Co-infection of Sexually Transmitted Infections in Adult Male Patients with Urethral Discharge: A need to strengthen surveillance in HIV-1 infected patients.

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Short title: Concomitant mixed sexually transmitted infections

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Abstract
The study aimed to evaluate sexually transmitted pathogens in a high risk sample population of men with urethritis. Urethral samples were collected from 400 men with urethritis and processed for common sexually transmitted pathogens such as Neisseria gonorrhoeae and Chlamydia trachomatis. Plate culture, microscopy and tissue cultures, were used to detect the presence of Neisseria gonorrhoeae, Chlamydia trachomatis and Herpes simplex virus (HSV). Screen for syphilis (Rapid Plasma Reagin-Becton Dickinson) and Human immunodeficiency virus (HIV) (Determine, Abbott, USA) were performed according to manufacturer’s instructions. Neisseria gonorrhoea (84%; OR 2.0, 95% CI: 1.0-3.0) was the major causative organism followed by Chlamydia trachomatis (65.5%; OR 1.9; 95% CI: 1.3-2.8). Serological results for syphilis on HIV positive patients reflected 17.5% with antibodies. There was a significant association with N. gonorrhoeae/C trachomatis and the number of sexual partners encountered (p = 0.03). In conclusion, the sample population revealed high rates of sexually transmitted infections. There were significant associations between STIs and continued high risk sexual practices in men. Sexually transmitted infections (STI) encourage risk factors for HIV infection, when it breaches’ the protective mucosal barriers and invade the immune system. Therefore, these findings support the need for studies that confirm the percentage of STIs using both “point of care” methodologies and clinical laboratory tests.

Keywords: sexually transmitted infections, HIV; sexual risk, urethral.

Introduction
Sexually transmitted infections (STIs) present a major public health concern in both industrialised and developing countries. Urethral discharge, a common presenting symptom, is preceded by an inflammation of the urethra and may present as either gonococcal urethritis (GU) or non gonococcal urethritis (NGU). The condition is primarily sexually acquired and multifactorial in its pathological approach and has been associated with an increased risk of HIV-1 infection [1, 2, 3, 4, 5]. Accordingly, it was demonstrated that Neisseria gonorrhoeae (N gonorrhoeae), due to its antigenic variation, evades the immune system which allows the organism to infect a high risk population of individuals [6]. The emergence of a penicillin resistant strain (lack of production of beta-lactamase) has further compounded the infection. The majority of individuals at risk of acquiring STIs are young adults due to unprotected coitus and multiple sexual contacts [7]. Specific urethral infections, due to Chlamydia trachomatis (C trachomatis) and/or other non-gonococcal pathogens are often under-diagnosed when appearing together with N gonorrhoeae. NGU may be due to C trachomatis, Herpes simplex virus (HSV), syphilis and other pathogens acquired sexually. It was observed that the most common STIs include a prevalence of N gonorrhoeae (9.5%), Chlamydia (5%) and syphilis (9.5%) which, increased at the time of HIV diagnoses [8, 9]. The facilitation of HIV transmission is completed by breaking the mucosal barriers and coming into contact with susceptible cells of the immune system [10].
Neisseria gonorrhoeae, the organism most frequently concomitant with Chlamydia trachomatis infections, involves the lower genital tract through direct infection of the columnar epithelium of mucosal membranes. Independent of their role as risk factors in Human immunodeficiency virus (HIV), STIs and their complications result in substantial morbidity and mortality [11, 12]. It has become imperative to gather details to understand and overcome the challenge of investigating and treating STIs in developing countries. Therefore, we examined the premise that increased genital infections, resulting from co-occurring STI, were prevalent in males infected with HIV.

Methods

Patients

This is a retrospective study carried out from 2008 to 2009. During this period, patients were recruited, specimens collected and all analyses performed. Clinical examinations, to confirm the presence of urethral discharge, were done. All men, with complaints of urethral discharges, were included into the study. However, patients who had received antibiotic treatment, in the past two weeks, were excluded from the study. Altogether, a total of 400 adult black male patients attending the Sexually Transmitted Infection (STI) clinic in KwaZulu-Natal, South Africa, were recruited. Ethical approval was received from the University of KwaZulu Natal and informed consent was obtained from all participants.

Specimen Collection

Swabs: Swabs were consecutively inserted 2-4 cm into the urethra and the urethral mucosa was gently scraped. A total of 4 swabs were done. Smears were made, with the first swab, on clean slides for Gram’s stain using microscopic examination. Modified New York City (NYC) medium was inoculated with the second swab for the isolation of N gonorrhoeae. The NYC plates were streaked and immediately placed into candle extinction jars and transported to the laboratory for incubation at 37°C in 6% CO2 atmosphere. Swabs collected thereafter, were placed separately into Chlamydial Transport Medium (CTM) for Chlamydia trachomatis and into Viral Transport Medium (VTM) for HSV culture.

Serological Specimens: Serum was aseptically separated from whole blood (5-10mls) and tested for antibodies to HIV and to Treponema pallidum.

Transport and Storage of Specimens

Within 2 to 4 hours of collection all CTM and VTM samples held at 4°C on ice, were transported to the laboratory. At the laboratory, specimens in CTM were inoculated onto prepared McCoy cell monolayer’s for the isolation of C trachomatis and the VTM specimens were cultured onto monolayers of Green monkey kidney cell lines for HSV.

Laboratory Methods

Direct Smear Examination: Gram Stain

A direct Gram stain was performed on all smears made at the point of collection. Air dried slides were heat fixed and stained using the standard Gram stain technique. Thereafter, slides were examined using 100X (oil immersion) objective, for the presence of pus cells, typical intracellular Gram negative diplococci and other bacteria.

Detection for Neisseria gonorrhoeae using Culture Technique

Modified New York City agar, were directly incubated at the site of specimen collection and incubated for 48 hours at 37°C in 6% CO2. N gonorrhoeae colonies were identified by their colonial morphology, as Gram negative diplococci which were oxidase positive (Organon Technica). The kidney shaped organism was confirmed by the carbohydrate fermentation test. Isolates were screened for penicillinase production, using chromogenic cephalosporin (nitrocephin - 0.5% w/v) as substrate (Oxoid).

Detection of N gonorrhoeae using Polymerase Chain Reaction

N gonorrhoeae cells, from swabs, were treated with a detergent solution to release gonococcal DNA. A second detergent solution was, thereafter, added to prepare the specimen for amplification. The target selection of N gonorrhoeae, based on sequence homology, was processed according to amplification, hybridization and detection sequences. Internal control amplification (biotin labeled CT/NG Internal Control) was utilised to permit optimal identification of processed specimens, containing substances that may interfere with polymerase chain reaction (PCR) amplification. The procedure was carried out according to manufacturers’ instructions (Roche Diagnostics). Briefly, the programme was as follows: Processed specimens were added to the amplification mixture reaction tubes containing master mix, in which PCR amplification occurred. Forty cycles of amplification was performed in a DNA thermal cycler (Perkin Elmer). The denatured amplicon was used in the hybridisation reaction and thereafter, detection of reactants using Avidin-Horseradish Peroxidase Conjugate (AHPC), was noted. The hybridization of amplicon, to the target-specific probe, increased the overall specificity of the test. APHC bound biotinylated amplicon, hybridized to the plate-bound, target-specific oligonucleotide probe, for N gonorrhoeae. After a series of washings, a coloured complex was formed and the reaction was stopped by the addition of a weak acid. The optical density was read at 450 nm and the measured absorbance was compared to a specific, predefined cut-off value for the detection of N gonorrhoeae DNA.

Tissue Culture for Chlamydia trachomatis

Collected specimens were cultured, in duplicate, onto
monolayers of cycloheximide treated McCoy cells in shell vials containing 12 mm round coverslips (Sterilin) and growth medium (Eagles Minimum Essential Medium with 10% foetal calf serum). The shell vials were incubated for 72 hours at 37°C in 5% CO₂ atmosphere. Post incubation the vials were aspirated, the cells mounted onto clean slides, coverslips were fixed in 10% methanol, air dried, and stained with immunofluorescent stain according to manufacturer’s instructions (Syva Co, USA). The slides were examined for C trachomatis at 40X magnification and morphology confirmed at 100X (oil immersion) magnification (Olympus Fluorescence Microscope, BH2). Positive and negative control slides were stained with each batch of slides so as to verify the quality of the stain.

Culture for Herpes simplex virus

Vervet monkey kidney cells were cultured to isolate Herpes simplex virus in urethral specimens. Supernatant of 200 µl were inoculated onto cell monolayers, done in duplicate. The inoculated cells were incubated at 37°C and observed daily for cytopathic effects (CPE), for up to 14 days. The observation of characteristic CPE was considered a positive result and the presence of HSV was confirmed by staining with fluorescein isothiocyanate labeled monoclonal antibody (MA Bioproducts) against HSV (Types Iand 2).

Serological Methods

Syphilis: Screening test for the detection of antibodies to syphilis was performed, according to manufacturer’s instructions, using the non-specific, Rapid Plasma Reagin (RPR) test (Becton Dickson). All tests, which were positive, were serially diluted and the diluted sera were further tested, using the semi quantitative tests for RPR. Treponema pallidium haemagglutination antibody test (TPHA), specific for syphilis, was performed on all sera according to manufacturer’s instructions (Omega Diagnostics). This was done to confirm all positive RPR results and to give an indication as to the number of patients with past exposure to syphilis infections. Agglutination of test cells but not control cells was indicative of the presence of specific antibody to Treponema pallidum. When TPHA reading pattern could not be conclusively interpreted, the indirect fluorescent treponemal antibody absorption (FTA-Abs) test using treponemal antigen, and fluorescein labeled anti-human immunoglobulin conjugate (BioMereux) was used as a specific test for confirmation of syphilis.

Human Immunodeficiency Virus: Antibodies to HIV-1 was performed on all sera using a routine ELISA tests (Determine, Abbott, USA) and Vironostika HIV Uni-form II Organon Teknika). If sera tested positive on both the ELISA tests, the patients were considered positive. Discrepant results between the first and second tests were further tested by an indirect immunofluorescent antibody technique and Western blot (Biotechnology Diagnostics).

Statistical Analyses

SPSS statistical software was used for all descriptive analyses in the study. Correlations were assessed using Pearson’s correlation test. The Chi Square test was used for assessing probabilities between variables and categories. A p-value <0.05 was considered statistically significant. Linear regression was used to determine the significance of inter-related interactions and risk ratios. Variables which did not alter the risk ratio were eliminated. Variables which changed the risk ratio were maintained and extensively evaluated.

Results

Demographic Data of Patients

The mean number of lifetime sexual partners was 4 with a mean age of 34.5 years (range: 15-55 years). A total of 330 (82.5%) were single and 70 (17.5%) were married. Single partner relationship were stated by 40 (10.0%). Positive

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were diagnosed in 274 (72%) of the sample population. Of 400 patients 380 (95.0%) had a urethral infection. A combination of 6 (1.6%) patients had urethral infections as well as antibodies to syphilis and HIV, whilst N gonorrhoeae and C trachomatis was seen in 222 (58.5%) patients (Figure 1). There was a significant association with N gonorrhoeae/C trachomatis and the number of sexual partners encountered (p = 0.03). Antibodies to HIV only, were detected in 12 (3%) patients. The most common infecting organism, in this study population, was N gonorrhoeae (84.0%; OR 2.0, 95% CI: 1.0-3.0) followed by C trachomatis (65.5%; OR 1.9; 95% CI: 1.3- 2.8). Syphilis was detected in 27.0% whilst 82.0% were HIV positive (OR 3.21, 95% CI: 1.32- 9.0). HSV was excluded in the logistic regression table, due to non infection in the current population of male samples (Fig 1).

Intracellular Gram negative diplococci were seen in 292 of 336 (86.9%) specimens. The remaining 44 (13, 1%) specimens were picked up as additional culture positive urethral exudates. In addition, beta-lactamase, producing strains were observed in 34 (10.1%) culture positive N gonorrhoeae isolates. A further 44(11%) was recorded using PCR, which brought the N gonorrhoeae value to 95% when compared to 84% culture positive.
Ninety three percent of men (138 of 148) with antibodies to HIV had concomitant mixed infections (Figure 2). *C trachomatis* and *N gonorrhoeae* were detected in 96% (242 of 252) men who did not demonstrate antibodies to HIV. There was no significant differences between patients who had gonococcal and/or chlamydial infections and the presence/or absence of antibodies to HIV-1. However, a significantly higher percentage of sexually transmitted infections were demonstrated in the population sample. Serological results for syphilis, on HIV positive patients, showed that 70 of 164 were positive (Figure 3).

### Table 1: Overall Results of Sexually Transmitted Pathogens in Men with Urethritis. N=400

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Number Positive</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N gonorrhoeae</em> (Culture)</td>
<td>336</td>
<td>84.0</td>
</tr>
<tr>
<td>Gram stain</td>
<td>292*</td>
<td>73.0</td>
</tr>
<tr>
<td>β-lactamase positive strains</td>
<td>34*</td>
<td>10.1</td>
</tr>
<tr>
<td>PCR</td>
<td>380</td>
<td>95</td>
</tr>
<tr>
<td><em>C trachomatis</em></td>
<td>262</td>
<td>65.5</td>
</tr>
<tr>
<td>Serology:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syphilis</td>
<td>54</td>
<td>13.5</td>
</tr>
<tr>
<td>HIV</td>
<td>164</td>
<td>41.0</td>
</tr>
<tr>
<td>HSV (Type II)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
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*All 292 Gram stained smears which showed Gram negative, intracellular, diplococci on microscopy were grown on culture. * Represents 34/336 culture positive *N gonorrhoeae* results, from men with urethritis.

**Figure 1:** Single and Mixed Infection of *N Gonorrhoeae* and *C trachomatis* in Men with Urethritis

**Figure 2:** Human Immunodeficiency Virus Status in Men with Concomitant *C trachomatis* and *N gonorrhoeae* Infections. N=400

![Diagram](http://www.jabsdirect.com)
Discussion

The symptoms, experienced by male patients are, usually one of dysuria, penile tingling or urethral discharge [13]. On investigation, the primary pathogens isolated are N gonorrhoeae and C trachomatis. The findings in this study, more specifically, shows that mixed gonococcal/chlamydial infections account for up to 95% of STI clinic males. At the site of collection a presumptive diagnosis of N gonorrhoeae was made, based on observation of gram-negative intracellular diplococci in gram-stained smears of urethral discharges from men. Confirmatory identification, using biochemical techniques, revealed that 85% men with urethritis were infected with N gonorrhoeae. An additional 44 (11%) patients were identified, with N gonorrhoeae, using the PCR technique. Recorded percentage, by the PCR technique, may be attributed to non-viable fragments of a previous infection, since this is a high risk population. Internationally, the range is from 11% to 38.2% [14]. When compared to other sub-Saharan Africa of 15% to 20%, the current study reveals that 65.5% of males are infected with C trachomatis [15, 16, 17, 18]. Overall, the values showed an increase in male urethritis when compared to other studies. One explanation for this phenomenon may be attributed to the high risk of acquiring a STI when diagnosed with HIV. There appears to be an association between HIV and the acquisition of urethritis. Sexually transmitted diseases such as N gonorrhoeae, C trachomatis, HSV and syphilis can increase the risk of HIV transmission [19]. It is also well documented that HIV negative individuals, who are infected with an STI, are at increased risk of becoming infected with HIV-1[20, 21]. This study reports that approximately 41% of males were HIV positive and were co-infected with other STIs. These infections are likely to be particularly important in promoting the sexual transmission of HIV-1 and should therefore, be the focus of HIV prevention strategies. A less common pathogen, identified in patients with urethritis, was Herpes simplex virus. The patient population appeared to be less at risk of acquiring HSV-2, when compared to N gonorrhoeae and C trachomatis.

Risk factors which increase the potential for exposure to infected sex partners, and thus infection, include the number of multiple sex partners over the individual’s lifetime [22]. Our study reflected a significant association with increased number of partners (p=0.03). Further contributing factors are the magnitude of the epidemic, the potentially high risk in the number of sexual contacts and the abstinence of condoms, particularly with men. Based on findings of the analyses, the prevalence of syphilis was made in two sets of patients. Patients were either HIV positive with antibodies to syphilis (17.5%) or HIV negative with antibodies to syphilis (16.5%). We observed that the value exceeded a meta-analysis study, which reflected a prevalence of 9.5% [3] However, the meta-analysis was done on studies available in both developed and developing countries. Our study reflects data obtained in a developing country, experiencing a limitation in adequate resources. It is evident that N gonorrhoeae and C trachomatis are the leading causes of STI infection. The risk associated with other STIs is further complicated with HIV. Further studies are necessary to determine changes in the relationship of STIs and HIV infection in the male population. Whilst infections in this study, is fairly comprehensive, it is also probably true for developing countries, that there are at present no reliable statistics on the true prevalence of sexually transmitted infections. The correct diagnosis and treatment for these diseases are inadequate in developing countries, where major
public health services are underfunded and over utilised. The need for control of STI is urgent, since it is recognised as independent risk factors for the acquisition of HIV. Failure to diagnose and treat infections such as N gonorrhoeae, C trachomatis and syphilis do have major health and social challenges, with respect to male infertility, amongst other causes [23]. Therefore, there is a need for formal monitoring and surveillance of STIs.

Conclusion
The syndromic management of STIs has been documented to achieve different ranges of success [24, 25]. Studies have shown that the number of lifetime sexual partners is a major measure of risk in acquiring STIs, including urethritis. The similar factors shared by both STIs and HIV, should encourage individuals to modify their sexual behaviour and practices thereby, reducing the incidence of STIs [26, 27, 28]. The aim of treatment, include prevention of complications, in patients and identifying the sexual contacts between partners. Partner treatment is imperative in reducing the transmission rate of co-infections and the risk of recurrence should be address with empiric therapy. Behavioural changes are important and should be advocated at all times.

Conflict of Interest: None Declared

Acknowledgement
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References