Paper IV
Herpes simplex virus infection and genital ulcer disease among STI patients in Dar es Salaam, Tanzania

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Short title: HSV and GUD in Tanzanian STI patients.

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Abstract

The relative importance of Haemophilus Ducreyi and Treponema pallidum in genital ulcer disease (GUD) has recently decreased in Africa, whereas that of HSV-2 has increased. We have analysed 301 lesional specimen from Tanzanian GUD patients for the presence of Haemophilus Ducreyi, Treponema pallidum and herpes simplex virus types 1 and 2 (HSV-1, HSV-2) by performing a separate PCR for each pathogen. Infectious agents were detected in 211 (70%) of the cases. A single pathogen was found in 191 samples and two or more pathogens in the remaining 20. HSV-2 represented 83% of all identified pathogens, HSV-1 8%, Treponema pallidum 5% and Haemophilus Ducreyi 5%. HSV-1 was identified as a single pathogen in 4 samples, in combination with others in additionally 14. Thus, HSV-1 can be the cause of GUD also in Africa. Regular surveillance of GUD aetiology is important in programs for management of GUD and HIV in Africa.

Key words: HSV-1, HSV-2, genital ulcer disease, STI, Tanzania

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Introduction

Sexually transmitted infections (STI) often present as genital ulcer disease (GUD). STIs represent a worldwide problem, and the annual global incidence is estimated to exceed 20 million cases (1). Genital ulcers are more common in Africa than in Western Europe and North America (2). Previously, the leading cause of GUD in African countries used to be *Haemophilus Ducreyi*, followed by *Treponema pallidum* (3-5). However, the situation changed markedly from the late 1980s so that the relative importance of *Haemophilus Ducreyi* and *Treponema pallidum* decreased significantly, whereas that of HSV-2 increased (6, 7). Recent studies from Africa have confirmed that most genital ulcers are now caused by HSV-2 infection (8, 9). However, the aetiology of GUD may still show geographical variations and change from time to time within the same area.

HSV-2 is considered to be mainly sexually transmitted, and infectious virus can be shed during symptomatic as well as asymptomatic periods. In contrast, HSV-1 most often causes orolabial lesions, and transmission is primarily by non-genital personal contact. However, both viruses are capable of causing either genital or orolabial infection and can produce mucosal lesions that are clinically indistinguishable (10). In recent years an increasing proportion of genital herpes in Western Europe and the USA is caused by HSV-1 infection (11-14), whereas HSV-1 does not seem to be the causative agent of GUD in Africa.

The traditional strategy for managing GUD has relied on individual patient diagnosis and treatment. However, due to scarce laboratory facilities for diagnosing GUD in resource-poor settings, syndromic management has been the most commonly used approach in
Africa. Selection of treatment is then based on which causative agent is expected to be most common in a particular area.

Different laboratory methods such as direct antigen detection, virus isolation by cell culture and polymerase chain reaction (PCR) are commonly used for the diagnosis of genital herpes. PCR has markedly improved the laboratory diagnosis of genital herpes (15-18) as well as of *Treponema pallidum* and *Haemophilus Ducreyi* (19-24). Culture of *Haemophilus Ducreyi* or of HSV is time-consuming and may require facilities that are not always available, when resources are limited. *Treponema pallidum* cannot be grown in vitro and detection of antibodies in the serum has relatively low sensitivity in primary syphilis and also may not distinguish between current and previous infection.

Control of STIs has recently become a greater priority in global public health campaigns throughout the world, due to the association between STIs – particularly genital ulcers - and the acquisition of HIV infection (25-27). Although associations between HIV and chancroid or syphilis are statistically significant (28), the link to HSV-2 seems even more important (25, 28-30). The latter relationship emphasises the need for effective management of genital herpes as part of strategies for HIV control. Effective treatment of STI is indeed associated with reduction of HIV infection (26).

In Tanzania, recent studies conducted on small population groups with genital ulcers suggest that HSV-2 is the leading agent (8, 9, 31). The current study was designed to include larger numbers of Tanzanian patients at risk for STIs, to span a longer period of time, and also to include HSV-1.
MATERIALS AND METHODS

Study population

STI patients with GUD attending the infectious Disease Clinic in Dar es Salaam, Tanzania between 1999 and 2001 were invited to participate in the study. Verbal consent was given by 319 consecutive patients. All participants were interviewed to respond verbally to a questionnaire. The study was approved by the Tanzanian ethical clearance committee.

Clinical specimens

Sterile dacron swabs were used to collect material from the bases of the ulcers. The swabs were immediately put into a liquid transport medium (Copan, Brescia, Italy) and stored at – 80°C until further analysis. Serum samples were obtained for analysis for antibodies against HIV, Treponema pallidum, HSV-1 and HSV-2.

Preparation of positive controls for PCR

Baby hamster kidney (BHK) cells were cultured in Eagles minimal essential medium supplemented with 2% calf serum and antibiotics, then infected with either HSV-1 17syn+ 8 (32) or with HSV-2 HG52 (33). Propagation of virus and isolation of crude virus preparations were performed as described by Sathananthan et al (34). The pellets were dissolved in a small volume of medium and the number of plaque-forming units per ml measured. DNA was extracted according to the method of Slomka et al (17) and used as control in the PCR reactions.

Haemophilus Ducreyi, kindly provided by the National Institute of Public Health in Oslo, Norway, was grown on chocolate agar. DNA was prepared from one colony of bacteria as described by Mohn et al (35).
Treponema pallidum DNA was generously provided by Centres for Disease Control and Prevention, Atlanta, USA. The material was a crude extract from rabbit testicular tissue containing approximately $10^8$ Treponema pallidum organisms per 50 ul.

**PCR**

DNA was extracted from the clinical samples using the QIA amp Mini Kit from Quiagen (Germany) according to the blood and body fluid spin protocol as described by the manufacturer. The sequences for all primers are specified in the quoted articles describing the PCR methods used.

Nested PCR for detection of HSV-1 DNA and HSV-2 DNA, respectively, were performed as described by Cinque et al. (16), except that the two reactions were run separately and the annealing temperature was reduced from 57°C to 55°C. The primers were targeted to a non-coding region in the 3’-end of the gene for glycoprotein D of HSV-1 or to a portion of the coding region of the glycoprotein G-gene of HSV-2 (8, 15, 16) The amplified products from the inner primers were 101 bp for HSV-1 and 139 bp for HSV-2. Thus, the PCRs distinguished between HSV-1 and HSV-2.

Primers for detection of Haemophilus Ducreyi were located to the 16S rRNA gene, and nested PCR was performed as described by Bruisten et al. (1), except that the primer concentration was reduced to 0.06 μM. The product from the inner primers was 309 bp long.

The PCR for Treponema pallidum amplified a 377 bp fragment of the DNA polymerase I gene (pol A). The method described by Marfin et al. (24) was slightly modified by adding
be attain to a final concentration of 1 M, to facilitate the reaction. This yielded more distinct bands in the agarose gel.

Analysis of control DNA showed that the PCR methods detected 2-3 plaque-forming units (pfu) of HSV-1, 1 pfu of HSV-2, 4-5 pg of *Haemophilus Ducreyi* DNA and approximately 20 DNA copies of *Treponema pallidum* DNA. DNA was omitted from negative controls for the PCR reactions, and positive controls contained the appropriate types of control DNA.

Amplified sequences were separated by electrophoresis in agarose gels, and products of correct sizes identified by comparison with a DNA ladder standard (Gene Ruler 100bp ladder from MBI Fermentas, Lithuania). The agarose concentration was 3%, 2% and 1.5% for detection of DNA from HSV-1 or HSV-2, *Haemophilus Ducreyi* and *Treponema pallidum*, respectively.

Various combinations of PCRs were tested to detect two or more microorganisms simultaneously. However, all combinations reduced the sensitivity as compared to separate PCRs, which consequently were used for all four pathogens.

**Statistical methods**

To test the hypothesis of no bivariate association between identified pathogens and information given in the questionnaire Pearson chi square test was used. When appropriate the strength of associations was estimated by calculating the odds ratio (OR).

In analyses involving continuously variable (as age, duration of ulcer etc) the $\chi^2$ test for trend (linear by linear association) was applied. All statistical tests were performed at a significance level of 0.05.
Data analysis was performed using SPSS for Windows release 10.0 (SPSS Inc. Chicago, IL, USA).

RESULTS

Participants and data collection
The study included 319 persons of whom 301 responded to the questionnaire and allowed lesional sampling. Characteristics of the study population are shown in Table 1. Ninety-one of the respondents were women and 210 were men. The age spanned from 17 to 60 years, with a mean of 29.9 years. Nearly all of the participants identified themselves as heterosexual individuals. The number of sexual partners during the last 12 months varied from 0 to 10, but 50% of the participants reported 2-4 partners. Females reported significantly lower numbers of sexual partners than did men. Approximately half of the participants denied any previous STI, and a similar number reported genital ulcers for the first time. The duration of the ulcers before attending the STI clinic varied from 1-2 days up to several weeks, but around 50% of the individuals had had ulcers for more than one week.

Detection of pathogens in lesional material
The detection of pathogens is based on the 301 patients responding to the questionnaire. Table 2 shows that pathogens were detected in 211 (70%) of the patients. HSV-2 was by far the dominant agent, as 91.6% of the single infections were caused by this virus. In contrast to our previous study from the same region (8) we now found HSV-1 as a single causative agent in a few (4) cases. Similar small numbers were observed for Treponema
pallidum and Haemophilus Ducreyi. In 20 cases (9.5%) there was a combination of two or more pathogens, one of these contained HSV-1, HSV-2 and Treponema pallidum. The most frequent combination was HSV-1 and HSV-2 (11 cases).

The relative importance of the 4 pathogens is shown more clearly in Table 3. This table lists all cases in which a specified pathogen is detected, so that the total number is larger than in Table 2. HSV-2 was present in 82% of the cases. Although the numbers for the other pathogens are small, HSV-1 was apparently at least as frequently identified as Haemophilus Ducreyi and Treponema pallidum.

Analyses for associations between pathogen and demographic/behavioural factors

We found very few statistically significant associations between identified etiological agents or antibodies and demographic or behavioural factors (gender, age, coitarchal age, sexual preference, number of sexual partners (recent and life-time), marital status, educational level, duration and number of ulcer, previous STI or previous genital ulcer). Hence, the results are not tabulated, but will be discussed later.

DISCUSSION

We have analysed material from 301 genital ulcers in Tanzanian STI patients and the infectious agents were identified by PCR. Consistent with previous reports (6, 8) HSV-2 was found to be the dominant cause. This virus was detected in 83% of the cases in which one or more pathogens were identified (Table 3), in 92% of the samples where a single pathogen was observed (Table 2) and in 64% of all patients with genital ulcers. The latter percentage is very similar to what has previously been reported from studies of smaller cohorts in the same area (8, 9). As in other African regions, Haemophilus Ducreyi and
*Treponema pallidum* were quantitatively of minor importance. One possible explanation for the relative decline of syphilis and chancroid is that the syndromic treatment approach has been targeted to bacterial, but not viral genital infections. Improved microbiological diagnostic methods for identification of HSV might also have played a role. The PCR methods used were slightly modified and also run separately, to increase their sensitivities and specificities. Nevertheless, no pathogen was detected in 30% of the ulcers. This is consistent with other reports (9, 36). Ulcers with unidentified aetiology might be caused by pathogens other than those searched for in the present study. In 13% and 12% of Behets cases (36) granuloma inguinale or lymphogranuloma venereum, respectively, was diagnosed clinically. Furthermore, the likelihood of defining an etiological diagnosis might decrease with increasing duration of the ulcer, particularly in HSV infections. This was also evident in our patients where shorter duration of genital ulcer was associated with higher proportion of identified HSV-2 cases (p=0.002) (data not shown). Overall, in 1-4 days old ulcers we could identify an etiological agent in 79.3% of the cases, whereas this figure dropped to 66.2% if the ulcer had been present for more than one week (data not shown).

The trend in Western Europe and the USA, that HSV-1 has become increasingly important in genital infections, was now observed at a significant — but still low level (8%) — in Tanzania. We were not able to demonstrate any statistically significant age differences between HSV-1 and HSV-2 PCR positive patients. Males were less likely than females to have HSV-2 infection, however, the difference was not statistically significant (p=0.12)(data not shown).

We could not identify any demographic or behavioural differences between patients with one or more identified agents. Male patients seem more likely than female to have
syphilis and chancroid, however, the numbers are small, and statistical significance was not reached (*Treponema pallidum*: Odds Ratio (OR) 4.05, 95% Confidence Interval (CI) 0.51-32.45, *Haemophilus Ducreyi*: OR 5.00, 95%CI 0.64-39.32.)

By accident, the sera were lost before being analysed for antibodies against HSV-1, HSV-2 and *Treponema pallidum*. Almost half of the patients (45.3%, females (F) 55.7%, males (M) 40.8%) had antibodies against HIV-1. In a similar cohort of STI patients in Dar es Salaam we have recently observed antibodies against HIV-1 in 33% (F 43%, M 26%) and against HSV-2 in 70% (F: 77%, M: 66%) (37, 38). Furthermore, in that cohort there was a strong and statistically significant association between HSV-2 seropositivity and HIV infection. An association between HIV transmission and HSV-2 seropositivity has been shown in numerous studies (30).

Epithelial disruption in symptomatic genital HSV-2 infection can be a portal of entry for HIV. However, in a prospective study in high-risk HIV-negative individuals, higher HIV transmission rates have also been demonstrated in HSV-2 seropositive individuals irrespective of genital lesions (39). Consequently, different or additional mechanism may be involved in the interplay between HSV-2 and HIV (40). It has been shown that HSV regulatory proteins may upregulate HIV replication and thereby increase titre of mucosal HIV shedding (40).

HSV-2 infections have increased in African countries (37, 41, 42). In the present situation with predominantly viral GUD aetiology, the approach of syndromic diagnosis and treatment, focusing on bacterial infections, will not be effective. WHO has recently issued new guidelines for the syndromic management of genital ulcer disease that include antiviral treatment for lesions consistent with genital herpes. Optimal management of HSV-2 infections, particularly in areas with high prevalences of HSV-2 and HIV, is
therefore important, including regular surveillance programs of genital ulcer disease. The present work indicates that such programs should also include HSV-1.

Acknowledgements

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of a PCR method for detection of Treponema pallidum in clinical specimens using  

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Table I. Characteristics of the study population

<table>
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<tr>
<th>Response to questionnaire</th>
<th>Women No/%</th>
<th>Men No/%</th>
<th>Total No/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Answered (numbers)</td>
<td>91</td>
<td>210</td>
<td>301</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>28.7</td>
<td>30.4</td>
<td>29.9</td>
</tr>
<tr>
<td>Coital age (mean)</td>
<td>16.4</td>
<td>17.6</td>
<td>17.2</td>
</tr>
<tr>
<td>Sexual preference (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heterosexual</td>
<td></td>
<td></td>
<td>97.3</td>
</tr>
<tr>
<td>Homosexual</td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>Bisexual</td>
<td></td>
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<td>2.3</td>
</tr>
<tr>
<td>Sexual partners last 12 months (%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>9.0</td>
<td>3.4</td>
<td>5.1</td>
</tr>
<tr>
<td>1</td>
<td>58.4</td>
<td>26.6</td>
<td>36.0</td>
</tr>
<tr>
<td>2-4</td>
<td>32.6</td>
<td>61.8</td>
<td>52.9</td>
</tr>
<tr>
<td>5-10</td>
<td>0</td>
<td>8.2</td>
<td>5.6</td>
</tr>
<tr>
<td>Life time sexual partners (mean number)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>2.2</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>1</td>
<td>48.4</td>
<td>10.5</td>
<td>22.0</td>
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<td>2-4</td>
<td>41.8</td>
<td>51.7</td>
<td>48.7</td>
</tr>
<tr>
<td>5-10</td>
<td>6.6</td>
<td>30.8</td>
<td>23.4</td>
</tr>
<tr>
<td>&gt;10</td>
<td>8.8</td>
<td>32.8</td>
<td>25.1</td>
</tr>
<tr>
<td>Previously STI other than GUD (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>41.8</td>
<td>57.1</td>
<td>52.5</td>
</tr>
<tr>
<td>Yes</td>
<td>52.7</td>
<td>41.9</td>
<td>45.2</td>
</tr>
<tr>
<td>Do not know</td>
<td>5.5</td>
<td>1.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Previously GUD (%)</td>
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<tr>
<td>No</td>
<td>44.0</td>
<td>55.2</td>
<td>51.8</td>
</tr>
<tr>
<td>Once</td>
<td>17.6</td>
<td>26.2</td>
<td>23.6</td>
</tr>
<tr>
<td>Several times</td>
<td>38.5</td>
<td>18.6</td>
<td>24.6</td>
</tr>
<tr>
<td>Duration of the present ulcers (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2 days</td>
<td>3.3</td>
<td>6.7</td>
<td>5.6</td>
</tr>
<tr>
<td>3-4 days</td>
<td>27.5</td>
<td>21.4</td>
<td>23.3</td>
</tr>
<tr>
<td>5-7 days</td>
<td>26.4</td>
<td>18.1</td>
<td>20.6</td>
</tr>
<tr>
<td>1-2 weeks</td>
<td>15.4</td>
<td>25.7</td>
<td>22.6</td>
</tr>
<tr>
<td>&gt;2 weeks</td>
<td>27.5</td>
<td>28.1</td>
<td>27.9</td>
</tr>
<tr>
<td>HIV seropositive</td>
<td>55.7</td>
<td>40.8</td>
<td>45.3 (p=0.019)</td>
</tr>
</tbody>
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Table 2. Number of patients with unknown, single or multiple pathogens

<table>
<thead>
<tr>
<th>Unidentified</th>
<th>Identified</th>
</tr>
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<tbody>
<tr>
<td>No (%)</td>
<td>No (%)</td>
</tr>
<tr>
<td>90 (30)</td>
<td>211 (70)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Single pathogen (No = 191)</th>
<th>More than 1 pathogen (No = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1</td>
<td>HSV-2</td>
</tr>
<tr>
<td>4</td>
<td>175</td>
</tr>
</tbody>
</table>

Tp=Treponema pallidum  HD=Haemophilus Ducreyi  HSV=Herpes simplex virus
**Table 3. Total number of infectious agents from genital ulcers**

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Detected</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No</td>
</tr>
<tr>
<td>HSV-1</td>
<td>18</td>
</tr>
<tr>
<td>HSV-2</td>
<td>192</td>
</tr>
<tr>
<td>Treponema pallidum</td>
<td>10</td>
</tr>
<tr>
<td>Haemophilus Ducreyi</td>
<td>12</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>232</td>
</tr>
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</table>