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Molecular Characterization of *Mycoplasma genitalium* detected among Sudanese Women with Genitourinary Infections

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Abstract

Background: The introduction of molecular diagnostic assays had helped the conduction of many clinical studies in developing countries. Improvement in laboratory detection methods, particularly with the introduction of the newer nucleic acid amplification tests had played an important role in elucidating the role of *Mycoplasma genitalium* (*M .genitalium*) among sexually transmitted pathogens.

Objective: To study the molecular characterization of *Mycoplasma genitalium* detected among Sudanese women with genitourinary infections.

Materials and methods: 200 first voided urine specimens and high vaginal swabs (HVS) were collected from each woman under the study. The DNA was extracted by micro-spin column extraction kit. Real-time PCR Tag MAN assay was used to detect and quantify *M. genitalium* DNA in each specimen. Positive specimens were tested by conventional PCR targeting the domain V region *23S rRNA* gene for detection of macrolide resistance and *MgPa* gene for genotyping. PCR products obtained were further purified, sequenced , blast at NCBI, protein translation and 3D protein model were done to locate mutations.

Results: *M. genitalium* was detected in 4% of the patients investigated. *MgPa* gene was characterized and submitted to the GenBank under accession numbers KF612736 to KF612738. First voided urine specimens were found to be highly sensitive for detection *M. genitalium* in comparison to HVS, and had a significance level ($p = 0.01$). Most infected women were in the age range 18-25 years. Patients residing in Jazeera State represented the highest residence frequency rate (16.7%). DNA sequence in this study aligned with of *M. genitalium* reference strain by CLUSTAL W four substitution mutation were detected in SDN19 and two in SDN51 and SDN151. Phylogenetic analysis also revealed that SDN51 and SDN151 were similar to *M. gentalium* genotype 25 in same clustering but SDN19 in separate cluster closer to TX84. All Sudanese isolate sequences were susceptible to Macrolides resistance test targeting domain V region 23S rRNA. No significant association ($p>0.05$) were found between *M. genitalium* infection and symptoms.

Conclusion: Several amino acids substitutions mutations of *M. genitalium* were detected. Leucocyte esterase test was not a screening test for detection of *M. genitalium*.

Key words: Molecular characterization, *Mycoplasma genitalium*, Sudanese women.

Introduction

Mycoplasma genitalium was first isolated in 1981 by Tully, *et al* from two men with non-gonococcal urethritis. Two isolates were grown on SP4 transport medium which they had developed two years earlier. The strains were designated G-37 and M-30, and shown to be distinct from all other *Mycoplasma* species. These unique isolates were subsequently named *Mycoplasma genitalium*. The G-37 isolate has become an American Type Culture Collection (ATCC 33530) strain with its genome being fully sequenced in 1995 due to its slow cell replication and fastidious growth requirements, culture is not usually used for laboratory diagnosis of *M. genitalium*, hence few epidemiological studies were done in the years following its discovery[1].

As highlighted by this literature review, *Mycoplasma genitalium* is a serious public health problem in young women in Sudan and the true burden of infection and related sequelae are unknown. Considering *Mycoplasma* is often asymptomatic and testing rates for *Mycoplasma* remain low, the notification data for *Mycoplasma* are likely to underestimate the extent of *Mycoplasma* in the population. Further, there are few population based prevalence estimates and no incidence data for women. Internationally re-infection rates for mycoplasmas are very high which is concerning considering repeated infections are more likely to cause serious upper genital tract infections, including PID and tubal factor infertility[2].

The re-infection rates for *Mycoplasma* in Sudan are also unknown. It will be essential for developing clinical guidelines, in particular retesting guidelines and partner management for women who test positive. Re-infection rates and incidence data will be important data for the development of *Mycoplasma* control strategies. International reports suggest *M. genitalium* is like *Chlamydia* is commonly asymptomatic, and as a consequence the burden of disease attributable to *M. genitalium* in young Sudanese women is currently unknown[3].

The aim of this study was to justify the sort of clinical specimen to be collected (urine or high vaginal swab) for the laboratory diagnosis of *Mycoplasma genitalium.* It investigated the role of *Mycoplasma genitalium* in the aetiology of genitourinary infections in Sudan. It also studied the incidence of *M. genitalium* among Sudanese female patients. It assessed the role of leukocyte esterase test as a primary screening tool for the diagnosis *M. genitalium*. Through the recent molecular techniques, this study is in the characterization of the Sudanese *Mycoplasma* genotype strain and whether it is different from other strains prevalent in other geographical regions worldwide. Further, it conducted molecular sensitivity of the Sudanese *M. genitalium* strains to the macrolide drugs that are used in treatment of *Mycoplasma* infections.

Materials and methods

This was a qualitative, prospective, hospital-based, analytical, descriptive, and cross-sectional study. It was carried out during the period from June 2011 to July 2014 at Khartoum North Teaching Hospital, Omdurman Military Teaching Hospital, and Al Hayat Charity Health Center

(Khartoum, Sudan). Population investigated was non-pregnant, married women with symptoms of genitourinary infections, covering different age groups. Inclusion criteria were non-pregnant, married women with symptoms of genitourinary infections. Exclusion criteria were pregnant women, non-married women, menstruating women, women on antibiotic therapy, and women without symptoms of genitourinary infections. Confidentiality of information obtained from patients investigated was maintained. Consent of the patients was taken before being enrolled in the study. Laboratory results of specimens collected were handed to all participants included in the study or dispatched to physicians treating those participants. Permission to collect the specimens was obtained from Khartoum North Teaching Hospital, Omdurman Military Teaching Hospital, and Al Hayat Charity Health Center (Khartoum). Approval to conduct the study was granted by Sudan University for Science and Technology, Khartoum, Sudan. Complete information regarding risk factors, if any, was handed to all participants under the study and no concealment what so ever. The software used for the analysis of data was the Statistical Package for Social Sciences (SPSS) program (version 14). For categorical variables, proportions were compared by the Chi-square test as appropriate. The means and medians of the continuous variables were compared by Student's t test program depending on the sample distribution. Frequencies, percentages, tables and graphs were used for presentation of the data.
Sampling adopted was a probability purposive sampling type. The sample strategy was a convenience type where patients were chosen on the basis of accessibility. The sample size was 200 urine and high vaginal swabs.
Demographic and clinical data were collected using a structured questionnaire with a written informed consent. 100 high vaginal swabs were collected by insertion of a non-lubricated sterile disposable plastic speculum (Welch Allay Klein speculum) into vagina and high vaginal swab was collected from the posterior vaginal fornix. The collected swab was inserted in a tube containing 5ml Tris-HCl buffer. The mixture was centrifuged at 2000 rpm for 10 minutes and the pellet was transferred to cryogenic tube and kept at -70°C until DNA extraction. The Tris-HCl buffer was prepared by adding A 2.24 g Tris to 100 ml of distilled water, B 1.7 ml hydrochloric acid in 100 ml distilled water, and 25 ml of A+13.4 of B (ph 8.0) made of up to 100 cm^3 with distilled water.
20 ml first voided urine was collected in sterile leak-proof urine containers. Then 10 ml of the urine specimen were transferred to clean Falcon centrifuge tubes, and centrifuged at 2000 rpm for 10 minutes. The pellet was suspended in one ml phosphate buffer saline (PBS), and kept at -70° C for later PCR investigation [4]. The phosphate buffer saline was prepared by dissolving 800g NaCl, 20g KCl, 144g Na_2PO_4-$2H_2O$, 24g KH_2PO_4,and 8 liters of distilled water. After complete mixing, final solution was topped up to 10 liters. The pH of the 10x stock was approximately 6.8, but when diluted to 1x PBS it was 7.4. The solution was autoclaved for 15 min. at 121° C and dispensed in one ml cryogenic tubes and stored at room temperature.
Urine leucocytes esterase detection test was used for screening of urinary infection. It detects white blood cells associated with infection. The test may be positive even if leucocytes had lysed. Leucocytes esterase is an enzyme present in granulocytes and hydrolyzes indoxyl carbonic acid esterase to produce indoxyl, which reacts with a diazonium salt to form a purple color

within two minutes. For best results, two strip-reagents were used as a quality control namely: Uristik strip (United Kingdom) and Combostik strip (Korea).
The DNA was extracted from specimens according to manufacturer's instructions (Aidlab Biotechnologies, China). The DNA so extracted was stored at -20° C. The extracted DNA from both HVS and urine specimens was subjected to Real Time PCR detection using the primers:
* *MgPa*-355F (5'- GAGAAATACCTTGATGGTCAGCAA-3')
* *MgPa*-432R(5'-GTTAATATCATATAAAGCTCTACCGTTGTTATC-3')
* *MgPa*-380 FAM (5'-ACTTTGCAATCAGAAGGT-3) MGB Probe
These primers were used to detect a 78pb fragment of the *MgPa* operon sequence (Accession No.M31431). They were designed by Jensen[5] for TaqMan assay.
The components of PCR reaction mixture were: 1X PCR buffer (20 mM Tris-HCl-pH 8.4), 50 mM KCl, Platinum, Invitrogen, Carlsbad, and California).Together with 5 mM MgCl2; 1 uM each primer MgPa-355F; MgPa-432R; TaqMan probe; 75 nM FAM-labeled MgPa TaqMan MGB probe; 62.5 uM of each dATP, dGTP, and dCTP;125 uM dUTP; 10% glycerol (Sigma-Aldrich Denmark A/S, Copenhagen, Denmark); 1 ul of 6-carboxy-x'-rhodamine reference dye (Invitrogen); and 2 U of *Taq* DNA polymerase (Platinum *Taq*, Invitrogen).
An ABI 7500 Real-Time PCR instrument (Applied Biosystems) was used with a 96-well block and MicroAmp Optical 96-well reaction plates covered with ABI prism Optical Adhesive Covers (Applied Biosystems).
Detection of *M. genitalium* in both HVS and urine specimens was confirmed by the amplification of the *23S-rRNA* gene in the *MgPa* TaqMan real time assay positive samples using the primers:
* Mg23S-1992F (5-CCATCTCTTGACTGTCTCGGCTAT-3)
* Mg23S-2138R (5-CCTACCTATTCTCTACATGGTGGTGTT-3)
Flanks mutations found in the V-region of the *23S-rRNA* gene and producing a 147-bp amplicon.
The primers used in the study were in the form of lyophilized powder purchased from Macrogen (Korea). Before preparation of primers their sequences were first checked for quality assurance. Primer was spun for few seconds, the required volume of sterile water was added according to manufacturer's instructions in a bio-safety hood using automatic pipette with sterile filter tips, labeled with date of preparation. Then the primers solution was mixed well and kept in a refrigerator at 4°C overnight. The solution was vortex, and 2-3 primers aliquots were made. Primers dilution was made by taking 10 µl of primers stock (100 pmol/ µl) and added to 90 µl sterile distilled water in a sterile Eppendrof tube to get a final concentration of 10 pmol/µl. After dilution, the primer was labeled and stored at -20°C until later use in PCR reactions. Specimens were denatured at 95°C for 1 min. A total of 35 cycles were performed. In the 35thcycle, the extension time was increased to 6 min. and primers were annealed at 65°C for 1 min. and extended at 72°C for 1 min. The 4ul specimen to be analyzed was adjusted to a total volume of 20 µl Maxime PCR PreMix (iNtRON Technology, Korea). Ready composed i-Tag DNA polymerase (5U/ µl) equal to 2.5 U, dNTPs 2.5 mM each, reaction buffer (10X) 1X and gel loading buffer 1X.
One µl of each primer was added to 14 µl nuclease- free water to get a final volume of 20 µl

which was entered an automated DNA thermal cycler (Convergys ® TD Peltier thermal cycler-Germany). This cycler was programmed to run at 95°C for 2 min. followed by 40 cycles each consisting of incubation at 95°C for 15 seconds. Combined with the 60 seconds, annealing and extension steps were performed at 60 °C[6].

20 ul of the amplified PCR product were analyzed on a 2% agarose gel, stained with ethidium bromide, and photographed by UV rays. Specimens showing a band of 147- bp were considered positive. The agarose powder was obtained from Ambion, USA; and the 1X TBE buffer was obtained from AppliChem. The gel was electrophoresed at 100V for 25min (Serva Blue Power 500, Germany); and it was visualized by UV trans-illuminator(Genius, UK).

All Real Time PCR positive specimens were selected and subjected to the conventional PCR testing using primers of:

1. *MgPa-1*: 179 to 206

* Forward primer (5'AGTTGATGAAACCTTAACCCC7TGG3')
* Reverse primer: (5'CCGTTGAGGGGTTTTCCATTTTTGC3')

2. *MgPa-3*: 435 to 460

* Primers correspond to the sequence base of coding strand of the same coding for the 140-kDa adhesion protein gene of *M .genitalium.*

3. *MgPa-B* gene:

* Amplification of this gene was developed by Jensen [7].

Interpretation of PCR results by drawing a blotting chart to compare the size of each amplicon against the DNA marker 100 bp (Vivantis, Malaysia). Positive results of *M. genitalium* will produce a band of 281 bp *MgPa.*

The PCR products of both 23S-rRNA and MgPa-B gene were subjected to purification using the QiaQuick PCR purification kit (QIAGEN, Hidlden ,Germany).500 µl of PB buffer were added to100 µl PCR specimen. The QiaQuick spin column was placed in the provided 2 ml collection tube to bind DNA. Then the specimen was applied to the QiaQuick column and centrifuged for 30-60 seconds. Flow-through was discarded, and the QiaQuick column was returned back to the same tube. The QiaQuick column was washed by adding 0.75 ml PE buffer and centrifuged for 30-60 seconds. Flow-through was discarded; the QiaQuick column was returned again to the same tube, and centrifuged for an additional one min. at a maximum speed. Then the QiaQuick column was placed in a clean 1.5 ml micro-centrifuge tube. The DNA was eluted by adding 50 µl EB buffer (10mM Tris-Cl, pH 8.5) or H_2O to the center of the QiaQuick membrane, and centrifuged for one min.

Sequencing of *23S-rRNA* gene and *MgPa-B* gene was performed by ABI Big Dye Terminator kit v. 2.0 (Applied Biosystems, Foster City, USA), and read by ABI 3100 genetic analyzer (Applied Biosystems, Foster City, USA). Both strands of amplified fragments were sequenced using the same PCR primers (SSI, Denmark).

The nucleotides sequences and amino acid sequences of *MgPa-1-3* were (SDN19,SDN51 and SDN151). They were aligned and compared with reference strain of *M .genitalium* G-37 (GenBank, Accession number NC000908) using CLUSTAL W 2.1 and MEGA5.2.2 software. This typing method was developed by Hjorth[8].

Sequences of the *23S rRNA* gene were assembled, consensus files were aligned with *Mycoplasma genitalium* wild-type positions 2,058/ 2,059, and analyzed by software packages. Sequence of *23S-rRNA* gene was as follows:

CCATCTCTTGACTGTCTCGGCTATAGACTCGGTGAAATCCAGGTACGGGTGAAGA
CACCCGTTAGGCGCAACGGGACGG*AA*AGACCCCGTGAAGCTTTACTGTAGCTTAAT
ATTGATCAA
AACACCACCATGTAGAGAATAGGTAGG
(Mutation binding sites are underlined and their positions "2,058 and 2,059" are marked in Italic)

Codons sequences were translated to correspondence putative protein using online Genemark software. Alignment of protein sequences was made using MEGA and CLUSTAL W software , to detect the presence of mutation.
The protein sequences were first blasted into NCBI to find 3D structure model with ID number. If the homologies models were not found, another online CHP software were used by coping and submitting the sequences. Furthermore the obtained homologies protein structure in Query pbd format were opened by Chimera software to locate the position of mutation in 3D structure protein.

Results:

As shown in Table (1), out of the 100 women investigated in this study, *M. genitalium MgPa* gene was detected in 4 urine specimens of which 2 were also detected positive in HVS. All positives were confirmed by detecting the *23S rRNA* gene. HVS revealed higher mean DNA load (640.92 geg/ml) of *M. genitalium* as compared with urine specimens (10.35 geg/ml).
3 peculiar *MgPa* genome sequences strains were detected in this study and were named SDN19, SDN51, and SDN151. These 3 strains were sent to the Gene Bank with the accession numbers: KF612736, KF612737, KF612738. The Gene Bank aligned the amino acids sequences of these three strains with the G37 *Mycoplasma genitalium* strain reference sequences (Fig. 1, 2, 3).

Table (1) Screening for *M. genitalium MgPa* gene in HVS and urine specimens

Specimen	No. of specimens	Positive *MgPa* genes	Negative *MgPa* genes	% Positives
HVS	100	2	98	2 %
Urine	100	4	96	4 %

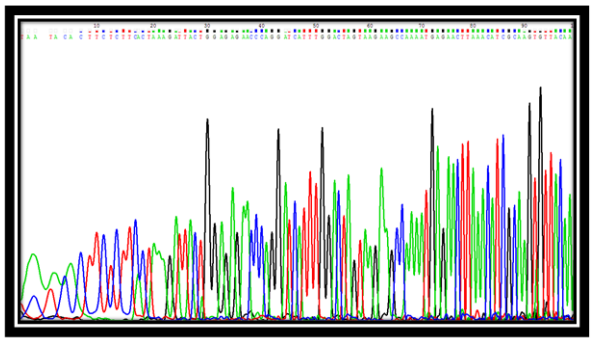

Fig. (1) Chromatogram showing the SDN19-*MgPa* (forward) sequence

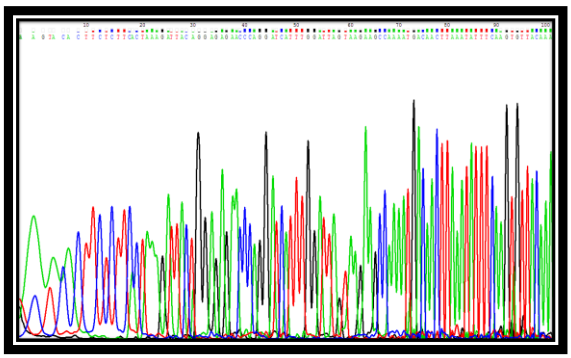

Fig. (2) Chromatogram showing the SDN51-*MgPa* (forward) sequence

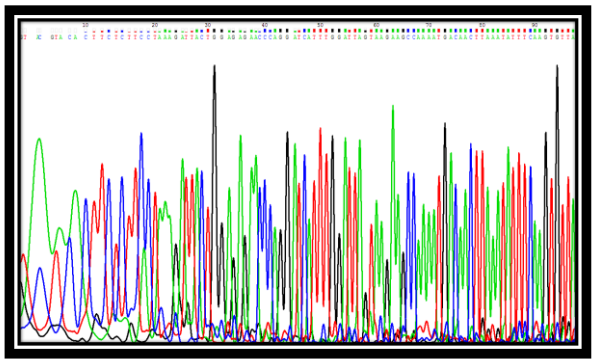

Fig. (3) Chromatogram showing the SDN151-*MgPa* (forward) sequence

The GeneBank spotted the following mutations in our SDN strains:
*** 4 mutations in SDN19 strain at positions: (D96/E, S101/A, S107/V, A117/S)**
*** 2 mutations in SDN51 and SDN151 strains at positions: (S107/V, G124/D)**

However, the GeneBank spotted no mutations in the nucleotides positions (20-058 and 20-059) in our SDN strains. Hence the GeneBank suggested to conduct more studies before considering our SDN strains as new emerging variants (Fig. 4).

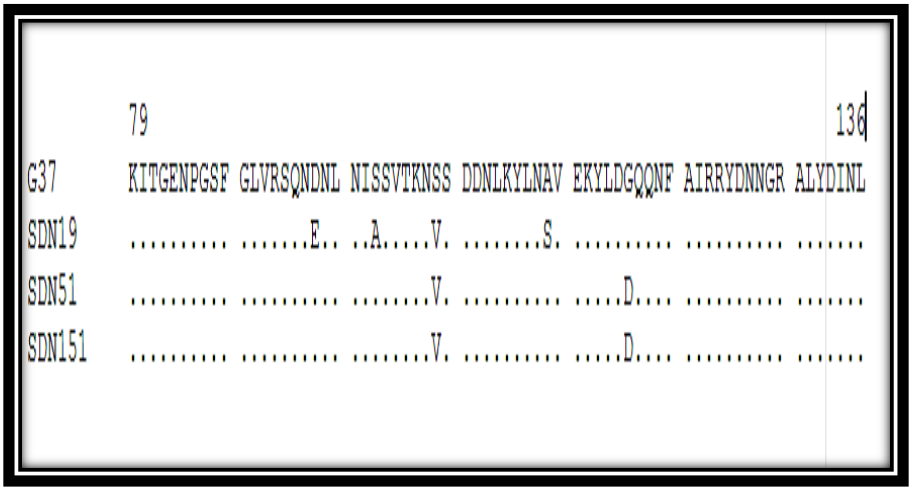

Fig. (4) Mutations of *M*gPa gene of Sudan sequences (SDN19, SDN51, SDN151) at positions 79–136. G37 is *M. genitalium* reference strain G37

With reference to *M. genitalium* G37 gene, the Gene Bank finalized the phylogenetic analysis of our SDN strains as follows:
*** SDN51 and SDN151 strains were found clustering with *M. genitalium* genotype 25.**
*** SDN19 was found clustering with *M. genitalium* genotype TX84 (Fig. 5).**

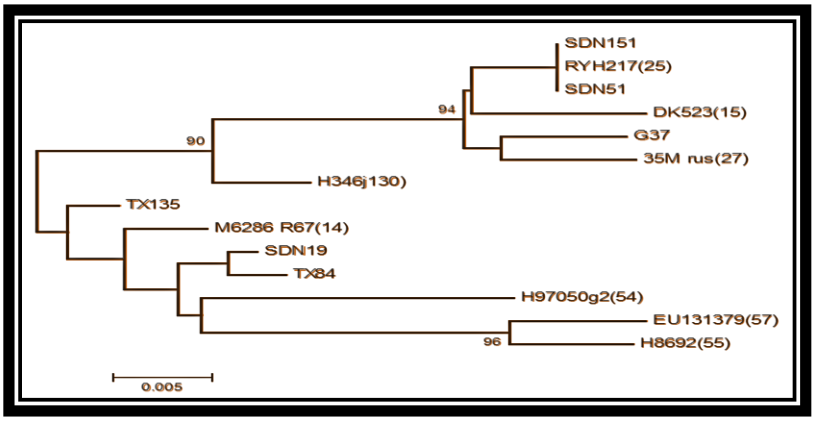

Fig. (5) Phylogenetic analysis of SDN151, SDN51, SDN19

Discussion

This is the first report conducted in Sudan to show the role of *M. genitalium* in the causation of genito-urinary tract infections among Sudanese women. The prevalence rate of *M. genitalium* among Sudanese women was found to be 4%. This finding was similar to that reported in Mozambique and Norway, but lower than that reported in United Kingdom (4.3%) and Kenya (26.3%) [9].

The quantification of *M. genitalium* DNA load was found higher in vaginal swabs than that found in first void urine. Rebecca *et al* (2011) [10] also reported that vaginal swabs had exhibited higher DNA load in 85.7% of cases. However, first-voided urine was found more sensitive (100%) in detection of *M. genitalium*.

When aligning our SDN sequences with *M. genitalium* wild-type, development of macrolide resistance had occurred. Resistance was revealed by the presence of mutations at positions 20,058 and 20,059. This may explain azithromycin treatment failure reported in Sudanese patients.

Also high frequency rates of *M. genitalium* infections were reported in USA (9.5%) and India (6.5%)[11]. The molecular technique applied in this study to amplify MgPb gene that encoded for MgPa protein which mediated attachment to ciliated epithelium of human fallopian tubes[12].

Significant difference ($p = 0.001$) in the DNA load were found in vaginal swab in comparison to the first void urine specimens. This may either due to contamination or due to colonization of *M. genitalium* in the vagina.

The mean age of infected women was found was 31years (age range18-44 years). The younger age women infected in this study was in good agreement with a recent study performed in United States of America where the association with *M. genitalium* infection in younger age decreases by10% for each year[13].

However the low level of education (high secondary) reported in this study ($p > 0.05$) was in disagreement with that reported in west African where there was a significant association ($p < 0.050$) between *M. genitalium* infection and low-level education[14].

In this study the leucocytes esterase screening test showed a 50 % frequency rate of *M. genitalium* infection among the women investigated. This result was inconsistent (76%) with other studies[15]. The four substitution mutations observed in SDN19 (D^{96}/E, S^{101} /A, S^{107}/V ,A^{117} /S) and two in SDN51and SDN151were (S^{107}/V, G^{124}/D).These mutations may indicate local predominant clinical variant of *M. genitalium* among Sudanese patients need to be confirmed by further studies. This finding was confirmed by the presence of 65 different MgPa-13 genotype variants recognized in 267 gene sequences found in nine countries.

Also other 8 different strains were identified among female prostitutes in Kenya[16 (93)]. These identical MgPa-13 variants showed clearly the existence of several common genotype sequences world-wide. This may be due to extensive sequence variability resulting from recombination between repetitive elements of MgPa-B and MgPa RS, which was recently found associated with clinical strain and *M. genitalium* strain G37[16].

In 2011, the frequency rates of macrolide resistance were 21%, 40% and 100% in Sweden, Denmark, and Greenland respectively[17]. In this study, all DNA sequence strains of

M. genitalium were found susceptible to macrolide antibiotics. This may be due to proper prescribed treatment of *M. genitalium* infections in Sudan. The obtained Id number 3D protein structure 3H49 pbd was found 30 % identical but not matching the *M. genitalium* model. It was belonging to other bacterial species such as *E.coli*. This may be due to similar protein domains. The obtained Id number was taken from the CHP server; using Chimera1.8 software; and the mutation was further located in a 3D model.

From this study it may be recommended that molecular diagnostic techniques of *M. genitalium* should be available in microbial laboratories. Screening programs of asymptotic pregnant women for *M. genitalium* should be adopted. Further studies are recommended to reveal more new *M. genitalium* variants among the Sudanese population; and to verify our SDN strains detected in this study.

Conclusion: The 23S-rRNA mutant strains of *M. genitalium* are susceptible to the macrolide antibiotic. Several amino acids substitutions mutations of *M. genitalium* were revealed by alignments analysis techniques. Leucocyte esterase test may be used as screening test for the detection of *M. genitalium.* Urine specimens were highly sensitive in detection of *M .genitalium* in comparison with high vaginal swabs.

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