. Martinez MA, Das K, Saikolappan S, Materon LA, Dhandayuthapani S. 2013. A serine/threonine phosphatase encoded by MG_207 of *Mycoplasma genitalium* is critical for its virulence. BMC Microbiol 13:44. <https://doi.org/10.1186/1471-2180-13-44>.
 36. Dhandayuthapani S, Blaylock MW, Bebear CM, Rasmussen WG, Baseman JB. 2001. Peptide methionine sulfoxide reductase (MsrA) is a virulence determinant in *Mycoplasma genitalium*. J Bacteriol 183:5645–5650. <https://doi.org/10.1128/JB.183.19.5645-5650.2001>.
 37. Wood GE, Iverson-Cabral SL, Patton DL, Cummings PK, Cosgrove Sweeney YT, Totten PA. 2013. Persistence, immune response, and antigenic variation of *Mycoplasma genitalium* in an experimentally infected pig-tailed macaque (*Macaca nemestrina*). Infect Immun 81:2938–2951. <https://doi.org/10.1128/IAI.01322-12>.
 38. Patton DL, Kuo CC, Wang SP, Brenner RM, Sternfeld MD, Morse SA, Barnes RC. 1987. Chlamydial infection of subcutaneous fimbrial transplants in cynomolgus and rhesus monkeys. J Infect Dis 155:229–235. <https://doi.org/10.1093/infdis/155.2.229>.
 39. Hjorth SV, Bjornelius E, Lidbrink P, Falk L, Dohn B, Berthelsen L, Ma L, Martin DH, Jensen JS. 2006. Sequence-based typing of *Mycoplasma genitalium* reveals sexual transmission. J Clin Microbiol 44:2078–2083. <https://doi.org/10.1128/JCM.00003-06>.
 40. Horner P, Thomas B, Gilroy C, Egger M, McClure M, Taylor-Robinson D. 2003. Antibodies to *Chlamydia trachomatis* heat-shock protein 60 kDa and detection of *Mycoplasma genitalium* and *Ureaplasma urealyticum* are associated independently with chronic nongonococcal urethritis. Sex Transm Dis 30:129–133. <https://doi.org/10.1097/00007435-200302000-00007>.
 41. Oakshott P, Aghaizu A, Hay P, Reid F, Kerry S, Atherton H, Simms I, Taylor-Robinson D, Dohn B, Jensen JS. 2010. Is *Mycoplasma genitalium* in women the “new Chlamydia”? A community-based prospective cohort study. Clin Infect Dis 51:1160–1166. <https://doi.org/10.1086/656739>.
 42. Tully JG, Taylor-Robinson D, Rose DL, Furr PM, Graham CE, Barile MF. 1986. Urogenital challenge of primate species with *Mycoplasma genitalium* and characteristics of infection induced in chimpanzees. J Infect Dis 153:1046–1054. <https://doi.org/10.1093/infdis/153.6.1046>.
 43. Taylor-Robinson D, Tully JG, Barile MF. 1985. Urethral infection in male chimpanzees produced experimentally by *Mycoplasma genitalium*. Br J Exp Pathol 66:95–101.
 44. McGowin CL, Spagnuolo RA, Pyles RB. 2010. *Mycoplasma genitalium* rapidly disseminates to the upper reproductive tracts and knees of female mice following vaginal inoculation. Infect Immun 78:726–736. <https://doi.org/10.1128/IAI.00840-09>.
 45. Lichtenwalner AB, Patton DL, Klebanoff SJ, Headley CM, Hillier SL. 2000. Vaginal myeloperoxidase and flora in the pig-tailed macaque. J Med Primatol 29:36–41. <https://doi.org/10.1034/j.1600-0684.2000.290105.x>.
 46. Rodwell AW, Mitchell A. 1979. Nutrition, growth, and reproduction, vol 1, p. In Barile MF, Razin S (ed), The mycoplasmas. Academic Press, New York, NY.
 47. Huppert JS, Bates JR, Weber AF, Quinn N, Gaydos CA. 2013. Abnormal vaginal pH and *Mycoplasma genitalium* infection. J Pediatr Adolesc Gynecol 26:36–39. <https://doi.org/10.1016/j.jpog.2012.09.005>.
 48. Lokken EM, Balkus JE, Kiarie J, Hughes JP, Jaoko W, Totten PA, McClelland RS, Manhart LE. Recent bacterial vaginosis is associated with acquisition of *M. genitalium*. Am J Epidemiol, in press.
 49. Bouvet JP, Gresenguet G, Belec L. 1997. Vaginal pH neutralization by semen as a cofactor of HIV transmission. Clin Microbiol Infect 3:19–23.
 50. Fox CA, Meldrum SJ, Watson BW. 1973. Continuous measurement by radio-telemetry of vaginal pH during human coitus. J Reprod Fertil 33:69–75. <https://doi.org/10.1530/jrf.0.0330069>.
 51. Patton DL, Halbert SA, Kuo CC, Wang SP, Holmes KK. 1983. Host response to primary *Chlamydia trachomatis* infection of the fallopian tube in pig-tailed monkeys. Fertil Steril 40:829–840. [https://doi.org/10.1016/S0015-0282\(16\)47489-3](https://doi.org/10.1016/S0015-0282(16)47489-3).
 52. Patton DL, Kuo CC, Wang SP, Halbert SA. 1987. Distal tubal obstruction induced by repeated *Chlamydia trachomatis* salpingeal infections in pig-tailed macaques. J Infect Dis 155:1292–1299. <https://doi.org/10.1093/infdis/155.6.1292>.
 53. Patton DL, Wolner-Hanssen P, Cosgrove SJ, Holmes KK. 1990. The effects of *Chlamydia trachomatis* on the female reproductive tract of the *Macaca nemestrina* after a single tubal challenge following repeated cervical inoculations. Obstet Gynecol 76:643–650.
 54. Van Voorhis WC, Barrett LK, Sweeney YT, Kuo CC, Patton DL. 1997. Repeated *Chlamydia trachomatis* infection of *Macaca nemestrina* fallopian tubes produces a Th1-like cytokine response associated with fibrosis and scarring. Infect Immun 65:2175–2182.
 55. Mestecky J, Alexander RC, Wei Q, Moldoveanu Z. 2011. Methods for evaluation of humoral immune responses in human genital tract secretions. Am J Reprod Immunol 65:361–367. <https://doi.org/10.1111/j.1600-0897.2010.00923.x>.
 56. Taylor-Robinson D, Furr PM, Hanna NF. 1985. Microbiological and serological study of non-gonococcal urethritis with special reference to *Mycoplasma genitalium*. Genitourin Med 61:319–324.
 57. He J, You X, Zeng Y, Yu M, Zuo L, Wu Y. 2009. *Mycoplasma genitalium*-derived lipid-associated membrane proteins activate NF- κ B through Toll-like receptors 1, 2, and 6 and CD14 in a MyD88-dependent pathway. Clin Vaccine Immunol 16:1750–1757. <https://doi.org/10.1128/CVI.00281-09>.
 58. McGowin CL, Popov VL, Pyles RB. 2009. Intracellular *Mycoplasma genitalium* infection of human vaginal and cervical epithelial cells elicits distinct patterns of inflammatory cytokine secretion and provides a possible survival niche against macrophage-mediated killing. BMC Microbiol 9:139. <https://doi.org/10.1186/1471-2180-9-139>.

59. Manhart LE, Critchlow CW, Holmes KK, Dutro SM, Eschenbach DA, Stevens CE, Totten PA. 2003. Mucopurulent cervicitis and *Mycoplasma genitalium*. *J Infect Dis* 187:650–657. <https://doi.org/10.1086/367992>.
60. Pepin J, Labbe AC, Khonde N, Deslandes S, Alary M, Dzokoto A, Asamoah-Adu C, Meda H, Frost E. 2005. *Mycoplasma genitalium*: an organism commonly associated with cervicitis among west African sex workers. *Sex Transm Infect* 81:67–72. <https://doi.org/10.1136/sti.2003.009100>.
61. Patton DL, Sweeney YC, Rabe LK, Hillier SL. 1996. The vaginal microflora of pig-tailed macaques and the effects of chlorhexidine and benzalkonium on this ecosystem. *Sex Transm Dis* 23:489–493. <https://doi.org/10.1097/00007435-199611000-00009>.
62. Phillips E, Nash P. 1985. Culture media, p 1051–1092. In Ballows HW, Shadomy HJ (ed), *Manual of clinical microbiology*, 4th ed. American Society for Microbiology, Washington, DC.
63. Horner PJ, Gilroy CB, Thomas BJ, Naidoo RO, Taylor-Robinson D. 1993. Association of *Mycoplasma genitalium* with acute non-gonococcal urethritis. *Lancet* 342:582–585. [https://doi.org/10.1016/0140-6736\(93\)91411-E](https://doi.org/10.1016/0140-6736(93)91411-E).
64. Jensen JS, Bjornelius E, Dohn B, Lidbrink P. 2004. Use of TaqMan 5' nuclease real-time PCR for quantitative detection of *Mycoplasma genitalium* DNA in males with and without urethritis who were attendees at a sexually transmitted disease clinic. *J Clin Microbiol* 42:683–692. <https://doi.org/10.1128/JCM.42.2.683-692.2004>.
65. Tully JG, Whitcomb RF, Clark HF, Williamson DL. 1977. Pathogenic mycoplasmas: cultivation and vertebrate pathogenicity of a new spiroplasma. *Science* 195:892–894. <https://doi.org/10.1126/science.841314>.
66. Tully JG, Taylor-Robinson D, Rose DL, Cole RM, Bove JM. 1983. *Mycoplasma genitalium*, a new species from the human urogenital tract. *Int J Syst Bacteriol* 33:387–396.