BRIEF REPORT

Identification of *Mycoplasma genitalium* from clinical swabs by direct PCR [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

Mycoplasma genitalium is one of the smallest self-replicating organisms. It is an obligate parasite found in the human genital tract. In men, the bacteria cause both acute and chronic non-gonococcal urethritis (NGU). In women, it has been associated with pelvic inflammatory disease and cervicitis among other related infections. Treatment of *M. genitalium* related infections has been effective using antibiotics such as the macrolides (e.g. azithromycin) and fluoroquinolones. However, there have been recorded cases of resistance to these antibiotics in various parts of the world as a result of a mutation in the 23SrRNA gene, although the antibiotic resistance has not been well established. The aim of this study was to detect *M. genitalium* in 352 swab samples collected from a clinic for sex workers in Nairobi, Kenya. DNA was extracted from the swabs and stored as a crude extract at -31°C. The swab lysates were subjected to direct polymerase chain reaction using primers that specifically target the 16S rRNA gene for *M. genitalium*. A total of 29 samples tested positive for *M. genitalium*. The data results showed a *M. genitalium* prevalence of 8.24% among sex workers in Nairobi, Kenya.

Keywords

Direct PCR, *Mycoplasma genitalium*

Open Peer Review

Approval Status

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version 1		
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Any reports and responses or comments on the article can be found at the end of the article.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Introduction

Mycoplasma genitalium is an emerging sexually transmitted disease that was first identified and isolated in 1980¹ from men with non-gonococcal urethritis (NGU). Its epidemiology in connection to other STI syndromes been established since nucleic acid amplification assay development in the early 1990s². The bacteria have been detected in substantial amounts from men with urethritis and women with cervicitis³. *M. genitalium* prevalence in the general population has been studied and found to be ranging between 1–3%⁴.

M. genitalium is found in roughly 15% of men with NGU and in 22% of men with non-chlamydial NGU. However, the associated infections do not have unique clinical symptoms, making it difficult to use clinical signs as a mode of identification⁵. Cervicitis has been described as the female version of male urethritis. *M. genitalium* is found in 10% of women with cervicitis⁶. Chlamydial coinfections in women with cervicitis are also common in some settings⁷. *M. genitalium* is a very fastidious bacterium and culturing of the bacterium is exhaustive and time consuming.

The introduction of polymerase chain reaction (PCR) assays has provided the necessary data for its clinical prevalence⁸. Many assays have been developed for the detection of *M. genitalium* in human specimens^{8–17}. Most of these assays are mainly based on the PCR detection technique. Use of these PCR tests have shown that the disease spectrum is similar to those caused by *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in both males and females¹³. However, these assays differ in their target DNA sequences, specimen preparation and amplicon detection methods. Many of these detection methods target the 16S rRNA and the *MgPa* protein genes. Conventional and, more recently, real-time PCR assays have been applied. Most of the detection studies have been conducted in the U.S.A, Europe and Australia, with various strains being discovered. In line with the detection of the bacterial species and its related infections in Africa, more studies need to be conducted on possible strains and their epidemiology. Whether the bacteria have links with other sexually transmitted infections can also be investigated. In this study, it is shown that direct PCR can be applied to the detection of *M. genitalium* from crude DNA extracts. *M. genitalium* prevalence and characteristics among female sex workers have been studied in Kenya and Uganda^{18–21}. Its prevalence has also been studied among males who underwent circumcision in order to prevent HIV acquisition in Kisumu, Kenya²². However, most of these studies have focused on conventional, real-time PCR or transcription-mediated amplification assays for the detection of *M. genitalium*. This study reports the use of direct PCR for *M. genitalium* detection from crude DNA extracts using specific primers that target the 16S rRNA gene.

Methods

Ethical statement

This study was approved by the Jomo Kenyatta University of Agriculture and Technology Institutional Ethics Review Committee (JKUAT-IERC): reference number JKU/2/4/896B. The swab samples were collected with written informed consent for the performance of further analysis.

Source of samples

The samples used in this study were collected as part of the sex workers outreach program (SWOP) central business district clinic in Nairobi, Kenya. As part of this program, patients who showed STI symptoms and consented to the study were sampled by taking vaginal swabs. The specimens were then put into sterile containers and transported to the Pan Africa Hub Laboratory (NUTM-KEMRI) within an hour and stored at -80°C. Anonymized samples were retrieved for use in this study.

Sample preparation

The 352 swab lysates were prepared using the MightyPrep reagent for DNA (TAKARA BIO INC, Kusatsu, Shiga Prefecture, Japan; Cat No: 9182) using the manufacturer's protocol with a slight modification. Swabs were cut and put into 1.5ml Eppendorf tubes. A total of 200µL of the MightyPrep reagent was added to the tubes and later centrifuged at 15krpm for one minute. The tubes were then transferred to a heated block at 95°C with shaking at 800rpm for 15 minutes. Later, the tubes were cooled down by lowering the heat block temperature to 25°C, followed by hard vortexing of each tube for one minute and, finally, centrifugation at 15krpm for two minutes before storage at -31°C.

Direct PCR

The master mix was prepared using the manufacturer's protocol with slight modifications (Hotstar Taq® Master Mix Kit 2.5 Units, Qiagen; Cat No: 203443). 100µM primer concentration was achieved by adding 303µl and 353µl of Tris EDTA (Nippon Gene Company Ltd, Japan, Cat No: 314-90021; 10mM Tris-HCl [pH 8.0], 1mM EDTA [pH 8.0]) to the forward and reverse primers (Table 1; Sigma-Aldrich, Darmstadt, Germany), respectively. The primers (Table 1) targeted the 16S rRNA gene²³ giving 433bp amplicon size fragments. Master mix components were: RNase- free water (1x = 7.84µl); primer mix (1x = 0.08µl, 0.2µM of each primer); and HotStar Taq® master mix (2x), comprised of 2.5 units HotStarTaq DNA polymerase (1x = 10µl), 1x PCR buffer (1x, contains 1.5mM MgCl₂) and 200µM of each dNTP.

After vortexing the master mix for five seconds, 18µl was aliquoted to each of the labeled 96 PCR tubes. 2µl of the swab lysates was added to each tube to make a final reaction

Table 1. Primer sequences.

Forward primer	Reverse primer
MG16-45F (TACATGCAAGTCGATCGGAAGTAGC)	MG16-447R (AAACTCCAGCCATTGCCTGCTAG-3')

volume of 20µl and the tubes were finger tapped for five seconds to mix the contents. A positive control (*M. genitalium* positive sample) and negative control (PCR water) were used. The PCR tubes were placed in the SimpliAmp™ Thermal Cycler (Applied Biosystems) and run under the following reaction conditions.

An initial antibody inactivation step was carried at 95°C for 15 minutes, followed by 35 cycles of: denaturation at 94°C for 60 seconds, annealing at 67°C for 60 seconds and extension at 72°C for 60 seconds. A final extension step was carried out at 72°C for 10 minutes, followed by the final hold at 4°C for ∞.

Agarose gel electrophoresis

The PCR products were subjected to gel electrophoresis using 2.5% agarose gel SeaKem® GTG® agarose (Lonza, Rockland, ME, USA; Cat No: 50074) at 100V for 45 minutes. 6x loading dye (Nippon Gene; Cat No: 314-90261) was diluted with sample to make 1x and loaded onto the gel. The 100bp GelPilot® Ladder marker (Qiagen; Cat No: 239035) was used. The gels were stained with 2x GelRed™ Nucleic Acid Gel Stain (Biotium; Cat No: 41003) for one hour on a shaker. The image was viewed using the UltraSlim UV Transilluminator (Maestrogen).

Amplification of the PCR products

The PCR products were subjected to another PCR. Master mix components were as described above. 18µl was aliquoted into the PCR tubes. 1µl of the sample products was added to the tubes to make a 19 µl final volume. The PCR tubes

were placed in the SimpliAmp™ Thermal Cycler (Applied Biosystems) and run under the following reaction conditions.

An initial antibody inactivation step was carried out at 95°C for 15 minutes, followed by 30 cycles of: denaturation at 94°C for 60 seconds, annealing at 69°C for 60 seconds and extension at 72°C for 60 seconds. A final extension step was carried out at 72°C for 10 minutes, followed by the final hold at 4°C for ∞.

The products were run on a 2.0% agarose gel at 100V for 40 minutes. A 3000bp ladder (Solis BioDyne, Tartu, Estonia) was used. The gels were stained using 2x GelRed for one hour and viewed under an UltraSlim UV Transilluminator.

Results

A total of 352 lysates were analyzed in this study. The results show evidence for the presence of *M. genitalium* from swabs taken from the female sex workers who were sampled. 352 lysates were prepared using the MightyPrep reagent.

M. genitalium detection

M. genitalium was detected among the 352 swab lysates. Examples of *M. genitalium* detection are shown in Figure 3 to Figure 4. Figure 1 shows clear bands at positions 1, 2, 9, 10 and 17 on a 26-well agarose gel. The same kind of bands can be seen in Figure 2 at positions 9, 10, 11 and 18. The positive and negative controls are at positions 24 and 25, respectively, on each gel. A 600bp ladder was used at positions 1 and 26 to track the *M. genitalium* amplicon sizes of interest.

Figure 1. First representative gel for *Mycoplasma genitalium* detection using direct PCR. Ultraviolet camera gel image showing 22 samples run on a 26-well gel. The ladder is at positions 1 and 26 (Gel Pilot®). Clear *Mycoplasma genitalium* positive bands can be seen at positions 1, 2, 9, 10 and 17 (Sexually transmitted infection lysates S099, S100, S108, S109 and S116, respectively). The positive control (PC) and negative control (NC) are at positions 24 and 25, respectively.

Figure 2. Second representative gel for the detection of *Mycoplasma genitalium* using direct PCR. UV camera gel image showing the 22 samples run on a 26-well gel. The first and last wells represent the ladder (GelPilot®). Clear *Mycoplasma genitalium* positive bands can be seen at positions 9, 10, 11 and 18 (lysates S137, S138, S139 and S146, respectively). The positive control (PC) is shown at position 24 and the negative control (NC) at position 25.

Figure 3. First representative gel showing the amplification of the PCR products. The selected PCR products were amplified: the above image shows the first nine PCR products run on a gel after the amplification was conducted. The ladder (Solis BioDyne) is at the first and last lanes. The positive control (PC) is at lane 11, while the negative control (NC) is at lane 12.

The PCR products were subjected to another amplification reaction. After the reaction, the products were run on a 13-well agarose gel. A 3000bp ladder was loaded on positions 1 and 13, with the positive and negative controls at positions 11 and 12, respectively, as can be seen in Figure 3 and Figure 4. Out of the 352 swab lysates used, 29 tested positive for *M. genitalium*.

M. genitalium prevalence

M. genitalium prevalence among the cohort of female sex workers was found to be at 8.24% (29/352), showing one out of eight patients had *M. genitalium* related infections.

Discussion

Recently, DNA amplification protocols using PCR have been employed in the detection of *M. genitalium*. To investigate the presence of *M. genitalium* from the clinical swab samples collected from a clinic for sex workers in Nairobi, Kenya, a direct PCR technique was used for the detection of *M. genitalium*. This technique involves the use of target-specific primers to select the DNA of interest from a crude extract. The study detected *M. genitalium* using primers that bind to the 16SrRNA gene from the crude DNA extract. Jensen and his colleagues²³ developed a wide range of primers

Figure 4. Second representative gel showing the amplification of the PCR products. This gel shows PCR products of the samples that were selected for amplification. The first and last lane contain the ladder marker (Solis BioDyne), the PCR products run from lanes 2 to 10 and the positive control (PC) is at position 11, while the negative control (NC) is at position 12.

that target the 16S rRNA gene, producing different amplicon sizes. A novel PCR was used²⁴ to detect *M. genitalium* using oligonucleotide primers that corresponded to sequences along its 16S rRNA gene.

The study was able to detect 29 *M. genitalium* positive samples out of the 352 lysates. However, the challenge experienced with this method was non-specific amplification, realized from the multiple fragments produced. A possible solution to this is in the use of more precise target-specific primers to prevent the amplification of genes with closely related sequences. Application of this method can be a remedy to the constant loss of DNA due to long extraction processes, at the same time maintaining its quality for further downstream analysis.

M. genitalium prevalence was shown to be at 8.24%. This shows that one out of every eight patients sampled was positive for *M. genitalium* related infections. Balkus and colleagues in 2018 were able to detect *M. genitalium* from 25 out of 221 (11.3%) women from Kenya and the US²⁵. Prevalence rates of 12.9%¹⁸ and 16%¹⁹ have also been reported among sex workers in Nairobi, Kenya. The prevalence obtained in this study therefore does not show any significant drop in *M. genitalium* infections. Despite better and improved access to healthcare, *M. genitalium* infections seem to continue to be a burden. Possible reasons might be due to having multiple sex partners²⁶ or antibiotic resistance to drugs of choice such as macrolides and fluoroquinolones^{27,28}

Overall, the prevalence results suggest that more measures need to be taken to control *M. genitalium* infections. Awareness campaigns need to be carried out to sensitize people on preventive measures rather than taking potential risks that may lead to exposure to the infection. Studies need to be done to investigate *M. genitalium* drug resistance. This will be helpful in informing policy and practice. As a result, screening can be done in patients to check for resistance before prescribing medication.

Data availability

Underlying data

Figshare: Detection of *Mycoplasma genitalium* using Direct PCR. <https://doi.org/10.6084/m9.figshare.10282691.v1>²⁹.

This project contains the following underlying data:

- Direct PCR 1.docx – Direct PCR 4.docx (lists of the samples tested for *Mycoplasma genitalium* in four sets of 88 samples)
- Exp 1 Gel 1.JPG - Exp 4 Gel 4.JPG (gel electrophoresis of PCR products; 100bp GelPilot® Ladder marker [Qiagen] at positions 1 and 26, samples from positions 2 to 23, positive control at position 24 and negative control at position 25)
- Amplification Gel 1.JPG - Amplification Gel 3.JPG (gel electrophoresis of the amplified products; 100bp ladder [Solis BioDyne] at positions 1 and 13, samples from positions 2 to 10, positive control at position 11 and negative control at position 12)
- Amplification Gel 4.JPG (gel electrophoresis of the amplified products; 100bp ladder [Solis BioDyne] at positions 1 and 13, samples from positions 2 to 7, positions 8, 11 and 12 contain no samples [blanks], positive control at position 9 and negative control at position 10).

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

Acknowledgements

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This paper presents a laboratory method used for detection of *Mycoplasma genitalium* in swab samples collected from sex workers. The authors provide descriptions of the method used and the results obtained. I consider that, in order to be indexed, the paper needs some revisions and explanations.

Abstract

The paragraph on treatment and antibiotic resistance is not necessary because they are not the subject of this article.

Introduction

The phrase "Most of the detection studies have been conducted in the U.S.A, Europe and Australia, with various strains being discovered" requires citations from literature to exemplify what is stated.

The phrase "In this study, it is shown that direct PCR can be applied to the detection of *M. genitalium* from crude DNA extracts." should not be placed in this section. It must be moved to the Discussions section.

Methods

In Sample preparation and Direct PCR sections, the authors declare that they used the manufacturer's protocol "with slight modifications". What are these changes and for what purpose were they made?

A positive control (*M. genitalium* positive sample) was used. Where does it come from? Why two PCR amplifications were made? The authors should explain why they have amplified PCR products.

Results

I think two Figures are enough: a representative gel for direct PCR and a representative gel for the

amplification of the PCR products.

There are mistakes in the legends of the Figures regarding the positive positions: e.g. in Figure 1 "The ladder is at positions 1 and 26" and "Clear *Mycoplasma genitalium* positive bands can be seen at positions 1, 2, 9, 10 and 17".

How do you explain that in Figure 4 the band corresponding to sample S163 is at a different level than the others?

Discussion

There is too little discussion about the technique used.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: molecular biology, microbiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 12 Oct 2021

Robinson Irekwa, Pan Africa University Institute of Basic Sciences, Technology and Innovation (PAUSTI-JKUAT), Juja, Kiambu, Kenya

Thank you for the comments given, will work on the sections. Truly appreciate

Competing Interests: No competing interests were disclosed

Reviewer Report 25 February 2020

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Hans Fredlund

World Health Organization Collaborating Centre for Gonorrhoea and Other Sexually Transmitted Infections (STIs), National Reference Laboratory for STIs, Department of Laboratory Medicine, Faculty of Medicine and Health, Örebro University, Örebro, Sweden

A commercial PCR method to detect *M.genitalium* was used in this study and a prevalence of 8% was found in female sex workers in Nairobi, Kenya. The manuscript is well written. It is of interest to perform such studies in many countries and cities globally to follow and understand the epidemiology of this STI. Of that reason the study can be accepted for indexing as it is. What is not studied is the antibiotic sensitivity of *M.genitalium*. In coming studies this has to be performed.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Infectious diseases and microbiology, epidemiology especially in the area of *Neisseria* sp and STI.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 12 Oct 2021

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Thank you Dr. Huns Fredlund.

Competing Interests: No competing interests were disclosed.

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