Molecular Detection of *Mycoplasma genitalium* among Sudanese Women presenting with Genitourinary Infection

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Abstract

Background: *Mycoplasma genitalium*(*M .genitalium*) is a common known cause of non-gonococcal urethritis in men and women. Infection with *M. genitalium* is reported to be a worldwide sexually transmitted infection.

Objective: To determine the frequency of *M .genitalium* among Sudanese women presenting with genitourinary infection, to explore its macrolide antibiotic resistance by detection of mutations found in region Vof the 23S rRNA gene and partial characterization of mgpB gene.

Materials and Methods: Two hundred urine and high vaginal swab specimens were collected from one hundred women complaining of genitourinary infections. Specimens were first screened for the MgPa gene using real time polymerase chain reaction (PCR). Positive specimens were further assayed for 23S rRNA gene using PCR confirmatory assay.

Results: The frequency rate of *M. genitalium* among the Sudanese women investigated was 4%. No significant association was observed between the clinical symptoms and *M. genitalium* infection. All *M. genitalium* DNA sequences partially characterized in this study had revealed susceptibility to macrolide antibiotics.

Conclusion: *M.genitalium* was successfully amplified in the clinical specimens collected, and the frequency rate of *M. genitalium* was 4%. *M. genitalium* strains were susceptible to macrolide antibiotics. Results reported provide a base for further research work on *M. genitalium* infections in Sudan.

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Introduction

*M. genitalium*is an intracellular, urogenital tract, gram negative, flask-shaped bacterium, that belongs to the *Mycoplasmataceae* family, in the *Mollicutes* class [1,2]. It is associated with cervicitis [3],and pelvic inflammatory disease [4] in women that leads to tubal factor infertility if not treated [5,6]. Traditional procedures for diagnosis of *M. genitalium* infections suchas culture and serology, were not able to identify its role in the disease for many decades. In contrast, the molecular diagnostic methods such as polymerase chain reaction (PCR), and especially the quantitative detection of *M. genitalium* nucleic acid has strongly facilitated the molecular characterization of this pathogen as a sexually transmitted and one of the common causes of non-gonococcal urethritis [7, 8].

M. genitalium urogenital infection in low-or high -risk populations indicates an overall prevalence of 2% to 7%,respectively worldwide [9]. The prevalence of *M. genitalium*has been reported to be high in some African countries. It is 26 %, 16 %,14 % and 4 % in West Africa, Kenya, Uganda and Mozambique respectively. Risk factors associated with high prevalence were attributed to promiscuity along with sero-positivity for HIV of the populations studied [10,11,12,13].

In Sudan the prevalence of *M. genitalium* and or its partial genome characterization had never been reported before to the best of our knowledge. Therefore, this study was aimed to investigate *M. genitalium* prevalence among Sudanese women presenting with genitourinary infection, to explore its macrolide antibiotic resistance based on detection and characterization of *Mycoplasma genitalium* protein mgpB and 23S rRNA gene.

Materials and Methods

This study was carried out during the period of June 2012 to July 2013 in Khartoum North Teaching Hospital(Sudan).Approval to perform the study was granted by the Ethical Committee of this hospital. Two hundred urine and high vaginal swab specimens were collected from one hundred women complaining of genitourinary infections, and seeking treatment at the departments of obstetrics and gynecology. After a written consent of patients, a structural questionnaire was filled with patients' demographic data, clinical symptoms such as vaginal discharge, itching, burning micturition, lower abdominal pain, and history of abortion**.**

A sterile non-lubricated, disposable, plastic speculum was inserted into the vagina, high vaginal swabs were then collected from the posterior fornix without touching the vaginal wall to avoid contamination. The collected vaginal swabs were then immerged in tubes containing sterile 5ml Tri-HCl solution (ph7.4). Tubes were further centrifuged for 10 minutes at 2000

rpm. The pellet was then transferred to a sterile cryogenic tube and stored at -80°C until further investigations.

Twenty ml of first void urine was also collected in sterile wide-mouth, leak- proof and screw-cap container; then centrifuged in 15 ml Falcon tube(Fisher Scientific) for 10 minutes at 2000 rpm.The resultant pellet was suspended in 2 ml cryogenic tube containing 1ml of phosphate buffer saline (ph 7.4)and stored at -80°Cfor further testing by PCR.

Molecular detection of *Mycoplasma genitalium*

DNA was extracted from both vaginal and urine specimens by the commercial DNA extraction kit (Aidlab Biotechnologies, China) according to the manufacturer's instructions. The extracted DNA was then stored in small aliquots at -80°C until further analysis by PCR.

The extracted DNA was subjected to real time PCR screening using the primers: MgPa-355F (5'- GAGAAATACCTTGATGGTCAGCAA -3'),MgPa-432R (5'-GTTAATATCATATAAAGCTCTACCGTTGTTATC-3'), and MgPa-380 FAM(5'-ACTTTGCAATCAGAAGGT-3'). These primers were designed for TaqMan assay to detect a 78pb fragment of the MgPa operon sequence (Accession no.M31431)[15].

PCR reaction mixture consisted of:1 x PCR buffer (20 mM Tris-HCl, pH8.4 and 50 mM KCl); 5 mM $MgCl_2$; 1 µM each primer MgPa-355F and MgPa-432R; Taqman probe; 75 nM FAM-labeled MgPa TaqMan MGB probe; 62.5 uM each dATP, dGTP(from Platinum Invitrogen, Carlsbad, Calif.); and dCTP;125 uM dUTP; 10% glycerol (from Sigma-Aldrich Denmark A/S, Copenhagen, Denmark); 1 ul of 6-carboxy-x'-rhodamine reference dye (from Invitrogen); and 2 U of *Taq* DNA polymerase (from Platinum *Taq*; Invitrogen).

An ABI 7500 real-time PCR thermocycler (from Applied Biosystems) was used with a 96-well block and MicroAmp Optical 96-well reaction plates covered with ABI prism Optical Adhesive Covers (from Applied Biosystems).

All tests were performed in with 5 µl of template DNA at 50°C for1sec (stage 1), 95°C for 10 min. (stage 2), and 50 cycles of denaturation at 95°C for 15 sec,annealing and extension at 60°C for 1 min.Standard curves were produced by analyzing 10-fold dilutions of *M. genitalium* DNA containing5to 500,000 genome equivalents (geq). The *M. genitalium* DNA was diluted in TE buffer (pH 8.0) containing 1 µg of calf thymus DNA per ml (from D-8661; Sigma-Aldrich).

23S rRNA gene confirmatory assay

10 µl DNA of the clinical specimens found positive by the real time MgPa gene PCRscreening were used in the 23S rRNA gene confirmatory assay. Primers of Mg23S-1992F (5-CCATCTCTTGACTGTCTCGGCTAT-3) and Mg23S-2138R (5-CCTACCTATTCTCTACATGGTGGTGTT-3) targeting a 147bp of the V region of the *M. genitalium* 23S rRNA gene were used[16].

PCR reaction mixture consisted of 1x PCR buffer (from PlatinumTaq; Invitrogen) containing: 20 mmol/L Tris-hydrochloride with a pH of 8.4 and 50 mmol/L potassium chloridewith 1.5 mmol/L magnesium chloride), 0.4 mmol/L of each primer, 125 µmol/L each of dATP, dGTP,

and dCTP and 250 µmol/L dUTP, and 2ul of Platinum *Taq* DNA polymerase(from Invitrogen)[16].

PCR was run at a total final volume of 100µl in Convergys® TD Peltier thermocycler (Germany) programmed to run at 95°C for 2 min. followed by 40 cycles each consisting of 95 °C for 15 sec and 60°C for 1 min. for both annealing and extension followed by final extension at 72°C for 7 min. PCR products were analyzed by electrophoresis in 2 % agarose gels in TAE buffer, stained with ethidium bromide and visualized under UV light(Genius, UK).

Amplification of *M.genitalium* mgpB gene by conventional PCR

All real time PCR MgPB gene positive specimens were selected and subjected to conventional PCR analysis using the primers: MgPa-1 forward (5'-AGTTGATGAAACCTTAACCCCTTGG-3') and MgPa-3 reverse (5'-CCGTTGAGGGGTTTTCCATTTTTGC-3')[17].

Specimens were run in a PCR final volume of 20 µl, denatured at 95°C for 1 min. followed by 35 cycles consisting of 95°C for 1min.at 65°C for 1 min. and at 72°C for 1 min. followed by a final extension at 72°C for 6 min.PCR products (281-bp) were analyzed by electrophoresis in 1.5 % agarose gels in TAE buffer, stained with ethidium bromide and visualized under UV light.(Genius, UK).

Sequencing and analysis of sequence data

The PCR products for both 23S rRNA and mgpB genes were purified using the QiaQuick PCR purification kit (from Qiagen,Hidlden,Germany).Sequencing was performed with ABI prism Big Dye terminator reaction kit v2.0(from Applied Biosystem,Foster City,CA) using the 23S rRNA and mgpB conventional PCR primers(SSI, Denmark). Alignment of amino acids sequences of *M. genitalium* characterized in this study together with reference isolates G37 were performed using ClustalW [18,19].

For detection of macrolide resistance, sequences of the 23S rRNA gene amplified in this study were assembled, consensus files were aligned with *M. genitalium* wild-type positions: 2,058/ 2,059[16]; and analyzed by DNA baser sequence assembler software v3.3.5 (Heracle BioSoft S.R.L).

Results

Out of the 100 women investigated in this study, real-time PCR successfully detected *M. genitalium* mgpB gene in 4 urine specimens of which two were also detected positive in high vaginal swabs. Positive results were further confirmed by detection of the 23S rRNA gene of *M. genitalium*. High vaginal swabs revealed higher mean (640.92 geg/ml) of *M. genitalium* DNA load when compared with urine specimens (10.35 geg/ml)(Table1).

No association was observed with clinical signs or history of abortion with *M. genitalium* infection among the patients studied. The *M. genitalium* mgpB detected in the four specimens found positive by the real time PCR were successfully amplified using conventional PCR. The three mgpB genome sequences characterized in this study(SDN19, SDN51, and SDN151) were submitted to the GenBank with accession numbers: KF612736 - KF612738.

When the amino acid sequences of the mgpB gene of the three strains characterized in this study were aligned with the G-37 reference sequences, four mutations were detected in SDN19 strain position (D^{96}/E,S^{101}/A, S^{107}/V ,A^{117}/S)and two mutations in strain SDN51 and SDN151 position (S^{107}/V, G^{124}/D) Fig 1.

When the wild-type M.*genitalium* was aligned with Sudan sequences (SDN19,SDN51, and SDN151) characterized in this study, no detectable mutation were observed at nucleotides at position 20,058 and 20,059 (*Escherichia coli* numbering) data not shown.

Table (1): Screening for *M.genitalium* MgPa gene by real-time PCR in high vaginal swabs and urine specimens

Specimen	No. of specimens	Positive MgPa	Negative MgPa	Percentage of positives
High vaginal swab	**100**	**2**	**98**	**2%**
Urine	**100**	**4**	**96**	**4%**

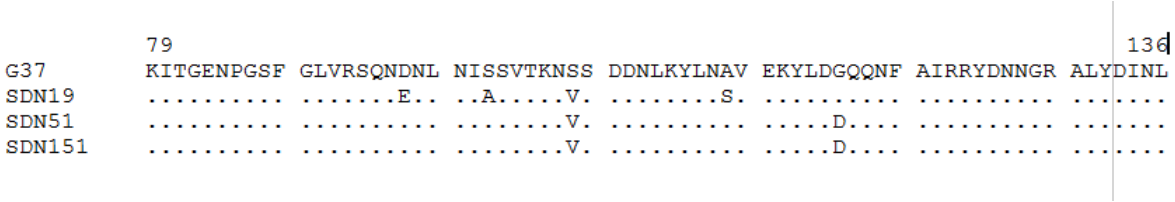

Fig. (1): Amino acids aligment of *M.genitalium* mgpB gene (position 79 - 136)of Sudanese sequences (SDN19,SDN51 &SDN151) together with *M.genitalium* reference strain G37.

Discussion

Mycoplasma.genitalium is an emerging cause of sexually transmitted diseases and has been implicated in urogenital infections of men and women around the world.[20]This study represents the first report conducted in Sudan to find out the occurrence of M .*genitalium* as a sexual transmitted disease among Sudanese women . Vaginal and urine swabs from women patients were first screened by real time PCR and positive samples were further confirmed by PCR confirmatory assay and sequencing.

The prevalence of M.*genitalium*a among Sudanese women was found to be 4% .These finding are in a accordance with previous results reported in Mozambique [13] and Norway [21].High incidence of M.*genitalium* 26.3%were also reported in different countries such as west Africa [10], Kenya [11], Uganda [13] USA[22] and India [23].In this study no significant association between M .*genitalium* infection and symptom such as vaginal discharge ,itching , buring micturation and history of abortion were observed. This may indicates that M.*genitalium* infection in women is more likely to be asymptomatic or have few slight symptoms which may easily be ignored and undiagnosed as reported before[24].

The quantification of *Mgenitalium*DNA load was found to be higher in vaginal swabs(640.92 geg/ml+STD±314.7) than first void urine (10.35 geg/ml+STD±5.53) among Sudanese women under the study . Rebeeca et al, 2011, also reported that vaginal swabs were with higher DNA load (85.7 %) where as urine was more sensitive in detection of M.*genitalium* 100% [25].

In this study four substitution mutations of amino acids were observed in SDN19 isolate when aligned with reference strain G37,on other hand two mutations were detected in SDN51 and SDN151 S^{107}/V G^{124}/D. The effects of silent mutations on protein folding and the role of MgPa as a major adhesin and its antigenicity have been demonstrated, but the exact positioning of the protein in the mycoplasma membrane, as well as the role of individual domains or regions, has not been detailed. Thus the impact of detected mutations on structural or functional properties of MgPa necessitates additional investigation.[26]

All DNA sequences of *M,genitalium* obtained from Sudanese women under the study were susceptible to macrolide, this was revealed by the absence of mutations at position 20,058 and 20,059 (*Escherichia.coli* numbering), in the Sudanese sequences when aligned with M.genitalium wild-type . Development of macrolide resistance was shown to correlate with subsequent azithromycin treatment failure [16].In contrast the susceptibility to macrolide observed here may be due to proper and sufficient prescription dose for treatment of M .*genitalium* infections, however this may be revealed by conducting further systemic investigations .

Conclusion

This is the first report of molecular detection of *M,genitalium* in Sudan .The mgpB gene was successfully amplified in clinical specimens. The prevalence of *M,genitalium* was found to be 4%. All isolates partially characterized in this study were found susceptible to macrolides

antibiotic. Results reported here will can provide a base to further research in *M genitalium* infections in Sudan.

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