Mycoplasma genitalium infections

Diagnosis, clinical aspects, and pathogenesis

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1. INTRODUCTION

M. genitalium is one out of the so far 15 named mycoplasma species of human origin. It is flask-shaped and is found predominantly in the urogenital tract. Due to the many features shared with the well-known respiratory pathogen *M. pneumoniae*, it was suspected already from its first isolation in 1980 to be a human pathogen (10).

Dark-field microscopy of fresh urethral smears from men with NGU often reveals motile bacteria with spiral forms (11). These are not *Treponema pallidum*, but resemble spiroplasmas, which are spiral shaped mollicutes with a significant agricultural importance. They are often transmitted between plants via insect vectors, but some species may also multiply in vertebrates at 37°C (12). Knowledge about these mollicutes were dramatically increased after the development of a new growth medium designated SP4 (13). The SP4 medium also improved the isolation rate of *M. pneumoniae* (14), and it has become one of the most widely used medium formulations in mycoplasmology. With the intention to isolate spiroplasmas, David Taylor-Robinson transported urethral swab specimens from 13 men with NGU to Joe Tully's laboratory at NIH in Maryland in 2SP medium, commonly used for transport of specimens for *C. trachomatis* culture (15). Using the SP4 medium, developed by Tully and colleagues (13; 14), evidence of growth was observed in two of the specimens as indicated by a colour change of the broth medium. The acidic colour-change indicating glucose fermentation appeared very slowly, taking initially more than 50 days, but after several passages, the strains were adapted to the medium and produced colour-change in about a week. Growth on agar medium was also very slow initially, but classical fried-egg colonies were observed after the strains were adapted (10). The strains designated G-37 and M-30 were shown to be serologically distinct from all other known mycoplasma species and were later named *M. genitalium* (16).

1.1. TAXONOMY AND THE GENERAL FEATURES OF THE MOLLICUTES

Mycoplasmas belong to the class *Mollicutes* (meaning soft skinned). Mollicute or mycoplasma is often used in a trivial way to refer to any species in the class. From a human perspective, the order *Mycoplasmatales* with a single family *Mycoplasmataceae*, is the most important. *Mycoplasmataceae* contains two genera, namely *Mycoplasma* and *Ureaplasma*. The genus *Mycoplasma* contains more than 100 species, of which 13 at present are considered a part of the human flora. Two *Ureaplasma* species *U. urealyticum* and *U. parvum* (previously known as *U. urealyticum*, parvo biovar) are commonly found in the human urogenital tract.

For many years, mycoplasmas were believed to be viruses, because they could pass through filters considered to block the passage of bacteria, i.e. 0.45µ or even 0.22µ pore size. In the 1930s, the concept of viruses became clearer, and mycoplasmas were now believed to be bacteria. During the fifties, some mycoplasma species were grouped with the bacterial L-forms, which are classical bacteria deficient in cell-wall formation. Finally, in the early sixties, the issue was settled, and the mycoplasmas found their current place in the taxonomic system. The filterability and lack of formation of a cell wall after incubation in medium without antibiotics, have now become one of the key properties defining new species as mycoplasmas (17; 18).

The mycoplasmas are the smallest prokaryotes capable of self-replication. They are believed to have evolved from Gram-positive bacteria most closely related to the clostridia by degenerative evolution leading to genomic reduction (19). Mycoplasmas, particularly species with the smallest genomes, have high mutation rates, suggesting that they are in a state of rapid evolution (20). *M. genitalium* is the species with the smallest genome of all *Mollicutes* studied thus far with a genome of only 580 kb.

In 1995 the *Haemophilus infuenzae* genome was published as the first complete genome sequence from a free-living organism (21). Later the same year, the *M. genitalium* genome sequence was published (22), making it the second bacterium to have its genome fully sequenced. After the larger 816 kb. *M. pneumoniae* genome was fully sequenced (23), it was shown that *M. genitalium* contains a subset of the *M. pneumoniae* genome complement (24), with all coding *M. genitalium* genes having their counterpart in the larger *M. pneumoniae* genome. Based on comparison of the *M. genitalium* 16S rRNA gene sequence, *M. genitalium* belongs to the *M. pneumoniae* cluster (**Figure 1**) with the closest relatives being *M. pneumoniae, M. alvi, M. gallisepticum, M. imitans, M. pirum,* and *M. testudinis.* A newly isolated glucose fermenting mycoplasma species from the human respiratory tract (25) also belongs to this cluster. This species is probably going to be named *M. amphoriforme*.

Most mycoplasma genomes have a low guanine plus cytosine (G+C) content, within the range of 24-33% G+C. The *M. genitalium* genome has a comparatively high G+C content of 32% (22), although some of its genes have a significantly higher G+C content i.e. the rRNA genes with a G+C content of 44%.

Mycoplasmas are difficult to study by classical genetic tools, both because of their fastidious growth requirements, and as a consequence of the absence of selectable markers. Furthermore, in contrast to other bacteria but similar to mitochondria, *Mycoplasma* use the UGA codon to code for tryptophan instead of the common STOP signal. This means that expression of mycoplasma genes in

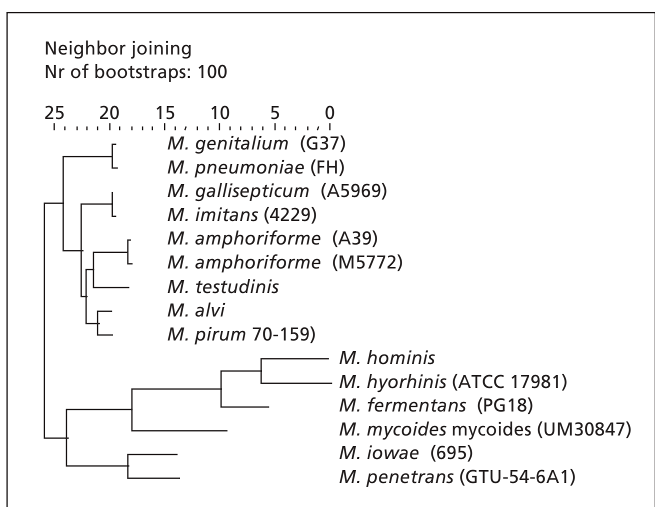

Figure 1. Phylogenetic tree of 16S rRNA gene sequences showing the *M. pneumoniae* and the *M. hominis* cluster. *M. amphoriforme* M5772 is a Danish isolate from our laboratory. Tree kindly provided by Dave Pitcher, PHLS, Colindale, UK.

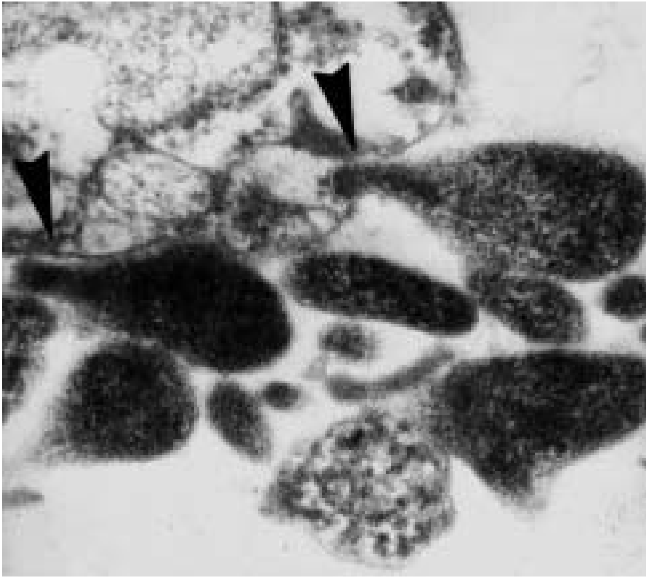

Figure 2. EM micrograph showing *M. genitalium* M 2300 (4;28) adhering to Vero cells with the specialised tip structure (arrow). EM performed by Jens Blom, Statens Serum Institut.

common bacterial systems is complicated due to the synthesis of truncated proteins. *M. genitalium* is a motile mycoplasma species (26), and like most of the other motile species, *M. genitalium* is flask shaped and uses its terminal tip-like structure to attach to surfaces and provide a gliding motility (**Figure 2**). The speed averages about 0.1 μ/s which is slower than that recorded for *M. pneumoniae* but faster than that of the avian species *M. gallisepticum* (26; 27). Attachment is mediated mostly by the main adhesin protein MgPa, the features of which is described in chapter 2.

The adhesin proteins may also have important functions during cell division. In *M. pneumoniae*, the P1 adhesin protein (the counterpart of the MgPa protein in *M. genitalium*) is located at one cell pole in all adhesive cells. By immunostaining and DNA content determination, it has been shown, that cells with a single P1 focus at one cell pole had a lower DNA content than cells with two foci. Those with one focus at each cell pole had the highest DNA content, suggesting that the nascent attachment organelle is formed next to the old one and migrates to the opposite cell pole before cell division (26).

2. PATHOGENESIS

Mycoplasmas could be considered the optimal parasites, *i.e.* they rarely kill their host, and fulminant infections are uncommon, rather, they tend to follow a more chronic course.

Mycoplasma pathogenesis has been studied intensively for several years, but among the human mycoplasma species, *M. pneumoniae* has been more thoroughly studied than *M. genitalium.* Due to their close genetic relationship, some features can probably be generalised.

Mycoplasmas are primarily considered surface parasites of mucous membrane cells. They usually have strict host and tissue specificities, although both species and tissue barriers may occasionally be broken down. This has been observed in a few cases where humans have been infected with animal mycoplasma species (29-31). Breaks in the tissue tropism within the host has also been reported as demonstrated by the isolation of *M. pneumoniae* from the urogenital tract (32) and *M. genitalium* from the respiratory tract (33).

The urogenital tract appears to be the primary tissue infected by *M. genitalium.* No information is available, however, about specificity towards particular urogenital cell types, but *M. genitalium* adherence does not appear to be restricted to epithelial cells *in vitro.*

The tissue damage caused by mycoplasmas can be accounted for only partially by mycoplasmal toxins and harmful metabolites such as hydrogen peroxide and superoxide metabolites known to be secreted by *M. genitalium* (34). Much of the tissue damage seen by infection with *M. pneumoniae* appears to be caused by the host cell response (35), and it could be anticipated that the same is the case in *M. genitalium* infections.

Mycoplasmas have been found to interact with many components of the immune system, inducing macrophage activation and cytokine production. Some mycoplasmal cell components may act as superantigens, and several autoimmune manifestations have been observed (recently reviewed in (36)). The discovery of mycoplasma adhesins responsible for attachment to host cells has provided important new insights into the pathogenesis. Furthermore, within recent years, evasion of the host immune system by antigenic variation of surface components has been intensively studied for a range of species. Demonstration of the ability of mycoplasmas to enter host cells, and to cause cell fusion, apoptosis, and even oncogenic effects (36), has further stimulated research.

2.1. ADHESION

Adhesion of *Mollicutes* to host cells is a prerequisite for colonisation and subsequent infection. The lack of a cell wall and cell wall associated structures such as fimbriae often responsible for adhesion of other bacteria indicates that this process is mediated by membrane bound components, which have been termed adhesins. Adhesins have been intensely studied both in *M. pneumoniae* and in *M. genitalium.* Both species bind erythrocytes from a variety of species (38) with their adhesin molecules clustered at the tip structure of the polar cell (39). Both species also attach to eucaryotic cells such as Vero cells (28), as shown already in the initial description of the species (10), but more importantly, *M. genitalium* attaches to the epithelial cells of human fallopian tube (37) (**Figure 3**)

A variety of techniques have been applied in the identification of the adhesins. SDS PAGE of mutant strains incapable of haemadsoption has been most widely used in the characterisation of proteins involved in adhesion (40). Monoclonal or polyclonal, monospecific antibodies to missing proteins have then been used to document the importance by blocking the adhesion (41-43). In addition, the similarity between *M. pneumoniae* and *M. genitalium* has been used to look for cross-reactive antibodies or cross-hybridising DNA probes from already identified *M. pneumoniae* adhesins (44). Based on characteristic lipoprotein-specific features, 21 putative lipoprotein genes have been identified in the *M. genitalium* genome (24), but only a minority have been characterised as adhesins (22). Recently, adhesion has been studied by transposon mutagenesis using Tn 4001 to produce knock-out mutants deficient in adhesion (45). Using this method, some of the accessory proteins involved in clustering of the adhesins have been characterised, but the disadvantage of this approach is the non-random insertion of the transposon in specific hot spots in the genome. Therefore, the development of methods for targeted disruption of genes based on homologous recombination,

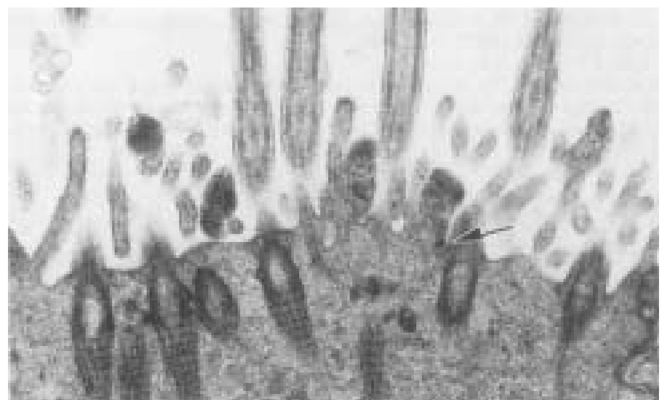

Figure 3. Electron microscopy of human fallopian tube obtained from women undergoing laparoscopic tubal ligation. Tissue grown *in vitro* inoculated with *M. genitalium*. Cross-sectioned *M. genitalium* cells attaching near the base of the cilia are marked by arrow. Reproduced from (37) with the authors' permission

which has been demonstrated for *M. genitalium* gene MG218 appears to be an important tool in the further investigations (46).

The main adhesin P1 of *M. pneumoniae* closely resemble the *M. genitalium* counterpart MgPa (ORF-2 or *mgpB* of the MgPa operon). The genes for both adhesins are organised in operons with three genes (47; 48). The *M. genitalium* MgPa operon consists of ORF-1 (29 kDa protein), ORF-2 (MgPa), and ORF-3 (MG192; 114 kDa protein). The function of the ORF-1 gene and the equivalent ORF-4 in *M. pneumoniae* is not known. The protein from the *M. pneumoniae* ORF-4 gene has been expressed in *E.coli* and used to immunise mice, but the antibodies were unable to recognise any antigen in immunoblots of *M. pneumoniae* proteins (49). In *M. pneumoniae*, the ORF-6 gene product appears to be cleaved in a 40- and a 90 kDa component (49). In contrast, the *M. genitalium* ORF-3 gene is present in full length in the protein profiles (50). The MgPa and the ORF-3 gene product are immunogenic both in immunised animals and in humans (50) and both have repeated sequences in the chromosome.

Another adherence component of the tip is designated P30 in *M. pneumoniae* (51) and P32 in *M. genitalium* (44). The genes encoding P30 and P32 are located in operons located distant from the MgPa and P1 operons, and are expressed together with adherence accessory proteins designated HMW3 (for high molecular weight) and P69, respectively (44). These accessory proteins, together with the other HMW proteins in *M. pneumoniae* and their analogues in *M. genitalium*, are important for clustering of the adhesin at the tip, and for maintaining the shape of the cell, acting like a cytoskeleton (39).

An additional membrane associated and surface exposed protein in *M. pneumoniae* has been designated P116, and is highly immunogenic. P116 may also be adherence associated, as antibodies directed against it abolish adhesion (43). This gene has its counterpart in MG075 (22), but protein expressed from this gene has not been detected by 2D PAGE (52), or among immunoreactive *M. genitalium* proteins identified by mass spectroscopy (50). Interestingly, the MG075 gene appears to be variable among strains as demonstrated by amplified-fragment length polymorphism fingerprinting (AFLP) (53). The variability has been shown widespread and not only limited to the restriction enzyme recognition site (Branko Kokotovic, personal communication, 2003).

The receptor for *M. pneumoniae* adhesion is believed to be long-chain sialo-oligosaccharides on the host cell (54), but other structures such as a glycoprotein isolated from human lung fibroblasts by Geary *et al.* (55; 56) may also be involved, since neuraminidase treatment does not completely abolish adherence. The same purified fibroblast glycoprotein was also capable of blocking the adhesion of *M. genitalium* to MRC-5 fibroblasts (56). Recently, surfactant protein D, considered being a part of the innate immune system and present in the lung alveoli, was shown to bind *M. pneumoniae*, presumably by a non-protein interaction mechanism (57). Thus, it appears that *M. pneumoniae* lipids may act as adhesins and that it is able to use the host's defence mechanisms for pathogenesis. However, whether *M. genitalium* uses similar receptors in the urogenital tract is still an open question. Recently, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key component of the glycolytic pathway, was shown to be one of the ligands involved in binding *M. genitalium* to human vaginal and cervical mucin (58). GAPDH exhibits its main function in the cytoplasm as a housekeeping component. Finding that it may also serve as an adhesin may be yet another way to rationalise the limited genomic information available in *M. genitalium*.

In conclusion, the presence of several adhesins in *M. genitalium* is well documented, and information about their interaction with host cells is accumulating. However, the interaction with host cell receptors and the relative importance of the different types of adhesin/receptor interactions remains to be determined.

2.2. EVASION OF THE HOST IMMUNE RESPONSE BY ANTIGENIC VARIATION

Mycoplasmas are dependant on an intimate contact with the host cells. In order to evade the immune response of the host, and to adapt to changing environments, they have a need for phenotypic plasticity, such as variation in the antigenic composition of the surface components. Other mechanisms, such as mimicry of host antigens, and survival within professional phagocytes, may help the parasite to survive (reviewed in (59)). The antigenic variation can be brought about by two basically different mechanisms. Either, the pathogen may regulate the expression of virulence factors in response to changes in the environment by signal transduction pathways, or the microbial population as a whole may spontaneously and randomly generate distinct new phenotypes that will survive the host response (59). Since mycoplasmas have few regulatory genes that could serve as sensors to environmental stimuli, and a few genes encoding transcriptional factors, the most important means for phenotypic plasticity in mycoplasmas is antigenic variation caused by molecular switching events (36).

Surface exposed membrane proteins are the major antigenic determinants of mollicutes, and a variety of mechanisms have evolved to generate high frequency intragenomic changes in nucleotide sequence or DNA conformation at selected chromosomal loci (60; 61). The functional consequence is, that these alterations in genotype can rapidly promote phenotypic heterogeneity, even in small, clonal populations of bacteria, such as the limiting inoculum that initiates an infection (62). The phenotypic changes do not occur more often when they are useful than when they are not (63). The capability to generate genetic variation has apparently evolved at particular loci to promote random phenotypic variation as a result of a constantly changing host environment. Thereby, the adaptive potential has been maximised without compromising household functions. Interestingly, host organisms generate random diversity in B lymphocytes by similar mechanisms (64).

Multiple copies of partial gene sequences have been reported for many pathogenic bacteria. Considering the very limited genetic information that mycoplasmas contain, the number of mycoplasmal genes involved in diversifying the surface exposed antigens is remarkably high (60).

2.2.1. Sequence variation of the MgPa gene:

In *M. genitalium*, nine repetitive elements, which are composites of non-contiguous regions of the MgPa operon, have been identified (3; 22). The sequence identity between the repeats and the operon sequences ranges from 78-90% and together with the sequence of the MgPa operon, they represent 4.7% of the total genomic sequence (22). Considering the evolutionary pressure towards a minimal genome (65), it would seem reasonable to expect such repeats to have an important function. The repeated MgPa sequences were identified by probing *M. genitalium* chromosomal DNA with a probe prepared from fragments of the MgPa operon (66). It was shown, that approximately half of the gene was present as multiple copies. The restriction fragments used for probe preparation were designated A-J (**Figure 4**), and although this division did not correl-

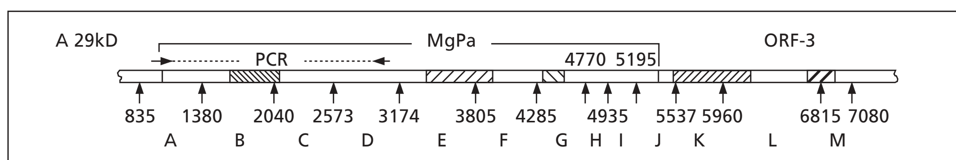

Figure 4. Nomenclature of the MgPa operon. Regions with repeated sequences are shaded. Reproduced from (3).

ate well with boundaries between repeated and single copy parts of the MgPa gene, this nomenclature has been used and expanded in subsequent work (3).

During the development of the MgPa based PCR (1), sequence variability in the amplified sequence was evident when clinical specimens were examined in contrast to the sequence conservation observed when the cultured stains available at that time was examined. When PCR amplified fragments of the MgPa gene from the

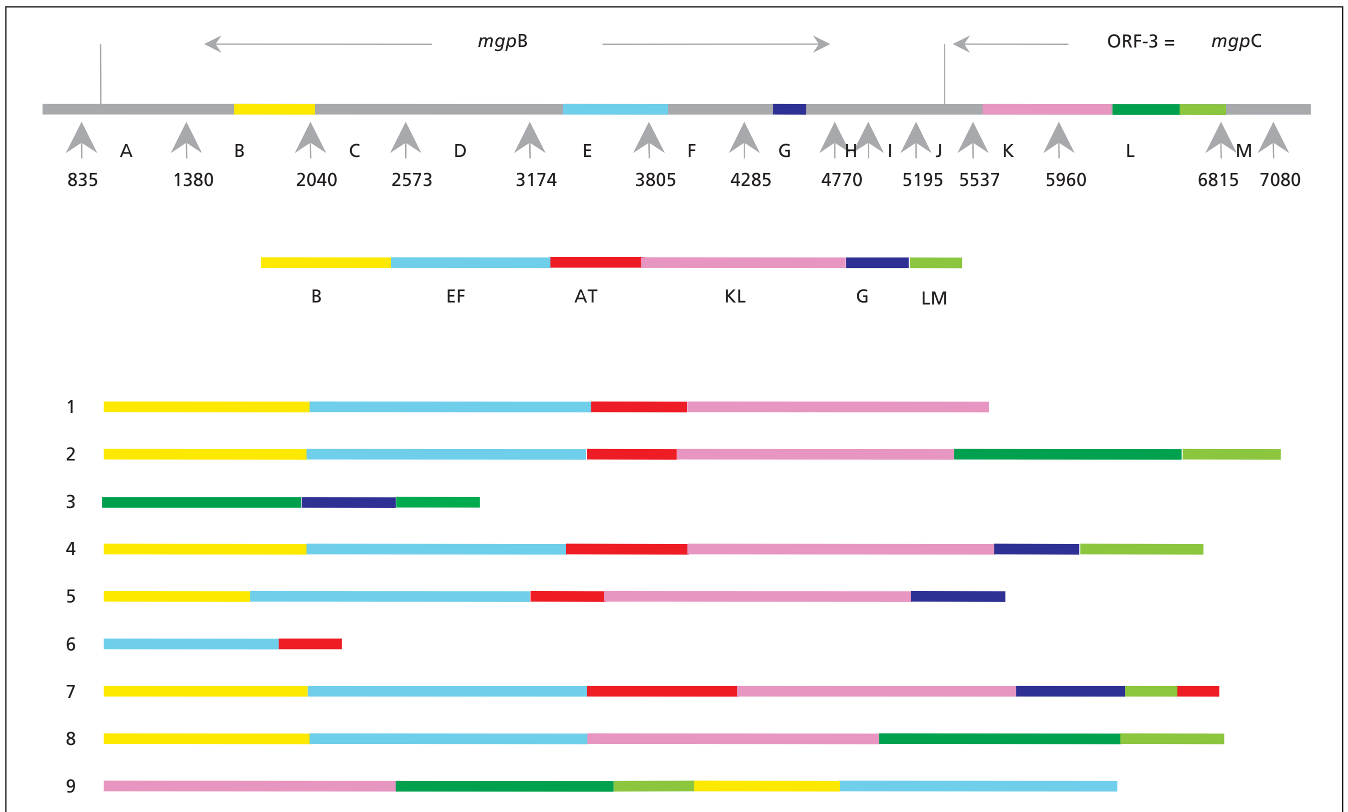

Figure 5. Positions of repeat regions in the MgPa gene, general structure, and structure of the nine MgPa related genome repeats (67).

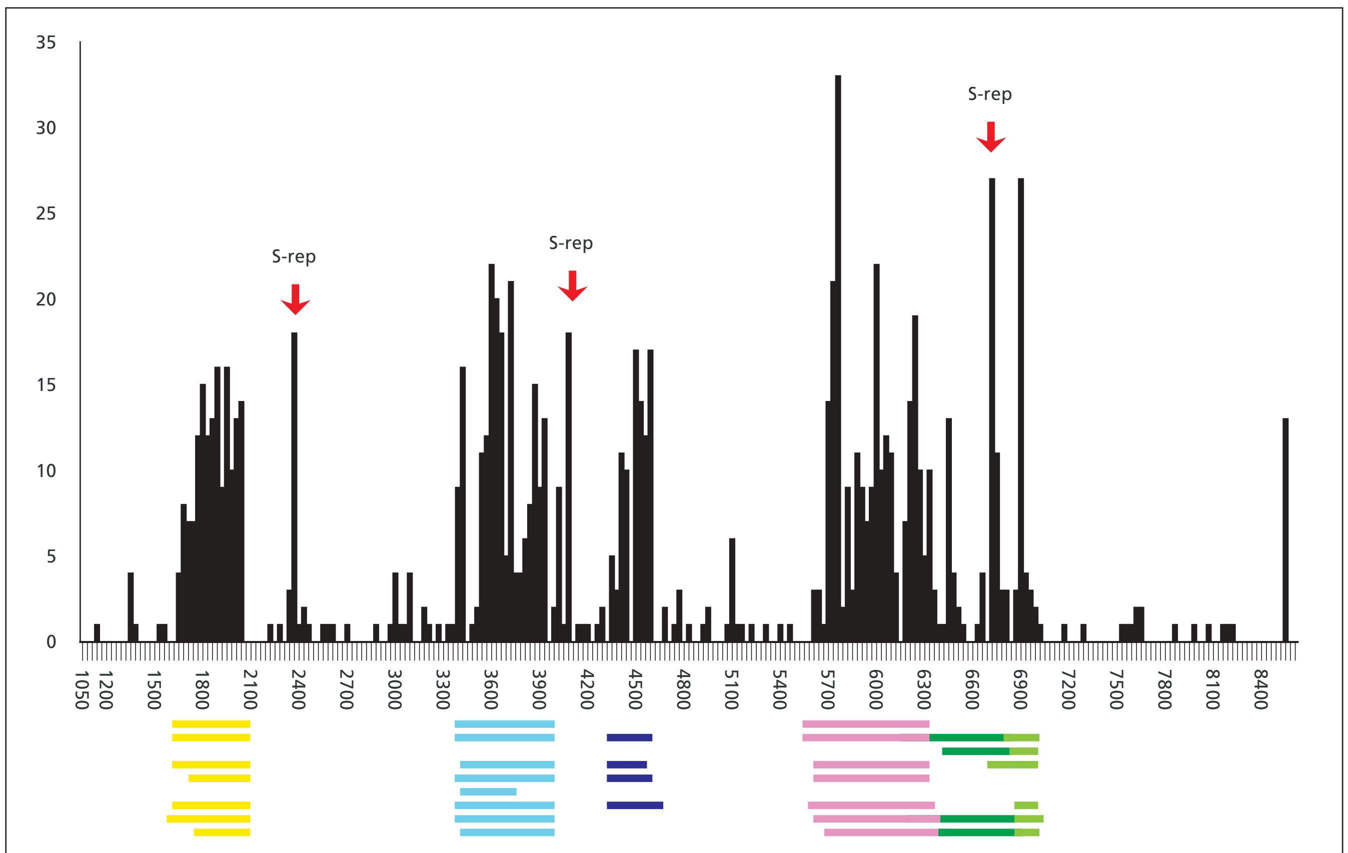

Figure 6. Number of base differences per 30 bp between *M. genitalium* G37 and the Danish strains M2288 and M2300. Positions of serine repeats marked with arrow. The position of the nine genomic MgPa repeats are marked below the graph (67).

four new Danish isolates (4) were analysed, it was found, that they were different from each other and different from the previously isolated strains.

At the IOM meeting in 1994, Camella Bailey presented the initial analysis of some of the MgPa related genomic repeat sequences and at the same meeting, our preliminary data showing the variability of the MgPa genes from the four Danish *M. genitalium* strains was presented. By a joint effort, we were able to show, that the B-region of the MgPa gene had five genomic repeats (later seven repeats were detected) and that the same region in the MgPa gene of the Danish strains was hypervariable (3). Furthermore, a difference in the B-region between the early passage strain of *M. genitalium* used in our laboratory (designated G37-DK), compared to the G37-US strain identical to the strain subsequently deposited in the ATCC was found. However, a sequence matching exactly the variant sequence in G37-DK was found in one of the repeats of the G37-US strain, indicating that the repeat sequences provide a source for sequence variation in the operon (3).

After the genome sequence of *M. genitalium* was published (22), the general structure of the genomic repeats was shown to comprise sequence similar to the MgPa operon segments B, E and F, followed by a stretch with an unusual high A+T percentage (around 80%). Then, a rather long stretch of the ORF-3 gene K and L regions, was in some cases interrupted by a G-region sequence from the MgPa gene, followed again by an ORF-3 sequence from the border between the L and M regions (**Figure 5**) (67).

The complete MgPa sequence and part of the ORF-3 sequence of the two Danish strains M2288 and M2300 were determined, and aligned with the sequence of the G37 (US) strain (67). When the number of base differences per 30 bases was calculated and related to the sequence position, it was shown (**Figure 6**), that relatively conserved areas were interrupted by hypervariable regions. Most often, these regions were confined to areas where MgPa genome repeats were present. However, in a few positions, spikes with a high variability were detected. By inspection of the sequence data, it was found, that these hypervariable regions were caused by repeats of AGT base triplets coding for serine. Three of these serine repeats were found, two in the MgPa gene, and one in the ORF-3 gene with repeat lengths varying from strain to strain from five to 11 serine codons. The function of the serine repeats is not clear, but it could be speculated, that they are important in the three-dimensional structure of the molecule, functioning as spacers between the adherence mediating epitopes (67). Interestingly, Opitz and Jacobs (42) found, that most of the adherence mediating epitopes of *M. genitalium* G37 are found in the hypervariable regions, primarily in the B and EF regions. They used overlapping octapeptides covering the MgPa protein to map the epitopes of adherence inhibiting monoclonal antibodies. In contrast, out of three polyclonal monospecific rabbit antisera produced by immunisation with recombinant MgPa fragments generated from the N- terminal, middle, and C-terminal parts of MgPa, only the antibody against the C-terminal part was able to block adhesion (43). The reason for this discrepancy is not clear, but obviously, the experimental set-ups are different, with the latter system being closer to the *in vivo* situation. The observation, that the C-terminal part is more immunogenic is also substantiated by the observation that human antibodies, primarily recognise the C-terminal fragment when the same fragments were reacted with human sera (50).

Ongoing work indicates that antigenic variation occurs also *in vivo*. A different pattern in restriction enzyme analysis (REA) of the MgPa gene was found between two isolates obtained from a patient with recurrent non-chlamydial non-gonococcal urethritis (NCNGU) with an 11-week interval. A different REA type was demonstrated in the 3' end of the MgPa gene (the G-region) and in the ORF-3 gene (the KLM region) (67). Furthermore consecutive isolates of *M. genitalium* obtained from male chimpanzees inoculated intraurethrally with *M. genitalium* during the early animal experiments (68) accumulated an increasing amount of mutations after five weeks post inoculation (Chimp A52 used as an example in **Figure 7**). However, all mutations were located within the hypervariable regions, and all the mutations could be accounted for by the sequences present in the MgPa-reps (69). The conserved sequence

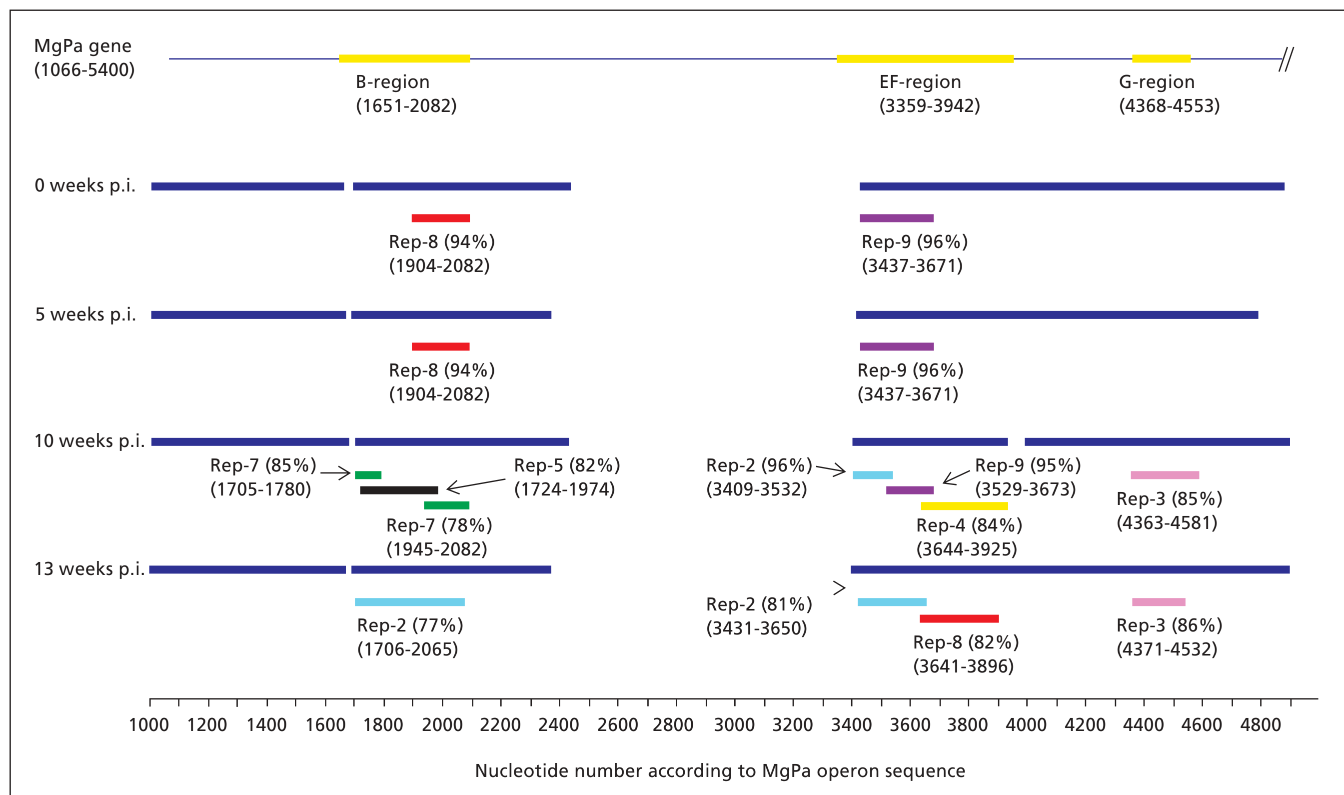

Figure 7. Sequence variation in four consecutive *M. genitalium* isolates from Chimpanzee A52 (68) inoculated with an early passage strain for *M. genitalium* G37. The time after inoculation (p.i.) to sampling extended until 13 weeks. The percentage of identity with the operon sequence (given for each MgPa-rep sequence) was calculated using the entire length of the matching MgPa-rep sequence as the denominator (69).

found during the first five weeks of infection correlates closely with the absence of antibodies in the chimpanzees during the first five weeks of infection (68). This finding strongly supports the notion, that the selection of *M. genitalium* strains expressing different epitopes within the adherence mediating domains is driven by selective pressure from the immune system (69).

2.2.2. Other variable antigens

As demonstrated by AFLP fingerprinting, the MG075 gene appears to be variable among strains (53). However, since the gene product is unknown, the effect of the variation is not known.

Recently, Ma and Martin (70) amplified a fragment of the putative lipoprotein gene MG309 from 42 specimens from 31 patients and demonstrated that this gene had a variable number of tandem repeats. They found 11 different types of these repeats varying in number from 7 to 17. In five patients, a mixture of 2 or 3 types were detected, suggesting that the *M. genitalium* population was changing from one type to another. Lipoproteins are usually membrane associated, but the specific function and location of the MG309 gene product is not known. The gene is expressed, however, as shown by reverse transcriptase PCR of broth grown *M. genitalium* cells (David H Martin, personal communication).

In conclusion, antigenic variation occurs *in vivo*. This has been shown to take place in the MgPa operon as demonstrated by the recombination between the MgPa-reps and the operon. Recombination with the MgPa-reps may generate almost unlimited variation in these epitopes allowing unique opportunities to evade the immune response and to adapt to various cell surfaces. Other variable antigens are currently under study and may prove to offer additional variation.

2.3. INTRACELLULAR LOCATION OF *M. GENITALIUM*.

From the early days of mycoplasmology, surface versus intracellular location has been disputed. During the 70's and 80's most evidence favoured an extracellular location. The studies of Lo *et al.* on the virus-like infectious agent (71), which was later shown to be a strain of *M. fermentans* (72) demonstrated quite convincing, that structures resembling mycoplasmas could be observed intracellularly by EM. Later, the same group discovered a new mycoplasma species isolated from patients with AIDS (73) which appeared to be capable of invading eucaryotic cells by means of a specialised arrow-shaped tip structure justifying its naming as *M. penetrans* (74). Most importantly, this phenomenon was also demonstrated in urothelial cells from infected patients, providing evidence that it occurred also *in vivo* (75).

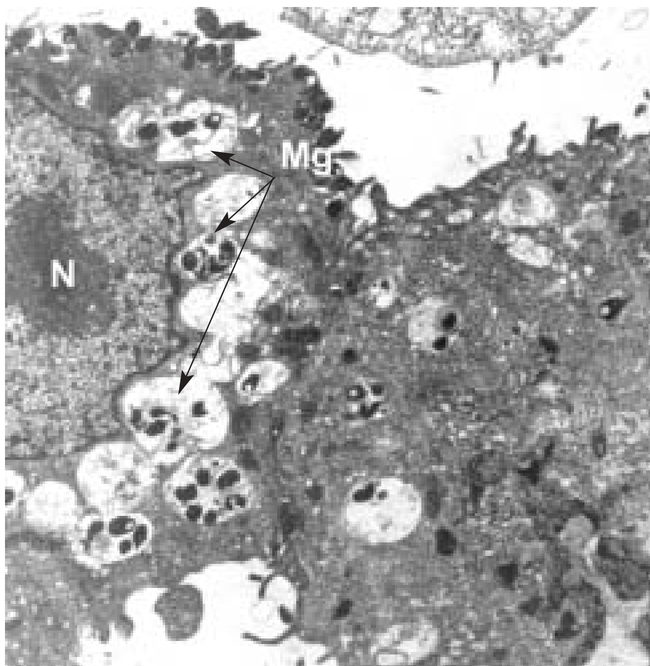

Figure 8. Two Vero cells with adherent and intracellular *M. genitalium* cells. Mycoplasmas in intracellular vacuoles (marked by arrows) are seen close to the nucleus (N). Modified from (28). EM photo provided by Jens Blom, Statens Serum Institut.

Documenting intracellular persistence of mycoplasmas could explain the severe difficulties experienced in eradicating them with antibiotics, which has been an enigma for years. Both in cell cultures and in humans, mycoplasmas may be isolated after antibiotic treatment with relevant doses of antibiotics to which the strain is highly susceptible *in vitro* (76).

Using electron microscopy (EM), the morphology of the Danish *M. genitalium* strain M2300 was studied during its isolation by coculture with Vero cells (28). Like *M. penetrans*, it appears that *M. genitalium* enters the cell through its specialised tip structure. Intracellularly, they were located in membrane bound vacuoles, often near the nucleus (**Figure 8**). Only 10% of the Vero cells revealed invasion of mycoplasmas; whether this reflects the age of the cell or just an uneven distribution within the cell causing the section to appear empty is not clear (28). EM studies are difficult to interpret because two-dimensional sections of a three dimensional structure is studied. It could be argued, that what was interpreted as mycoplasma cells in vacuoles were actually the bottoms of crypts formed by invagination of the membrane. This issue could be addressed by staining the cells with ruthenium red, which stains the mucopolysaccharide surface coats of the cells. Taylor-Robinson *et al.* successfully used this approach to demonstrate, that the intracellular vacuoles with *M. hominis* and *M. fermentans* seen in their studies did not communicate with the surface of the cells (77; 78). However, in the *M. genitalium* study, this approach was not successful, possibly because the Vero cells were grown in a medium without foetal calf serum (28).

Mernaugh *et al.* used the G37 type-strain and human lung fibroblasts to study the kinetics and molecular mechanism involved in invasion. The adhesion to the cell surfaces occurred almost instantaneously, as the cells were covered with mycoplasmas already at "zero" time. After one hour, mycoplasma cells were seen invaginating the cell membrane with a morphology similar to clathrin-coated pits (40). Clathrin-coated pits are important in the signal modulation in cells being one of the main pathways for inactivation of membrane receptors by endocytocis (79). It has also been suggested that *C. trachomatis* enters the cell by means of these structures (80). After 12 hours, a significant number of *M. genitalium* cells were observed in vacuoles, and at 96 hours, lysis of the infected cells was almost complete (40).

Baseman *et al.* labelled *M. genitalium*, *M. pneumoniae*, and *M. penetrans* with $DiIC_{18}$, a fluorescently labelled cationic lipophilic tracer that can be used to label the mycoplasma membrane yet retaining mycoplasma viability (81). Labelled mycoplasmas were grown with cell cultures, and by laser confocal microscopy, thin sections of the cells could be visualised, showing clusters of fluorescently stained mycoplasmas without contact with the eucaryotic cell membrane. Another interesting finding was the existence of *M. pneumoniae* mutants capable of cytadherence, but incapable of invasion. Unfortunately, these mutants have not been further characterized. Subsequent work by the same group showed, that *M. genitalium*, *M. pneumoniae*, and *M. penetrans* were able to persist intracellularly for extended periods of time (82). Intracellular survival was documented for *M. pneumoniae* and *M. genitalium* by culturing HEp-2 cells in a medium containing a high concentration of gentamycin for up to six months (82). Since both mycoplasma species are susceptible to gentamycin, and since gentamycin does not cross the HEp-2 cell membrane, only those mycoplasmas persisting inside the cells would survive. Survival and even multiplication of *M. pneumoniae* was documented by weekly cultures of fractionated HEp-2 cells showing an increasing ratio of mycoplasma cfu per cell. For *M. pneumoniae* the increase was more pronounced in the cyto-

plasmic fraction than in the nuclear fraction. Interestingly, *M. genitalium* could not be subcultured from its intracellular state, but persistence was documented by PCR, which remained positive for all 6 months. Furthermore, DNA replication of both *M. pneumoniae* and *M. genitalium* was shown by incubating the cells with medium containing radiolabelled thymidine in the presence of an inhibitor of eucaryotic DNA polymerase. After 48 hours, total DNA extracts were subjected to restriction enzyme digestion with *Eco*RI, and autoradiographed to demonstrate the mycoplasma specific restriction enzyme pattern (82). *M. penetrans* was also capable of prolonged persistence, but since this species is resistant to gentamycin, the experimental design regarding this species was less convincing. However, an interesting observation was made during attempts to eradicate *M. penetrans* from the cell culture with ciprofloxacin: Two weeks after the inclusion of the antibiotic to the medium, cultures turned negative, but even though the treatment was maintained for one month, viable *M. penetrans* cells were recovered after one week without antibiotic. Surprisingly, the mycoplasmas had maintained their ciprofloxacin susceptible phenotype (82).

In conclusion, it appears that *M. genitalium* behave like a facultative intracellular pathogen, *i.e.* it is capable of retaining viability both extracellularly and intracellularly *in vitro*. Whether the same occurs *in vivo* has not been proven. However, from clinical experience with patients receiving multiple courses of highly active antibiotic treatment with temporary improvement but subsequent relapse, it seems likely that such mechanisms may act in at least in a subset of patients.

3. DIAGNOSTIC ASPECTS

In infectious diseases, the ability to detect and clearly identify the infecting microbe is essential. Two fundamentally different principles apply for infectious disease diagnostics. The first is the direct detection of the infectious agent by microscopy, culture, antigen detection, or detection of nucleic acids by a variety of methods. The second is the indirect evidence provided by the detection of antibodies.

3.1. CULTURE

M. genitalium was first isolated in 1980 from two of 13 men with NGU using the SP4 medium. Evidence of growth occurred extremely slowly; initially more than 50 days were needed for a colour-change to occur (10). The two new strains were designated G-37 and M-30. G-37 was later selected as the type strain of *M. genitalium* (16).

Even though strains G-37 and M-30 were re-isolated from the original specimens after they had been refrozen and transported back to the UK, later attempts using culture of urogenital specimens have not been very successful. Taylor-Robinson *et al.* failed to isolate *M. genitalium* although they reported "presumptive isolation" based on colour-change in the mycoplasma broth from men with NGU (83). However, a colour change in the mycoplasma broth could also be caused by other bacteria, including unknown mycoplasma species, or by human cells, which may grow in the rich SP4 medium.

Samra *et al.* (84) studied a variety of clinical specimens using SP4 and a modified medium with horse serum. They also observed acidification of the medium in about 15% of the specimens, but were unable to subculture any microbes. Among the 513 specimens studied, 33 were urethral specimens from men with NGU, and 83 were cervical swab specimens from women suspected of an STI. However, no isolates of *M. genitalium* were obtained. Although not clearly described, the swab specimens appear to have been transported in Stuart's transport medium and inoculated mostly within three hours. Although the viability of *M. genitalium* in Stuart's medium was found to be five days, this contrasts with experience from our lab. We observed a poor recovery of *M. pneumoniae* from clinical specimens transported in this medium (unpublished observation), and ascribed it to the presence of methylene blue, which inhibits the growth of *M. pneumoniae* and some other species. Whether *M. genitalium* is sensitive to growth inhibition by methylene blue is at present not clear.

In 1988, Joel Baseman reported on the isolation of four strains of *M. genitalium* found among 16 cultures of *M. pneumoniae* isolated from recruits during a vaccine trial for an *M. pneumoniae* vaccine (33). This publication made mycoplasmologists rethink the dogma that *M. genitalium* was restricted to the urogenital tract. At approximately the same time, and in the same laboratory, an isolate of *M. pneumoniae* isolated from the synovial fluid of a patient with pneumonia and polyarthritis was investigated actively for the presence of a mixed infection with *M. genitalium* by epi-immunofluorescence on agar-grown colonies (85; 86). Approximately 1-2% of the colonies were found to react more strongly with the *M. genitalium* polyclonal antibody than with the polyclonal antibody against *M. pneumoniae.* Subsequently, the *M. genitalium* strain was recovered by selection of colonies within the inhibition zone of *M. pneumoniae* in a disc growth inhibition test (a principle closely resembling selection of resistant colonies from the zone around an antibiotic containing disc) (85; 86). Thus, five *M. genitalium* strains have been recovered from what was believed to be cultures of *M. pneumoniae* by the same group of persons within a short period of time. Since then, a lot of work has been spent on the importance of *M. genitalium* in the respiratory tract, but no other reports about respiratory tract isolates have been reported.

Eight strains of *M. genitalium* were reportedly isolated by culture in SP4 medium from urogenital tract specimens from STD clinic patients in China (87). Unfortunately, these strains have not been made available outside of China despite several requests.

One of the aims of the current thesis was to develop diagnostic tools to study the role of *M. genitalium* in human disease, and since culture is the mainstay of infectious disease diagnostics, an improved culture method was developed (4). Like many other laboratories with interest in human mycoplasma infections, attempts to isolate *M. genitalium* from urogenital specimens had also been made at SSI, however, without much success. During the first clinical study (2), specimens were collected in SP4 medium with the specific purpose of culture. However, despite incubation for 12-14 weeks and the use of three different medium formulations with quality-controlled ingredients, no isolates were obtained. Since it had been documented by PCR, that *M. genitalium* was present in some of the specimens, the development of a culture method was facilitated. Furthermore, growth of the bacterium could be followed by subjecting dilutions of the growth medium to PCR. Considering the susceptibility of cultured cells to mycoplasma infection with a variety of reported species, and also with the knowledge of propagation of fastidious strains of *M. hyorhinis* in cell cultures (88), attempts were made to grow *M. genitalium* in cell cultures (4). This approach proved to be very efficient but extremely time-consuming. Four new strains were recovered and cloned to ensure the purity of the strains. For most strains, at least six months were required from the cell-culture was inoculated, until the strain was ready for cloning (4). Furthermore, some strains did not grow on solid media after filtration and therefore, they were difficult to purify. Since the publication of the manuscript, three additional strains have been recovered (8); two of them were from the same patient attending an STD clinic in Bordeaux, France at several occasions with a recurrent *M. genitalium* urethritis. From this patient, three PCR positive specimens were kindly provided by Dr. de Barbeyrac, Bordeaux, and all three specimens grew in the cell culture system, but only two have been adapted to cell-free growth. An early cell-culture passage of one of the strains (M 2282) from the isolation study (4) was later successfully grown in cell-free medium and cloned. In **Table 1**, all currently available strains of *M. genitalium* have been listed.

The Ultroser HY serum substitute was shown to be superior to foetal calf serum, when isolation was attempted in Vero cells (4). This product was discontinued from the distributor, and only re-

Table 1. Publicly available strains of *M. genitalium.*

M. genitalium strain designation	Isolate from	Source of strain at SSI	Reference
G-37	Urethra	*David Taylor-Robinson*	(16)
M-30	Urethra	*J. G. Tully*	(16)
R 32G	Human throat	*J. G. Tully*	(33)
Tw 10-6G	Human throat	*J. G. Tully*	(33)
Tw 10-5G	Human throat	*J. G. Tully*	(33)
Tw 48-5G	Human throat	*J. G. Tully*	(33)
UTMB-10G	Synovial fluid	*J. G. Tully*	(86)
M2282	Urethra	*J. S. Jensen*	(86)
M2288	Urethra	*J. S. Jensen*	(86)
M2300	Urethra	*J. S. Jensen*	(86)
M2321	Urethra	*J. S. Jensen*	(86)
M2341	Urethra	*J. S. Jensen*	(86)
M6090	Urethra	Specimen provided by *Bertille de Barbeyrac*, Bordeaux, France. Isolated in Copenhagen, Denmark.	(8)
M6151	Urethra	Specimen provided by *Bertille de Barbeyrac*, Bordeaux, France. Isolated in Copenhagen, Denmark. Isolated from same patient as M6090.	(8)

cently has it again become available from another producer. However, cell-assisted growth of *M. genitalium* has also been reported with standard cell-culture medium containing foetal calf serum (George Kenny, personal communication and (89)) although it has not led to new isolates.

Recently, Baseman *et al.* again reported isolation of *M. genitalium* (90). Low-income minority women were enrolled in an intervention trial to reduce the recurrence of STDs. *M. genitalium* was isolated from cervical and vaginal swab specimens from 31 of 838 women using SP4 medium with 0.25 mg/ml of ciprofloxacin. The latter was stated to inhibit growth of contaminating flora, in particular *M. hominis.* However, since the MIC_{50} for *M. hominis* is 0.5 mg/ml (91), more than half of the strains would not be inhibited at all. The effect on wild-type *M. genitalium* strains is unpredictable as the MIC ranges from 0.25-10 mg/ml (92), meaning that at least some strains would be inhibited. Furthermore, the specimens were passed through 0.45μ filters known to reduce the viable count >10-fold. Evidence of growth of *M. genitalium* was observed already from 14 days and up to 20 days. This is a very rapid growth compared to the >50 days required for isolation of the original G37 strain. It was notable, that all isolations occurred over a period of 6 months. It is not described how many specimens were positive for each of the 31 women, however, if the same proportion of the 838 women were examined each month during the 22 month study period, 229 women had been examined in the 6-months successful period, resulting in a positivity rate of 13,5%. Another surprising finding was that only 12 (39%) of the 31 culture positive women were PCR positive at the time of culture. It should be noted, however, that the DNA extraction method applied on the clinical specimens included a washing step where the pellet from the centrifuged specimens was resuspended in sterile water and incubated on ice for 20 min. before a repeat centrifugation step. Bearing in mind that *M. genitalium* is a wall-less prokaryote, the washing step is a very efficient method of inducing osmotic lysis leading to false negative PCR results.

3.2. SEROLOGY

The cross-reactions between *M. pneumoniae* and *M. genitalium* have significantly hampered the use of specific serology for diagnosis and epidemiological studies. Over the years, several techniques have been applied, but in most studies, the diagnostic performance has not been validated.

Already in 1982, Klaus Lind demonstrated the significant serological cross-reactions between *M. pneumoniae* and *M. genitalium* (93) in both complement fixation tests, metabolism inhibition, indirect haemagglutination, and disc growth inhibition. The cross-reactions were found both with antisera from rabbits immunised with *M. pneumoniae* and *M. genitalium*, and with human sera from patients with *M. pneumoniae* infection. Only the metabolism inhibition test with sera from patients with *M. pneumoniae* infection appeared to discriminate between infections with the two pathogens in the three sera examined. In a more extensive study, where also microimmunofluorescence, crossed immunoelectrophoresis and heamadsorption inhibition tests were included, these findings were to some extent confirmed (34). Interestingly, cross-reactions with rabbit antisera have been reported by others to be less pronounced (94), particularly in the metabolism inhibition test. This test, involving the growth of *M. genitalium* in the presence of human or animal serum, however, is not very practical for large-scale studies and has only been used in a few human studies. Tully *et al.* (95) studied sera from HIV-positive and HIV-negative blood donors. They found that the *M. genitalium* titre was significantly higher for patients with AIDS than for HIV-negative blood donors, but the importance of this finding is not clear (95).

Taylor-Robinson *et al.* observed a four-fold or greater rise in antibodies to *M. genitalium* in men with NGU using a microimmunofluorescence assay but the correlation to presumptive isolations was poor (96).

As a part of a prevalence study (2), acute phase serum specimens were collected, and IgG, IgM, and IgA were measured in an ELISA. A method for antigen preparation, which had previously been shown to detect clinically relevant serological responses to *M. pneumoniae* (97) as well as immunoblotting methods were used, however, no correlation between the PCR status and the antibody response could be shown. It should be noted, however, that only acute phase sera were investigated, and since it has been shown in a chimpanzee model (68; 98), that antibodies are slow to develop after urogenital inoculation, these findings should be interpreted with caution.

Wang *et al.* (99) studied the seroprevalence of *M. genitalium* antibodies in different populations with Triton X-114 extracted Lipid-Associated Membrane Proteins (LAMP). The assay was validated with serum and urine specimens from 104 patients; 40 (38%) were LAMP ELISA positive. 15 (38%) of the LAMP positive patients were urine PCR positive for *M. genitalium*, and all PCR positive patients were detected. The LAMP positive, PCR negative could have been caused by previous exposure to *M. genitalium*, cross-reactions with *M. pneumoniae* or other species, or lack of sensitivity of the PCR test. The LAMP ELISA thus appears to be one the most promising of the serological assays applied on male patients.

Immunoblotting was also applied in a Danish study (100) of serum from different groups of infertile women, where it was shown, that women with tubal factor infertility reacted with the *M. genitalium* MgPa, the 114 kDa protein, and a recombinant truncated MgPa fragment more often than women with infertility due to a male factor or unexplained infertility. Even more importantly, this relation was observed also after controlling for the *C. trachomatis* serological status (100). The recombinant MgPa fragment has later been applied in an ELISA format, which provides a means for rapid screening of a large number of patients (50). However, the significant variability of the MgPa protein should be taken into account, before the test can be used more widely. Among the Danish strains, a significant number of mutations are present in the 5' part of the cloned sequence. If the sequence variations in the MgPa gene (3) are biologically meaningful, it would be expected that they involved exposed and antigenically relevant parts of the expressed protein.

Recently, women enrolled in the above-mentioned study by Baseman *et al.* (90) were followed with sequential serology over a 22-months follow-up period using a modification of the LAMP ELISA described by Wang *et al.* (99). Using an ELISA OD cut-off determined by testing serum specimens from pregnant women with a

low risk of STDs, 83% of the 29 *M. genitalium* culture positive women had positive ELISA results at some time during the 22 month observation period. However, the time of seroconversion correlated poorly with the time of isolation. Furthermore, the OD values appeared to be rather low and fluctuated over time, and no results were presented for culture negative high-risk women (90).

3.3. DNA PROBES AND POLYMERASE CHAIN REACTION

Before the development of PCR methods, now documented to be of tremendous importance in studies on the role of *M. genitalium* in human infections, a few attempts were made using DNA probes. The design of radiolabelled oligonucleotide probes targeting the 16S rRNA was reported to have a detection limit of approximately 1000 organisms (101) and could be tailored to detect only *M. genitalium*. Clinical studies using such probes have not been reported, but actually, the sequence information from this study was used in one of the first publications on PCR for *M. pneumoniae* (102). Risi *et al.* used a cloned 256 bp. fragment of unknown function, and used it to probe simulated female genital tract specimen and found a detection limit of approximately 10,000 genome copies (103). Results from clinical studies with this probe have not been published. In contrast, a whole-genomic, nick-translated probe with a reported detection limit of 10^4 to 10^5 genome copies was used to study urethral specimens from 203 men (104). No evidence suggesting an important role of *M. genitalium* in acute NGU was found, but men with recurrent NGU were more often positive. Considering that a high proportion of the patients have a very low DNA load in urogenital specimens (8), it is surprising, that Hooton *et al.* found an overall rate of 15% *M. genitalium* positive in their study. Thus, data obtained with the TaqMan quantitative PCR assay on urethral and FVU specimens found that 28% of the positive urethral swab specimens contained less than 10 genome equivalents (geq) per assay corresponding to less than 12,600 geq/swab. Because of the centrifugation step involved in specimen treatment of FVU specimens, approximately 280 geq/ml of urine would correspond to the 10 geq per assay (8). Thus, highly sensitive assays are needed for detection of *M. genitalium* with a high clinical sensitivity. At present, only nucleic acid amplification tests offers the sensitivity needed and only PCR based assays have been published thus far.

The first PCR based test was published in 1991 (1) and very shortly after, Helen Palmer from David Taylor-Robinson's group published their method (105). Both of the assays were based on the MgPa DNA sequence. At that time, it was believed, that an adhesin gene would be rather conserved, since it has an important role in the pathogenesis of the infection. It was therefore surprising, that the region of MgPa flanked by the MgPa-1 and MgPa-3 primers (1) appeared to be variable. An *Eco*RI site present in the G-37 strain, from which the MgPa sequence was determined, was present also in the M-30 strain and in the four respiratory tract isolates, but in none of the amplicons from PCR positive Danish patients. Other sequence variants were also found by restriction enzyme analysis, and later by sequencing (8). This observation led to further studies into the genetic variability of *M. genitalium* (3) and to the acknowledgement of the need for alternative PCR assays (2; 6). The assay developed by Palmer *et al.* was a hemi-nested PCR (105), and usually this type of assay is extremely sensitive, but very prone to contamination due to carry-over. In an assay targeting a variable gene sequence, though, there is also a risk of false negative reactions, if a mutation is present in one of the primer binding sites. In one of the few head to head comparisons of *M. genitalium* PCR assays, however, a slight modification of the original hemi-nested assay using a new outer forward primer, was compared with our MgPa-1/MgPa-3 assay (106) and the two assays were found to be in perfect agreement. Fortunately, both assays have primers located in rather conserved parts of the MgPa gene.

A modification of the MgPa-1/MgPa-3 assay was published by Totten *et al.* and used for their studies on male urethritis (89), mucopurulent cervicitis (107), and endometritis (108). Recently, a

Table 2. Published diagnostic *M. genitalium* PCR assays.

Target gene	Forward primer: Name	Forward primer: Sequence	Forward primer: Position	Reverse primer: Name	Reverse primer: Sequence	Reverse primer: Position	Reference
MgPa	MgPa-1	AGT TGA TGA AAC CTT AAC CCC TTG G	180 - 204	MgPa-3	CCG TTG AGG GGT TTT CCA TTT TTG C	436-460	(1)
MgPa	Mg1 (outer f)	TGT CTA TGA CCA GTA TGT AC	3837-3856	Mg2 (outer r)	CTG CTT TGG TCA AGA CAT CA	4191-4210	(105)
	Mg3 (inner f)	GTA ATT AGT TAC TCA GTA GA	3910-3929				
MgPa	Mg1a (outer f)	GTG TAA CTT ACC AGT GGC TTT GAT C	3864-3889	Mg2	same as (105)		(106)
	Mg3	same as (105)					
MgPa	G3A	GCT TTA AAC CTG GTA ACC AGA TTG ACT	3755-3781	G3B	GAG CGT TAG AGA TCC CTG TTC TGT TA	4236-4261	(128)
MgPa	MgPaW1	AAG TGG AGC GAT CAT TAC TAA C	-85 - -63	MgPaWR1	CCG TTG TTA TCATAC CTT CTG A	389-410	(129)
MgPa	MGS-1	GAG CCT TTC TAA CCG CTG C	38-56	MGS-4	GTT GTT ATC ATA CCT TCT GAT	388-408	(111)
				MGS-2	GTG GGG TTG AAG GAT GAT TG	691-710	
MgPa	MgPa-1-mod	TGA AAC CTT AAC CCC TTG G	186-204	MgPa-3-mod	AGG GGT TTT CCA TTT TTG C	436-454	(89) (109) mod. from (1)
MgPa	MgPa-476	ATG GCG AGC CTA TCT TTG ATC CTT TTA A	476-503	MgPa-903	TTC ACC TCC CCA CTA CTG TTC TTA TGC	903-929	(2)
MgPa	MgPa-355F	GAG AAA TAC CTT GAT GGT CAG CAA	355-378	MgPa-432R	GTT AAT ATC ATA TAA AGC TCT ACC GTT GTT ATC	400-432	(8)
16S	Mge 1	GAA TGA CTC TAG CAG GCA ATG GCT G	439-463	Mge 2	ATT TGC TCA CTT TTA CAA GTT GGC T	1224-1248	(122)
16S	Mg16S-45F	TAC ATG CAA GTC GAT CGG AAG TAG C	45-69	Mg16S-447R	AAA CTC CAG CCA TTG CCT GCT AG	447-469	(6)
16S	16SFG2	CCT TAT CGT TAG TTA CAT TGT TTA A	1096-1220	16SRG	TGA CAT GCG CTT CCA ATA AA	1417-1436	(110)
16S	My-ins	GTA ATA CAT AGG TCG CAA GCG TTA TC	520-545	MGSO-2	CAC CAC CTG TCA CTC GGT TAA CCT C	1012-1036	(123)
P115 (MG299)	p115-74	CCC ATC GTC AAG GTA CAA TGA TGA	74-97	P115-173	GCATTTTTCAAGTTCAACTG CAA AGG	149-173	(127)

modification of the assay adapting the detection format to microtiterplate hybridisation and incorporating an internal processing control was published (109). This assay modification facilitated high-throughput detection of *M. genitalium* and has the advantage of using the same procedures as the manual Amplicor® *C. trachomatis* PCR assay (Roche)

The MgPa-1/MgPa-3 assay was validated as a confirmatory assay as part of the development of a 16S rRNA gene PCR (6) where it was found to detect *M. genitalium* in 40 of 41 specimens positive in the 16S assay. In a recent head-to-head comparison of 64 FVU specimens with the MgPa-1/MgPa-3 assay and another 16S assay (110), it was found, that the 16S based assay detected nine positive specimens, and the MgPa-1/MgPa-3 assay detected eight of them. The modified hemi-nested PCR (105; 106) confirmed all nine, but since the original MgPa-1/MgPa-3 assay was changed, using only one tenth of the primer concentration that was recommend in the original publication, the sensitivity may have been impaired. As can be seen in **Table 5**, the MgPa-1/MgPa-3 assay together with the Palmer *et al.* hemi-nested PCR have been used most widely in the clinical studies published thus far. In **Table 2** are listed the different PCR assays that have been published and used for diagnostic purposes.

Using the MgPa gene as a target for diagnostic PCR could be challenging because of the genetic variation in this gene. This was clearly demonstrated in our MgPa-476/MgPa-903 assay used for the first clinical study (2). At the time of the study, detection of *M. genitalium* by PCR was a new method, and a confirmatory assay was needed to substantiate the findings with the MgPa-1/MgPa-3 assay. Using purified DNA from the *M. genitalium* G-37 strain and the six other strains available, the MgPa-476/MgPa-903 primer-set provided excellent results with an apparent low limit of detection. However, during the study, it became clear, that although a complete concordance was found between the results with the two primer-sets, the intensity of the amplicon produced by the MgPa-476/MgPa-903 primer-set was very low for some of the specimens clearly positive in the old assay (2). Furthermore, a remarkable variation in the restriction enzyme analysis of amplicons from the clinical specimens was found, and with some of the amplicons, an internal probe did not hybridise (2). The reason for this variation was later explained by the observation that the MgPa-903 primer was located in the B-region of the gene (described in more detail in chapter 2). This region is hypervariable due to the presence of repeat sequences scattered throughout the genome (3). As can be seen in **Figure 9** (reproduced and modified from (3)), only strain M 2321 of the four Danish strains, matched the primer perfectly, and even the Danish G-37 strain had a mutation in the 5' end of the primer.

Placing a primer in the hypervariable B-region probably also explains an observation by de Barbeyrac *et al.* (111). They found, that whereas their MGS-1/MGS-4 primerset (located outside the B-region) could detect *M. genitalium* in 8 (17%) of male urethral swab specimens, the MGS-1 primer in combination with the MGS-2 primer (placed in the B-region) was unable to detect any positives. Interestingly, the amplicons produced by the MGS-1/MGS-4 primer-set flanked the same *Eco*RI site as the MgPa-1/MgPa-3 primers, and all urethral specimens from French patients lacked this restriction site.

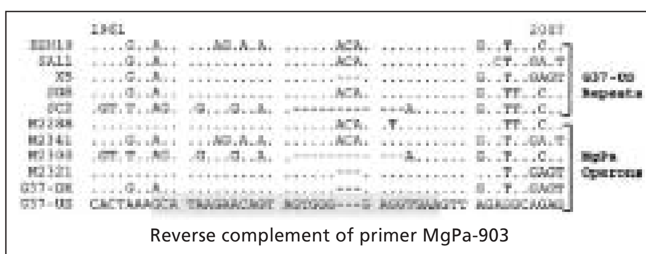

Figure 9. Alignment of MgPa genes from different *M. genitalium* strains and from MgPa repeat sequences found in the genome (modified from (3)). The position of the MgPa-903 primer (reverse complemented) is marked.

The experience with the MgPa-476/MgPa-903 primer-set provided further impetus to develop an assay based on the 16S rRNA gene (6). This gene sequence was not publicly available when the development of the assay began, and based on the experience with the MgPa gene, it was decided to sequence the gene from the *M. genitalium* G-37 strain, as well as from the four Danish isolates. As compared with the G-37 strain, the Danish isolates had a single base substitution at position 1430, included in the reverse primer of the Eastick *et al.* 16S assay (110) recently published. The *M. genitalium* 16S sequence was highly similar to that of *M. pneumoniae*, and, furthermore, 16S rRNA gene sequences are notorious for their secondary structures, so the task of finding efficient, specific primer-combinations was rather complicated. However, the MG16-45F/MG16-447R (Table 2) provided both a low limit of detection of *M. genitalium* DNA, and a good specificity towards other mycoplasma species, in particular to *M. pneumoniae.* An internal process control was developed, and this design has been used in most of the other assays developed in our laboratory since then. It was concluded, that screening with the 16S assay and confirming all positive results with the MgPa-1/MgPa-3 assay provided a good sensitivity and specificity (6), whereas the MgPa-476/MgPa-903 primer-set was more or less useless, at least with the modifications introduced between its development and the 16S validation study. Since 1994, the 16S PCR has been used as the primary assay in all clinical studies performed in our laboratory (4; 5; 7; 8; 112-120), and the protocol has been adapted in several other laboratories (89; 107; 121).

Another 16S rRNA gene based assay was described by Sasaki *et al.* (122). This assay, however, has only been used on simulated clinical specimens, and the reported limit of detection was about 1000 *M. genitalium* cells, which is insufficient for clinical use.

One of the more recent developments in diagnostic PCR is the homogeneous real-time PCR. The major advantage of this type of assay is the closed format, where amplification is detected without opening the PCR tube. Thereby, the risk of amplicon contamination is drastically reduced. Furthermore, real-time assays can be performed quantitatively with a wide dynamic range; the probe-based assays provide additional specificity to the assay, and the subjective reading of an ethidiumbromide stained agarose gel is no longer needed.

The first real-time PCR assay for *M. genitalium* was published by Yoshida *et al.* in 2002 (123). The assay was based on detection of the 16S rRNA gene, but the forward primer My-ins hybridised to almost all species of mycoplasma (>50 hits with 100% homology in a GenBank Blast search). The reverse primer MGSO-2 is a modification of the mollicutes specific primer described by van Kuppeveld *et al.* (124), which is used for detection of mycoplasma infection in cell cultures in many laboratories. The modified primer has 100% homology with all members of the *M. pneumoniae* 16S cluster. Consequently, the specificity resides only in the probe, which has only two mismatches with the *M. pneumoniae* sequence. It is, therefore, likely, that the primers will amplify 16S sequences from a wide variety of mollicutes including ureaplasmas, and even though the probe is specific for *M. genitalium*, the competition for primers and other reaction components is likely to influence accuracy of the quantitation of *M. genitalium* DNA. Questions could also be raised against the sensitivity of the assay, since it was shown that all five FVU specimens from men with a simultaneous *M. genitalium* and gonococcal infection were negative in the TaqMan assay, and the same applied to one of two specimens from men with asymptomatic *M. genitalium* infection. These specimens were previously found positive in an assay based on sequencing of PCR amplicons obtained with mollicutes 16S specific primers (125).

Due to the homology between the *M. genitalium* and *M. pneumoniae* 16S rRNA genes, and the predominance of secondary structures, the design of specific and sensitive primers and probes for a

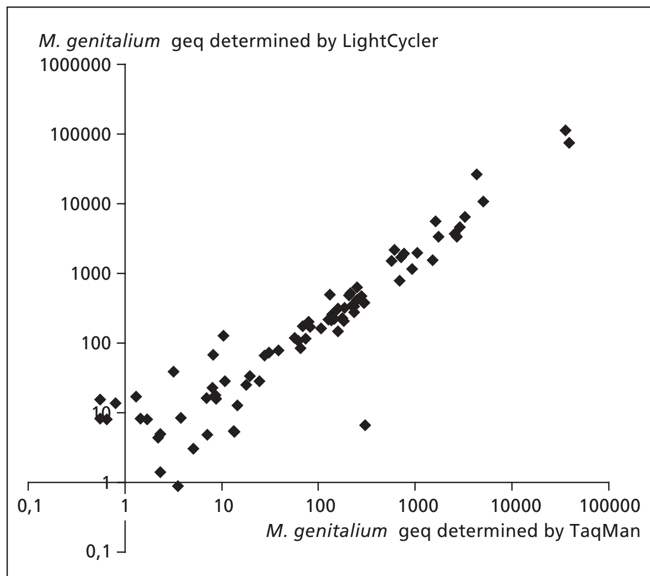

Figure 10. Comparison between *M. genitalium* DNA load in 83 *M. genitalium* 16S PCR positive urethral swab specimens determined by TaqMan MgPa assay (8) and Dual-probe Lightcycler *gap* gene assay (126). One specimen was negative in both assays (not shown).

TaqMan assay was found to be difficult. From previous experience, it was known that results obtained with the MgPa-1/MgPa-3 primer-set (1) correlated very well with those of the16S rRNA gene based assay (6). Since partial MgPa sequence information from four Danish strains was available (3), primers and probes from conserved regions of the MgPa gene were selected (8). An internal processing control was designed using the same principles as used in the 16S assay. The specificity of the new assay was validated against all human mycoplasmas and a range of phylogenetically related species. Sensitivity was validated by comparing the results of the 16S rRNA gene PCR, by analysing the standard curve in the presence of different clinical specimen types, and by adding known amounts of *M. genitalium* to clinical specimens to control for the matrix effect.

Using this assay it was shown, that men with urethritis had higher DNA loads in the FVU than men without urethritis. This relation was less obvious when urethral swab specimens from the same patients were tested, but if data for grey-zone and high-grade urethritis were combined, it could be shown that patients with urethritis had higher DNA loads than controls. The TaqMan assay appeared to be slightly more sensitive than the 16S PCR. This was shown by finding two TaqMan positive of 10 16S PCR negative urethral swab specimens from patients with a confirmed infection as documented by a positive FVU specimen. Surprisingly, the sequence of the forward primer was not completely conserved when more MgPa-1/MgPa-3 amplicons from patients were sequenced. However, comparing detection limits with the 16S assay and diluted specimens proved that this variation did not influence sensitivity. The TaqMan MgPa assay has been adapted to a SmartCycler® instrument in Karlstad, Sweden with very good results (Thomas Ahlquist, personal communication) and in Glasgow the RotorGene® instrument is being used also with good results (Jill Shepherd, personal communication).

In order to compare the quantitative results found with the TaqMan assay, 83 positive urethral swab specimens were tested in a dual-probe LightCycler® assay targeting the housekeeping gene *gap* (126). An excellent correlation between the two methods was obtained (**Figure 10**), and presently a larger head-to-head study is in progress.

Recently, a LightCycler assay detecting the P115 gene (MG299) with LNA (locked nucleic acid) probes was applied for detection of *M. genitalium* and for monitoring the response to treatment in nine infected men (127). Thus, important new insight in *M. genitalium* disease can be gained by the use of quantitative techniques.

Using the best PCR methods available is not sufficient, if the specimen is inadequate. In an attempt to determine the optimal type of diagnostic specimen both for *M. genitalium* and for *C. trachomatis*, the relative performance of FVU, urethral and cervical swab specimens was determined (9). Using a Chelex extraction method, FVU proved to be the optimal specimen type for men detecting >97% of the *M. genitalium* and *C. trachomatis* infections. In women, however, FVU detected only 71% of the *M. genitalium* infections and 90% of the *C. trachomatis* infections. Combining the detection from FVU with a cervical swab specimen increased these figures to 96% and 99%, respectively. Consequently, FVU specimens from women should always be supplemented with a cervical swab specimen for optimal sensitivity, whereas the urethral swab specimen, which is considered painful by most women could be avoided. Another important finding was that female FVU specimens appeared to be unstable at –20°C since 27% of the specimens subjected to sample preparation after storage were found to be false negative, whereas those extracted initially after receipt in the laboratory remained positive (9).

In conclusion, diagnosis of *M. genitalium* infections in the future should be based on real-time PCRs since these are highly sensitive and specific. The quantitative aspects may provide important information in relation to a variety of scientific questions, but appears to be of less importance from a diagnostic point of view. It is encouraging, that the TaqMan MgPa assay has proven so easy to adapt to other instrument platforms and until a commercially available kit is at hand, it may be one of the preferred methods for new laboratories inexperienced in "home-brew" PCR.

4. A *M. GENITALIUM;* A CAUSE OF UROGENITAL DISEASE IN MEN?

4.1. MODIFIED HENLE-KOCH POSTULATES

The original Henle-Koch postulates are a series of requirements that must be met before an organism can be considered causative for a given disease (130). The original postulates are listed in **Table 3**.

These postulates were established in 1840 by Jakob Henle and further developed by his pupil Robert Koch based amongst others on his observations of anthrax.

One of the important strengths of the Henle-Koch postulates is the fact, that if a given microorganism is established as the causative agent of a specific disease, it is implied that the disease can be treated and/or controlled by elimination of the organism or by preventing contact with the organism.

However, fulfilling the postulates is a very stringent requirement; for certain diseases and organisms, only some of the postulates can

Table 3. Original Henle-Koch postulates.

I	The organism must be present in every case of the disease in question and under circumstances, which can account for the pathological changes and clinical course of the disease.
II	The organism occurs in no other disease as a fortuitous and non-pathogenic parasite.
III	After being fully isolated from the body and repeatedly grown in pure culture, it can induce the disease anew

Table 4. Modified Henle-Koch postulates as suggested by David Taylor-Robinson (132) for establishing that a mycoplasma is a cause of disease.

I.	Epidemiology The organism should be detected more frequently and/or in larger numbers from patients with disease than from those without.
II.	Antibody response An antibody response, measured by any of several available techniques, should be demonstrated in the infected host.
III.	Response to treatment Clinical and microbiological cure after treatment with an antimicrobial agent to which the organism is susceptible *in vitro*.
IV.	Transmissibility The organism should infect an animal host from which they can be recovered and, in so doing, produce disease similar to that seen in man.

Table 5. Clinical studies of the prevalence of *M. genitalium* as determined by PCR in different categories of men.

Investigators	Country	Clinical setting	Specimen type	PCR system	Inclusion criteria for urethritis
Jensen et al, 1993 (2)	Denmark	STD clinic	Urethral swab in SP4	MgPa 1-3 + MgPa konf.	Urethritis sypmtoms and >4 PMNLs/hpf
Horner et al, 1993 (135)	UK	STD clinic	FVU	Mg 1-2 heminested Mg 3-2	Urethritis sypmtoms and >4 PMNLs/hpf
Blanchard et al, 1993 (152)	USA	STD clinic	Urethral swab in 2SP	MgPa 1-3	Urethritis sypmtoms and >4 PMNLs/hpf or *C. trachomatis* infection within 60 days
Deguchi et al, 1995 (142)	Japan	Urology	Urethral swab in 2SP	MgPa 1-3	Urethritis sypmtoms and >4 PMNLs/hpf
Janier et al, 1995 (141)	France	STD clinic	Urethral swab in 2SP	MgPa 1-3	Urethritis sypmtoms and >4 PMNLs/hpf
					Urethritis sypmtoms and >9 PMNLs/hpf in FVU
Lackey et al, 1995* (143)	USA	STD clinic	NA	NA	>4 PMNLs/hpf
Busolo et al, 1997 (144)	Italy	STD clinic	Urethral swab in SP4	Mg 1-2 Palmer	Complaint of discharge and/or dysuria
Uno et al, 1997 (153)	Japan	Department of Medicine	FVU	Mg 1-2 heminested Mg 3-2	
Maeda et al, 1998 (145)	Japan	Urology	Urethral swab in TE	Mg 1-2 heminested Mg 3-2	Urethritis sypmtoms and and >4 PMNLs/hpf
Björnelius et al, 2000 (5)	Sweden	STD clinic	Urethral swab in 2SP	Mg 16S + MgPa 1-3 konf.	Urethritis sypmtoms and >4 PMNLs/hpf
					>4 PMNLs/hpf
Gambini et al, 2000 (148)	Italy	STD clinic	Urethral swabs in PBS	MgPa 1-3	Urethritis sypmtoms and >4 PMNLs/hpf
Johannisson et al, 2000 (147)	Sweden	STD clinic	Urethral swabs in Roche medium	MgPa 1-3	>4 PMNLs/hpf
Keane et al, 2000 (146)	UK	STD clinic	FVU	Mg 1a-2 heminested Mg 3-2	>4 PMNLs/hpf or >9 PMNLs/hpf in FVU
Luo et al, 2000 (149)	China	STD clinic	Urethral swab in saline	Mg 16S or Mg 1-2 heminested Mg 3-2	Urethritis symptoms not specified
Totten et al, 2001 (89)	USA	STD clinic	FVU	5'truncated MgPa 1-3	Urethral discharge and >4 PMNLs/hpf
Pepin et al, 2001 (151)	7 West African countries	Primary health care	Urethral swab in Amplicor	MgPa 1-3 heminested MgPa 1-2	Complaint of discharge
Morency et al, 2001 (150)	Central African Republic	STD clinic/ primary health care	Urethral swab in Amplicor	MgPa 1-3 heminested MgPa 1-2	Complaint of discharge
Taylor-Robinson et al, 2001 (117)	South Africa	Primary health care	FVU	Mg 16S + MgPa 1-3 konf.	Complaint of discharge and/or dysuria
Yoshida et al, 2002 (125)	Japan	Urology	FVU	GPO1/MGSO heminested w. My-ins mollicutes 16 S rDNA with sequencing	>4 PMNLs/hpf
Anagrius 2002 (121)	Sweden	STD clinic	Urethral swab in 2SP	Mg 16S + MgPa 1-3 konf	>4 PMNLs/hpf
Mena et al, 2003 (129)	USA	STD clinic	Urethral swab in dry tube and FVU	MgPaW1 + MgPaWR1	>4 PMNLs/hpf and symptoms
Eastick et al, (110)	UK	STD clinic	FVU	16SFG2 + 16SRG	>4 PMNLs/hpf or >9 PMNLs/hpf in FVU
Dupin et al, (127)	France	STD clinic	FVU	P115-74 + P115-173	Urethritis sypmtoms and >9 PMNLs/hpf in FVU + threads
Total***					

*) Not published in peer reviewed journal
**) Assuming that the two patients infected with *C. trachomatis* and *N. gonorrhoeae* belonged to the group with discharge
***) For calculation of the total number of patients in each group, data used from Janier *et al.* and Björnelius *et al.* were for urethritis defined by urethral smear. Figures in shaded boxes not included in total.

No. with NGU	Mg+	% Mg+	No. with NCNGU	Mg+	% Mg+	No. w/o NGU	Mg+	% Mg+	Inclusion criteria for controls	Total no. studied
48	13	27.1	34	12	35.3	47	4	8.5	Asymptomatic men attending same institution	99
103	24	23.3	58	16	27.6	53	3	5.7	No symptoms <5 PMNLs/hpf and <10 PMNLs/hpf in FVU	164
64	9	14.1	NA	NA	NA	NA	NA	NA	NA	
114	17	14.9	76	14	18.4	28	0	0	Heterosexual volunteers No symptoms <5 PMNLs/hpf in FVU	142
100	29	29.0	82**	29	35.3	96	8	8.5	Urethritis sypmtoms and <5 PMNLs/hpf	273
74	32	43.2	58	32	55.2	123	5	4.1	Urethritis sypmtoms <10 PMNLs/hpf in FVU	
62	20	32.3	NA	NA	NA	62	11	18	<5 PMNLs/hpf	190
52	6	11.5	NA	NA	NA	44	0	0	Asymptomatic men attending same institution	100
NA	NA	NA	NA	NA	NA	187	2	1.1	Asymptomatic men attending Dept. of Medicine for checkup; w/o genital complaints	187
76	10	13.2	34	9	26.5	21	0	0	Asymptomatic men attending same institution	97
40	12	30	29	12	41.4	44	5	11.4	No symptoms v5 PMNLs/hpf	101
50	13	26	36	13	36.1	51	5	9.8	<5 PMNLs/hpf	
178	52	29.2	110	27	24.5	23	1	4.3	No symptoms, no history of urethritis last 3 years <5 PMNLs/hpf	201
115	17	14.8	74	16	21.6	118	1	0.8	<5 PMNLs/hpf	233
36	12	33.3	22	10	45	11	1	9.1	<5 PMNLs/hpf and <10 PMNLs/hpf in FVU No symptoms	47
116	28	24.1	NA	NA	NA	67	3	4.5	Asymptomatic men attending same institution	183
121	27	22.3	85	24	28.2	117		4.3	No urethritis sypmtoms and <5 PMNLs/hpf	246
251	41	16.3	209	37	17.7	339	0	8.8	Asymptomatic men attending same institution	998
127	53	41.7	NA	NA	NA	100	15	15	Asymptomatic men attending primary health care	510
96	17	17.7	81	16	20	185	16	8.6	Every fifth asymptomatic man attending same institution	367
93	14	15.1	46	9	19.6	42	1	2.4	Asymptomatic men attending same institution	190
279	27	9.7	254	26	10.2	222	3	1.4	<5 PMNL/hpf	501
52	16	30.8	32	9	28	184	14	7.6	Asymptomatic men attending same institution	285
54	9	16.7	46	7	15.2	NA	NA	NA		54
74	16	21.6	57	16	28.1	60	0	0	Urethritis sypmtoms <10 PMNLs/hpf in FVU	193
						50	1	2	Asymptomatic men attending same institution	
2261	470	20.8	1336	290	21.7	2107	124	5.9		

be fulfilled; e.g. the causative agent for leprosy, *Mycobacterium leprae*, has not been isolated in pure culture and subsequently used to induce experimental disease. According to a rigorous interpretation, viruses could never fulfil the postulates since they cannot be propagated without cells. Furthermore, the postulates leave no room for factors other than the implicated microorganisms playing critical roles in the causation of disease, such as host susceptibility or interaction with other organisms. Moreover, the model is not suited to manage diseases that may be due to several different causative agents like pneumonia and urethritis.

As a consequence of these shortcomings of the Henle-Koch postulates, many revisions of the postulates have been proposed (reviewed in (131)). David Taylor-Robinson suggested modifications of the postulates (132), that should be fulfilled before regarding a mycoplasma species as a cause of disease (**Table 4**).

The degree to which *M. genitalium* fulfils these criteria in the case of male NGU and NCNGU will be discussed in the following.

4.2. EPIDEMIOLOGY

Due to the difficulties in culturing *M. genitalium*, epidemiological studies have been few in the years after the discovery of the organism. In a study using culture and serology, Taylor-Robinson *et al.* (83) failed to isolate *M. genitalium* although they reported "presumptive isolation" based on colour-change in the mycoplasma broth from 7 (32%) of 22 men with NGU, from 5 (42%) of 13 men with NCNGU, and from 2 (10%) of 22 men without urethritis. *M genitalium* was also presumptively isolated from rectal swabs from both homo- and heterosexual men. Using a microimmunofluorescence (MIF) antibody test, a significant rise in antibodies was detected in 4 (29%) of 14 patients with NGU and in 2 (12%) of 17 without urethritis. Disappointingly, only one of the four NGU patients responding serologically had presumptive isolations, and none of the two men without urethritis, who seroconverted, had presumptive isolations (83). Using colour change in the mycoplasma broth without further identification as evidence of *M. genitalium* growth may have a low specificity since it could be caused by other bacteria, including unknown mycoplasma species. Several years later, some of the specimens remaining from their study were examined with PCR, and the presumptive isolations from the urethral specimens were to some extent corroborated, since *M. genitalium* was detected in 9 (50%) of 18 NGU specimens, and 1 (14%) of 7 specimens from men without urethritis. No details were given, however, on the precise correlation between culture and PCR results (133; 134). Hooton *et al.* (104), using a nick-translated probe prepared from genomic DNA of *M. genitalium*, studied 203 men attending a sexually transmitted disease clinic, and detected *M. genitalium* in the same proportion of men with acute gonococcal urethritis (14%; 3/21), acute *C. trachomatis* positive NGU (10%; 3/30), acute NCNGU (13%; 4/31); and in 12% (10/84) of patients without urethritis, but in 27% (10/37) of men with persistent or recurrent NGU. *M. genitalium* was found more often in homosexual than in heterosexual men, which led the authors to suggest that *M. genitalium* may reside in the gastrointestinal tract (104).

Further clinical studies were made possible due to the development of PCR assays (1; 105).

In the first clinical study applying the PCR technique, urethral, rectal, and throat samples from 99 male STD clinic attendees were studied and *M. genitalium* DNA was found in 17 (17%) of the urethral swabs, but in none of the rectal and throat swabs indicating that the urogenital tract is the primary site of infection (2). Significantly more men with NGU (27%; 13/48) were positive for *M. genitalium* than were those without urethritis (9%; 4/47). In men with urethritis, *M. genitalium* was found more often in *C. trachomatis* negative NGU (35%; 12/34) than in those with chlamydial NGU (7%; 1/14) indicating that the two microbes may act as separate causes of urethritis (2). Almost simultaneously with the publication of the Danish data, Horner *et al.* from David Taylor-Robinson's

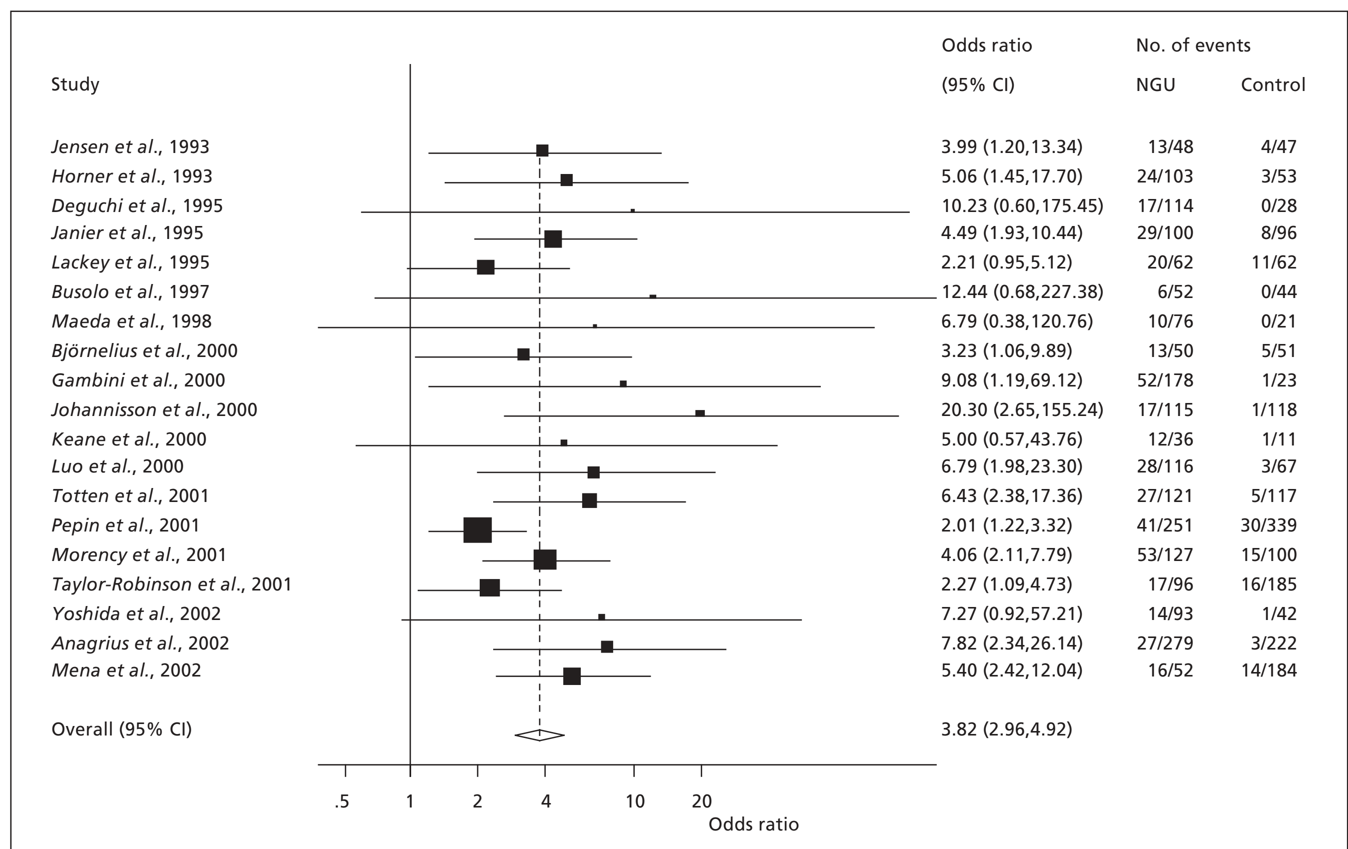

Figure 11. Odds ratios and 95% confidence intervals in epidemiological studies of *M. genitalium* PCR positivity and non gonococcal urethritis (NGU). DerSimonian - Laird random effects analysis. Box size represents weight (number of patients studied) of each study in the meta analysis. Forrest plot prepared in Stata 8.0 using the Metan module by Jannik Helweg-Larsen (reproduced from (154)).

group published a study with the same conclusions (135). The Danish study was criticised for not having examined the asymptomatic men for microscopic signs of urethritis (136). At the time of the study, asymptomatic men attending the STD clinic in Copenhagen did not routinely have urethral smears examined for microscopic signs, so the chosen inclusion criteria would be more true to the situation in everyday clinical life (137). Furthermore, the conclusions regarding the role of *M. genitalium* would be biased only in direction of decreasing the importance of *M. genitalium*. Later studies (5; 138; 139) have actually demonstrated that *M. genitalium* is more closely associated with symptomatic than with asymptomatic urethritis. Only one (10%) of 10 men with asymptomatic urethritis was *M. genitalium* positive (5) and Horner and Taylor-Robinson found only two (8%) of 25 men with asymptomatic urethritis positive for *M. genitalium*, a prevalence closely matching that found in male STD clinic attendees without urethritis (138). Horner *et al.* recently presented further evidence that *M. genitalium* is associated with discharge using data from the original study (135) and applying multivariate analysis to control for confounding factors (139) and Taylor-Robinson *et al.* (140) studied 52 men with persistent or recurrent NGU and found 11 (21%) to be *M. genitalium* positive.

Since the two original studies (2; 135), several PCR based studies have been presented (5; 89; 110; 117; 121; 125; 127; 129; 141-153). A total of 5455 patients have been examined in 23 published studies including three studies without controls (summarised in Table 5) showing an overall *M. genitalium* prevalence of 20.8% (470/2261) among men with NGU and 5.9% (124/2107) among the control patients. In 20 studies, a case-control design was used. Although there were important differences between the studies in terms of design, clinical criteria used for diagnosis of urethritis, and different PCR methods used, the heterogeneity between studies was not found to be statistically significant, thus allowing for calculation of a pooled odds ratio. However, bearing the differences in mind, the pooled odds ratio should be interpreted with caution. Of the 2143 patients with NGU, 452 (21.1%) were *M. genitalium* positive as compared to 122 (6,4%) of the 1920 patients without NGU resulting in a DerSimonian-Laird random effects analysis pooled odds-ratio of 4,2 (95% CI 3.1-5.5; p < 0.0001). In all studies, *M. genitalium* has been detected more often in men with NGU than in those without (Summarised in **Figure 11**).

In 16 of the clinical studies, it is possible to calculate the prevalence of *M. genitalium* in men with NCNGU (2; 5; 89; 117; 121; 125; 127; 129; 135; 141; 142; 145-148; 151) (summarised in **Figure 12**). In these studies, the *M. genitalium* prevalence in the NGU group was 19.3% (345/1786) compared to a *C. trachomatis* prevalence of 27.7% 496/1786). Except for the studies of Gambini *et al.* (148) and Mena *et al.* (129), the *M. genitalium* prevalence has invariably been higher in the NCNGU group than in the total NGU group. Among the NCNGU patients, 21.9% (283/1290) were *M. genitalium* positive compared 6.0% (93/647) of the patients without urethritis resulting in a DerSimonian-Laird pooled odds-ratio of 5.8 (95% CI 3.9-8.7; p < 0.0001). *M. genitalium* was found less often in chlamydial NGU (12.5%; 62/496) than in the NCNGU group (p <0.0001, Fisher's exact test).

Thus, *M. genitalium* behaves largely independent of *C. trachomatis* and with a prevalence of about two thirds that of *C. trachomatis* among men with urethritis. It is interesting to note, that in the studies performed on black African men (117; 150; 151), the odds ratios for being *M. genitalium* positive both in NGU and in NCNGU tend to be somewhat lower. This could partly be explained by the syndromic definition of urethritis used in these studies where microscopy of urethral smears was not performed, and absence of symptoms was used to define the control group. However, the higher prevalence in men without symptoms may also reflect true variation in the relative importance of *M. genitalium* in populations with a very high burden of STIs.

Finding *M. genitalium* in higher numbers in patients with NGU than in those without was another of the revised Henle-Koch postulates (Table 4). Recently, data supporting the presence of a higher number of *M. genitalium* DNA copies in FVU from patients with NCNGU than from asymptomatic men was presented (123). These researchers applied a quantitative TaqMan 5'nuclease assay based on

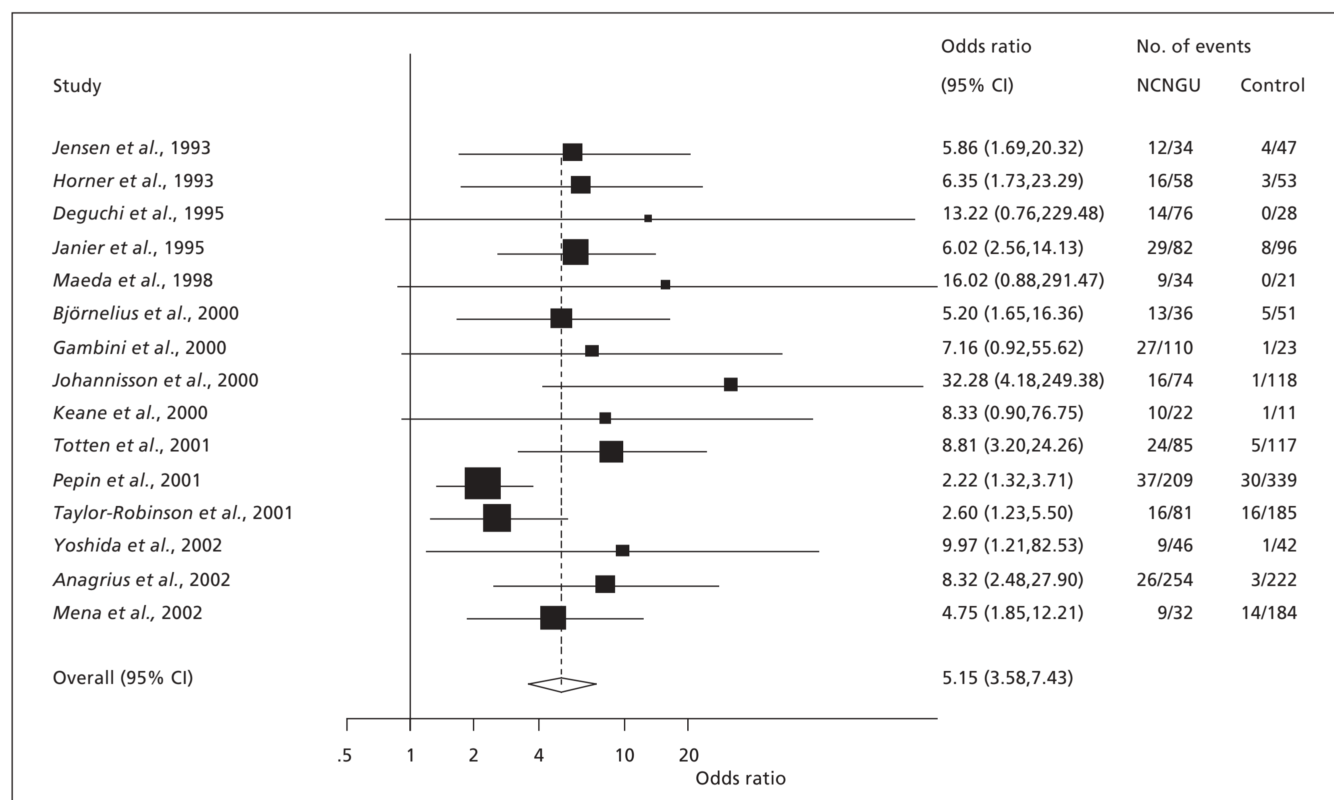

Figure 12. Odds ratios and 95% confidence intervals in epidemiological studies of *M. genitalium* PCR positivity and non chlamydial, non gonococcal urethritis (NCNGU). DerSimonian – Laird random effects analysis. Box size represents weight (number of patients studied) of each study in the meta analysis. Forrest plot prepared in Stata 8.0 using the Metan module by Jannik Helweg-Larsen. (reproduced from (154))

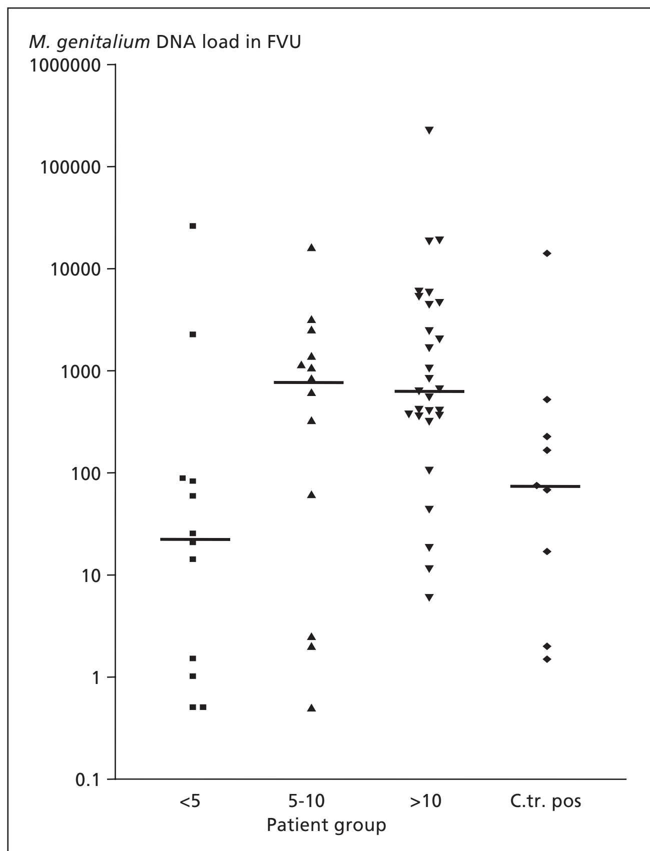

Figure 13. *Mycoplasma genitalium* DNA load in FVU from patients without urethritis (<5 PMNL/hpf), low-grade NCNGU (5-10 PMNL/hpf), high-grade NCNGU (>10 PMNL/hpf) and patients with a concomitant *C. trachomatis* infection.

16S rRNA gene sequences to *M. genitalium* positive specimens. Both the sensitivity and the specificity of the assay could, however, be questioned (discussed in more detail in chapter 3.3). Only two asymptomatic men were examined and one of them was actually negative in the TaqMan assay.

The TaqMan quantitative PCR assay (8) (described in more detail in chapter 3.3) was applied to test FVU and urethral swab specimens and showed that there was a significantly higher number of *M. genitalium* genome equivalents in the FVU from men with urethritis than from those without (**Figure 13**).

In conclusion, the first of the modified Henle-Koch postulates has been fulfilled with a high degree of certainty both for NGU and for NCNGU.

4.3. ANTIBODY RESPONSE

As described in chapter 3.2, the cross-reactions between *M. pneumoniae* and *M. genitalium* have significantly hampered the use of serology for diagnosis and epidemiological studies. Using a MIF assay (96), Taylor-Robinson *et al.* observed a four-fold or greater rise in antibodies to *M. genitalium* in 29% of patients with NGU and in 12% of those without. However, the correlation between antibody response and presumptive isolation of *M. genitalium* from the urethra was not very convincing (83; 133). As a part of the first Danish prevalence study (2), acute phase serum specimens were collected. IgG, IgM, and IgA was measured by ELISA and immunoblotting, but a clear correlation between the PCR status and the antibody response could not be shown (chapter 3.2). Using Lipid-Associated Membrane Proteins (LAMP), Wang *et al.* (99) studied the seroprevalence of *M. genitalium* antibodies in different populations. They collected serum and urine specimens from 104 patients (68 HIV infected asymptomatic individuals and 36 intravenous drug users), and found that 40 (38%) were LAMP ELISA positive. 15 (38%) of these were urine PCR positive for *M. genitalium*, indicating that antibodies from previous exposure were persisting. On the other hand, none of 64 LAMP ELISA negative were PCR positive demonstrating an apparent satisfactory sensitivity. The correlation between a positive *M. genitalium* PCR test and detection of *M. genitalium* antibodies was highly statistically significant. Whether the PCR positive patients had signs or symptoms of urethritis was not mentioned. Considering the apparent performance of the LAMP assay, it is surprising, that it has not been studied more thoroughly.

Although further studies are needed, particularly with collection of follow-up serum specimens from PCR positive patients, the second of the modified Henle-Koch postulates appears to be at least partly fulfilled.

4.4. EFFECT OF TREATMENT

Considering the amount of evidence linking *M. genitalium* to NGU, and in particular to NCNGU, it is surprising, that randomised controlled clinical trials aimed at determining the optimal antibiotic treatment regimen have not been conducted. The relevance of *in vitro* studies can be questioned, since the number of strains is small. Furthermore, in view of the fact that seven of the strains circulating seem to have a clonal origin (53), the conclusions that can be drawn from most of the published studies are very limited. The only study including the four strains isolated from Danish patients (92) is, therefore, the most relevant one to consider. Furthermore, this study gives both the usual initial minimal inhibitory concentration (MIC), defined as the MIC at the time the control broth culture shows colour change, and a final MIC read one week later. From a clinical point of view, the final MIC would seem more appropriate, since it reflects the presence of *M. genitalium* cells capable of surviving the antibiotic. The surviving cells could be either a resistant subpopulation or an effect of the slow growth of *M. genitalium*, allowing resting cells to survive the effect of bacteriostatic antibiotics. The main conclusions from Hannan's study (92) were, that the *M. genitalium* strains tested were highly susceptible to the macrolide group of antibiotics with erythromycin showing a final MIC of max. 0.1 mg/L, and azithromycin with a final MIC of max. 0.005 mg/L. The only other group of antibiotics capable of suppressing the growth of all *M. genitalium* strains was the newer quinolones with extended Gram positive spectrum exemplified by Sparfloxacin with a max. final MIC of 1 mg/L. Most importantly, however, the final MIC for the tetracylines reached 10 mg/L for the least susceptible strains (92). This is a very important observation, since tetracylines are the drugs of choice for treatment of NGU in most countries due to the low cost and high clinical efficacy towards *C. trachomatis* infections. Furthermore, since the G37 type-strain and the clonal derivatives of this strain frequently used in the evaluation of new antibiotics is susceptible to tetracyclines, the erroneous conception that *M. genitalium* is susceptible *in vitro* to tetracyclines is widespread (155)

Several studies have indicated that treatment for *C. trachomatis* urethritis with tetracyclines or quinolones is not always sufficient to eradicate *M. genitalium* (summarised in **Table 6**).

In all studies, the concordance between persistent positive *M. genitalium* PCR and clinical treatment failure has been a common feature. Horner and colleagues (135; 156) found that seven of seven men who had *M. genitalium* detected after doxycycline treatment had urethritis at follow-up. Likewise, Johannisson *et al.* (147) found that eight of 13 men (62%) still harboured *M. genitalium* after treatment with tetracyclines for 10 days and that six of them had urethritis.

In a study of Swedish STD clinic patients (7; 113), 10 of 17 men (59%) treated with tetracyclines were *M. genitalium* positive at follow-up. The patient's symptoms responded to treatment, but the urethritis remained. In a subgroup of *M. genitalium* positive men with urethritis, 16 received treatment with tetracyclines and six received 1.5 g of azithromycin distributed as a 500 mg loading dose and 250 mg in the following four days. Whereas all patients treated

Table 6. Response to treatment of *M. genitalium* infected men in open studies.

Study	Treatment	Follow-up time	No. available for follow-up	Persistent positive by PCR	Urethritis in persistent positive
Horner et al. (135; 156)	Doxycycline 200 mg × 1 + 100 mg × 13 days	10-21	14	7 (50%)	7 (100%)
Johannisson et al. (147)	Tetracycline 500 mg × 2 × 10 days	>21	13	8 (61%)	6 (75%)
Falk et al. (7; 113)	Doxycycline 200 mg × 1 + 100 mg × 8 days or				
	Lymecycline 300 mg × 2 × 10 days	28-35	16	10 (63%)	8 (80%)
	Azithromycin 500 mg × 1 + 250 mg × 4 days	28-35	8	0	-
Gambini et al. (148)	Doxycycline 200 mg × 7 days	14	35	2 (6%)	2 (100%)*
	Azithromyxin 1 g stat	14*	17	3 (18%)	3 (100%)*
Maeda et al. (157)	Levofloxacin 100 mg × 3 × 14 days	14	12	8 (67%)	1 (13%) 5 (63%) recurrent NGU
Dupin et al. (127)	Minocycline 100 mg × 7 days or				
	Doxycycline 100 mg × 7 days	15-35	8	5 (63%)	3 (60%)

*) Not clearly stated in the article

with azithromycin were PCR negative at follow-up, 10 (63%) of the 16 patients treated with tetracyclines were *M. genitalium* positive at follow-up (p=0.015)(7; 113).

In contrast, Gambini *et al.* treated 35 *M. genitalium* positive patients with doxycycline (200 mg x 7) and 17 positve patients with azithromycin (1g stat) and found that only two (6%) of those treated with doxycycline and three (18%) of those treated with azithromycin had "partial clinical response" and remained *M. genitalium* positive one week after end of therapy. Those patients responding microbiologically, were also clinically cured (148). Whether the higher cure rate after doxycycline resulted from use of a 200 mg daily dose for seven days, as compared to the other studies using doxycycline in a lower dosage remains to be determined but appears unlikely based on the clinical experience in Scandinavia. The early follow-up could also be questioned, but no systematic studies regarding clearance of *M. genitalium* DNA after treatment have been conducted.

The effect of quinolones could also be questioned. Maeda *et al.* examined 12 *M. genitalium* PCR positive men treated for NGU with levofloxacin (the *l*-isomer of ofloxacin) of whom eight (66%) remained PCR positive after treatment; one had persistent NGU, and of the seven that appeared to be cured, two did not return and five returned with recurrent *M. genitalium* PCR positive NGU. Interestingly, in a subsequent study by the same group (158), the *M. genitalium* load was measured by quantitative TaqMan PCR. The *M. genitalium* DNA load increased from below the detection limit one week after initiation of therapy to $>10^4$ DNA copies/ml in four of the patients at the time of recurrence.

Considering the diversity in the dosage of tetracyclines and azithromycin that has been used in the published studies, a randomised controlled trial should include at least two arms comparing doxycycline 100 mg bd for seven days and azithromycin 1 g stat as recommended in the clinical effectiveness guidelines (159; 160). However, considering the slow growth of *M. genitalium*, which may call for a longer duration of treatment, the azithromycin regimen comprising 500 mg in a single dose on day one, followed by 250 mg od for four days, which has provided excellent cure rates, should also be considered. That a longer dosage of azithromycin would be beneficial, was actually provided in a much-overlooked study from 1991 (161). Here it was shown, that azithromycin 500 mg in a single dose on day one followed by 250 mg od for two days was superior both to azithromycin 1 g stat and doxycycline 200 mg daily for seven days for treatment of male urethritis.

In conclusion, although the published studies are small and could be confounded by reinfection from untreated partners and by the persistence of DNA from non-viable *M. genitalium* cells, it seems as if there is a strong correlation between eradication of *M. genitalium* and clinical cure. Those patients with microbiological treatment failure appear to suffer from persistent or recurrent disease. It would therefore be reasonable to accept that the third demand of the modified Henle-Koch postulates is fulfilled.

4.5. TRANSMISSIBILITY AND ANIMAL STUDIES

The transmissibility of *M. genitalium* has been studied both in animal experiments, and recently, data regarding transmission between sexual contacts has begun to accrue.

4.5.1. Animal models

The use of an animal model for male urethritis has severe limitations. Many microbes have narrow host specificities and furthermore, it is impossible to control the urination of the animal, which may disturb the interpretation of urethral smears for detection of inflammatory cells. The best animal studies of experimental male urethritis caused by *M. genitalium* have been performed in chimpanzees. Two such studies have been published (68; 98). In the first study, four animals were inoculated intra-urethrally with the G37 type-strain. Two of the animals became persistently infected for 13 weeks; they developed a measurable antibody response after 5 weeks, and a urethral inflammatory response was noted. The animals were treated with a tetracycline drug resulting in microbiological eradication of *M. genitalium* and normalisation of the urethral cellular response. Six months after successful treatment, one of the animals was re-challenged with an isolate from the other animal, but whereas a naïve animal was infected with an identical inoculum, the re-challenged animal remained uninfected. Although a very limited basis for conclusions, this finding may indicate some degree of protective immunity (68). In a second experiment (98), six male chimpanzees were inoculated with strains re-isolated from the two animals in the first experiment. All animals became infected, but two of them cleared the infection spontaneously after one and five weeks, respectively. The remaining four were colonized for up to 18 weeks before treatment was commenced. Five of the six animals showed an inflammatory response in the urethral secretions. There was no correlation between the presence of a urethral PMNL response and the development of a detectable antibody response, since one animal without urethritis had a systemic antibody response, whereas the single animal without seroconversion had a clear urethral PMNL response and high titres of *M. genitalium* in the urethra. Two animals had *M. genitalium* recovered from blood cultures (98). The isolations from the bloodstream demonstrate the invasive potential of *M. genitalium* but may also give rise to speculations regarding haematogenous spread to other organs. Such a spread might explain some cases of sexually acquired arthritis, and the detection of *M. genitalium* DNA in the synovial fluid of patients with arthritis (162).

Lower primates may have a decreased susceptibility to infection. This was clearly demonstrated in a study using both of the human strains G37 and M30 (10) and the same two strains after re-isolation from intra-urethrally inoculated chimpanzees. The strains isolated directly from humans were not capable of establishing an infection in any of six rhesus monkeys (*Macacca mulatta*) and only two of four cynomolgus monkeys (*Macacca fascicularis*) were colonised and shed small numbers of *M. genitalium* organisms until they

spontaneously cleared the organisms after 9 and 28 days, respectively. No antibody response was noted. Challenge with the chimpanzee passaged strains resulted in colonisation/infection with an inflammatory response. However, only a poor antibody response was recorded (98).

4.5.2. Sexual transmission

Only limited information about the important question as to whether *M. genitalium* is sexually transmitted is available. Keane *et al.* (146) studied 39 men with NGU (index patients) and their female partners. *C. trachomatis* was detected in six (43%) female partners of 14 *C. trachomatis* positive men. *M. genitalium* was detected in seven (58%) female partners of 12 *M. genitalium* positive men. Thus, the concordance rate for *M. genitalium* seems to be as high as that for *C. trachomatis. M. genitalium* was also detected in two (9%) of 22 female partners to *M. genitalium* negative men; the corresponding figure for *C. trachomatis* was four (11%) of 37°*C. trachomatis* negative men (146). In a recent study from Sweden, Anagrius and Loré (121) attempted partner tracing in 27 male and 26 female index patients infected with *M. genitalium.* Out of 38 reported male partners to female index patients, 26 were examined for *M. genitalium* and 10 (38%) were positive. Corresponding figures for 22 of the 30 reported female partners to male index patients were 10 (45%) infected (121). In another Swedish study, 17 female partners of 18 *M. genitalium* positive men were traced and 12 (71%) of them were *M. genitalium* positive (one of the women was given as the steady partner for two men) (Lars Falk, personal communication). Transmission rates of *C. trachomatis* have recently been established with the use of PCR, and rates of transmission have been in the order of 26% to 68% in large series (163; 164). Thus, *M. genitalium* appears to be transmitted at least as efficient.

Although further studies regarding the sexual transmission of *M. genitalium* are needed, particularly on the transmission from female index patients to men, the chimpanzee studies have documented that the fourth criterion of the modified Henle-Koch postulates is fulfilled.

4.6. CONCLUSIONS

Using the modified Henle-Koch postulates for establishing that *M. genitalium* is a causative agent in urogenital tract disease, it seems reasonable to conclude, that *M. genitalium* can cause NGU. *M. genitalium* is sexually transmissible with transmission rates similar to those of *C. trachomatis.* Compared to the number of patients with urethritis caused by *C. trachomatis*, the share of patients with urethritis caused by *M. genitalium* is probably smaller, but not insignificant. In a range of studies, *M. genitalium* has been found in 10-45% of men with NCNGU, and considering the poor microbiological cure rate after the conventional treatment with tetracyclines, a significant number of patients may develop recurrent or persistent urethritis. Randomised controlled clinical trials aiming at determining the optimal treatment of the infection, are urgently needed.

5. *M. GENITALIUM;* A CAUSE OF UROGENITAL DISEASE IN WOMEN?

Compared to the number of studies in men, fewer studies on the role of *M. genitalium* in women have been published. Although the first papers applying PCR for detection of *M. genitalium* documented the presence of the bacterium in both cervical and urethral specimens (1; 165), more detailed information about correlation with disease has been slow to accrue. Applying the modified Henle-Koch postulates described in chapter 4, the current literature will be reviewed

5.1. EPIDEMIOLOGY

5.1.1. Prevalence studies without controls

As part of the validation of the MgPa1-3 PCR assay (1), *M. genitalium* was detected in 5 (7%) of 74 women having specimens submitted for *C. trachomatis* culture. Palmer *et al.* found *M. genitalium* in 10 (18%) of 57 women attending an STD clinic using their heminested PCR (165) and Tsunoe *et al.* from Japan found, that among 174 female commercial sex workers, *M. genitalium* was detected in 22 (13%) as compared to 1 (1%) of 90 pregnant women (166). This prevalence should be judged against a *C. trachomatis* prevalence of 19% and a prevalence of *N. gonorrhoeae* of 33% in this group. In contrast, de Barbeyrac *et al.* did not find *M. genitalium* in any of 55 female urogenital tract specimens from STD clinic patients (111). In an ongoing prospective study from Örebro, Sweden, an *M. genitalium* prevalence of 6% was found among 464 women attending the STD clinic. There was no significant difference between patients with *M. genitalium* and patients with *C. trachomatis* in regards to signs and symptoms. However, *C. trachomatis* was found more frequently, being detected in 10% of the women (Falk *et al.* submitted).

5.1.2. Cervicitis

A few studies have addressed the correlation between *M. genitalium* infection and cervicitis. One of the major problems in interpreting these studies has been the varying definitions of cervicitis. Some studies have considered the presence of 10 PMNLs in a cervical smear significant, whereas others demand 30 PMNLs/hpf to define cervicitis. The finding of more PMNLs than epithelial cells in a vaginal wet smear is commonly used and the specificity of these signs has not been thoroughly validated. Less objective measures such as the presence of yellow mucopus from the cervical canal collected on a white cotton swab have also been included. One problem is the lack of exact definitions of what is normal and abnormal. In a study of 59 women between 22 and 26 years of age who participated in a Papanicolau smear screening, none of the women were *M. genitalium* PCR positive, and only one (2%) was *C. trachomatis* positive. The standard definition of <4 PMNL/hpf was used for urethritis and cervicitis was defined as more PMNL than vaginal epithelial cells in the vaginal wet smear. Surprisingly, 22% of the women had signs of urethritis and/or cervicitis demonstrating a low specificity of these signs (167).

One early study by Uno *et al.* defined cervicitis by endocervical discharge and ≥20 PMNL/hpf and detected *M. genitalium* in 5 (8%) of 64 women as compared to none of 80 asymptomatic pregnant women (168).

In a recent US study using archived cervical secretions from 719 women collected in 1984-86 on filter paper, Manhart *et al.* found that *M. genitalium* was strongly associated with cervicitis being detected in 7% of all the women examined but in 11% of women with cervicitis (≥30 PMNLs/hpf) and in 5% of women without cervicitis (p=0.004). In a multivariate logistic regression analysis correcting for other factors found to be associated with cervicitis, and excluding the 172 women infected with *N. gonorrohoeae* and/or *C. trachomatis*, *M. genitalium* remained strongly associated with cervicitis (OR 3.1; 95% CI 1.5-6.8), further supporting an independent role for *M. genitalium* as a cause of cervicitis. The attributable risk percent (estimated by OR-1/OR) was 70%, suggesting that among women with cervicitis and *M. genitalium*, 70% of cervicitis can be attributed to *M. genitalium* (107).

In a study from Sweden (121) including 445 women, an overall prevalence of *M. genitalium* of 6% was found. *M. genitalium* was significantly associated with signs of urethritis or cervicitis as defined by ≥5 and ≥30 PMNLs/hpf, respectively, since 21 (10%) of 217 women with cervicitis/urethritis were *M. genitalium* PCR positive as compared to 6 (3%) of 218 without (p=0.003) (121). In contrast, Casin *et al.* were unable to demonstrate any correlation between the presence of *M. genitalium* and clinical, demographic or microbiologic data in 170 consecutive women with genital symptoms attending an STD clinic in Paris (169). However, only 25 asymptomatic women attending a private gynaecological practice were included

and 4 (16%) were *M. genitalium* positive. Surprisingly, 65 (38%) of the symptomatic women were positive in the MgPa1-3 PCR assay, which is remarkable when compared with a *C. trachomatis* prevalence of 8%. This raises the suspicion that the specificity has not been optimal which may obscure the relationship between symptoms and the infection.

5.1.3. Pelvic inflammatory disease

Pelvic inflammatory disease (PID) is the clinical syndrome caused by the spread of microorganisms from the lower to the upper genital tract. A range of bacteria including those found in bacterial vaginosis, *C. trachomatis*, and *N. gonorrohoeae* can cause PID. Establishing a connection between *M. genitalium* and upper genital tract infection is of major importance in determining the significance of the infection. PID is more likely to lead to severe disease with loss of working days and to long-term sequelae such as tubal factor infertility, ectopic pregnancy, and chronic pelvic pain. The fundamental problem in PID research is concerned with case definition and diagnostic accuracy. Laparoscopy is considered the "gold standard", but is not a realistic possibility in daily clinical practice (170). Studies using laparoscopy for case definition are likely to be biased towards a more severe spectrum of the disease and, therefore, other invasive methods such as endometrial biopsies for detection of plasma cell endometritis may play a role in research, but is not applicable as a standard diagnostic procedure. In a study of 115 women presenting to an STD clinic in Kenya with acute pelvic pain (108), plasma cell endometritis was found in 58 and *M. genitalium* was detected in cervical swabs or endometrial biopsies in 16% compared to only 2% of women without endometritis (p=0.02). As many as 33% of the women studied were HIV positive, but *M. genitalium* was not detected significantly more often in the HIV infected women. In another study of women with clinically suspected PID (171), *M. genitalium* was detected more often in HIV positive women (19% vs 5%), but the data presented does not allow an evaluation of the importance of this finding. Whether the results of the two studies could be generalised to HIV-negative Caucasian populations is not clear.

In a recent case-control study, Simms and colleagues (172) examined 45 women with clinically diagnosed PID and found *M. genitalium* DNA by PCR in endocervical swabs from nine (16%) as compared to none of 37 control patients. It should be noted, however, that the control population consisted of women attending for bilateral tubal ligation. These women were significantly older, and would be expected to have considerably less risk taking behaviour regarding STIs.

Earlier studies relying on serology have been controversial. Møller *et al.* (173) studied 166 women with clinically suspected acute PID admitted to hospital in Århus, Denmark. Serum was collected at 0, 1, and 4 weeks after admission; 31 women with negative serology for *C. trachomatis* and *M. hominis* as determined by MIF and indirect heamagglutination (IHA) tests, respectively, were examined for antibodies to *M. genitalium* by MIF. Using a cut-off titre at ≥16, 12 (39%) showed a ≥4 fold change in titre between acute and convalescence sera. However, no information regarding the changes in *M. genitalium* titre among women with *C. trachomatis* PID or without PID was given, and therefore, the significance of the findings is difficult to interpret. In contrast, Lind *et al.* (174) examined 95 patients seen in Odense, Denmark with clinically suspected PID. After laparoscopy, 39 women were considered not to have salpinigitis. From these women seven had paired serum specimens available, and none showed a change in titre. Likewise, none of 21 patients with gonococcal or chlamydial salpingitis seroconverted, and only one (4.8%) of 21 patients with salpingitis but without evidence of gonococcal or chlamydial aetiology responded with a ≥4-fold rise in an IHA test for *M. genitalium*. Since the difference in test principle could be important, sera from 10 of the patients from whom three or more sera were available were examined in a MIF test comparable to that used by Møller *et al.* (173). However, only in one was a fall in titre from 80 to 10 against both the *M. genitalium* and the *M. pneumoniae* control antigen observed (174). The reason for the discrepancy between the two studies is difficult to determine exactly. However, the number of sera from Odense studied with the MIF test was small, and the difference not statistically significant.

5.1.4. Bacterial vaginosis

Bacterial vaginosis (BV) is a very common clinical syndrome with characteristic clinical, biochemical, and microbiologic features. BV is usually diagnosed according to the Amsell criteria (175) demanding at least two out of four criteria fulfilled: 1. Thin, malodorous homogeneous, grey, nonpurulent vaginal discharge; 2. pH of the discharge >4.5; 3. Presence of "clue cells" on wet mount examination, and 4. "Fishy" odor when mixed with 10% potassium hydroxide. Another definition is provided in the Nugent criteria by which Gram stained vaginal secretions are scored according to the microbial flora (176) or in modifications of the criteria described by Spiegel (177). The Gram scoring systems are based on the relative amount of the *Lactobacillus* morphotype (large Gram-positive rods) and of the *Gardnerella* morphotype (small Gram-variable rods). *M. hominis* is found more often in women with BV with isolation rates up to 75%, and remains significantly associated even after adjustment for co-infection with other BV-associated flora (178; 179). To a lesser extent such a correlation is also found for *U. urealyticum* (179) with isolation rates as high as 95%, but the frequent isolation in women without BV (around 40%) obscures the connection. In one of the early reports on the *M. genitalium* PCR assay developed by Palmer *et al.* (165) *M. genitalium* was found in 4 (16%) of 25 unselected women and in 3 of 10 women with BV. More recently, Keane *et al.* (180) studied 15 women with BV, none of whom were *M. genitalium* positive, as were only 2 (12%) of 17 women without BV attending an STD clinic. This lack of association between BV and *M. genitalium* was also reported as part of the cervicitis study mentioned previously (107). Thus, also in this respect, *M. genitalium* behaves very much like *C. trachomatis*.

5.1.5. Adverse pregnancy outcome and infertility

There is little information as to whether *M. genitalium* can cause adverse pregnancy outcome, either as preterm labour, abortion or stillbirth. One study has been published where *M. genitalium* was detected in only 4% of midtrimester vaginal swabs from 124 women delivering preterm (181). This is probably slightly higher than the prevalence in a normal population of pregnant women as estimated from a recent study (182) where only 0.7% (6/915) of women pregnant in the first trimester were *M. genitalium* positive. However, even with a significant difference in the two prevalences it is not going to be an important factor. *M. genitalium* was more common in women aged <20, those with concurrent bacterial vaginosis, women of Afro-Caribbean or Black African ethnic origin, women in social classes 3-5, and single women. Only one woman with *M. genitalium* infection miscarried, and none of those followed until term had a preterm birth but numbers were small (182)

These findings were corroborated in a recent case-control study of 1014 women who gave birth or aborted at the national reference hospital of Guinea-Bissau (183) of whom 6% were *M. genitalium* positive, but it was not shown to have any effect on the outcome of pregnancy.

Regarding infertility, only indirect evidence has been presented. In a Danish study (100), sera from 308 infertile women were investigated for antibodies to *M. genitalium* by immunoblotting against whole cell proteins of *M. genitalium* and *M. pneumoniae* as well as against a recombinant MgPa antigen. Reactions in patients' sera were evaluated against the MgPa band in the whole cell antigen preparation, and reactions with the cloned MgPa fragment was used for confirmation. Among the women with tubal factor infertility, 22% (29/132) were seropositive as compared to 7% (11/176) of women with either male factor infertility or unexplained infertility

with normal tubes. The recombinant MgPa fragment has later been used as antigen in an ELISA test with promising results (Helle Friis Svenstrup, personal communication). Such an ELISA test would facilitate seroepidemiological studies and be a very valuable tool if it proves to be sufficiently sensitive and specific.

In conclusion, the first of the modified Henle-Koch postulates seems to be fulfilled with some degree of certainty for cervicitis and PID. Only indirect evidence exists for infertility, whereas BV and adverse outcome of pregnancy does not appear to be associated with *M. genitalium*. The clinical studies of *M. genitalium* in different diseases are summarised in **Table 7**.

5.2. ANTIBODY RESPONSE

The two studies of salpingitis (173; 174) and the Danish study of patients with infertility (100), have been discussed above. Only recently has one study been published (90) looking at the development of antibodies in women with acute infections documented by PCR. However, as discussed in chapter 3.2 severe criticism could be raised against the methodological approach of the study. More studies are therefore needed in order to fulfil the second of the modified Henle-Koch postulates. Particularly in PID studies, the simultaneous detection of *M. genitalium* in the lower genital tract and the development of an antibody response could be very informative.

5.3. EFFECT OF TREATMENT

No studies specifically aimed at determining the treatment efficacy in women have been published. However, during a prospective study of the prevalence of *M. genitalium* in Swedish STD patients (7), 26 *M. genitalium* PCR positive women were identified. Among 14 women treated with tretracyclines in standard dosages, 10 (71%) were still PCR positive at follow-up, and without improvement in symptoms and signs. In contrast, out of 12 women treated primarily with azithromycin (500 mg day 1 and 250 mg the following 4 days) due to a known *M. genitalium* positive partner all of 10 women returning for follow-up were PCR negative. The 10 patients with treatment failure after treatment with tetracyclines were subsequently treated with azithromycin and all nine who returned for follow-up were PCR negative. Obviously, this study was not designed as a treatment study, but the results emphasise that a randomised controlled trial is needed in order to document the fulfilment of the third of the modified Henle-Koch postulates.

5.4. TRANSMISSIBILITY AND ANIMAL STUDIES

5.4.1. Animal models

To some extent, animal studies of female *M. genitalium* infections have been more rewarding than those performed in male animals. Female squirrel monkeys became heavily colonised shedding more

Table 7. Clinical studies of the prevalence of *M. genitalium* as determined by PCR in different categories of women.

Investigators	Country	Clinical setting	Specimen type	PCR system	Inclusion criteria
Jensen et al, (1)	Denmark	Random specimens for *C. trachomatis*	Urethral swab in SP4	MgPa 1-3	Random specimens for *C. trachomatis* culture
Palmer et al, (165)	UK	STD clinic	Cervical swabs in PBS	Mg 1-2 hemi-nested Mg 3-2	Unselected female STD clinic attendees
Blanchard et al, (152)	USA	STD clinic	Cervical swab in 2SP	MgPa 1-3	Cervicitis symptoms or >10 PMNLs/hpf or *C. trachomatis* infection within 60 days
Tsunoe et al, (166)	Japan	STD clinic	Cervical swab in ELISA medium	Mg 1-2 hemi-nested Mg 3-2	Female commercial sex workers
de Barbeyrac et al, (111)	France	STD clinic	Urethral swab in 2SP	MGS1/MGS-2 and MGS1/MGS-4	Random specimens for *C. trachomatis* culture
Falk et al, (2004 submitted)	Sweden	STD clinic	Cevical swab in SP4 and FVU	Mg 16S + MgPa 1-3 konf.	Unselected female STD clinic attendees
Uno et al, (168)	Japan	Department of Ob-gyn.	Cervical swab in TE	Mg 1-2 hemi-nested Mg 3-2	Mucopurulent discharge or >20 PMNL/hpf
					PID (fever, leucocytosis, tenderness)
Manhart et al, (107)	USA	STD clinic	Cervical secretions on filter paper	5'truncated MgPa 1-3	Cervicitis: Yellow mucopus or >30 PMNLs/hpf
Anagrius et al, (121)	Sweden	STD clinic	Urethral swabs in 2SP	Mg 16S + MgPa 1-3 konf.	Cervicitis >30 PMNLs/hpf or urethritis >4 PMNLs/hpf
Keane et al, (180)	UK	STD clinic	Vaginal and cervical swab in PBS	Mg 1a-2 hemi-nested Mg 3-2	BV (Spiegel criteria)
Casin et al, (169)	France	STD clinic	Urethral and cervical swab in 2SP	MgPa 1-3	Vaginal discharge
Cohen et al, (108)	Kenya	STD clinic	Cervical swab + endometrial biopsy in Amplicor STM	5'truncated MgPa 1-3	Pelvic pain and plasma cell endometritis
Lu et al, (181)	USA	Prenatal health care	High vaginal swabs in PBS	MgPa 1-3	Subsequent spontaneous abaortion
Labbé et al, (183)	Guinea-Bissau	Dept of Ob-gyn.	Cervical swab in Amplicor STM	MgPa 1-3 heminested MgPa 1-2	Adverse pregnancy outcome (Spontaneous abortion, stillbirth, pre- or dysmaturity)
Simms et al, (172)	UK	Dept of Ob-gyn.	Cervical swab in LCR medium	16SFG2 + 16SRG	Clincal PID (Hager criteria)

than 10^5 ccu/ml 2-3 weeks following intravaginal inoculation. A serum antibody response was detected as late as 10-11 weeks after the inoculation (98). Six female marmosets were inoculated intravaginally. Four of the animals were infected as indicated by repeated recovery of the organisms on vaginal swabbing, and the infection persisted for up to 149 days. In addition, the infected marmosets exhibited a serum antibody response detectable by MIF, and a persistent vaginal PMNL response not seen in two uninfected (inoculated with a 100-fold dilution of the inoculum received by the other animals) and in two uninoculated animals (184).

All four female chimpanzees inoculated intravaginally with *M. genitalium* reisolated from male chimpanzees shed organisms for 12 to 15 weeks, at which time-point they were treated with tetracycline. In contrast to the male chimpanzees, none of the animals had *M. genitalium* recovered from the blood. A vaginal PMNL response was documented, but overt discharge was not observed. All inoculated chimpanzees developed a clear-cut antibody response, in some detected after only 3 weeks.

In experiments where *M. genitalium* was inoculated directly into the oviducts, marmosets and grivet monkeys developed a moderate to severe endosalpingitis with infiltration of inflammatory cells into the tubal epithelium and luminal exudates. Adhesions very similar to that seen after inoculation of *C. trachomatis* were also observed. Antibody responses were observed 25 to 32 days after infection of the marmosets, but the titres did not appear to be maximal before 8 weeks after the infection. A remarkable finding was the failure to recover *M. genitalium* from the fallopian tubes. This may be indicative of problems when studying human material (185).

5.4.2. Sexual transmission

The demonstration that *M. genitalium* is sexually transmissible between partners has been described in chapter 4.1.4.

In conclusion, the results of animal experiments offer substantial evidence for the pathogenicity of *M. genitalium* for the urogenital tract of subhuman primates and the agent is sexually transmitted between partners. Thus, the fourth of the modified Henle-Koch postulates would appear to be fulfilled.

5.5. CONCLUSIONS

Using the modified Henle-Koch postulates, it seems reasonable to conclude, that *M. genitalium* can cause mucopurulent cervicitis although the demonstration of an antibody response is lacking. *M. genitalium* is sexually transmissible with transmission rates similar to those of *C. trachomatis.* The role of *M. genitalium* in women is less well established than in men, and further studies are needed to address the relation to PID and late sequelae such as infertility.

No. examined	Mg+	% Mg+	No. w/o disease	Mg+	% Mg+	Inclusion criteria for controls	Total no. studied
74	5	7					74
57	10	18					57
50	3	6					50
174	22	13	90	1	1	Pregnant women No symptoms	264
55	0	0					55
464	39	6	59	0	0	Women attending for pap smear	523
64	5	8	80	0	0	Pregnant women No symptoms	200
56	4	7					
215	24	11	504	26	5	Women w/o MPC attending same institution	719
217	21	10	218	6	3	Women w/o urethritis or cervicitis attending same institution	445
17	0	0	21	2	12	No BV	38
170	65	38	25	4	16	Asymptomatic women in gyn. private practice	195
58	9	16	57	1	2	Pelvic pain, no endometritis	115
124	5	4					124
414	27	7	600	36	6	Delivery of term neonate >2500g	1014
45	6	13	37	0	0	Women undergoing tubal ligation	82

Only indirect evidence exists for infertility, and BV and adverse outcome of pregnancy does not appear to be associated with *M. genitalium* infection. As in men, randomised controlled clinical trials aiming at determining the optimal treatment of the infection are needed.

6. EXTRAGENITAL INFECTIONS

Accumulating evidence has shown that *M. genitalium* should be considered a pathogen primarily of the urogenital tract. However, as has been shown for other urogenital pathogens such as *C. trachomatis*, extragenital infections may occasionally occur.

6.1. RESPIRATORY TRACT INFECTIONS.

As mentioned in previous chapters, respiratory tract infection with *M. genitalium* has been the subject of much controversy. The best evidence stems from the four *M. genitalium* isolates obtained as mixed cultures from *M. pneumoniae* isolates (33). In the early years of PCR, de Barbeyrac *et al.* studied 75 BAL specimens from adult patients with pulmonary infiltrates of unknown aetiology (111). Four (5%) were PCR positive, and three of the patients were at the same time positive in a *M. pneumoniae* PCR. Most interestingly, all four respiratory tract amplicons contained the *Eco*RI restriction enzyme recognition site found in the G37 type strain, whereas none of eight amplicons from urogenital specimens possessed this site. It would seem highly unlikely, that all respiratory tract strains had one restriction enzyme type as opposed to the urogenital tract strains having another. Unfortunately, no attempts to confirm the findings were reported, whereas all urogenital tract specimens were confirmed with another primer-set (111).

We have used PCR methods to study respiratory tract specimens from different patient populations (114); 50 recruits with pneumonia of whom 14 had evidence of *M. pneumoniae* infection by serology and/or PCR; 13 *M. pneumoniae* culture positive patients, 29 patients with respiratory tract infection and a positive *M. pneumoniae* complement fixation test, but PCR and culture negative. However, no *M. genitalium* PCR positive patients were identified.

Considering the risk of having mother-to-child transmission of *C. trachomatis* during birth causing neonatal pneumonia, very little is known about transmission of *M. genitalium.* In a study of 47 high-risk pregnant women (186), 8 neonates were studied for transmission of urogenital mycoplasmas including *M. genitalium*. Transmission of *M. genitalium* from mother to the newborn child was documented in one premature child with acute respiratory distress. However, the data presented did not include control patients and it is not clear how the infants were selected. Neonatal pneumonia is definitely a disease entity that should be studied in the near future.

6.2. ARTHRITIS

Infection with *M. genitalium* may give rise to symptoms from the joints either because of a direct invasion causing, in principle, a septic arthritis, or it may cause a reactive arthritis, where the pathogen may not necessarily need to be present in the joint. Because of the similarities in disease manifestations with *C. trachomatis*, it has been speculated that *M. genitalium* may give rise to sexually acquired reactive arthritis (SARA).

Indirect evidence for *M. genitalium* being a cause of seronegative arthritis was provided by serology, where it was shown, that five of 10 women had antibodies to *M. genitalium* as compared to none of the controls (187). Later, two of 13 synovial fluid specimens were found to be *M. genitalium* PCR positive; in one patient *C. trachomatis* negative urethritis was documented whereas the other had a chronic polyarthritis (162). The isolation of *M. genitalium* from a synovial fluid isolate of *M. pneumoniae* gave further support to a possible association between *M. genitalium* and arthritis (85; 86). Using PCR, *M. genitalium* was detected in nine (35%) of 26 specimens from the temporomandibular joint (188). In the same study, *C. trachomatis* was detected in 42%, but no control tissues were analysed, thus raising questions about the specificity of the laboratory methods used.

6.3. OCULAR INFECTION

SARA is to some extent overlapping with Reiter's syndrome. One of the symptoms of Reiter's syndrome is conjunctivitis, and since one of the patients having a positive *M. genitalium* PCR from synovial fluid had Reiter's syndrome (162), it would have been interesting to test a conjuctival specimen for *M. genitalium* DNA. Recently a male patient with NCNGU and unilateral conjunctivitis was found to be positive for *M. genitalium* in both an FVU specimen and a conjunctival specimen (189). Conjunctival symptoms had been ongoing for five months and no relief from local treatment with anti-allergic eye-drops was obtained. He reported intermittent dysuria for several months and was treated with doxycycline 100 mg bid for 10 days and improved. The sequence of the MgPa1-MgPa3 amplicon from both sites were identical suggesting that the eye infection could be a result of self-inoculation

Neonatal conjunctivitis should also be studied further. Considering the low prevalence of *M. genitalium* among pregnant women, mycoplasmal conjunctivitis may not be numerically important, but since *M. genitalium* is resistant to chloramphenicol (final MIC 10-25 mg/l) (92) it could have therapeutic implications.

7. CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

The fact that more than 50% of all cases of NGU, and an even higher percentage in the female counterpart MPC have an unknown aetiology has been an enigma for decades. The discovery of a new mycoplasma species from the urogenital tract in 1980 gave hope for explanation of at least a part of the unexplained urogenital infections. Due to the diagnostic difficulties, however, the first decade after its isolation led to more frustration than knowledge about the clinical importance of the infection.

The first PCR assay for *M. genitalium* developed as part of this thesis work has been widely used in clinical studies published within the last decade. Comparison with the 16S rRNA gene PCR has documented that the two assays have a comparable performance, but possibly, the recent TaqMan PCR assay may have a marginally increased sensitivity.

Much has been learned about the epidemiology of the infection, and for male NGU, the modified Henle-Koch postulates have been fulfilled. In women, less complete information has been collected, but the evidence for MPC is convincing, and within recent years, data on upper genital tract infections has begun to accrue.

The pathogenicity is being intensely studied, and new tools for targeted gene interruption will probably contribute to even more knowledge. However, the link from the *in vitro* to the *in vivo* situation lags behind, and the host factors need more attention.

We still have much to learn about *M. genitalium* infections; in that sense, it is still a new disease. Future research should focus both on the clinical and diagnostic aspects.

Availability of commercially available assays is needed to take the investigations out of specialised research laboratories. However, in order to provide valid results, these assays need to have an extremely low limit of detection, which may be difficult to obtain in a robust kit format.

The available serological assays should be validated against patient populations with a known infection status. Sensitive and specific assays are needed both to fulfil the second of the modified Henle-Koch postulates and to gain new insight in infections such as PID, where the infectious focus is not easily accessible.

Determination of the optimal treatment of *M. genitalium* infections has a high priority. Although there is a clear impression that macrolides are better than tetracyclines, randomised controlled trials are urgently needed. These trials should include long-term follow-up in order to monitor recurrence. Along the same line, new

strains should be recovered and their antibiotic susceptibility profile should be determined. Such isolation studies are extremely time-consuming and require access to quality-controlled mycoplasma media. Furthermore, expertise in both mycoplasma culture techniques and PCR, preferably quantitative, is needed. Good antibiotic susceptibility studies also require experience and collaboration.

More studies on upper genital tract infections using properly chosen control populations should be performed. Preferably, long-term follow-up should be established on a large cohort in order to determine the possible late sequelae e.g. chronic abdominal pain and infertility. In order to accelerate this process, nested case-control studies with follow-up of women included in the various Scandinavian research projects could be considered, but would probably have to be performed as registry based research due to the confidentiality issues. If upper reproductive tract sequelae can be documented, the infection would probably gain more interest also outside research settings.

Complications such as epididymitis and prostatitis should be studied and the role of *M. genitalium* infection in male infertility also deserves attention.

Extragenital *M. genitalium* infections should be studied systematically. In particular, studies of SARA would benefit from good serological assays.

Neonatal infections should be investigated. Both pneumonia and conjunctivitis are relevant disease entities to be considered, but neonatal meningitis should also be thought of, bearing in mind the apparent invasive potential of *M. genitalium*.

It would probably be rewarding to study further the genetic mechanism behind the antigenic variation shown during the present study. Adherence inhibiting antibodies to the adhesins could be used to document, that changes in the adhesin gene are brought about by the need for evasion of the immune system.

Even if we acknowledge, that *M. genitalium* is a cause of urogenital tract infection, and accept that it may account for up to 25% of the cases of NGU and MPC, we still miss the etiologic agent in more than one third of the infections. Although the advent of modern diagnostic techniques has made research in new suspected pathogens easier, there is still a very long way to a complete understanding of these complex diseases.

8. ENGLISH SUMMARY

Mycoplasma (*M.*) *genitalium* is a rather newly discovered bacterium, first isolated in 1980 from two of 13 men with urethritis. It is extremely difficult to isolate by culture, hence, knowledge about its pathogenicity has been slow to accrue. Only after the advent of the first PCR-based detection method that we published in 1991, has clinical studies been possible. The diagnostic methods were improved, and the latest development is a quantitative TaqMan PCR assay. Using this method, we were able to document that men with non-gonococcal urethritis (NGU) had higher DNA loads in first-void urine (FVU) and urethral swab specimens than had *M. genitalium* positive men without urethritis.

The diagnostic methods developed as part of this study have been used in several clinical studies addressing the relationship between *M. genitalium* and NGU. In the first published study on *M. genitalium* in male NGU, we were able to detect *M. genitalium* significantly more often in men with NGU than in those without symptoms of urethritis. Furthermore, the *M. genitalium* prevalence in men with non-chlamydial NGU (NCNGU) was significantly higher than in men with chlamydial NGU. This may indicate that *M. genitalium* and *C. trachomatis* may act as separate causes of urethritis. Later studies have uniformly shown that *M. genitalium* is associated with NGU, and most of them have documented that the prevalence in men with NCNGU is even higher.

In order to determine the most sensitive and least expensive strategy for detection of *M. genitalium* from urogenital tract specimens, we compared the diagnostic performance of FVU with male urethral swabs and found that significantly more infections were detected from FVU than from urethral swab specimens. In women, the results were not as clear-cut, since FVU was less suited as a diagnostic specimen alone, but should be supplemented with a cervical swab in order to obtain a satisfactory sensitivity. Furthermore, it was shown that some of the *M. genitalium* positive female FVU specimens lost reactivity if frozen without previous DNA extraction. This observation may have important implications for studies based on stored specimens.

Taking advantage of the knowledge about the PCR status of specimens collected during the first clinical study, we developed a method for isolation of *M. genitalium*, based on enrichment in Vero cell cultures grown in a medium without foetal calf serum and subsequent passage in cell-free media. Using this method, we were able to isolate *M. genitalium* from urethral specimens for the first time since the initial isolations.

The new urogenital isolates were mutually genetically different, and different form the type strain and the four strains isolated from *M. pneumoniae* cultures from the respiratory tract as well as from a strain isolated from a synovial fluid culture of *M. pneumoniae.* The homogeneous genetic structure among the older isolates as compared to the marked variability of the MgPa gene of the Danish strains may raise concern that the respiratory tract isolates were laboratory contaminants with the G37 strain. This suspicion is supported by the finding that an early passage of one of the primary urethral isolates (M30) had a markedly different MgPa gene sequence from that of the passage later deposited in the ATCC.

Partial sequences of the MgPa gene encoding the main adhesin were obtained. It was shown that sequences varying between strains were clustered in regions of the gene also present as incomplete repeated regions scattered throughout the chromosome. Such sequences make up for 4.7% of the total genomic content of this otherwise very limited genome. It seems likely, therefore, that such sequences may serve as a reservoir for antigenic variation. This was substantiated by finding such genetic variations in consecutive isolates from experimentally inoculated male chimpanzees.

Antimicrobial susceptibility studies including the Danish *M. genitalium* strains have shown that some strains are resistant to tetracyclines and/or quinolones. The clinical relevance of these findings were documented in a study, in which we were able to show, that the response to treatment of male NGU was significantly better if the patients were treated with 1.5g of azithromycin given over five days than if they were treated with doxycycline.

The studies included in this thesis have significantly contributed to the recognition of *M. genitalium* as a cause of male NGU, and has provided tools for further studies of infections caused by this extremely fastidious microbe.

LIST OF ABBREVIATIONS

ATCC	American type culture collection
BAL	Bronchoalveolar lavage
BV	Bacterial vaginosis
CSW	Commercial sex worker
ELISA	Enzyme linked immunosorbent assay
FVU	First void urine
geq	Genome equivalent
hpf	High power field
IHA	Indirect haemagglutination
LAMP	Lipid associated membrane protein
MIC	Minimal inhibitory concentration
MIF	Microimmunofluorescense
MPC	Mucopurulent cervicitis
NCNGU	Non-chlamydial non-gonococcal urethritis
NGU	Non-gonococcal urethritis
PAGE	Polyacrylamide gel electrophoresis
PID	Pelvic inflammatory disease

PMNL	Polymorphonuclear leucocytes
SARA	Sexually acquired reactive arthritis
SDS	Sodium dodecyl sulphate
STD	Sexually transmitted disease
STI	Sexually transmitted infection

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