UNIVERSITAT ROVIRA i VIRGILI

DEGREE IN BIOCHEMISTRY AND
MOLECULAR BIOLOGY

FINAL DEGREE PROJECT

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Macrolide resistance mutations in *Mycoplasma genitalium* in the region of Tarragona in 2021: prevalence and associated features.

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Tarragona, June 2021

This work is based on my internship carried out in the Clinical Analysis Laboratories of the Joan XXIII Hospital of the Tarragona Area, directed by Gemma Recio Comí.

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ABBREVIATIONS

AZT: Azithromycin

BASHH: British Association for Sexual Health and HIV

CDC: Centers of Disease, Control and Prevention

CE: *Conformité Européenne* (European Conformity Framework)

CFU: Colony-forming units

CI: Confidence Intervals

CRO: Ceftriaxone

CT: *Chlamydia trachomatis*

Ct: Polymerase Chain Reaction Cycles

DOX: Doxycycline

FDA: Food and Drug Administration

HIV: Human Immunodeficiency Virus

i.m.: intramuscular

IC: Internal Control

ICS: *Institut Català de la Salut* (Catalan Institute of Health)

IVD*: in vitro* Diagnosis

LDT: Laboratory-Developed Test

MG: *Mycoplasma genitalium*

MICs: Minimum Inhibitory Concentrations

MRMM: Macrolide Resistance-Mediating Mutations

MSM: Men Sex Men

MSW: Men Sex Women

NAATs: Nucleic Acids Amplification Tests

NCNGU: Non-chlamydia Nongonococcal Urethritis

NG: *Neisseria gonorrhoeae*

NGU: Nongonococcal Urethritis

PCR: Polymerase Chain Reaction

PID: Pelvic Inflammation Disease

RT-PCR: Real Time Polymerase Chain Reaction

SARA: Sexually Acquired Reactive Arthritis

SEIMC: *Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica* (Spanish Society of Infectious Diseases and Clinical Microbiology)

SNP: Single Nucleotide Polymorphism

STI: Sexually Transmitted Infections

TLR: Toll-Like Receptors

TMAs: Transcriptional-Mediated Amplification Test

TOC: Test of Cure

TV: *Trichomonas vaginalis*

TWSM: Transsexual Women Sex Men

WHO: World Health Organization

WSM: Women Sex Men

ABSTRACT

Introduction: The *Mycoplasma genitalium* (MG) is a micro-organism that causes uncommon sexually transmitted infections (STI) in the general population, but it stands out for being the main cause of nongonococcal urethritis in men. Since the discovery of the recombination techniques, it has been possible to begin to study more extensively. Currently, it is considered an emergent problem worldwide. Its prevalence and the rate of macrolide resistance-mediating mutations (MRMMs) are each time higher, and administered therapies are declining in effectiveness worldwide among the years.

Hypothesis: The studies related to the azithromycin resistances in *M. genitalium* are limited and they vary greatly depending on the geographical region and the population studied. More resistance studies in Spain, to know the features and rates of affectation in the population, would help direct towards an optimized therapy and thus, minimize the mutations associated with therapies.

Aims: To determinate the prevalence of the azithromycin resistance in a positive MG population in the region of Tarragona and the different characteristics related to a greater probability of suffering them.

Materials and Methods: A cross-sectional study was carried out with 66 positive MG patients who underwent MRMMs detection test by PCR. Specific characteristics were also analysed in each patient, including the age, gender, sexual orientation, coinfections, STI in the last year, symptomatology and administered treatment.

Results: In the study, MRMMs were reported in 14,49% of the cases. The most prevalence was the A2059G mutation (80%), followed by A2058G (10%) and A2058T (10%). Most of the patients had less than 30 years (77,8%), symptoms related with this STI and only a 37,5% of patients received a treatment appropriate to their features.

Conclusions: The MRMMs detection in positive MG patients would considerably improve the orientation to an optimal and effective therapy and would reduce the probability to produce resistances. Moreover, more studies are needed worldwide to achieve more updated data on prevalence of this infection and mutation rates.

KEYWORDS: Sexually Transmitted Infections (STI), *Mycoplasma genitalium,* macrolide resistance mutations

RESUMEN

Introducción: El *Mycoplasma genitalium* (MG) es un microorganismo causante de ITS poco común en la población general, que destaca por ser la principal causa de uretritis no gonocócica en hombres. Desde el descubrimiento de las técnicas de recombinación se ha podido empezar a estudiar de forma más extensa. Actualmente, se considera un problema emergente a nivel mundial. Su prevalencia y la tasa de mutaciones de resistencia a macrólidos (MRMs) cada vez es mayor, y las terapias administradas van disminuyendo su eficacia con los años a nivel mundial.

Hipótesis: Los estudios relacionados con las resistencias a azitromicina en *M. genitalium* son limitados y varían mucho según la zona geográfica y la población estudiada. Un mayor número de estudios de resistencias en España, para conocer las características y la tasa de afectación en la población, ayudaría a direccionar hacia una terapia óptima y minimizar así las mutaciones asociadas a la terapia.

Objetivos: Determinar la prevalencia de las resistencias a azitromicina en una población MG positiva en el área de Tarragona y diferentes características relacionadas con una mayor posibilidad de padecerlas.

Materiales y Métodos: Se realiza un estudio transversal con 66 pacientes MG positivos a los que se les realizó una prueba de detección de MRMs mediante PCR. También se analizaron características concretas en cada paciente entre las que se incluyen la edad, sexo, orientación sexual, coinfecciones, ITS pasadas, sintomatología y tratamiento administrado.

Resultados: En el estudio se detectaron MRMs en un 14,49% de los casos. La más prevalente fue la mutación A2059G (80%) seguida de A2058G (10%) y A2058T (10%). La mayoría de estos pacientes tenían menos de 30 años (77,8%), síntomas relacionados con esta ITS y solo un 37,5% recibieron un tratamiento acorde a sus características.

Conclusiones: La detección de MRMs en pacientes MG positivos mejoraría considerablemente la orientación hacia una terapia óptima y eficaz y reduciría la probabilidad de generar resistencias. Además, se necesitan más estudios a nivel mundial para tener unos datos más actuales sobre la prevalencia de esta infección y de la tasa de mutaciones.

PALABRAS CLAVE: Infecciones de transmisión sexual (ITS), *Mycoplasma genitalium,* mutaciones de resistencia a macrólidos.

INTRODUCTION

The Sexually Transmitted Infections (STI) are pathologies caused by infection agents or parasites in which sexual transmission pathway has epidemiological importance.[1] Currently, STI are one of the principles health problems worldwide. [2, 3] The high morbidity and mortality load produce physic, psychologic and social consequences. They end considerably up compromising the quality of life inside the infected people, as well as sexual and reproductive health, and the health of neonates and children. [4]

The effects and possible disabilities, which are originated by the lack of an early diagnosis and adequate treatment, could lead to a series of serious complications, like pelvic inflammation disease (PID) or infertility. [1, 3] Some of the pathogens that cause STI have been related to carcinomas. In the pregnant population, they can produce serious complications in gestation, important effects on birthing or during the first weeks of life, because of the close relationship with the mother. [1]

Moreover, the human immunodeficiency virus (HIV) and STI are clearly interrelated. [2] There is an increased risk of HIV infection between 2-5 times greater if you have other STI. [1] The STI indirectly make easier the HIV transmission because they share transmission mechanisms and risks. [4]

On the other hand, the professionals on the health area should have an optimal knowledge about the right STI management, both in prevention, diagnostic approach, treatment, patient follow-up and contact search. [2]

According to World Health Organization (WHO), more than a million of STI are acquired worldwide every day, usually between 15-49 years old. [5] This is approximately equivalent to 357 million new STI worldwide each year. [4]

The registered increase in the incidence of STI is due to a relaxation of the preventive measures during sexual intercourse, due to the contraceptive use, the existence of antiretroviral therapies against HIV infection, which makes a chronic infection and not a lethal one, etc. The increase in the risk sexual behaviour is appreciated mainly among a focused population groups, such us the young or some men sex men (MSM) population. [6] The concern is so much so that the WHO establishes global strategies for the decrease of the STI incidence, like the proposal from 2016 to 2021. [3, 4, 5]

More than 30 bacteria, viruses, and parasites are known to be transmitted by sexual contact. Among them, several species of mycoplasma, who are capable to produce STI, have been located and each time, they are generating an increasing interest. [3]

Mycoplasmas belongs to a peculiar group of bacteria and they are identified as the smallest free-living micro-organisms capable of self-replication in the world. Their size is used to be around 0,2-0,8 micrometres, being a problem specially for cell culture techniques, because they are capable to cross some antibacterial filters (HEPA filters). [7, 8]

They are mainly characterized by not presenting a rigid cell wall and by being surrounded by a simple plasmatic membrane with sterols, which allows them to adapt to different environmental

niches. The wall absence and the lack of peptidoglycans are the principal differences compared with other bacteria. Especially against the β-lactam antimicrobial activity, such us penicillin, to which they are intrinsically resistance. As a visual feature, the cultures on soft agar plates have a fried-egg appearance of colonies. [7, 8, 9]

The genome, like the organism in general, is the smallest size that can be given to be a free-living organism. These features have led to a loss of many metabolic pathways, needing a host for their survival. The most of them are parasites, more than 100 different species with pathogenic ability in humans, animals or plants are being located. The main needed nutrients are amino acids, fat acids and above all, cholesterol, which is the principal compound for the external cover. [7]

Unlike viruses, the mycoplasmas are capable to live in different biologic liquids o tissues without killing the host cells. However, the competition for nutrients with the cells creates functional alterations even at the genetic level, aberrant mutations, etc. [7]

In general, the infections caused by mycoplasmas and ureaplasmas have a limited length, silent and with a non-specific symptomatology. [10] In the urogenital tract, the most relevant human-isolated species are *Mycoplasma genitalium, Ureaplasma urealitium, Ureaplasma parvum* and *Mycoplasma hominis.* [9] The genital microbiota is varied between individuals and in many cases, these species are part of the element of the normal flora, which makes difficult their diagnosis as infections. [10]

Mycoplasma genitalium. CHARACTERISTICS.

Taxonomically, *Mycoplasma genitalium* (MG) is a bacterium belonging to the *Mycoplasmataceae* family, *Mollicutes* class. [1, 11] MG was first isolated in 1980 from a subset of samples from two men with urethritis grown in cell cultures. Growth was detected after 50 incubation days; colonies were identified due to a colour change in the medium caused by the fermentation of glucose. [8]

Currently, it is increasingly appreciated as a pathogenic cause of sexually transmitted infections (STI) in the population, and it is considered the main cause of nongonococcal urethritis (NGU) in men worldwide. [12]

This micro-organism is a fastidious obligate intracellular bacterium with self-replicative ability. It is suggested to come from a Gramm positive bacterium, through a degenerative evolutionary process. [7]

Morphologically, it is a mobile micro-organism thanks to the use of a specialized structure, also named "*tip".* This structure is a slightly curved terminal organelle who it is distinct in shape from the other mycoplasma species. [8]

The *tip* is mostly composed by phosphoproteins and membrane proteins and it allows the adhesion, motility both inside and through the cells, and is involved in cell division. Its structure is remarkably complex, containing an electron-dense core and surface-exposed proteins visible

in electron micrographs. Besides, the basal structure thought to be the motor, producing energy for motility of the organism. [8]

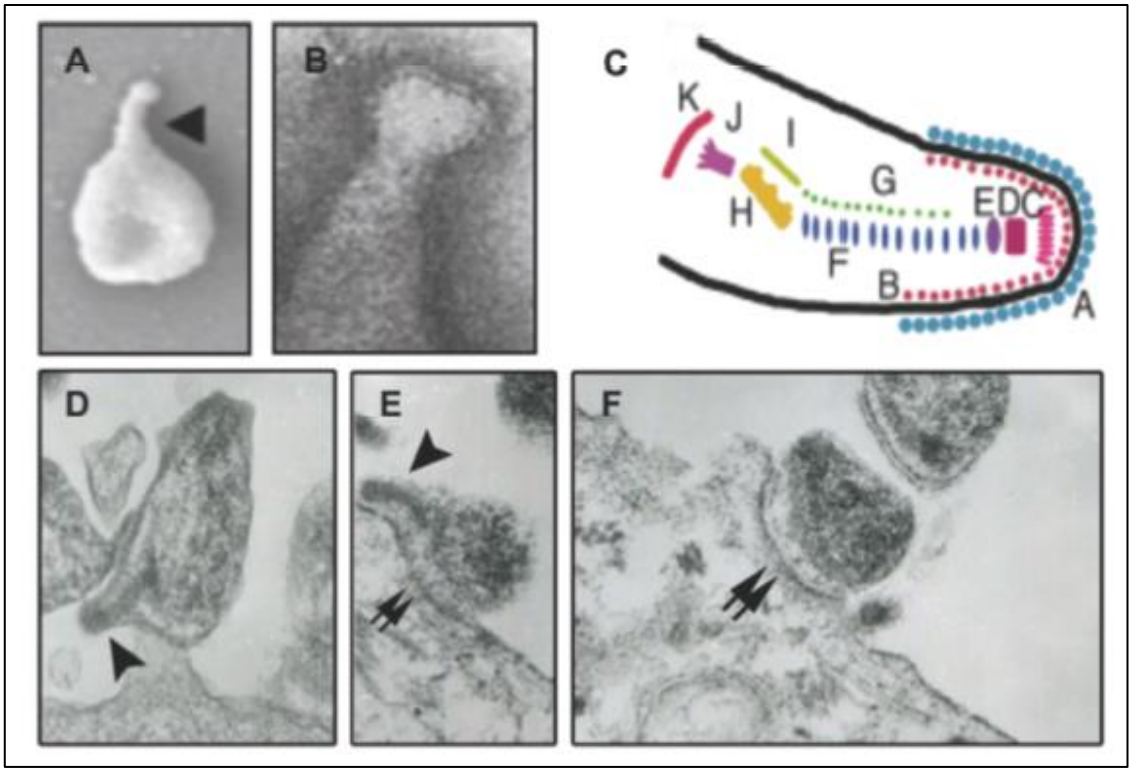


Figure 1: *Mycoplasma genitalium* structure with the organelle tip and its interaction with the human reproductive tract epithelial cells. **A, B.** Electron micrograph imagen where it can appreciate the terminal curved organelle tip (pointed with an arrow) **C.** Terminal organelle of *M. pneumoniae*, letters indicate different protein complexes of *M. pneumoniae* which are homologous in *M. genitalium*. **D-F.** Interaction between *M. genitalium* and human cells. In the two first images (D and E), tip and its interaction with cells can be clearly appreciated (arrowheads) and in the images E and F, it can be seen that the adhesion involves more surface proteins and not only tip. (double arrows).
Imagines obtained from [8]

This organelle is key for the MG pathogenic process. The tip surface-exposed proteins, called MgpB and MgpC, end up shaping an adhesin termed MgPar. This last one mediates the attachment to the eukaryotic cells. [8]

The genes, who are making up MgPar, are all organized in the same operon and they are three, *mgpA, mgpB* and *mgpC*. All of them are subject to phase and antigenic variations, which make infection possible. Overall, the adherent proteins are the main virulent factors of this pathogen. [7, 8, 13]

Their biological features have predisposed it to be a pathologic bacterium, although in many cases it appears in an asymptomatic form. The *M. genitalium* lacks genes for the amino acids synthesis and it has few genes for the nucleic acids synthesis, vitamins and fat acids. It relies on the obtaining of many growth factors for its metabolism, requiring the acquisition of all these nutrients from the environment where it lives, which it is normally the host. MG doesn't have neither genes for the oxidative metabolism (Krebs' Cycle), gluconeogenesis nor enzymes who

protect it from the oxidation, such as catalase or peroxidase. All these strict growth factor requirements have been hindering *in vitro* laboratory studies, slowing down the research. [7, 8]

In addition, *Mycoplasma genitalium* was further distinguished from other mycoplasma species by its inability to hydrolyse arginine and urea, and because it can be inhibited by thallium acetate. [8]

Because of their genetic and metabolic features, its biosynthetic abilities are too low and its growth rate too slow and fastidious, as in the most of mycoplasmas. In addition, its morphologic limitations make it quite sensitive to environmental conditions. The lack of cell wall become it osmotically much sensitive. Sexual transmission makes easier the less contact with external environments because it can only live in constant osmotic environments. [7]

In this aspect, it is likeness *Lactobacillum* and *Clostridium,* it is from human origin and it is mainly into the urogenital tract, although some cases has been found it into the respiratory system. [7]

MOLECULAR GENETICS

The *M. genitalium* genome is extremely tiny, the tiniest between all the free-living organisms who are known, with 580kb long. It is considered the minimal cell model in the absence of a smaller organism with self-replication ability. [7, 14]

The *M. genitalium* was the second bacterial genome to be fully sequenced, in 1995. This sequence was termed G37 and it was selected as the type stain in phenotypic studies. This fact made the genomic structure, the energetic metabolism and the evolution understood. However, the phylogenetic analysis isn't yet established, there aren't a link between the origin countries and the years when the bacterium was isolated. Certain studies showed that the nucleotide divergence is less than 0,5% compared to G37. All the sequences have the same protein-coding genes and there aren't accessory regions in any analysed case, suggesting the *M. genitalium* stains are similar worldwide. [8, 11]

It is a specie with a double-stranded circular DNA where it contains the full genetic information. No extrachromosomal DNA or plasmids have yet been presented. [8] Similar to other mycoplasmas, the average guanine-cytosine content is 31-32%, approximately and less of 500 protein-coding genes. The general absence of regulation systems and the low number of transport systems can be appreciated at the genetic material. [11]

One interesting characteristic is the modification of the genetic code in which UGA encodes a tryptophan rather the common translational stop codon. [7, 8, 14]

This pathogen despite the minimal genome, it is characterized by presenting a very heterogeneous and complex genome. The first sequenced and distinguished gen was the *mgpB.* After the full sequencing, both 9 partial noncoding genomic loci, called MgPar regions and hypervariable regions of adjacent genes *mgpB* and *mgpC* was discovered. These two last genes encode the surfaced proteins MgpB and MgpC. The among of these genes are the responsible of the genomic heterogeneity. Remarkably, the *mgpB/mgpC* operon and their homologous

regions at the MgPar sites allocate the 4,7% of the genome, highlighting the great importance of this system into the micro-organism. [8]

The *mgpB* and *mgpC* are functionals and expressed at a single site within the genome, flanked by MgPar4 upstream and MgPar5 downstream. These genes encode a cyto-adhesin and certain proteins involved in cyto-adhesion. While the MgPar regions are non-functional fragments of specific genes, forming a family of repetitive chromosomal repeats. [11]

On the other hand, potential regulatory genes are very important to respond to the changing metabolic environment and the formation of niches within the human host. The most highly regulatory system in bacteria is the recombination, which it wasn't identified in *M. genitalium* until 2014 when Burgos and Totten discovered the MG428 protein, [15] which is a positive regulator of the *mgpB*/MgPar recombination. This recombination is what occurs in *M. genitalium* and it is explained in detail in section "*Action Mechanisms and Pathogenesis*". [8]

Moreover, this MG428 protein coordinates other recombination regulatory genes, such as *recA*, *ruvA* and *ruvB*. The protein is considered an alternative sigma factor that controls the gene expression involved in physiological processes. This protein is the responsible for the direct binding of the RNA polymerase to a subset of genes containing promoters targeted by sigma factor. [8]

Other new regulatory mechanism is through the RecA protein, which is found in *M. genitalium,* yet it isn't part of other types of bacteria. [8]

The activity of MG348 and the functions of the RecA in *mgpBC/*MgPar recombination is crucial for the pathogenesis of this human pathogen. [8]

ACTION MECHANISMS AND PATHOGENESIS

The registered cases of nongonococcal urethritis, which was the beginning of the knowledge of MG, already demonstrated inflammation ability of this pathogen. Thanks to the detection of *tip* organelle, previously mentioned, it was discovered that surface proteins and some genetic sequencies had a high homology with those of *M. pneumoniae* therefore, it is possible that the virulence factors have a high similarity too. [7, 8, 11]

The urogenital tract colonization is the first target of infection, the mycoplasma begins to reproduce actively and consequently, the first symptoms begin to appear. The caused tissular harm had been shown that is like those of the *M. pneumoniae,* which is mediated by the hydrogen peroxide production and superoxide metabolites. First, the bacterium attached itself to the cell with its cyto-adhesin and then, the peroxide is realised damaging the cell. [7]

The binding between *Mycoplasma genitalium* and the epithelial cells, already elicits signals for the development of a subsequent acute inflammation. The innate immune sensors of the epithelial cells, like Toll-like receptors 2 and 6 (TLR2 and TLR6), by binding to mycoplasma surface lipoproteins, induce together the activation of NF-κB cascade. This fact results in the induction of genes involved in host defence. [8]

The primary stimulus, which induces the immune response, are the pathogen surface lipoproteins because toxins or secretion of virulence factors aren't found. The products derived from mycoplasma metabolism and the host local response promote the accumulation of reactive oxygen species (ROS), which has a cytopathological impact on epithelial cells. [7, 8, 13]

First, a proinflammation response mediated by local macrophages and monocytes of the own human reproductive tract was developed. Lastly, the high chemokines concentrations induce the leucocyte recruitment at the infection site. These end up promoting an adaptative response mediated by systemic antibodies and the own genital mucosa against the MgpB and MgpC membrane proteins, which behave as a superantigens. [7, 8, 13]

The antibody clearance isn't elucidated nor if the antibodies may protect from future infections. The most possible is that they can´t, due to the higher probability of a new infection if you had suffered a previously STI infection. [8]

The *Mycoplasma genitalium* shows an incredible ability to avoid this immune response promoted by the host. The cases of persistent or even chronic urogenital infection are common in both male and females. [8]

The survival is possible thanks to a non-uniform recombination mechanism which induces a high genetical variability. [8] On average, the 50% of genome is predicted to be involved in the recombination process, having chromosome regions with a clear higher rate of recombination, termed *hotspots.* [11]

The *mgpB* and *mgpC* genes are assembled by both conserved regions and variable regions, the last ones are B, EF, G in the *mgpB* gene and KLM in the *mgpC* gene. The recombination key is that homologous copies of the variable sequencing regions are presented in multiple MgPar sites distributed around the chromosome. [8, 11]

Not every MgPar sites contain all the repeated sequences. It can be found as a complete unit, as a truncated sequence or as a split one. For instance, the G segment can be placed after KLM sequence or inserted between the two split KLM fragments (KLM-1, KLM-2). This fact makes that it is a reservoir of very heterogeneous variations with quick introduction in *mgpBC,* which produces a very high antigenic diversity. The MgPar regions loci are clearly *hotspots.* [8, 11]

In the presence of certain environmental stimulus, reciprocal recombination events are yielded; changes in regions of *mgpBC* sequence are accompanied by a change with their corresponding homologous region at some MgPar site. This produces variations in the aminoacidic sequences of the surface proteins, which at last are the antigenic compounds, allowing to avoid the immune response. This process is known as *mgpBC/*MgPar recombination. [8] Moreover, the MgPar regions are capable to produce recombination between their different loci, causing an incalculably high internal diversity. [11]

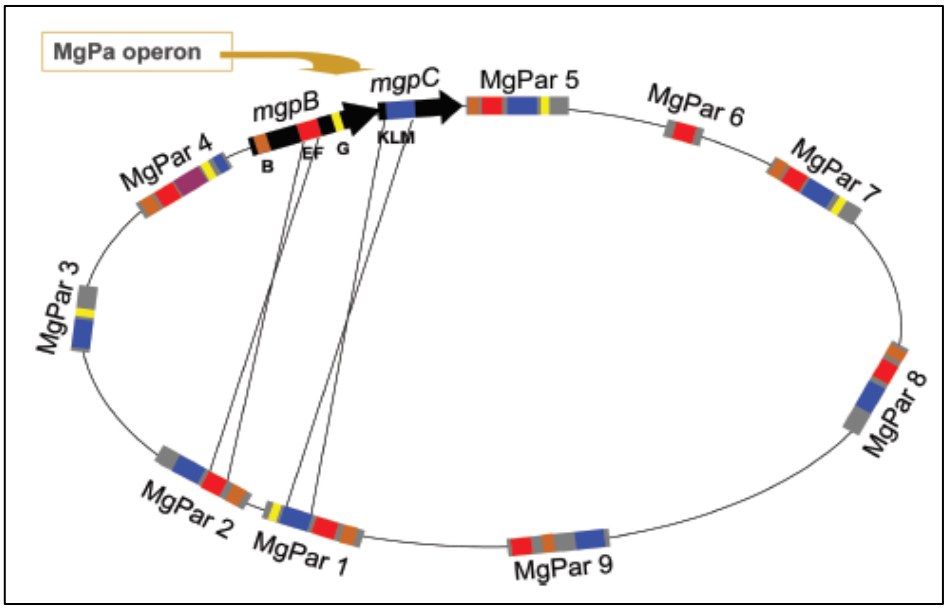


Figure 2: Representation of *Mycoplasma genitalium* genome, where *mgpB* and *mgpC* are within the same operon, and the 9 MgPar homologous sequencies can be observed. The reciprocal recombination between mgpBC and the homologous sequencies in the MgPar sites is also represented.
The conserved regions are represented in black, the variable region B in orange, EF in red, G in yellow and KLM in blue. These variable regions are found in *mgpB* and *mgpC* with homologous copies in the MgPar regions, where the sequences may be completed or not. The gray regions represent adenine-thymine-rich zones of the MgPar sequences.
Imagine obtained from [8]

In addition of these MgPar sites, it is located other recombination blocks subjects to recombination in other genes involved in cytoadherence and lipoprotein formation too, such as *hmw2, hmw3* and *hmw1.* [11]

Two types of gene variations in *M. genitalium* are discovered: antigenic variations and phase variations. The last ones are due to the loss the ability to adhere to epithelial cells, which end up generating a dissemination to the blood and binding to red blood cells. Non-adherence is because of the non-expression MgpB and/or MgpC proteins. This functional loss is a consequence of multiple recombination resulting in the translocation of a part of the *mgpBC* sequence to the participating MgPar sites, leaving an incomplete *mgpBC* operon. [8]

The lack of many recombination enzymes in MG, because of its little genome, makes the RecA, RuvA, RuvB and RecU proteins, encoding by regulatory sequences, essentials for the *mgpBC/*MgPar recombination. [8]

Understanding the recombination, its regulation, and the environmental factors, which produces it, are essential to understand the molecular strategies and the pathogenesis of *Mycoplasma genitalium.* [8]

TRANSMISSION

Mycoplasma genitalium is primarily transmitted by direct genital-genital contact, how it isn't surprising being a STI infection. Other important transmission way is the genital-anorectal and it may play a fundamental role during so many infections. [9] The rectal MG may play a role as a reservoir, it has been shown in the anal mucosa, being asymptomatic so many rectal carries. [16]

The oral-genital contact is less likely to contribute to the infection, the bacterial charge in the oro-pharynx is insignificant. The vertical transmission (mother-to-child transmission) is unknown, and it has not been studied enough, as a result there aren't significant facts to prove it. Nevertheless, in some cases the pathogen has been detected in the respiratory tract of new-born children, so more studies might be needed. [9]

On the other hand, the infection prevalence of *M. genitalium* is lower than the prevalence of *C. trachomatis,* it could be considered slightly less contagious. Furthermore, the contagious ages in MG are higher in both genders. Also, *M. genitalium* infected patients appear to peak 5 years later than *C. trachomatis* cases. [9]

PREVENTION

The principal aims in the presence of an infection is to avoid the spread between individuals, to stop to the maximum the incidence and to prevent the development towards persistent infections or reinfections. The infected patients should abstain from any type of sexual contact without protection until they and their sexual partners have completed the treatment, the symptoms have resolved, and the posttreatment test of cure gives a negative result. [6, 9] The contact studies are beneficial for the individuals and within the public health area, cutting off the chain of transmission at population level. [2]

In addition, other possible STI should be screened in patients with a MG-positive diagnosis, in order to reject a coinfection and to know the seriousness of the specific case, including at least *C. trachomatis, N. gonorrhoeae,* syphilis, HIV and *T. vaginalis.* [9]

The information about the infection, the details about transmission, prevention and possible complications should be given to every patient with *M. genitalium* pathology. So as to increase the awareness about the STI seriousness and perhaps thus, reduce their risk behaviour. [9]

The more common STI among the population are frequently screened. However, routine screening of *M. genitalium* are not recommended as optimal preventive strategy, despite the similar prevalence with *C. trachomatis.*[2] This is due to the lack of evidence and knowledges about the natural history of the infection, which may be a sexual transmission or stand by in the natural genital microbiota of the person. In addition to the lack of data on the cost-effectiveness of the test. [16] Similarly, screening is not recommended within the asymptomatic population due to the imperative of treating them with the problems of resistance and toxicity of the treatment.[6]

In the most vulnerable risk population, such us men sex men (MSM) where the elimination of the infection is more complex, there are a lot of asymptomatic rectal carriers of the infection. The rectal MG as a reservoir is producing controversy as to how much it is necessary the detection of the infection and the management of treatment in both sexual partners in specific cases among the population with higher degree of incidence and vulnerability. [16]

SPECIMENS

The STI diagnosis is syndromic and the collection of samples necessary to carry out the pathogen detection could be so diverse. It will depend on the clinical appearance and the clinical history of the patient, usually attending the gender, their frequent sexual practices, [9] the symptomatology [17] or previous surgeries. [3]

In the case of men with symptoms, the priority of the specimens is variable. In every case could be taken by first void urine, but if urethritis or epididymo-orchitis diseases are present, it is always better the collection of a urethral swab sample if it is possible. [17] The urethral sample is also more appropriate if it will carry out the study of various pathogens at the same time. [3]

In the female population, the mycoplasma can be established within the endometrium, cervix, or vagina, [14] therefore there are larger sample diversity. In general, the samples with the highest sensibility are the vaginal or endocervical swabs, being the most useful and advisable. [9]

According to the proposal of homogenization of the screening and STI diagnosis of the Catalan Institute of Health (CIH, June 2018), [17] the vaginal swab is taken with a priority over the endocervical samples in the asymptomatic women because of practicability and the same diagnosis efficiency. In the symptomatic women, the sample mainly depends on the region of the female reproductive tract where the infection is established. Vaginal swab is collected by physician or self-collected in cases of vaginitis, [9] whereas endocervical swab is collected in case of cervicitis or inflammatory pelvic disease. [17]

The extragenital specimens depends on the sexual practices, mostly from the anal localization in *M. genitalium* infections. [17] Anal samples are useful and especially advisable in MSM or in women who have been in a sexual anal unsafe relationship. These samples are very relevant because they include up to 70% of the infections among these patient groups. [9]

No data is available regarding time of the sample stability to the diagnosis of *M. genitalium.* The similarities with *C. trachomatis* have led the same protocols being followed, in relation to stability and minimal incubation time between the exposition and the test. [9]

EPIDEMIOLOGY AND CLINICAL CHARACTERISTICS

The prevalence of *M. genitalium* is rather low in the general population, affecting only 1% to 3,3% and being the impact rate higher in men than in women. [9, 12] The age interval more affected is between 16-35 years in sexually active individuals. [1] The increase of prevalence is especially shown among the risk groups like MSM, both positive and negative HIV population. [6]

The international population research about this pathogen is low and they can be downwardly conditioned due to the limitations in the number of reliable diagnostic methods [11] and the high prevalence of asymptomatic cases. The infection is not detected in so many patients. This feature is so like *Chlamydia trachomatis* in which between 60-70% cases are also asymptomatic.[9] The epidemiological evolution is also limited because it is not a mandatory notifiable disease.[6]

However, the organism has been potentially associated with many disease outcomes despite being a less common sexually transmitted infection. Consequently, the detection has a high relevance in the clinical setting. [14]

This pathogen leads to urogenital infections where the reproductive tract is the principal damaged in both men and women. [9]

The main symptoms associated with *Mycoplasma genitalium* infection include urethritis and epididymo-orchitis in men under 50 years. In women, the pathogen may cause many adverse reproductive sequelae, including miscarriage o premature births with a multifactorial aetiology, as well as mucopurulent cervicitis, abnormal vaginal or cervical swab together with a risk factor for STI, postcoital or intermenstrual bleeding, acute pelvic pain and/or PID. All these disease outcomes are advised to carry out a diagnostic test in the laboratory. [9, 14, 18]

Another aspect to take into account, apart from the mentioned symptoms, are the risk factors to that the patients have been exposed. [9] The risk factor is increased with the number of new and total sexual partners and unsafe sexual practices. There are research studies, such us the carried out between 2010-2012 in Brittany NATSAL-3 (National Survey of Sexual Attitudes and Lifestyle) where the black race is included as risk factor. [12]

According to the 2016 European guideline, the risk factors are considered to be under 40 years and to have more than 3 new sexual contacts in the last year or more than 5 life-time partners and they have never tested for STI. Also, it is considered a risk factor to have sexual contact with positive STI or PID infected person, in particular contacts with MG positive result. People with a stable and monogamous relationship, who suffers some of the previous mentioned symptoms, are a risk factor too. Before termination of pregnancy is taken into account the cervical barrier break. The MSM population group is considered vulnerable due to the risk of increased HIV transmission and they are encouraged to test frequently. [9]

A lot of STI screen risk individuals for the speed treatment administration or for the induction of the contact studies. However, the screening is not recommended for *Mycoplasma genitalium* infections. [2]

CLINICAL SYMPTOMS IN MEN

The 70% of the *M. genitalium*-infected men are symptomatic, being the gender with the most affectation. [9]

In men population, the major clinical appearance is the nongonococcal urethritis (NGU). [12] The urethritis is a urethral inflammation, usually accompanied by dysuria and mucopurulent or non-purulent urethral discharges. [2]

Currently, MG is the cause from 10% to 25% of NGU in Europe and it could reach up to 35% in cases of non-chlamydia nongonococcal urethritis (NCNGU). [3, 16] Moreover, together with *C. trachomatis* and *T. vaginalis, M. genitalium* is one of the major agents related to recurrent or persistent NGU (more than 90 days with the infection, discarding a reinfection). *M. genitalium* is associated with the 40% of the cases of recurrent or persistent NGU, due to an escape of the immune system, which would allow to establish a prolonged infection. [2, 12] The complex MG infections is usually associated with age groups under 35 years. [1]

Despite urethritis, other causes can produce symptoms of dysuria and abnormal or excessive urethral discharges. It believes that this pathogen may produce balanoposthitis and inflammation of the glands and foreskin. However, there aren't clear evidence, needing more research studies to prove it. [9, 12]

Other clinical symptoms related to MG may be:

Proctitis: characterized by pain and/or increased rectal discharge. The bacteria load was significantly higher in men with proctitis compared to asymptomatic. [12] The implication of MG in this disease is being discussed, yet MSM with proctitis are more frequently positives for MG.[3]

Prostatitis: prostate gland inflammation which may be caused in small clinical events by a *M. genitalium* infection. [12]

Epididymo-orchitis: characterized by inflammation, pain, and oedema of the epididymis, and ipsilateral testicle may be affected too. This disease is frequent caused by *Chlamydia trachomatis* infections. Given the high association between both bacteria, it is to be expected that MG could produce this inflammation. However, *M. genitalium* may cause epididymitis, trough less commonly than *C. trachomatis.* [2, 12]

If the MG infection remains during large periods of time, it may cause difficulties and produce sexually acquired reactive arthritis (SARA) in some cases. [9]

The men sex men (MSM) usually present an increased prevalence of suffering STI. Some research studies have shown the strong association between *M. genitalium* and the risk for HIV infection, such as that of Bissessor et al. [19] or that of Soni et al. [20] As a result, if MSM are more likely to suffer HIV infection, a coinfection with MG will be more likely among this population group and it could lead to a worse prognosis. These cases should be treated more carefully due to the vulnerability of the group. [12, 14]

CLINICAL SYMPTOMS IN WOMEN

The women have less prevalence to suffer *M. genitalium* infections and among MG positive attendees, between 40-75% are asymptomatic. However, the symptomatology could be very varied in the people who presents it. The clinical appearances in this gender have been more questioned due to many fewer studies with less consistent evidence. There are doubts related to the female reproductive tract diseases. There aren't data enough to associate *M. genitalium* and the adverse pregnancy outcomes, premature births, ectopic pregnancy, miscarriages, or women urethritis. Although, its influence is not discarded, and they should be managed with more caution. [9, 18, 21]

However, some multiple research studies have shown an increased risk of suffering some pathologies with many adverse reproductive sequelae in women. [14] Such as:

Infections related to the cervical or urethral area. They are the cause for an increasement in the symptomatology, improving the altered vaginal discharges (<50%), dysuria (30%), leucorrhoea and less frequently menorrhagia, intermenstrual and post coital bleeding. [9]

Cervicitis: MG increases in a 70% the risk of suffering this infection. It is usually characterized by visible mucopurulent cervical discharge, clear inflammation evidence, it is more likely cervical or uterine neck. The disease might often be course asymptomatic. [2, 18]

Lower unspecific abdominal pain (<20%): this symptom is very important to take into account and treat it as soon as possible because it could lead to PID, which it is often accompanied by endometritis and salpingitis. [9]

Pelvic inflammatory disease (PID): it is a polymicrobial syndrome of the female upper genital tract. It is characterized by endometritis, salpingitis, pelvic peritonitis and tubo-ovarian abscess. Furthermore, it is a common cause of infertility. [18]

In 85% of the cases, PID is caused by a STI. [2] MG can be detected in the female upper genital tract of women with PID, and endometritis was found to be 12% more common around infected than health women. These facts made possible the correlation between the MG and the acute PID. This was already supported by the similarity in the prevalence of endometritis in *N. gonorrhoeae* (10%) and *C. trachomatis* (7%). [18]

Besides, it has seen that subclinical PID, which is more frequent than acute PID, may be caused by a *M. genitalium* infection. Women with either of the two pelvic infections have more risk of infertility due to the damage to the reproductive system.

This mycoplasma is not sure known if it can cause infertility, but its role in the PID was confirmed, consequently this could produce sterility. So, an indirect association was emerged. [18]

The infertility studies are complicated by the population variability, multifactorial aetiology and the methods used to determine infertility, which have a suboptimal sensibility and specificity. This is similar in the spontaneous miscarriages and premature births. The study results are not significant or there isn't clinical evidence in most of the cases due to the low prevalence of the mycoplasma in the population and the few studies conducted with pregnant women. [18]

If the MG infection remains during large periods of time, it may cause difficulties and produce sexually acquired reactive arthritis (SARA) or tubular factor infertility in some cases. In addition to PID. [9]

Generally, it has shown that MG infected women have so much increased proinflammation cytokine levels. The persistent infection caused by this mycoplasma is suggested to produce an impairment of female reproductive system due to the chronic inflammation, just like untreated chlamydia infections. However, vaginitis is not associated with *M. genitalium* although, it is a clinical appearance in other STI, like *Trichomonas vaginalis* and *Candida albicans.* This mycoplasma is not able to cause vaginitis, but it can worsen it in a situation of coinfection. [18]

The notified rectal or pharyngeal infections are asymptomatic in most cases. [9]

There are possible reproductive sequelae in women about which there aren't conclusions or significant association. The prevention and control systems are very important to keep the possible pathologies derived from a *M. genitalium* infection in among this gender. [18]

Table 1: Clinical characteristics, symptoms, and possible complications of *Mycoplasma genitalium* infection in both sexes.

Signs and Symptoms: Females	Signs and Symptoms: Males
Asymptomatic: 40%-75%	Symptomatic: 70%
Increased or altered vaginal discharge (<50%)	Urethritis (acute, persistent, or recurrent)
Dysuria or urgency (30%)	Dysuria
Occasionally intermenstrual bleeding or postcoital bleeding	Urethral discharge
Cervicitis	Proctitis
Lower abdominal pain (<20%)	Balanoposthitis
Complications: Females	**Complications: Males**
Pelvic inflammatory disease (endometritis, salpingitis)	Sexually acquired reactive arthritis
Tubal factor infertility	Epididymitis
Sexually acquired reactive arthritis	Rarely conjunctivitis in adults
Adverse pregnancy outcome	
Infertility (only indirect evidence)	

Adapted by [6] and [9]

DIAGNOSTIC ASSAYS

The mycoplasmas are characterized by an extremely low growth. This feature has considerably difficulted the screening and detection of this specie, for both clinical and research fields, *Mycoplasma genitalium* is not an exception, its growth in cellular cultures makes that 50 incubation days are necessary to identify the colonies, approximately. [14] Consequently, the traditional detection assays, through bacteriological cultures or biochemistry and serological techniques, are extremely laborious and they need an average of three months to achieve a confirmatory diagnosis. These methodologies make routine diagnosis of this pathogen unfeasible. [10]

The advances, which emerged with the appearance of the recombination techniques, have been possible the studies of many pathogenic bacteria though the molecular amplification. These new techniques include the polymerase chain reaction (PCR) or the transcriptional-mediated amplification tests (TMAs). Since the early 1990s, between the different advances it was possible to study and diagnose the *M. genitalium* pathogen in a routinary way. [14]

Currently, nucleic acids amplification tests (NAATs) are the only useful diagnoses assay for the diagnosis of a lot of infectious organisms, such as *Chlamydia trachomatis, N. gonorrhoeae, Trichomonas vaginalis* and *M. genitalium.* It is introduced in hospitals, research groups and more institutions for the routine identification assays. The improvement diagnoses have allowed a detection with a high sensibility and stability and with less response time compared to culture or immunological techniques, giving more reliable and speed results. [6, 9, 14]

Since the symptomatology of the pathogen could happen in other STI, it may give rise to clinical misunderstandings during the diagnosis. The use of multiplex diagnoses techniques, which are capable to detect more than one pathogen causing STI simultaneity, is totally recommendable.[6]

Although the diagnoses assays are carried out through NAATs worldwide, there aren't a commercially available standardized method, approved by the USA FDA or by other international organization. Consequently, it is extremely important that the laboratories perform quality controls and accurate evaluations of the particular techniques they are using and thus, the reliability of the results is guaranteed at all times. These type of control tests are generally known as a *laboratory-developed test* (LDT). The clinical laboratories, hospitals and academic institutions often develop these LDTs as testing services according to their own procedures. [9, 14]

The lack of a standardized method causes the development of a wide variety of different molecular methods in both clinical and research use, where are included conventional PCR, qPCR, Multiplex PCR, etc. [14] Between all the possibilities each one detects a different targeted sequence in the genome for the *M. genitalium* identification. Between the most used targeted regions highlight a fragment of the 16S rRNA gene or a region of the adhesin protein MgPar. [10] Each technique has their different individualized protocols and the sensibility and specificity, together with the recommendable type of sample, vary among the different procedures. [14]

The high variability between techniques led to the classification of the different techniques within the European conformity framework or CE (*conformité Européenne*). This is a

commercialization declaration which assures the compliance with the essential requisites of the relevant European protection legislation. This makes an assessment to meet high safety, strength, and environmental protection of the products. [14]

Moreover, according to the 2016 European guideline on *Mycoplasma genitalium* infections, the MG positive cases should be screened with an additional diagnostic test for detecting macrolide resistance-mediating mutations (MRMMs). This is strongly recommended due to the widespread increase in resistance to many antibiotics in Europe, as it is commenting in the following section. [9]

TREATMENTS

Although *Mycoplasma genitalium* is in most of the cases an asymptomatic pathogen, it has been shown an increased risk between 2 to 2,5 times more likely to suffer from certain diseases like cervicitis, PID and urethritis, among others. [18] Since it was possible to study in more detail, screenings have been conducted to see the susceptibility of the pathogen to various types of antibiotics. [21]

The peculiar characteristics of mycoplasma, like the absence of a cell wall and the fastidious growth in *M. genitalium*, causes that only a small group of antibiotics have activity against them. The research area has carried out multiple antimicrobial susceptibility studies to different types of antibiotics, evaluating the minimum inhibitory concentrations (MICs) in *M. genitalium.* It was isolated four classes of effective antibiotics: ketolides, macrolides, quinolones and tetracyclines with some slow MICs values, involving high sensibility of the pathogen. [9, 21]

In many STI treatment guidelines, the doxycycline and azithromycin 1g in a single dose are the most recommended therapies for the chlamydia treatment, as well as to treat urethritis and unknown aetiology cervicitis. [22]

According to the CDC (Centers of disease control and prevention) 2015 Sexually Transmitted Diseases Treatment Guidelines, the diagnostic changes should focus on cases of urethritis, cervicitis and PID, due to the high mutation rate. [21]

The CDC indicates that the doxycycline therapy in a dose of 100mg each day for 7 days or azithromycin 1g in a single dose is recommended in cases of cervicitis o NGU. The cure rate with azithromycin was fairly consistent, between 72-100% cured. However, doxycycline treatment demonstrated lower and more variable cure rates between patients. The differences between both therapies are considerably significant. [12, 21]

Table 2: Treatment procedure depending on the patient symptoms, indicating the treatment of choice and the recommended alternatives in concrete cases.

Symptoms	Of Choice	Alternative
Non-gonococcal Urethritis/ Cervicitis	Doxycycline 100mg/12h 7days (oral)	Azithromycin 1g in a single dose (oral) **If *M. genitalium* is isolated:** Azithromycin 500mg + 250mg/24h 4 days
Persistent or recurrent Urethritis	**If he firstly was treated with Doxycycline:** Azithromycin 500mg day one + 250mg/24h 4 days (oral) **If he firstly was treated with Azithromycin:** Moxifloxacin 400mg/12h 7-14 days (oral)	**If macrolide-resistant *M. genitalium* is isolated:** Moxifloxacin 400mg/12h 7-14 days (oral)

Adapted by [2]

On the other hand, the 2016 European Guideline on the management NGU [23] doesn't recommended the use of azithromycin 1g as first line of treatment for the NGU disease, due to the generation of resistance and because only the 87% of the macrolide susceptible NGU cases are cured. There is not considered effective enough compared with the against of resistances that would be produced. [12]

A lot of treatment guidelines don't consider *M. genitalium* in cases of PID, increasing the risk of severe disease of the female reproductive tract. [22] The doxycycline and azithromycin are often prescribed as common therapies. However, the first drug causes a high failure rate in the cure of *M. genitalium.* Moreover, the "short" treatments often produce a more probable reinfection in cases of PID. Consequently, organizations like CDC recommend longer duration therapies. [18]

Added to this problem, the CDC made a control randomized study, and they conclude that the therapies with these antibiotics were significantly decreasing their effectiveness over time. Both antibiotics have reported cure rates so much less in current studies compared with rates reported some years ago (2009). [21]

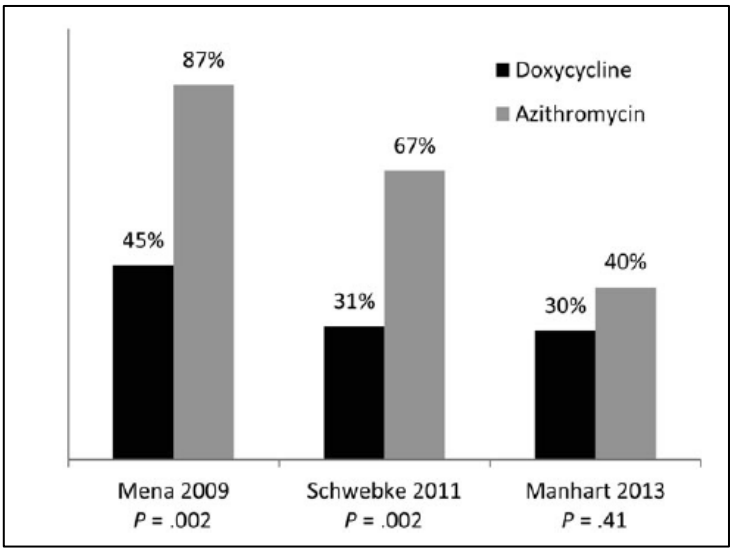


Figure 3: Representation of the efficacy in treating *Mycoplasma genitalium* infections by administering doxycycline (100mg/day 7 days) and azithromycin (1g in a single dose) over the years.

Obtained from [21]

The antimicrobial resistance development entails a difficult health therapeutical challenge. The geographical regions with azithromycin 1g in a single dose for the NGU disease have reported resistance percentages between 30-45% of the cases. In Spanish geographical zone, it is reported 16-35% resistance macrolide rates and 8% of the fluoroquinolones resistance, approximately. Among men the resistance rates are generally higher than women, being 22,2% and 4,6%, respectively. Furthermore, comparing the heterosexual population with MSM the rates are 17,6% vs 32,5%, respectively, being this last population group (MSM) in the spotlight. [6]

Another of the major agreement issues, on which there aren't a consensus, is the management of the asymptomatic people. Most of the MG cases are asymptomatic, as already mentioned before and what is more, spontaneous eradication of the infection is likely, not being necessary the treatment of these patients. Some occurred situations are from macrolide resistant strains. That is to say, the treatment would not have cured the infection in most cases, and it would have produced a severe infection that develops symptoms, significantly worsening the infection progression. [6]

The problem emerge due to the aim of the therapies is to avoid sexual transmission and to prevent the appearance of worst symptoms, besides curing the symptoms already present. Moreover, without treatment the *M. genitalium* infection could persist for up to more than a year. [22]

AZITHROMYCIN THERAPY

Azithromycin is an antibiotic of the macrolide family. Some years ago, the use of this drug was the first line treatment against a *M. genitalium* infection. [21, 22] The mechanism of action lies in inhibiting the bacterial proliferation by binding the drug to the ribosomal 50S subunit, this

subunit is formed by 5S and 30S. This binding allows the blocking of mRNA translation and consequently, protein synthesis is not produced. [14]

Although the evidence of the therapies with azithromycin 1g in a single dose which had the highest cure rates (85-95%) before 2005, diverse studies conducted to verify the effectiveness of the antibiotic epidemiologically and over time suggested a decline in the patient's recovery.[21, 22]

The different research suggested that the resistance is not presented in the pre-treatment mycoplasma and differences are not observed in the bacterial load of the patients who develop resistances compared to those who are cured. The selective pressure due to the drug exhibition are the responsible for the resistant strains created. As a result, it was concluded that the major cause of the decreased cure rate was the single dose of azithromycin regimen in itself. [21, 22]

MG azithromycin strains become resistant due to a single nucleotide polymorphism (SNPs) in the gene encoding the V region of 23S rRNA at the positions A2058 and A2059 (numbering of *E. coli*), which is the macrolide binding site. [11, 22, 24]

The mutations hinder the antibiotic binding, so the mRNA translation and protein synthesis go on. There are different possibilities being able to be transitions of adenine to guanine, cytosine, or thymine. Therefore, the resistance localizations are 5: A2058G, A2058C, A2058T, A2059G, A2059C, A2059T. [14]

At the beginning, the global prevalence of MRMMs strains was estimated between 4% to 38%. However, the cure rates have decreased from 84% in 2005-2007 to 69% between 2007-2009. In 2016, countries like UK and USA exceed the 50% of infections with resistant strains. [21, 24]

The biggest concern for the failure on the treatments led to proposing the treatment with azithromycin in an extended dose, instead of a single dose. This regimen consists in the administration of 500mg the first day and 250mg the four following days. Into the susceptible macrolide populations, differences on the healing times of the patients were not appreciated, but there was lower risk of resistance generation. [12, 21, 22]

However, in the presence of macrolide resistance, the extended dose gives a failure in the cures like the single dose regimen, although the patients who overcome the infection did so in less time with the extended regimen. [21, 22]

The problem is that the extended azithromycin dose also doesn't prevent the development of macrolide resistances. As a result, it is not a possible optimal treatment either. [22]

DOXYCYCLINE THERAPY

Doxycycline is an antibiotic of the tetracycline family. Its effectivity against a MG positive infection often is quite poor, having a cure rate between 30-40%. However, the development of resistance hasn't observed in any case and it is able to reduce the bacterial load. Therefore, it is considered more safety than other medicaments and it is fairly useful in patients with persistent infections and with severe multiple resistances complications, improving the symptomatology and reducing the risk of generation of resistance neither to macrolide nor to quinolone. [9, 12, 24]

According to International Union guideline against sexually transmitted diseases and to European Guideline on the management of NGU 2016, [23] it was recommended the use of doxycycline as first line treatment in cases of NGU. This is since between 35% to 45% of NGU treated with azithromycin have developed resistances. [9, 24] Furthermore, after doxycycline regimen as pre-treatment, it is believed that the full cure with extended azithromycin is more effective and with less mutation probabilities, supporting the infection solution. [12]

MOXIFLOXACIN THERAPY

Moxifloxacin is an antibiotic of the quinolone family. As a therapy against *Mycoplasma genitalium* is has been proven its effectivity in a 400mg daily for 7-10 days regimen, as indicated by various low MICs from different research studies. [22]

However, this drug is only recommended as an alternative to infections where other medicaments, like azithromycin or doxycycline, have not given good results. Even knowing its effectiveness, it isn't normally used as first line treatment because of its possible secondary effects. These effects are very severe and produce high liver toxicity, even be unusual. [22]

In cases of PID caused by *M. genitalium*, the regimen with moxifloxacin 400mg for 14 days is a good therapeutical option. [21]

The therapy with this drug didn't have virtually no failure of the cure rates of this STI between 2008-2012. However, more recent studies have reported cure rates ranging from 69% to 100%. Equal as azithromycin administration, the moxifloxacin administration started to produce cases with quinolone resistance associated with treatment failures. [21]

The selective resistances facing this drug are associated to mutations in the locations S83 and D87 (numbering of *M. genitalium*) of the *parC* gene in the quinolone resistance-determining region. Mutations disrupt the amino acidic sequence in the serine 83, where there is a substitution S83I, and in the aspartic acid 87, decreasing the binding of the IV topoisomerase enzyme. Mutations in *gyrA* has been shown too, but they seem to be less significant. [3, 11, 24]

The treatment failure is happening in just over 30% of the population, primarily in patients from the Asia-Pacific region where it is starting to give problems like the azithromycin ones. In Europe, this resistance is unusual. [9, 24]

OTHER ANTIMICROBIALS

The lack of fully effective treatments to cure *Mycoplasma genitalium* infections has carried out to alternative therapies research studies using other antibiotics and trying to improve the cure rates worldwide.

Josamycin is a drug of the macrolide family widely used in Russia, as an alternative to azithromycin. It has cure rates around 94%. However, it isn't capable to eradicate the generation of resistant strains, being also a macrolide. [9, 21]

Another effective macrolide is the pristinamycin. The use of this antimicrobial has activity against the pathogen in patients failing azithromycin, doxycycline, and moxifloxacin. Its use is

limited to France, but it isn't discarded its future use in other European countries. It is considered the last choice with therapeutical activity, being of great importance the compliance with the regimen in the doses. [9]

The increase in resistance strains caused by moxifloxacin made that Japan stated to use gatifloxacin and sitafloxacin as alternative treatments. They have cure rates ranging from 83 to 100% and from 79 to 100%, respectively. [21]

In Europe, the administration of metronidazole, normally used by *Trichomonas vaginalis* cases, is been recommended to treat persistent NGU with failure in the first treatment. [12]

TREATMENT PROTOCOL

After the analysis, the useful drugs to the eradication of *Mycoplasma genitalium* and having in account their effectivity degree, the adverse effects and the produced resistance rates, the therapeutical regimen assumed by the SEIMC (Spanish acronym *for Spanish Society of Infectious Diseases and Clinical Microbiology*) follows the recommendations of the European guidelines. [6] The 2016 European guideline on *Mycoplasma genitalium* infections have created a treatment protocol according to the most common features of the population. This recommendation should be followed in every infected patient for the optimal eradication of the infection. [9]

Table 3: Recommended treatments according to the European Guideline for the management of MG depending on the type of infection that the patients have and the presence/absence of resistance to macrolides.

<table>
<tr><th>Type of Infection</th><th>Macrolide resistance</th><th>First-line antibiotics</th><th>Second-line antibiotics</th><th>Third-line antibiotics</th></tr>
<tr><td rowspan="2">Uncomplicated Infection</td><td>No</td><td>Azithromycin or Josamycin</td><td rowspan="2">Moxifloxacin</td><td rowspan="2">Doxycycline or Pristinamycin</td></tr>
<tr><td>Yes</td><td>Moxifloxacin</td></tr>
<tr><td>Complicated Infection (pelvic inflammatory disease, epididymitis)</td><td colspan="4">Moxifloxacin 400mg once a day for 14 days</td></tr>
</table>

Adapted from [25]

Recommended therapy for uncomplicated MG infections and in the absence of MRMMs are extended azithromycin (500mg the first day followed by 250mg daily for 2-5 days) as first line treatment. In some cases, it would also administer josamycin 500mg three times daily for 10 days. This last one is especially used in Russia, but in Spain it is not usually a treatment option.[9]

For persistent infected cases, the second line treatment most recommended is moxifloxacin (400mg daily for 7-10 days). The continued use of azithromycin could produce a resistance, not being never a good choice. [9]

Facing a failure in both therapies, the available medicament options are considerably decreasing. It is recommended a regimen of doxycycline, 100mg twice daily for two weeks. The

cure rate is up to 30%, but it is the best option to avoid mutations. Other available option is the use of pristinamycin 1g four times daily for 10 days. Compliance with this treatment should be strict and the effectiveness research studies are not unanimous yet. [9]

Recommended therapy for uncomplicated and macrolide resistant MG infection are moxifloxacin (400mg daily for 7-10 days). This is the best option. It reports high cure rates, and it has been verified that it was very effective against cervicitis. The infections with serious complications, like PID or epididymitis, should also follow this therapeutical regimen. [9]

TEST OF CURE (TOC)

The test of cure (TOC) is a follow-up of the patient infection weeks after exposure to treatment.

This test was planned as a result of the knowledge of many infection's latency. The clinical experience pointed that many patients enter a stage of remission or asymptomatic after treatment, yet with persistent latent bacterial load which could lead to pathogen mutations and subsequent risk of more aggressive new outbreak. [9]

Mycoplasma genitalium has a very speed and high rate of mutations, so it is a good possible applicant to develop the commented phenomenon. Therefore, many experts are recommending the TOC to avoid a severe reinfection with a high antibiotic resistance.

There is not information about the time between the treatment administration to the pathogen disappearance, as a result it is taken as a reference *C. trachomatis*, because of their similarities. *C. trachomatis* takes an average of three weeks to disappear. [22] There aren't a unanimous consensus between guidelines that differs between the two weeks or 4-6 weeks after start of treatment. The most of them recommended not to take the sample until three weeks after the start of treatment. [3]

If the sample is taken before these three weeks, the result may be worthless. Sometimes patients responding treatment would have MG undetectable within a week, but at the same time, tests may become temporarily false negatives in patients failing treatment. [9]

According to diverse STI guidelines, tests of cure should be obligatory in every MG positive case due to the increase prevalence of macrolide resistance. [3, 9] In contrast, British association for sexual health and HIV (BASHH) and CDC doesn't recommend in any case the follow-up with TOCs within the asymptomatic population. [9] The majority of the countries don't obligate the sanitation to do TOCs, in most of them it is only a recommendation due to the divergence between guidelines.

To sum up, the lack of standardized detection methods not only is harmful to infected patient detection and spreading, but also benefits treatment failure. Due to the alarming increase in antibiotic resistance in *Mycoplasma genitalium,* recommendations about the need of mutation testing, both macrolides and quinolones, are the best solution in this moment, according to several studies. [11, 14, 21, 24] This would be vital for more specific diagnosis and a better monitorisation of patients. [21] In cases of not being able to detect the type of strain, is highly recommended carry out the TOC between 3-6 weeks after the start of treatment, to control the resolution of symptoms and the infection in every case. [2, 22]

HYPOTHESIS AND OBJECTIVES

The *Mycoplasma genitalium* is experimenting an increasing importance within the STI. Between main reasons stand out: the increase in the incidence and prevalence in the population, clinical manifestations related with this pathogen that were previously unknown, increasing on the diagnostic demand and the improvement in the detection techniques and overall, the description of resistances, which it is currently considered a serious problem of public health. [6]

Over the last years, the resistances of *Mycoplasma genitalium* have been increased worldwide due to the use of azithromycin as first line treatment. [24] Nevertheless, the number of research studies about this pathogen are limited and they widely differ depending on both the geographical localization studied and the population tested. The research studies related to resistance to azithromycin are even more limited. [26] Therefore, the need of research studies of resistance in Spain, to know the more vulnerably population characteristics and the affection rates, may help to obtain evidence about the importance of following the treatment protocols facing the infection. Consequently, this fact could lead to an optimal therapy and could minimize to the maximum mutations associated with current therapies administered.

To prove this hypothesis, the aims of this study are to determine the prevalence of the resistances to macrolides in a general MG-positive population belonging to the Sanitary Area of Tarragona, to evidence the high relationship between the obtained mutations and the administered treatments and to analyse other particular features that can also be related with mutations.

MATERIALS AND METHODS

STUDY DESIGN AND SOURCES OF INFORMATION

This cross-sectional study includes a total of 69 samples collected from patients with suspicious of STI who were finally diagnosticated positive for *Mycoplsma genitalium,* attending by the Laboratory of Clinical Analysis of the Joan XXIII Universitary Hospital of the Tarragona Area, during the period between October 2020 to March 2021. The samples were collected from five different locations depending on the sexual life and the symptomatology of each patient: endocervical, vaginal, urethral, first void urine and rectal samples.

After the discard of wrong samples (lack of sample or wrong labelling that cause the generation of an analytical test, which doesn't proceed) and grouping together the samples from the same patient, the study counts with 66 people who are between 16 and 59 years, and their clinical information was included in a common database specially created for this study. The inclusion criteria directed the patients to come obligatory from a multiplex STI analysis and to have a *Mycoplasma genitalium*-positive diagnosis. Later, the principal characteristic from each patient, which are useful for the study, were further analysed. For this study, the variables taking in account were: age, gender, sexual orientation, presence of coinfections, STI in the last year, reason of medical consultation and treatment administered.

All necessary general information on samples and patients were established from the EYRA®-specific data exploration program (EYRA® software system), together with other integration programs of the public health network of Catalonia, like the general computer system of hospital management SAP® and the database of the primary care centres eCAP®.

STI DIAGNOSTIC PROCEDURE

The simultaneous qualitative DNA determination of *Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, Mycoplasma genitalium, Mycoplasma hominis, Ureaplasma urealyticum* and *Ureaplasma parvum,* as pathogens associated with STI, are routinary tested at the Clinical Analyses Laboratories of Joan XXIII Hospital.

After the receipt and homogenization of samples, the DNA extraction is preferentially obtained using the STARlet® (Seegene); an automatic nucleic acid extraction and purification equipment, or using QIAcube® (QIAGEN, Germany) on the samples without transport medium, following the instructions of the "QIAamp MinElute Virus Spin Kit" extraction kit.

Samples already extracted are amplified and detected by the Multiplex Real-Time PCR technique, using the Allplex™ STI Essential Assay® (Seegene) kit and the analyser CFX96 Touch™ Real Time PCR Detection System® (BIORAD, USA), approved by the CE for *in vitro* diagnosis (IVD). This kit contains fluorophore-labelled primers of the specific target sequences for each of the seven DNAs of the aforementioned micro-organisms, and it is able to quantify each target in a simple fluorescent channel, respectively. [27, 28]

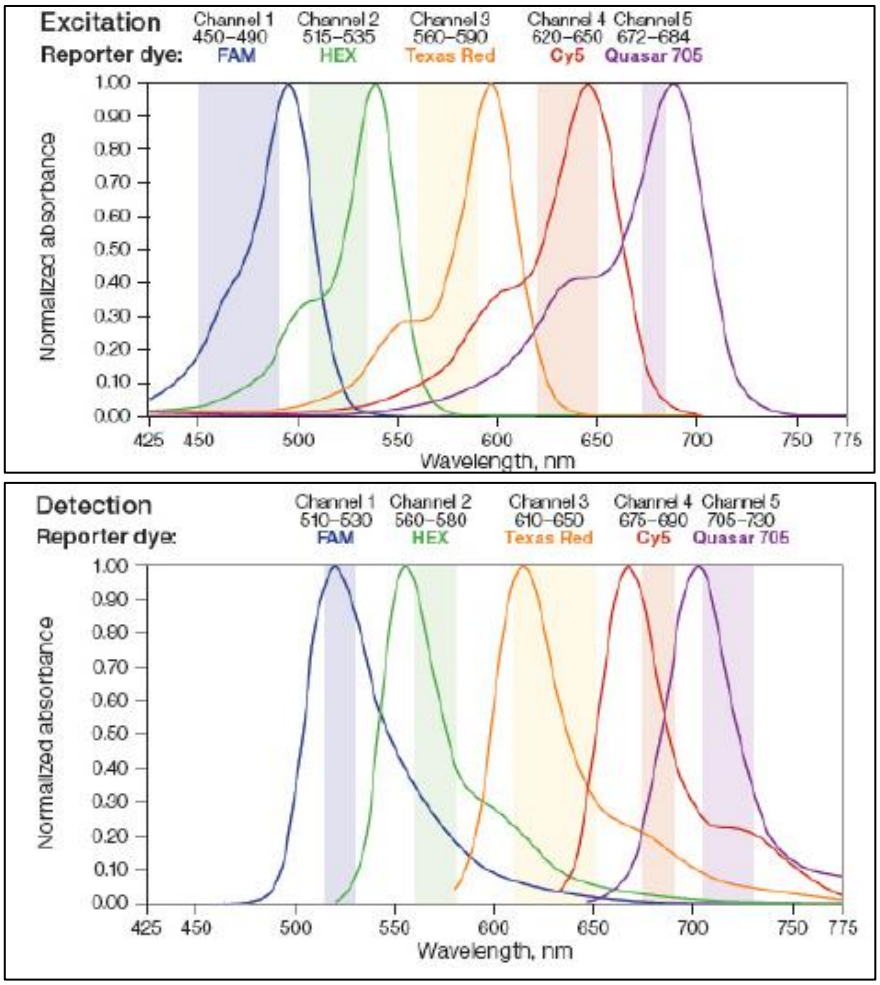


Figure 4: Representation of the excitation (the top imagen) and detection (the bottom imagen) spectra of the different florophores used in the Allplex™ STI Essential Assay® (Seegene) kit, according to wavelength.

An endogenous housekeeping gene of human cells, β-globin, is used as an internal control (IC) to supervise the process and to record any inhibition which could be occur in the PCR process. This IC is only added into the urine samples after DNA extraction. The other samples are genital discharges, so the endogenous β-globin of the own cells present in the sample are used as internal control. This gene is amplified together with target nucleic acids of the clinical sample. [29, 30] The PCR also contain a positive control, supplied by the manufacturer of the Allplex™ STI Essential Assay® (Seegene) kit, and a negative PCR water control, which both follow the same procedure as the problem samples to externally control the analyses. [29]

The multiplex PCR reaction is carried out in CFX96 Touch™, with a detection limit of 100copies/reaction, below which do not amplify. Previously, the PCR Mastermix was prepared in the flow hood, adding PCR buffer, the polymerase enzyme, PCR water and primers, nucleotides, and all mandatory thing for the operation of the PCR. When the mix was prepared, it was dispensed in a 96-well plate, adding in each of them 5µL of the DNA extraction and 15µL of the PCR mix specific for this determination. This technology allows the identification of multiple micro-organisms simultaneously. The signals may be identified in real time thanks to the receiver melting temperature. [27, 28, 29]

Table 4: Classification of the different micro-organisms that the Allplex™ STI Essential Assay® (Seegene) kit detects, according to the florophore attached to the specific primers for each micro-organism and the melting temperature at which it amplifies.
Ct: PCR cycles from which the pathogen is detected.

Reporter	Graph1 60ºC	Graph2 72ºC	Ct
FAM	*Ureaplasma urealyticum*	*Neisseria gonorrhoeae*	<40
HEX	*Mycoplasma hominis*	*Mycoplasma genitalium*	<41
CalRed 610	*Ureaplasma parvum*	*Chlamydia trachomatis*	<42
Quasar 670	*Trichomonas vaginalis*	Internal Control	<43

Obtained from [29]

The results obtained from this determination can be observed digitally. The program creates a graphic where the "*crude curves*" of the PCR cycles (Ct), generated for each sample, are shown.[29]

Lastly, a data analyse is carried out exporting the results of the "*crude curves*" obtained with the CFX96 Touch™. According to the manufacturer guidelines, the final results are shown and interpreted by the Seegene Viewer® software (Seegene). [27] The cut-off point for a positive test is Ct<40, except into ureoplasmas and *Mycoplasma hominis* in which the cut-off is 104 CFU/mL, equal to Ct<25 and Ct<21, respectively. [29]

AZITHROMYCIN RESISTANCE DIAGNOSTIC PROCEDURE

The extracts of the positive samples in *Mycoplasma genitalium* in the diagnostic test of STI, Allplex™ STI Essential Assay® PCR, were isolated from the rest for a subsequent determination of azithromycin resistance-mediating mutations. All the samples were stored in -80ºC the freezer, after the STI multiplex PCR until there was enough load to perform the resistances determination test.

The amplification and determination were performed by a multiplex Real-Time PCR assay using the Allplex™ MG & AziR Assay® (Seegene) kit. This kit is capable of qualitative establishing the presence of *Mycoplasma genitalium* and the mutations A2059G, A2059C, A2059T, A2058G, A2058C and A2058T in the 23S rRNA gene, for an *in vitro* diagnosis. These mutations are responsible of azithromycin antibiotic resistance. [30]

The basis of the technique is similar to STI determination assay. There are specific primers to each target sequence labelled with fluorophores that detect and sent out at different wavelengths, being able to quantify each target in a single fluorescent channel. The signals can also be distinguished by the receiver melting temperature. [30, 31]

Table 5: Classification of the different types of macrolide resistance mutations that the Allplex™ MG & AziR Assay® detects, according to the florophore attached to the specific primers from each type of mutation and the melting temperature at which it amplifies.
Ct: PCR cycles from which the mutation is detected.

Reporter	Graph1 60ºC	Graph2 72ºC	Ct
FAM	A2059T	A2058T	≤45
HEX	A2058C	A2058G	≤45
CalRed 610	A2059C	A2059G	≤45
Quasar 670	Internal Control (IC)	*Mycoplasma genitalium*	≤45

Obtained from [30]

This technique also has an internal β-globin control to supervise the sample collection process, nucleic acids extraction and possible PCR inhibitions. However, in our specific case it wasn't necessary, because all our extracts came from the STI determination where the internal control had already been added. On the other hand, the positive and negative controls are necessary. The positive one is a sample with all the antibiotic resistances whereas the negative is a PCR water (RNAse-free water). [30, 31]

First, the PCR Mastermix for this determination was prepared in a flow hood. For each sample analysed, it was added 5µL of the buffer (with BSA and glycerol), 5µL of polymerase enzyme and dNTPs buffer and 5µL of primers and reagents necessary for amplification and determination. The multiplex PCR takes place in the CFX96 Touch™ Real-Time PCR Detection System® (BIORAD, USA) analyser with a 96-well plate, where 15µL of PCR Mastermix and 5µL of nucleic acids from each sample are incorporated into each well. [30]

The obtained results appear in the digital program. In the program, you can see a graph where the "*crude curves*" of the cycles (Ct) performed are observed, together with the amplifications of each corresponding sample depending on the PCR cycle. [30]

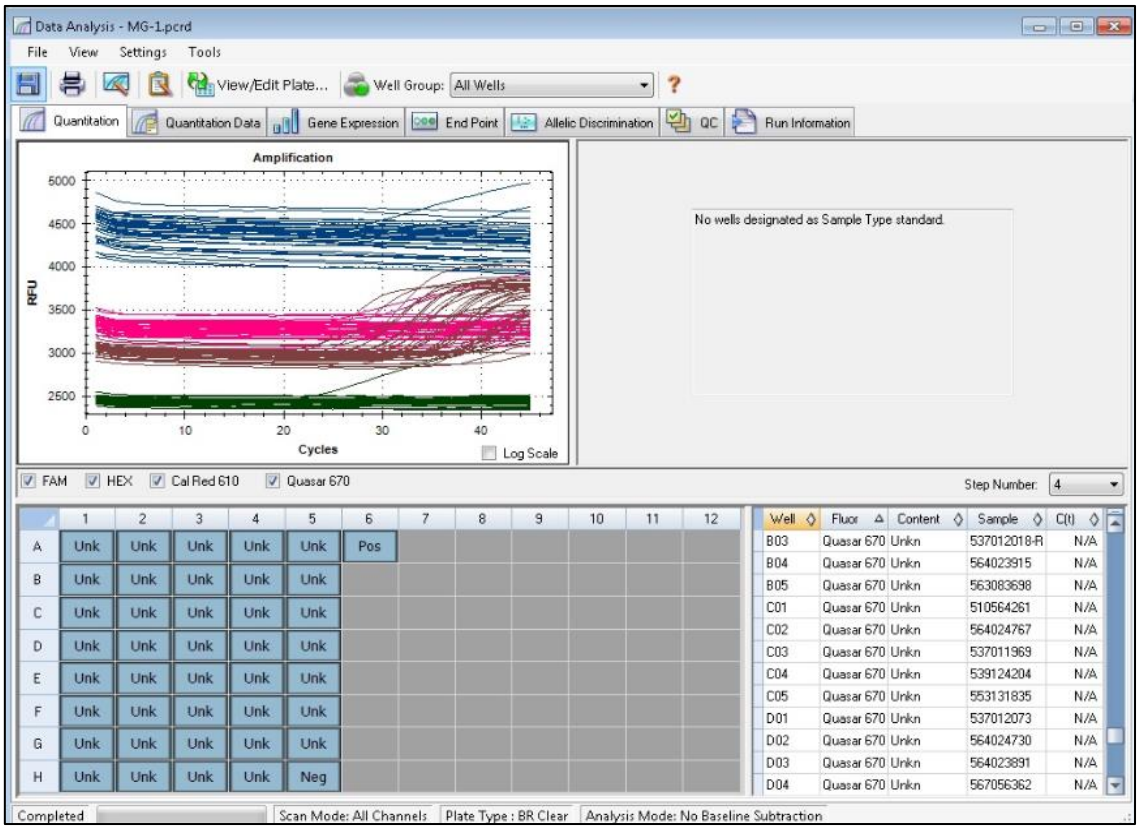
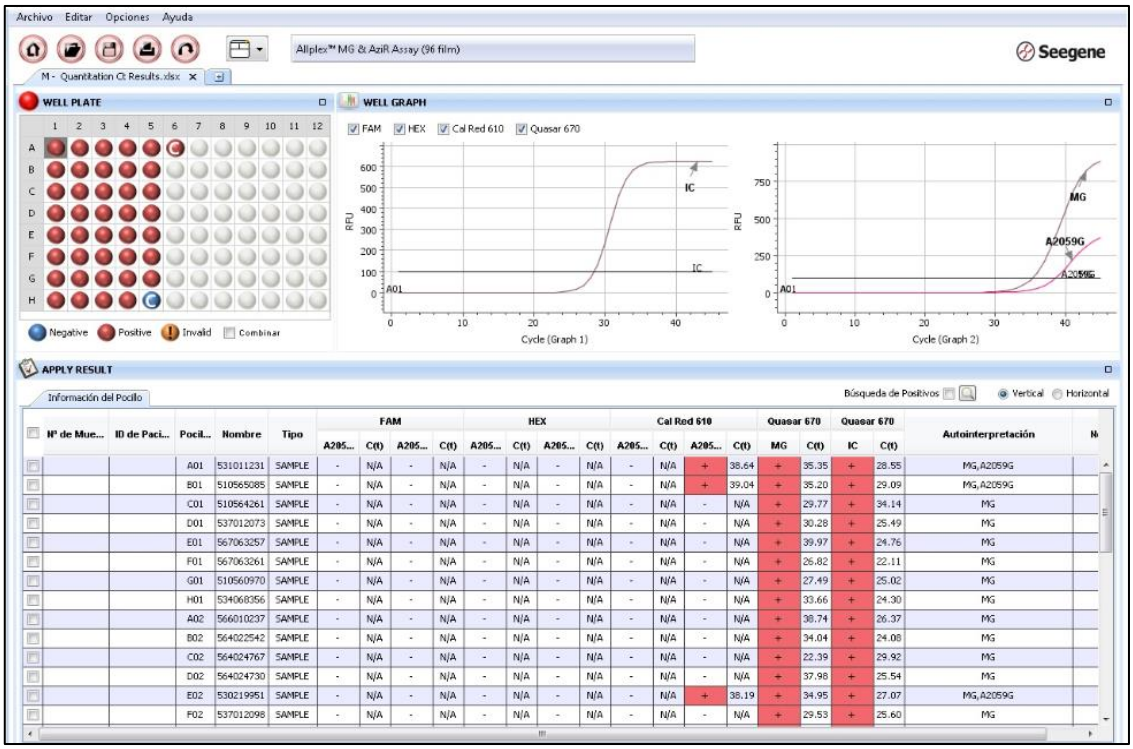


Figure 5: Representation of the results of the crude amplification curves obtained by the CFX96 Touch™ Real-Time PCR Detection System® analyser, after the PCR.

To analyse and interpret the data obtained, they are exported to the Seegene Viewer® software. This viewer shows you the amplification curves for each sample individually as you are clicking on the wells of the plate. Furthermore, every result appears simply at the bottom, indicating the interpretation of each sample. It points directly out which samples are MG positives and which mutation they present, if there are any. [30]



Nº de Mue...	ID de Paci...	Pocil...	Nombre	Tipo	FAM				HEX				Cal Red 610				Quasar 670		Quasar 670		Autointerpretación
					A205...	C(t)	A205...	C(t)	A205...	C(t)	A205...	C(t)	A205...	C(t)	A205...	C(t)	MG	C(t)	IC	C(t)	
		A01	531011231	SAMPLE	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	38.64	+	35.35	+	28.55	MG,A2059G
		B01	510565085	SAMPLE	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	39.04	+	35.20	+	29.09	MG,A2059G
		C01	510564261	SAMPLE	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	29.77	+	34.14	MG
		D01	537012073	SAMPLE	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	30.28	+	25.49	MG
		E01	567063257	SAMPLE	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	39.97	+	24.76	MG
		F01	567063261	SAMPLE	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	26.82	+	22.11	MG
		G01	510560970	SAMPLE	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	27.49	+	25.02	MG
		H01	534068056	SAMPLE	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	33.66	+	24.30	MG
		A02	566010237	SAMPLE	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	38.74	+	26.37	MG
		B02	564022542	SAMPLE	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	34.04	+	24.08	MG
		C02	564024767	SAMPLE	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	22.39	+	29.92	MG
		D02	564024730	SAMPLE	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	37.98	+	25.54	MG
		E02	530219951	SAMPLE	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	38.19	+	34.95	+	27.07	MG,A2059G
		F02	537012098	SAMPLE	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	-	N/A	+	29.53	+	25.60	MG

Figure 6: Representation of the results from the CFX96 Touch™ Real-Time PCR® test into the Seegene Viewer® programme.
In the upper right, the 96-well plate is represented, where the positive results appear in red and the negative results in blue. All our results are red because all are MG positives. The wells can be clicked and to their right appear the respective amplification curves of the clicked sample. Below, a table with the interpretation of each sample are shown.

The detection limit of Allplex™ MG & AziR Assay® kit is 10 CFU/mL for *Mycoplasma genitalium*, 100copies/reaction for A2059T and 50 copies/reaction in the other mutations, below these values the amplification reaction don't take place. The cut-off to consider the sample positive in this analyse is Ct ≤45 with a test sensibility of 100%. [31]

MAIN CLASSIFICATION OF THE POSITIVE Mycoplasma genitalium CASES

The positive *Mycoplasma genitalium* diagnosis is notified to clinics only in specific cases, following criteria established by the ICS. Therefore, the detection of azithromycin resistance mediating mutations should be done only in these situations. Most of the infections from this pathogen pass asymptomatically and its presence may not have pathogenic relevance, because it can be part of natural microbiota of some people. Hence, most of the cases are not informed so that a treatment, which may be not necessary, is not administered.

According to the Catalan Institute of Health (ICS) Report on the Screening, Diagnosis and Follow-up of Sexually Transmitted Infections published in 2018, [1] the cases that must be reported and resistances determined are:

- Pregnant women with endocervical or vaginal discharge sample.

- Contacts with *Mycoplasma genitalium* positive partner.
- Non-chlamydia nongonococcal urethritis with urine or urethral discharge samples.
- Epididymitis with urine or urethral discharge samples.
- Dyspareunia or postcoital bleeding with vaginal or endocervical discharge samples.
- Cervicitis with vaginal or endocervical discharge samples.
- Non-specific abdominal damages or pelvic inflammation disease with vaginal or endocervical discharge samples.
- Proctitis with rectal samples.

The patients have been classified in four groups depending on whether they had been reported the positive diagnosis or not and if they had been received some treatment. Moreover, inside these groups are analysed the presence/absence of resistances, the therapeutic guidelines administered and if these are appropriated according to the protocol previously explained in section "*Treatment protocol*". [1, 31]

In our specific case, resistances determination was made in all MG-positive samples between December 2020 and March 2021 for the correct conduct of the study, but routinely the performance of this analysis would not proceed in all cases.

RESULTS

This study focuses on analysing the prevalence of resistances to azithromycin in the Health Area of the Tarragona Region and the possible causes that favour a greater tendency to suffer these mutations. Moreover, the treatments administered to patients are monitored, due that the main guide to choose the optimal treatment for each patient is the presence/absence of mutations.

GENERAL CHARACTERISTICS OF THE STUDY POPULATION

Lastly, the study has a total of 66 people from both genders, 27 men (40,90%) and 39 women (59,10%), aged from 16 to 59 years. The average age of the studied population is 29 years (95% CI, 27-31). When grouping them by decades, it can be observed that the age range with the greatest involvement of *Mycoplasma genitalium* is between 20 and 29 years, where the 45,45% of the patients are found. The second most affected group, which also presents great differences with the others, is the decade of the 30s, where the 30,30% of the studied cases are located.

Table 6: Grouping of patients (n=66) depending on the age, the gender, the localization of the samples (n=69), the sexual orientation and the symptomatology.
ND: no data available.

		GENDER		
		Men (n=27)	**Women (n=39)**	**TOTAL (n=66)**
AGE (Decades)	**15-19**	1	6	7 (10,61%)
	20-29	10	20	30 (45,45%)
	30-39	12	8	20 (30,30%)
	40 or more	4	5	9 (13,64%)
SAMPLES (n=69)	**Urine**	16	0	16 (23,19%)
	Urethral	10	X	10 (14,50%)
	Vaginal	X	15	15 (21,74%)
	Endocervical	X	23	23 (33,33%)
	Rectal	2	2	4 (5,80%)
SEXUAL ORIENTATION	**MSW/WSM**	18	36	54 (81,81%)
	MSM/WSW	2	0	2 (3,03%)
	TWSM	X	1	1 (1,51%)
	ND	6	3	9 (13,63%)
SYMPTOMATOLOGY	**Asymptomatic**	16	20	36 (54,55%)
	Symptomatic	9	19	28 (42,42%)
	ND	2	0	2 (3,03%)

Of the total, 23 of the samples are endocervical (33,3%), 15 vaginal (21,7%), 10 urethral (14,5%), 16 first void urines (23,2%) and 4 rectal (5,8%). One sample are mislabelled, and its location is unknown.

In addition to taking in account these basic parameters about age and gender affected, a more exhaustive analysis about the characteristics of each patient was conducted to elucidate if the infection had diagnostic relevance and the degree of risk of the patient in a specific way.

In accordance with the sexual orientation, the 84,84% of the studied population are heterosexuals, 38 women who have sex with men (WSM) and 18 men who have sex with women (MSW). The homosexual population are consisted of only two men who have sex with men (MSM), representing the 3,03% of the cases. One of the patients are a transsexual woman who has sex with men (TWSM) and for the remaining 7 patients no data was found regarding this parameter.

Regarding the symptomatology, the 54,55% of the patients are asymptomatic, whereas the 42,42% present any symptom that could be related to a *M. genitalium* infection. If each gender is analysed separately, the 33,33% of the male population have some symptom, whereas in the female population the presence of symptoms occurs in 48,72%. The cause of consultation is unknown in two men, because they came from the penitentiary centre and there aren't access to this information, it is confidential.

The coinfection with other STI and the diagnosis of other STI episodes in the last year also take on a considerable relevance and they must be taken into account for MG diagnosis. The 27,27% of the patients have a coinfection of several pathogens and the 16,67% had suffered other case of STI in the last year.

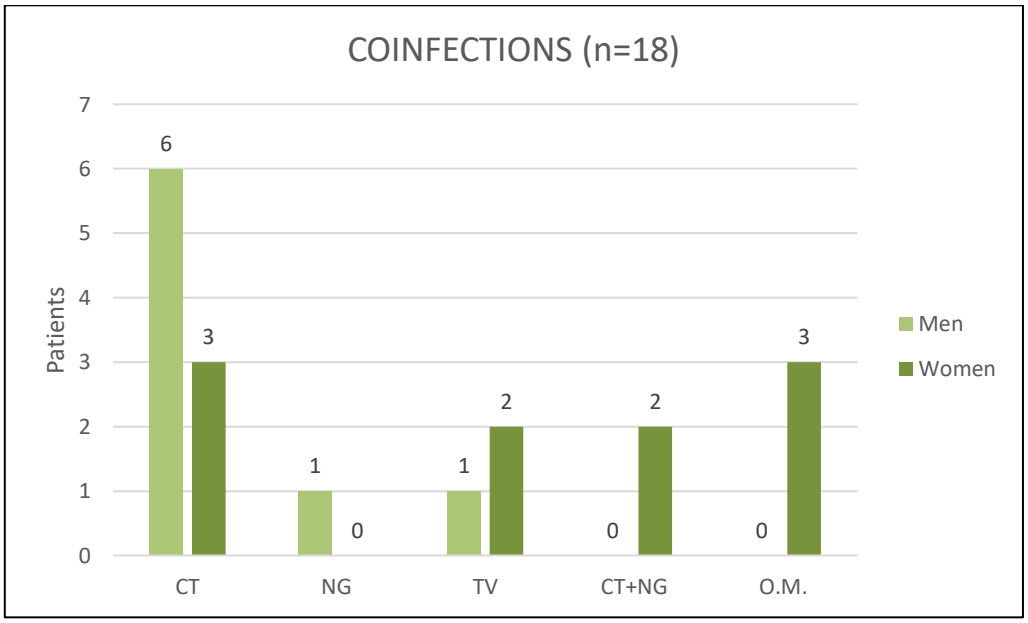


Figure 7: Representation of the cases of coinfection in both genders separately depending on the type of the isolated pathogen/s.
Abbreviations: CT: Chlamydia trachomatis; NG: Neisseria gonorrhoeae; TV: Trichomonas vaginalis; O.M.: Other micro-organisms. In this particular case, they are Candida albicans and Hemophilus parainfluenzae.

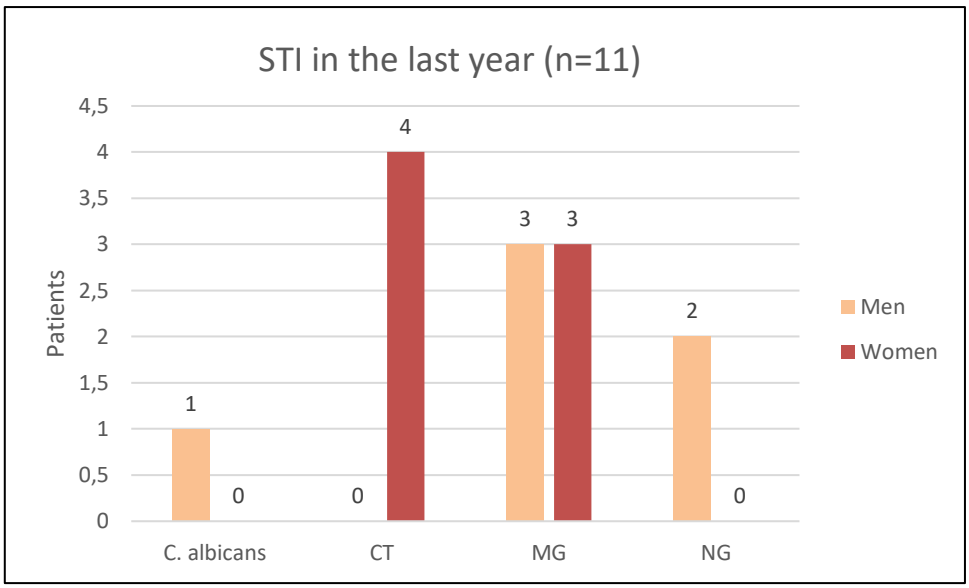


Figure 8: Representation of the cases with some STI diagnosis in the last year in both genders separately depending on the isolated pathogen/s. The total n indicates the number of patients who suffered a STI in the last year in general. While at the graph, it is indicated the number of patients who has had this specific pathogen in the last year. There are patients who has been infected by more than one pathogen, but not at the same time (there are not a coinfection).
Abbreviations: CT: Chlamydia trachomatis; NG: Neisseria gonorrhoeae; TV: Trichomonas vaginalis; MG: *Mycoplasma genitalium*

POSITIVE CASES OF MACROLIDE RESISTANCE MUTATIONS

After RT-PCR, ten samples with macrolide resistance mutations were detected (14,49% of the population, 95% CI, 6% to 22,98%). Three different mutation types were shown, where the most frequent was A2059G located in 8 samples, followed by A2058T and A2058G, each one found in one patient.

Six of the nine patients, who had found mutations, suffered some symptomatology related to the cases of mandatory clinical information mentioned in section "*Main classification of the positive Mycoplasma genitalium cases*", which corresponds to two thirds (2/3) of the cases of resistance.

Of the positive resistance cases, two of the samples, spaced apart in time, belonged to the same patient, who had a case of persistent *M. genitalium.* On the other hand, it was verified that other two patients with mutations came for a persistent infection. Of the three, two of them had been firstly treated with unsuitable azithromycin guidelines.

Of the remaining patients, two presented urethritis, one of them persistent. Other patient had recurrent MG infections and other was pregnant.

Moreover, one of the subjects despite suffering no apparent symptoms, he attended a consultation for HIV check-up, and he belongs to the MSM population group, both reasons

grouped him within the risk population. Only one of the nine individuals with resistances had an initial clinical case without apparent complications.

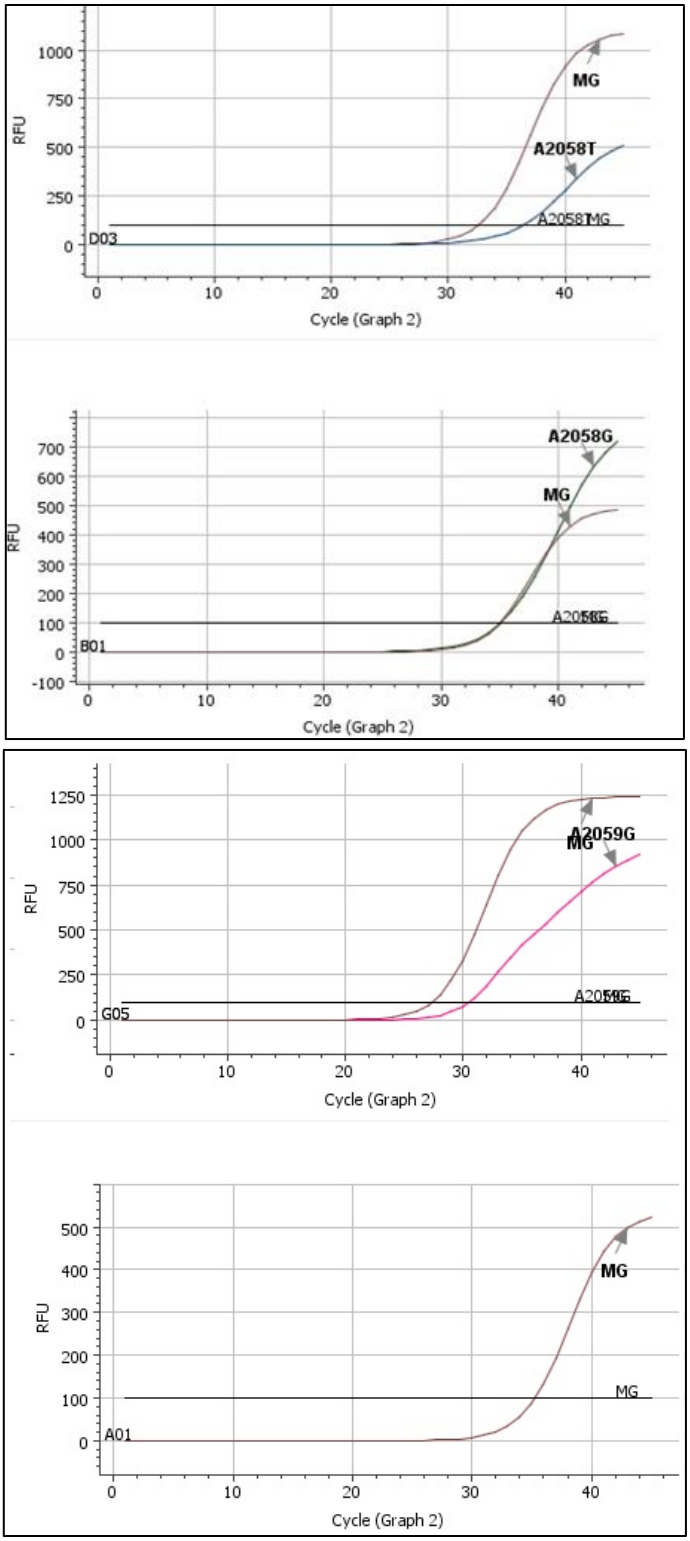


Figure 9: Representation of the PCR curves, acquired using the Seegene Viewer Software®, of the three macrolide resistance mutations obtained in the experiment together with a case of *M. genitalium* without mutation (the bottom graph). The graphs show the fluorescence intensity (axis Y) depending on the PCR cycle (axis X).

INFORMED PATIENTS AND ADMINISTERED TREATMENTS

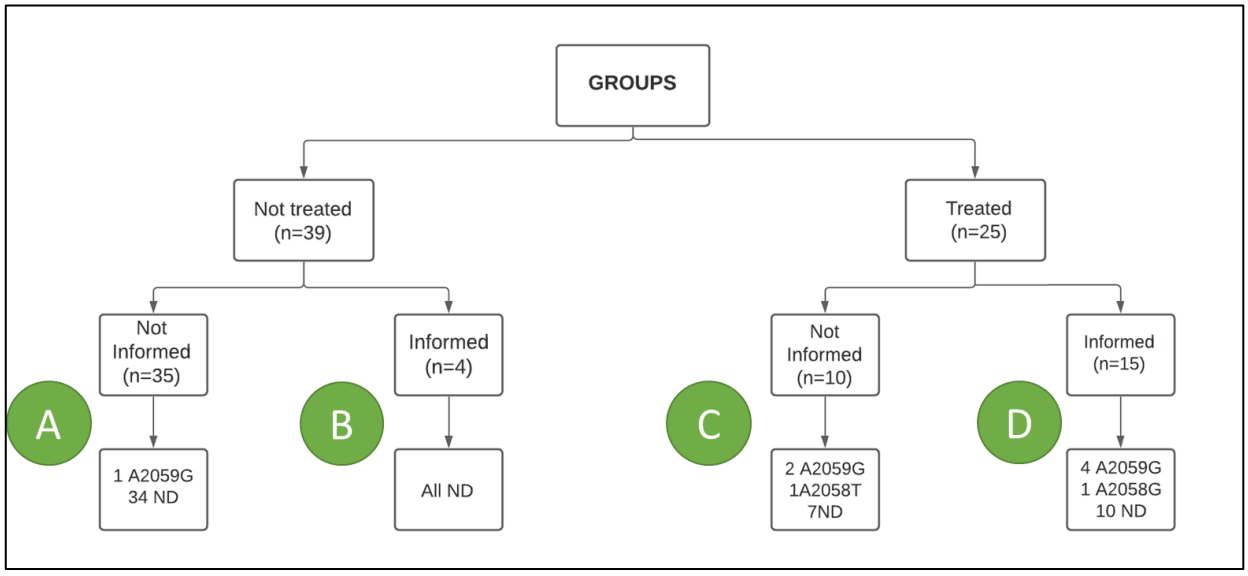


Figure 10: Classification of the patients in four different groups (A, B, C, and D) depending on the treatment administration and if the doctor was informed. In each group, it is indicated the number of cases of presence/absence of mutations.
Abbreviations: ND: no mutations detected.

In the study, only 19 of the 66 patients were related to some situation mentioned in the ICS 2018 report, section "*Main classification of the positive Mycoplasma genitalium cases"*. As a result, a 28,78% of the MG positive cases should have been informed to the clinicians.

However, 39,40% of the studied population had received treatment, that is to say, a total of 25 people. After reviewing each particular case, four of the patients had been treated before the diagnosis with empirical antibiotic treatment.

The patients were divided into four groups, depending on whether they were informed to clinicians and whether they received treatment:

Group A (n=35): Neither informed nor treated patients.

Group B (n=4): Informed but not treated patients.

Group C (n=10): Not informed but treated patients.

Group D (n=15): Informed and treated patients.

Regarding symptomatology, within the group A cases, most of them were asymptomatic and they came for STI screening in 37,14% of the cases or for contact with other STI in the 22,85%.

Any symptomatology was predominated within the group B, having one pregnant woman, one NG control study, one TV contact and one case of leucorrhoea.

Within the group C, most of the symptomatology came from other pathogens that cause STI, due that the 70% of the patients of this group (7/10) suffered a coinfection.

Finally, within the group D, the population with the most symptoms directly associated with *Mycopasma genitalium* was found. Of the 15 patients, five was pregnant women, three men had urethritis (20%), one of them persistent, four people came for contact with MG or persistent MG (26,7%) and three because of unspecified abdominal pain and dyspareunia.

Other very important factor to consider is the administrated therapeutic guidelines, which should follow the 2016 European Guideline explained at section "*Treatment protocol*". Mainly the therapies depend on: the symptoms of patients, because asymptomatic shouldn't be treated, the presence/absence of coinfections, because the other pathogens are preferably treated, and especially the presence/absence of mutations. As it can be seen in the diagram (Figure 10), five people of the nine who had mutations, were informed. However, eight patients with mutations received a therapy and only one of the cases was not treated. This last patient was asymptomatic and the only one belonging to the group A.

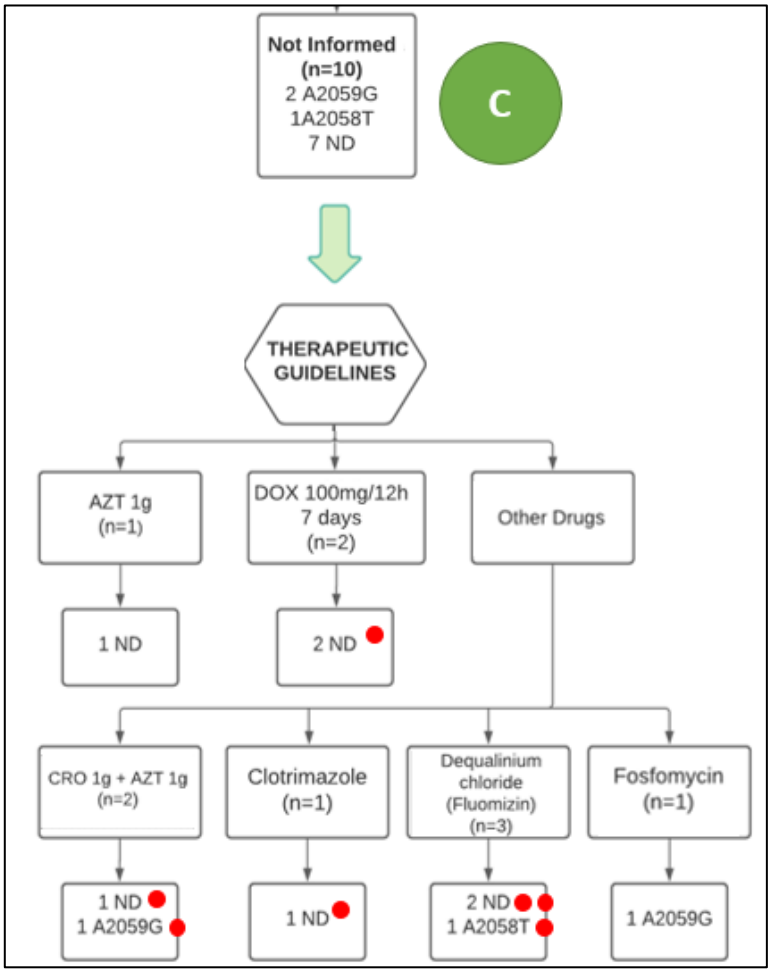


Figure 11: Classification of the treated but not informed patients (group C) depending on the therapeutic guidelines administered. In each regimen is indicated the number of cases of presence and absence of mutations and the type of mutation.
Abbreviations: ND: no mutation detected; AZT: azithromycin; DOX: doxycycline; CRO: ceftriaxone

- Presence of coinfection in this patient.

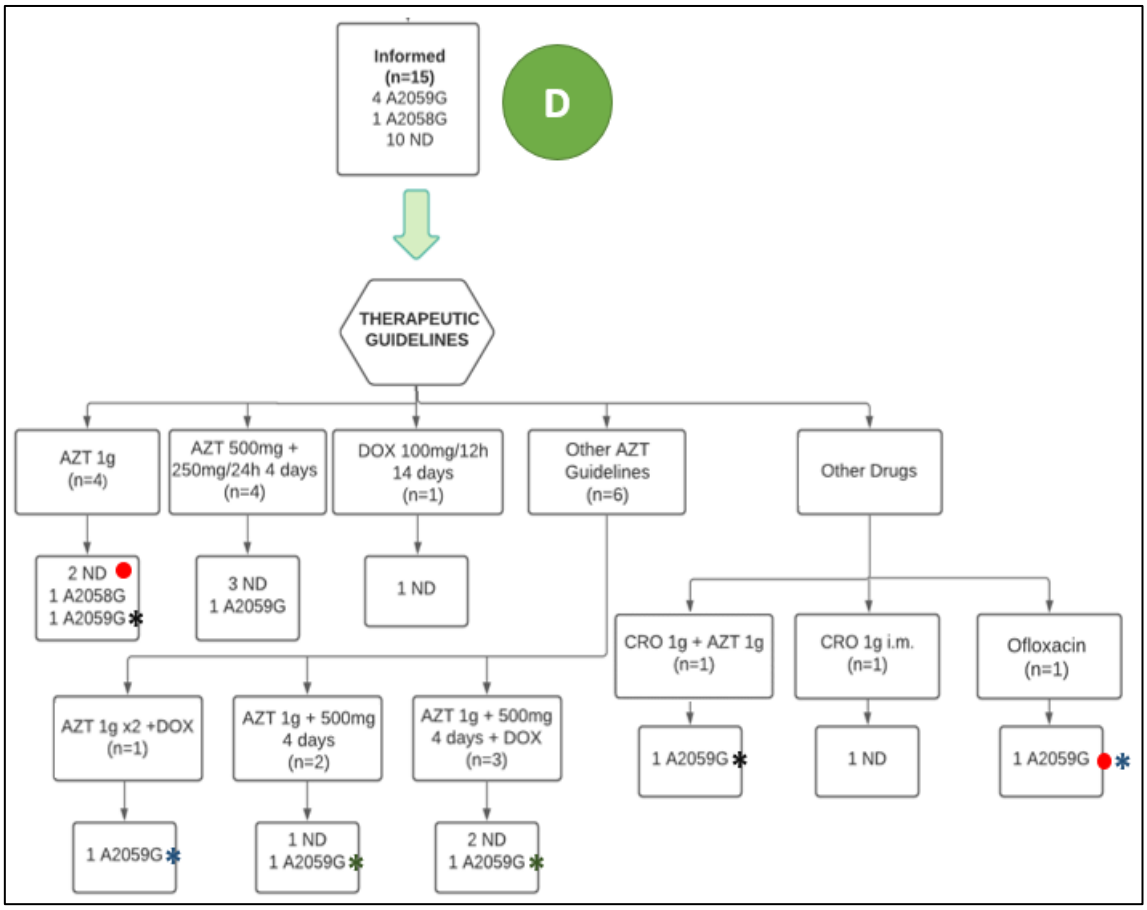


Figure 12: Classification of the treated and informed patients (group D) depending on the therapeutic guidelines administered. In each regimen is indicated the number of cases of presence and absence of mutations and the type of mutation.
Abbreviations: ND: no mutation detected; AZT: azithromycin; DOX: doxycycline; CRO: ceftriaxone
In patients who use DOX without specification, the regimen is 100mg/day 7 days.
*Same patient with two therapeutical guidelines for recurring medical consultation (persistent *M. genitalium*)
*Same patient with two therapeutical guidelines for recurring medical consultation (recurrent urethritis)
*Same patient with two therapeutical guidelines for recurring medical consultation (persistent *M. genitalium*)
- Presence of coinfection in this patient.

Six of the people with resistance and treated, all the group D and one of C, had received some azithromycin regimen, despite having a resistance to this drug. Within the group D, three patients were attended for not presenting improvements with the administered treatment. In one case a coinfection with *C. albicans* and *H. parainfluenzae* were detected and the treatment was changed to ofloxacin, which is a broad-spectrum antibiotic. In the other two cases a different treatment regimen was prescribed again, but also with azithromycin.

Of the three group C patients with mutation, two of them had a coinfection and therefore received treatment. Concretely, one of the cases with the A2059G gene presented *Neisseria gonorrhoeae* and consequently, he followed a therapy with ceftriaxone 1g i.m. together with 1g azithromycin, treatment recommended for this pathogen, and the patient with the A2058T gene

had *Gardnerella vaginalis,* so he followed a therapeutic regimen with fluomizin (dequalinium chloride), widely used to treat bacterial vaginosis caused by this pathogen. The patient without coinfection received a treatment with fosfomycin.

To sum up, within the patients with mutation only in the two cases of group C coinfections and in the group A patient without treatment, the European guideline for the therapy administration had been optimally followed. Although the treatment for NG improves the MG resistance.

Among the people who didn't have mutations and belonged to groups C and D, a total of 17 patients were treated for diverse causes. Various conclusions had been obtained after a specific analysis of each clinical case and the therapeutic regimens administered.

The therapeutic regimens administered had followed the guides in 8 cases, whereas in 9 of them the therapies had not been appropriated. For the analysis of the therapies in each patient, the presence of coinfections and if they had persistent or recurrent MG infections had been taken in account.

Within the 8 patients with well-managed therapeutic guidelines, three of them was treated with extended dose of azithromycin (500mg first day and 250mg/day the following 4 days). In two cases, the doxycycline regimen (100mg/12h for 7 or 14 days) was prescribed. The first one was from group D, with a clinical case of recurrent urethritis, so the second line treatment was administered. The other case belonged to group C and he would be treated with azithromycin because the patient had no history for this pathogen, but doxycycline also is a suitable drug for treating this infection, so therapy would be effective.

The three remaining cases had a coinfections and therapeutical regimens were prescribed for the other pathogens rather than treating *M. genitalium.* The CT coinfection was treated with the doxycycline regimen. In the case of NG coinfection, ceftriaxone 1g together with azithromycin 1g was administered and the case with *Candida albicans* received clotrimazole. These indications had been properly following the 2020 Antimicrobial Therapy Guideline. [32]

In the case of patients with the wrong medication, three was treated with azithromycin 1g in a single dose and of the remining six, three of them followed other azithromycin regimens in doses much higher than those recommended (a total of 3g in 5 days) and together with doxycycline in two of the three patients. The drugs administered to the other three patients, ceftriaxone to the patient in group D and fluomizin to both patients in group C, are not even part of the battery of drugs appropriate to treat *Mycoplasma genitalium.* One of them had a coinfection with TV and another with CT, but the antibiotic fluomizin is not indicated as a possible treatment against these pathogens. [32]

Consequently, after analysing each case specifically, it can be concluded that within the 25 treated patients only in 10 cases (40%) the treatment had been administered according to 2016 European Guideline and 2020 Antimicrobial Therapeutic Guideline. [9, 32] The 37,5% (3/8) of the patients with mutation and the 47,05% (8/17) of the patients without mutation.

DISCUSSION

THE PREVALENCE OF Mycoplasma genitalium INFECTION

The prevalence of *M. genitalium* have been experiencing a growing impact on the world population throughout the years. [33] Sufficient evidence has been seen among the last decade to place this pathogen as an important cause of infections related to the urogenital tract of men and women. [34] The symptomatology produced by this pathogen is not as frequent as in the case of other STI with which it shares clinical similitudes, like *Chlamydia trachomatis* or *Neisseria gonorrhoeae.* However, the steady increase of cases due to this pathogen place it currently among the STI with a high prevalence. Currently, there are a constantly change related to the management and control, being in the spotlight of the health services because it is undertested, underdiagnosed and each time there are more treatment difficulties. [35]

In this study (Table 6), no differences have been seen in the prevalence of MG between men and women, being 40,9% and 59,1%, respectively. The fact that there is a slightly higher of women would be related to a greater general habit among the female gender to go to periodic medical check-ups more frequently than men.

However, differences have been seen in the prevalence according to the ages (Table 6). The young population under 30 years represents the 56,1% of the cases and this value increase until 78,8%, if patients until 35 years are taken into account. As age increases, the prevalence of infection gets to decreasing in both genders more or less equally. The average age of the studied population is 29 years (95% CI, 27-31). In the case of male population, the average age is 31 years (95% CI, 28-34), whereas in the female population, it is slightly less, 28 years (95% CI, 23-31).

Regarding the symptomatology (Table 6), the 54,55% (95% CI, 42,54% to 66,56%) of the studied population is asymptomatic. Within the female population, the 51,3% (95% CI, 35,61-66,99%) are asymptomatic, agreeing with the European guide values, where it is indicated that 40-75% of the MG positive women are asymptomatic. However, the male population without symptoms rises until 59,26% (95% CI, 40,73-77,79%), being a value much higher than expected, because the European guide indicates that around the 70% of the male cases are symptomatic and in our studied population group only the 33,33% have symptoms, less than half expected.

The coinfections with other pathogens are located in 27,27% of the cases (Figure 7). As in the male population as in the female one, the most frequent cases of coinfection were with *C. trachomatis.* Among the female population, the coinfections presented a higher pathogen variability, being also relevant the coinfection with *T. vaginalis* or the multiplex coinfection with *C. trachomatis* and *N. gonorrhoeae.*

In the general population, the prevalence of *Mycoplasma genitalium* is estimated between 1,1% and 3,3%, being able to have a higher prevalence in symptomatic patients or risk population, like positive HIV people or sexual workers, ranging from 9% to >50% in some cases with a high risk. [34, 36, 37] The number of cases is highly variable depending on the geographical region and the population studied. In Spain, the prevalence of *M. genitalium* is positioned around 3,34% [26] with affectation similar to other countries, like Netherlands (4,3%), [38] France (4%) or Singapore

(4,3%). [39] However, in other countries like China, Switzerland, USA or UK, the high number of cases each time is increasingly worrying with prevalence of 7,4%, [36] 12,95%, [40] 16,6% [34] and 16,7%, [41] respectively.

MACROLIDE RESISTANCE MUTATIONS

This increase in the general prevalence is due to in part to the increase in the macrolide resistance mutations. The resistance to macrolides by a mutation in *Mycoplasma genitalium* was first reported by Bradshaw et al. in 2006. [42] Since then, studies have been carried out in different countries, showing very varied results when compared between them. However, in all the cases, an increasing in the prevalence of those mutations are seen worldwide over time. [24, 26, 34, 36-41, 43-50]

The differences in the mutation rates may also be due to the preferred treatments used depending on countries. The azithromycin in a single dose is the preferred treatment against nongonococcal urethritis in many countries like UK, Netherlands, USA, Australia, or Denmark. [37, 38] In some countries the administration of azithromycin against other infections like *C. trachomatis* is quite common, without excluding the coinfections with *M. genitalium,* while in others, other drugs are preferred. [36, 40, 45] Among the coinfections, the administration of azithromycin to treat one pathogen may indirectly benefit the appearance of resistant strains of *M. genitalium.*

The resistance rate obtained in the current study (14,5%) is lower than the theoretically expected in accordance with other research studies in Spain, where the results showed a higher prevalence of resistances, 20% in 2015 [26] and 36,4% between 2018 and 2019. [24] The disparity between the results may be influenced by the geographical areas where the studies are conducted. The two previous Spanish studies are carried out in Madrid [26] and southern Spain [24] regions, while ours belongs to patients from Tarragona region (northeaster of Spain) with more similar results with other nearby countries with a high interaction such as France with a resistance rate of 14,2% [43] or even Switzerland with a 13%. [40]

The cases of resistance obtained do not seem to present differences in relation to gender, being the ratio 1:1,25 (men:women). However, a higher number of resistances have been seen among the young population under 30 years, where the 77,8% of the cases were found. Only two patients with MRMMs were older than 30 years, with 31 and 44 years.

Among the patients with resistances, it is worth highlighting the data obtained on past STI in the last year. Within the six patients with a clinical case of MG in the past, four of them (66,7%) had a MRMM and two of these had also suffered a CT and NG infections, respectively. Other studies also suggest that the cases of macrolide resistance are more likely among patients who have a previous diagnosis or medical history of bacterial STI. [35] According to the data obtained, recurrence of MG infection makes the presence of a resistant strains more likely in these patients.

Table 7: Representation of the macrolide resistance rates (%) registrate at different countries. It is indicated the type of population on which the study has carried out and the types of resistances (%) reported.
General population and Unselected patients: they correspond to patients both symptomatic and asymptomatic.
NA: the types aren't analysed
*Patients with symptoms of urethritis, cervicitis, pelvic inflammation disease or proctitis.
[1]They don't specify the number of specific cases of each type of mutation
[2]The article from the resistance types is obtained is not the same one that provides the rest of the data. Netherlands [44] and USA [47].

COUNTRY	POPULATION	RESISTANCE ºRATE (%)	TYPES OF RESISTANCES (%)
Current Study	MG- positive unselected patients	14,49	A2059G (80%) A2058G (10%) A2058T (10%)
Spain [26]	General population	20	A2059G (50%) A2058T (50%)
Spain [24]	MG- positive unselected patients	36,4	A2059G (50%) A2058G (32%) A2058T (3,5%) A2058C (11%) A2059G+A2058G (3,5%)
France [43]	MG- positive unselected patients	14,2	A2059G (60%) A2058G (40%)
Switzerland[40]	General population	13	A2059G (65%) A2058G (29%) A2058C (3%) A2058T+A2058G (3%)
UK [41]	NGU males	41	A2059G (33%) A2058G (56%) A2059C (11%)
Netherlands[38]	General population	34	A2059G (33%) A2058G (38%) A2058T (29%) [44]
Denmark [37]	General population	38,2	A2059G (35%) A2058G (61%) A2058T (4%)
Russia/Estonia[45]	MG- positive unselected patients	5,3	A2059G (64%) A2058G (31%) A2058T (2,5%) A2058C (2,5%)
Europe [46]	General population	>50	NA
USA [34]	General population	48,31	A2059G (62,5%) A2058G (37,5%) [47]
Australia [48]	General population	63,1	A2059G (32%) A2058G (56%) A2058T (10%) A2058C (2%)
Australia [49]	Patients with genital symptoms *	62	NA
China [36]	Female unselected patients	42,1	All A2059G or A2058G [1]
Japan [50]	Male unselected patients	42,2	All A2059G or A2058G [1]
Singapore [39]	General population	25	A2059G (25%) A2058G (25%) A2058T (50%)

The prevalence of resistance is a reason of great concern in most countries, where the increase in these mutations is happening especially quickly, generating problems worldwide. [43, 50]

Consequently, the STI treatment guideline from the United States Centers for Disease Control and Prevention added it to the 2015 list of emerging issues. [47, 49]

Some countries such as Japan went from a prevalence around 5% before 2009 to one of 42% between 2010-2012, [50] or Netherlands where the cases of resistance doubled between 2014 and 2017. [38] Other countries such as USA, China, UK, or Australia have more than 40% of the population with resistances [34, 36, 41, 48] and one study developed in 2020 position Europe with a resistance rate over the 50%. [46] Spain is not between the countries with the highest affectation rates, which does not exclude that no measures should be taken in this regard. The frequency of mutations each time is increasing in the country and treatments will get decreasing their effectiveness if it isn't given the necessary importance and isn't taken appropriate preventive measures.

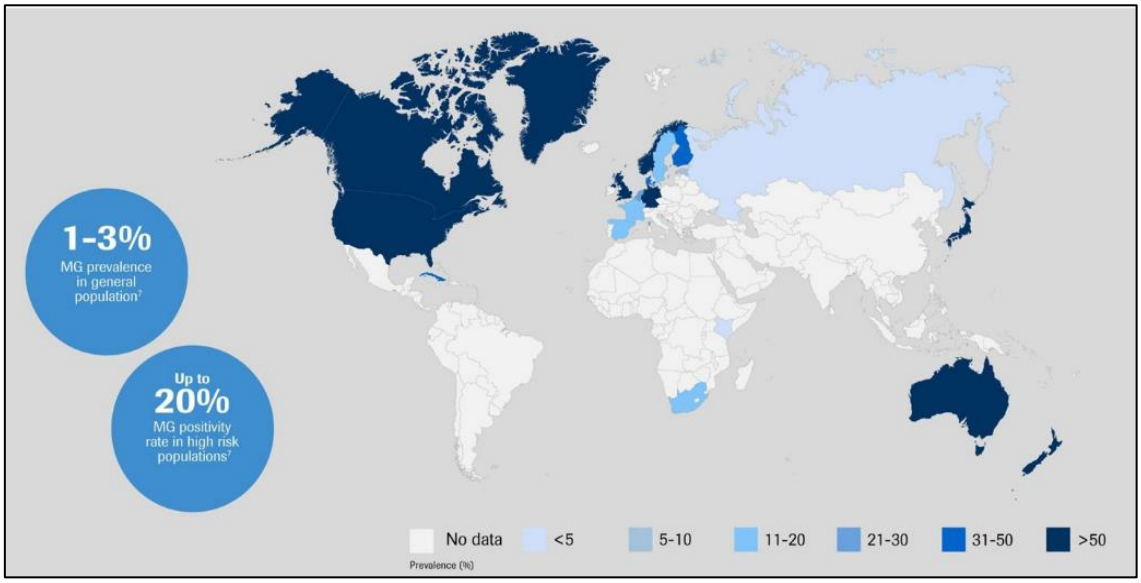


Figure 13: Representation of the prevalence (%) of macrolide resistance mutations around the world. *Obtained from Roche® Website that is based on data from [51].*

Mutations in the 23S rRNA gene that lead to macrolide resistances are associated with different SNPs of the A2059 and A2058 regions. This gives rise to 6 types of different mutations (A2058G, A2058C, A2058T, A2059G, A2059C and A2059T) of which the most common, practically worldwide, are A2059G and A2058G (Table 7).

In our case, the majority corresponds to A2059G SNP with a prevalence of 80%, followed by A2058G and A2058T, both with the same prevalence (10% each one). This predominance of A2059G gene was previously shown in Spain [24] and other neighbouring countries like France [43] or even Switzerland, [40] with whom it shares a reasonable interaction. However, in other geographical areas is more prevailing the A2058G gene as it could be observed in some norther European countries (Denmark, Netherlands, UK) [37, 38, 41] or Australia. [48] Only in one study carried out in Singapore was A2058T observed as the majority SNP with a prevalence of 50%. [39] It has been shown that despite having several types of SNPs generating resistances, there are no differences in the cure rates with azithromycin according to the type of mutation. [37]

The increase in macrolide resistances is believed to be due in large part to the suboptimal treatment with high doses of azithromycin for many years. [37, 43] Single dose 1g azithromycin was until recently, the first line treatment against a MG infection or nongonococcal urethritis in men and it still is the treatment against other pathogens such as NG (as part of the dual therapy) or CT in many cases. [36, 38] In the last decade, treatment failures with azithromycin to treat MG have seen increased with fast frequency. The cure rates in 2008 were over the 80%, while currently they have decreased up to 54% or less. In turn, this has improved the horizontal transmission of these bacterial resistant chains. [38, 41, 45]

All of this has promoted changes about the *M. genitalium* monitoring and treatment on many guidelines that currently, advise against this regimen. They point out that the first line treatment should be an extended azithromycin dose in susceptible strains and the moxifloxacin dose in macrolide resistant strains. [49]

Throughout this study, it could be seen that neither in cases of patients with mutations nor in the susceptible ones, the guidelines about treatments are strictly followed in most patients. Eleven patients were treated with single dose 1g azithromycin or with other azithromycin regimens in even higher doses, whereas the extended azithromycin regimens were only administered in four patients and one of them had a mutation and this drug should not have been given.

The cases with mutations should have followed a moxifloxacin therapy as first line treatment. Nevertheless, none of the studied patients received this treatment at any time, not even the three patients who recurrently came for consultation because they hadn't showed improvements. Furthermore, among the nine patients with mutations, the 67% received azithromycin as treatment at some time, being able to considerably worsen the symptoms of the infection and prolong it for longer.

Moreover, four treated patients received empirical antibiotic treatment before isolating the micro-organism who causes the infection. This practise is carried out with considerable frequency in the health field to speed up the process in cases where there are high indications to suspect a specific pathogen. However, this may promote the increase in resistances to some antibiotics, because of a suboptimal treatment, [36] and the infection persistence if the expected micro-organism is not finally diagnosed.

In general, within the 25 patients who received treatment, the 60% of cases had been prescribed with a regimen or drug that is not adapted to their needs (Figures 11 and 12), according to the indications of the guidelines above-mentioned.

This study is carried out before permanently introducing the technique of detection of resistance to macrolide by PCR in the hospital, therefore, the doctors were not informed about the mutation results.

This procedure is made in this way to demonstrate the usefulness and need of this determination to achieve a better clinical response, to avoid a persistence of the symptoms for longer because of an unfinished diagnosis and to guide the treatment in an optimal way with

more targeted therapies and more appropriate regimens in accordance with the clinical case of susceptibility of each patient.

The implementation of guided treatments based on the results of macrolide resistance test shows in this study, and have shown recently in others, [49] to have a considerable potential for improving the MG infection cure with the first line treatment.

The test of cure starting 3-4 weeks after treatment is also especially recommended to ensure that the infection has disappeared, according to several international guides. [36] Some studies, where these TOCs were carried out in the studied population, have been able to verify its great utility in detecting cases in which the antibiotic pressure has developed resistance to macrolides in strains primary sensitive. [40, 49] In our case, all the patients informed about the infection are suggested to treat only in symptomatic cases and if they are treated, a TOC is recommended to them, starting 4 weeks after the treatment.

In the cases of patients with persistent symptomatic infection, this helps redirect towards other therapy which may give better results. Moreover, the use of TOCs could favour a slight decrease in the number of positive cases, proving again its usefulness. [38]

STUDY LIMITATIONS

The results obtained in this study are limited to a very specific geographical area, where patients from only three clinical centres take part (San Joan University Hospital of Reus, Valls Hospital and Joan XIII University Hospital of Tarragona) and the collected samples belongs to a relatively short period of time. Consequently, the number of patients included in the study is relatively low (*n* low) for an epidemiological study. One larger-scale study would generate more accurate and reliable results.

Furthermore, being a random study where the samples are collected from a specific period of time, none type of population nor none of the infection-related characteristics analysed could be selected with the intention of having more conclusive data. This has led to have a very low number of the MSM population group (3,03%) and no relationship or conclusion can be established about the *M. genitalium* infection in these specific population group. It would have been interesting to have more patients of this group and to be able to analyse some issue such as the prevalence of mutations, symptomatology or the infection development in this risk group compared to others.

In addition, we have had few rectal samples (5,8%) of which would have been useful to analyse a bigger number and to be able to have significant data about infection on this location. Other studies suggest that the prevalence of *Mycoplasma genitalium* varies with the anatomical area where the infection occurs [47] and that the rectal location is the most likely to present mutated strains. [49]

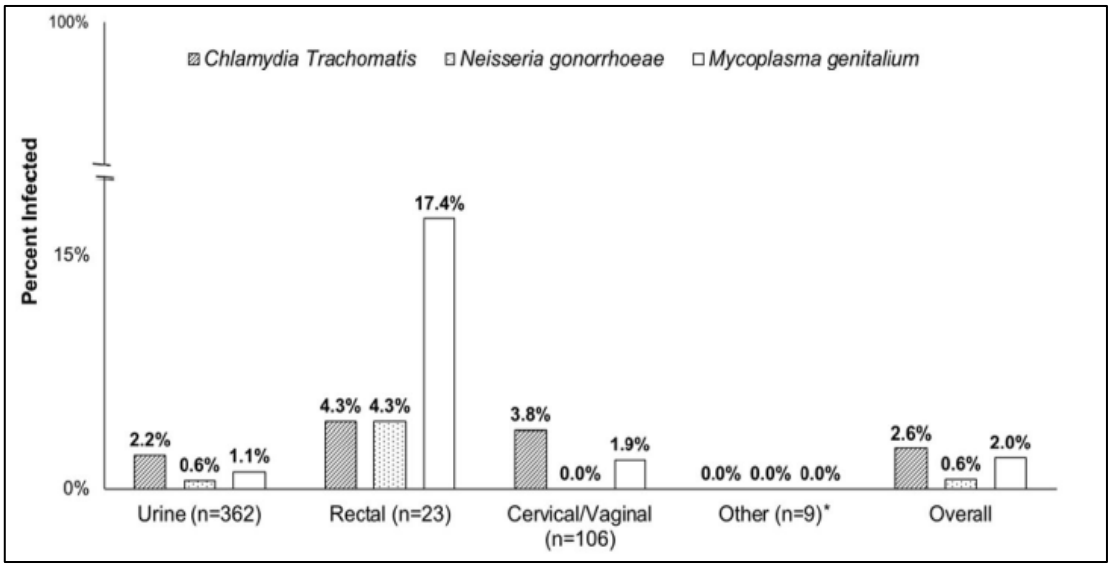


Figure 14: Prevalence of the *C. trachomatis*, *N. gonorrhoeae* y *M. genitalium* infection in specimens from men and women classified according to the anatomical site of the sample.
The positive-MG samples with resistance to azithromycin represented the 75% of the rectal samples, 75% of the urines and the 100% of the endocervical/vaginal discharges.
Obtained from [47]

In our study, the anatomical area, where more mutations were found, is the vaginal one in women (60%) and urethral one in men (50%). Although having a very low proportion of both MSM and rectal samples, the only rectal-located sample with a macrolide resistant mutation belongs to a man who has sex with men. This may be an indication of the high prevalence of this mutation in this anatomical site in the MSM population, but we don't have data enough to confirm it.

Regarding azithromycin treatment, in our case, we have examined all treatments administered against the current infection in the study and in relation with several characteristics (presence of mutations, coinfections, symptomatology, etc). However, in most of the cases, we don't know if any patient has had azithromycin treatment in the past, which may be useful to see if there are a relationship with the appearance of resistance mutations.

CONCLUSIONS

In conclusion, the results in this study show a lower prevalence of macrolide resistance than expected comparing to other national studies. Nevertheless, individually the results obtained show a resistance rate that could be considered high.

The data obtained suggests that the genotypic assays of macrolide resistance-associated mutations should be part of the diagnostic procedure and should be analysed before the initiation of the treatment. In fact, this would provide highly relevant data for guiding clinicians more precisely and thus, shorting the time of the initiation of an effective treatment, improving the antimicrobial stewardship. In the study, it can see that the lack of this resistance analysis may lead to many mistaken treatments and this fact could produce a persistence of the infection because of treatment failures, and it may also increase the antimicrobial resistances due to a high selective pathogen pressure.

According with studies carried out in other countries, all agree on a fast and constant increase both in the general prevalence of infection by *Mycoplasma genitalium* and in the rate of resistance to macrolides. However, the results obtained are very diverse depending on the population and geographic region studied. More exhaustive research on this infection is recommended to obtain more unanimous conclusions, in order to establish standardization diagnostic and therapeutic guidelines at international level.

Finally, it would be advisable a reappraisal of the antibiotic options used as the first-line treatment, due to the decrease in their effectiveness, and extensive research to evaluate the effectiveness of new treatment or even combination of therapies.

ACKNOWLEDGEMENTS

First, I would like to express my gratitude to Dr. Gemma Recio Comí, my internship supervisor, for her dedication, her appreciated and helpful suggestions and all the knowledge that has taught me as professional in health sector. Without her this project would not have been possible.

I would also show all the technical staff of the Clinical Analysis Laboratory of the Joan XXIII Hospital gratitude for letting me be part of their daily work and for their help.

Special thanks to my family who have always been supporting me in all my academic decisions, specially to my mother, for her eternal patience, her encouragement and for listening and motivating me whenever I have needed it, and to my cousin Elvira, for her time and knowledge.

Finally, I would express thanks to my friends, specially to my flatmates for their moral support, their originality, their advice and for being there raising my spirits in time of stress and sharing the day to day together.

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