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We standardized a serologic enzyme immunoassay (EIA) for human immunoglobulin G and M antibodies against Haemophilus ducreyi. We evaluated the performance of this test with respect to the time from acute chancroid and coinfection with human immunodeficiency virus (HIV). Antibody to a crude, soluble bacterial antigen of one H. ducreyi strain was detected in a panel of serum samples from clinically and microbiologically confirmed cases of chancroid and from controls. Test interpretation was standardized for optimal sensitivity and specificity. Performance of the EIA was enhanced in the period of early convalescence from acute primary chancroid and was not diminished in the presence of HIV coinfection. The EIA performed adequately as a serologic screening test for field evaluation and epidemiologic application in conjunction with sexually transmitted disease and HIV detection and control efforts.

Chancroid is a sexually transmitted genital ulcer disease (GUD) caused by Haemophilus ducreyi. It is the leading cause of GUD in many developing countries (1, 21, 26). It was once uncommon in industrialized nations, but there has been a rise in the incidence of chancroid in U.S. cities, and there have been sustained outbreaks since 1984. More than 5,000 cases were reported in 1987 (21). Outbreaks are associated with urban poverty, prostitution (2), and illicit drug use (8).

Medical diagnosis of chancroid has relied on clinical features and microbiologic culture (7, 9, 10, 12) in settings where the disease is known to exist and to be prevalent. With the inconsistent sensitivity of diagnosis by culture, other methods that use monoclonal antibodies, immunofluorescence, or immunologic detection have recently been reported (14, 15, 23) for diagnostic or epidemiologic application. Since these techniques are relatively expensive and depend on the availability of ulcer specimens for direct detection of bacterial antigen, their practicality for use as an epidemiologic screening test remains limited.

Within the past 10 years there has been a renewed public health interest in chancroid, principally because of a demonstrated association with human immunodeficiency virus (HIV) infection (2, 11, 27). Observational studies have shown a high rate of HIV transmission between sexual partners in the presence of chancroid (2) because of the increased transmissibility of HIV in the presence of GUD (11, 19, 27). In cases in which chancroid is identified in core groups, which are demographically identifiable epidemiologic reservoirs of high-frequency sexually transmitted disease (STD) transmitters, control of H. ducreyi infection is a feasible goal with a high potential impact on HIV transmission (3, 4).

Seroepidemiologic detection and measurement of the prevalence of chancroid in targetable reservoir or core groups and evaluation of targeted medical and educational public health STD control programs require a simple, inexpensive assay. A desirable serologic test must not only have technical simplicity and be of low cost but it must also have high or at least well-defined performance characteristics. We developed a sensitive assay that was standardized for the optimal detection of H. ducreyi antibody and characterized its performance.

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MATERIALS AND METHODS

Antigen preparation. Soluble bacterial antigen was prepared from H. ducreyi 35000, which was obtained from a 1975 outbreak of chancroid in Winnipeg, Manitoba, Canada (12). Bacteria were cultured on chocolate agar base supplemented with 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.)–1% bovine hemoglobin–5% fetal bovine serum. Plates were incubated at 33°C in 5% CO2 for 48 h. Bacterial lawns were harvested and suspended in 10 ml of sterile phosphate-buffered saline (PBS), and the suspension was washed four times by centrifugation at 1,500 × g for 15 min at 4°C. The pellet was suspended in 10 ml of PBS with 1% sodium dodecyl sulfate and sonicated three times by using 30-s pulses with 15-s pauses. The suspension was gently rocked on a shaker for 2 h at 20°C, and the supernatant was collected after centrifugation at 50,000 × g for 90 min. The protein concentration of the supernatant was determined for future adjustments by using a protein assay (Bio-Rad Laboratories, Richmond, Calif.), and the supernatant was stored in aliquots at −70°C.

Sorbent preparation. Respiratory isolates of Haemophilus parainfluenzae, Haemophilus parahaemolyticus, and Haemophilus influenzae obtained from a hospital laboratory were grown on gonococcal agar supplemented with 1% IsoVitaleX–1% hemoglobin and were incubated at 37°C in 5% CO2 for 24 h. Antigen was prepared in the same manner as described above for H. ducreyi. The protein concentra-
tion was adjusted to 250 µg/ml in PBS with 1% newborn calf serum–0.1% Tween 80 to minimize nonspecific reactivity. Equal volumes of each sorbent were mixed, and the mixture was stored at −70°C. Test serum samples were diluted to a concentration of 1:50 by adding sorbent, and the solutions were gently shaken for 1 h at 20°C and incubated overnight at 4°C.

**Enzyme immunoassay (EIA).** Ninety-six-well round-bottom plates (Polyorb U96; Nunc) were coated with 20 µg of *H. ducreyi* antigen per ml suspended in 0.1 M carbonate buffer (pH 9.6), incubated for 2 h at 37°C, and washed three times with washing buffer (PBS, 0.1% Tween 80). Plates were wrapped in foil and stored at 4°C for no longer than 2 weeks.

Adsorbed test serum (100 µl per well) was applied at final dilutions of 1:200 and 1:400 to PBS (1% newborn calf serum, 0.1% Tween 80), incubated for 30 min at 37°C, and washed five times with washing buffer. A total of 200 µl of a 1:2,000 dilution of peroxidase-conjugated goat anti-human immunoglobulin G (IgG) (Tago, Burlingame, Calif.) diluted in PBS (5% newborn calf serum, 0.1% Tween 80) was added to each well. A 1:1,400 dilution was used with peroxidase-conjugated goat anti-human IgM (Tago). The plates were incubated for 30 min at 37°C and were then washed five times with washing buffer. Bound conjugated antibody was detected by adding 100 µl of 0.36 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (Boehringer Mannheim) and 0.03% H₂O₂ dissolved in citrate buffer (0.1 M citric acid and 0.02 M sodium phosphate [pH 4.25]). The plates were kept at 20°C for 25 min and were read spectrophotometrically at 405 nm. Each plate included one positive and one negative serially diluted control serum sample pooled from sera from eight highly reactive cases and five Caucasian donors, respectively.

**Inhibition EIA and rheumatoid factor.** Control adsorbed EIA-positive sera from a selection of reacting sera from clinically and microbiologically proven cases of chancroid were pooled. Pooled positive control sera at a 1:1,000 dilution were adsorbed to seven serial twofold dilutions (160 to 2.5 µg/ml) of *H. ducreyi* antigen, and the solutions were incubated for 2 h at 37°C with gentle shaking. A total of 100 µl of pooled positive control sera that adsorbed to the soluble antigen of *H. ducreyi* was then tested as described above for extinction of EIA-positive reactivity.

Fifteen *H. ducreyi* IgM-positive serum specimens from patients with no history of STDs were tested for the presence of rheumatoid factor by using the Ortho rheumatoid antibody test (Ortho Diagnostic Systems, Beere, Belgium).

**Patient population and sera.** Characterized sera were avail-
able from male and female patient populations of the Nairobi City Commission Dermatovenerology Clinic. Male urethritis was the most common STD diagnosis (189 serum specimens), and 95% of the cases of GUD were clinically if not microbiologically diagnosed chancre (2). Patients with non-STD dermatologic conditions were also represented. Standardization of the EIA was performed by using a panel of 432 serum specimens that were characterized according to (i) the clinical or microbiologic diagnosis of an STD, (ii) non-STD diagnosis, (iii) HIV antibody serology as described previously (2), (iv) past history of genital ulceration as reported by the patient, and (v) for chancre, the time from the appearance of genital ulcers to the time of phlebotomy. Select positive control sera were used for standardization. These sera were from patients with primary H. ducreyi infection and included those with clinically acute chancre, an ulcer specimen culture positive for H. ducreyi, the absence of a past history of GUD, and HIV seronegativity. Negative control sera were obtained from non-STD clinic patients without STDs, with other infectious or dermatologic diseases, and no history of a past GUD.

**Standardization.** Standardization was carried out with positive sera that represented a primary H. ducreyi infection and negative sera as described above. Both IgG and IgM EIA were standardized by using receiver operator characteristic (ROC) curves (20). Arbitrary threshold values for each plate were generated by adding serial multiples of 1 standard deviation of the mean optical density for 30 plate negative controls (multiples of 2 to 20 for IgG and 1 to 10 for IgM), the negative control of the plate. Test results were evaluated by determining the arithmetic ratio of the optical density of a test serum sample to the threshold for that plate by ROC curve analysis. Positive test results were defined as those that had a ratio of 1.0 or higher. Sensitivities and specificities were calculated for each arbitrary threshold. ROC curves were constructed by plotting the specificity versus the false-positive rate. The accuracy and the positive and negative predictive values of the assay were calculated for the threshold that performed optimally (13, 20, 24).

**Comparative evaluation.** The performance of the EIA was compared by using the larger group of positive case serum specimens from patients with chancre with respect to the time of phlebotomy from the time of onset of acute chancre, and with respect to the HIV antibody serology of the same test serum specimen. The EIA was also evaluated for its ability to detect past GUD versus the absence of current or past GUD.

**RESULTS**

**Reactivities of control sera.** The mean ratio of positive control sera to negative plate control sera for the IgG EIA at a serum dilution of 1:400 was 9.0 ± 2; for the IgM EIA, it was 18.1 ± 3.3. For both the IgG and IgM EIAs, up to 80% of the positive reactivity was inhibited by preabsorption with the lowest amount of soluble crude antigen (Fig. 1A and B). No inhibition was produced by preadsorption with *Escherichia coli* O55:B5 lipopolysaccharide (Sigma Chemical Company, St. Louis, Mo.). None of the IgM-positive samples was positive for rheumatoid factor (data not shown).

**Standardization of the IgG and IgM EIAs.** Each EIA was standardized with ROC curves for optimal performance (Fig. 2; Tables 1 and 2). For the IgG EIA, optimal performance was identified for an arbitrary threshold of 10 standard deviations (0.180 optical density units) of the mean value for the negative plate controls (0.092 optical density units) above the negative control value for the plate. Improved performance was observed by using positive control sera taken at 4 weeks from the time of disease onset. The sensitivity was 100% at and after 4 weeks, whereas the overall performance was 94% and the specificity was 84% (Fig. 2A; Table 1).

For the IgM assay, optimal performance was similarly identified for a threshold 2 standard deviations (0.034 optical density units) of the mean value for the negative plate controls (0.061 optical density units) above the negative control value for the plate. The sensitivity was 92% for case sera at 3 weeks from the time of disease onset, whereas the overall sensitivity was 74% and the specificity was 64% (Fig. 2B; Table 2).

**Assay performance for detection of primary infections.** As described above, both IgG and IgM EIAs demonstrated time-related performance, with the peak sensitivity occurring at 3 weeks from the time of disease onset for the IgM EIA and after 3 weeks for the IgG EIA (Fig. 3A and B). The reduced sensitivity of the IgG EIA before 4 weeks from the time of onset of genital lesions (Fig. 3A) was not statistically significant. For the IgM EIA, there was a gradient in sensitivity, which peaked at 3 weeks from the time of disease onset; this was followed by a significant decline (P = 0.002) (Fig. 3B).

**HIV serology.** Comparison of overall assay performance between HIV-positive and HIV-negative sera revealed significantly but moderately improved specificity for the IgG EIA (Table 3) and significantly but moderately improved sensitivity for the IgM EIA (Table 4).
FIG. 3. Specificity (dashed boxes) and sensitivity (shaded boxes) of the IgG (A) and IgM (B) EIAs overall and at weekly intervals from the time of onset of genital ulcers to phlebotomy. Fractions refer to the number of case serum samples that were found to be positive at weekly intervals. The sensitivity of detection of IgG was greater at 4 weeks from the time of onset of genital lesions, but it was not significantly different from that at the other times of detection. The sensitivity of detection of IgM peaked at 3 weeks and was significantly different from that at the other times of detection ($P < 0.001$; chi-square test for trend).

sensitivity for the IgM EIA in HIV-seropositive case and control sera (Table 4).

Past GUD. By using the threshold as standardized for acute chancroid, the EIA detected IgG seropositivity with 90% sensitivity and 64% specificity in a panel that included 83 case serum samples from individuals who reported a past GUD and 261 control serum samples from individuals without a current or past GUD. This represented optimal
TABLE 3. IgG EIA performance by HIV serology*  

<table>
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<tr>
<th>EIA result</th>
<th>HIV positive (sera)</th>
<th>HIV negative (sera)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>STD-naive</td>
<td>Chancroid-positive</td>
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<tr>
<td></td>
<td>(n = 80)</td>
<td>sera</td>
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<tr>
<td>Positive</td>
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<td>52 (98%)</td>
</tr>
<tr>
<td>Negative</td>
<td>80 (100%)</td>
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</table>

* Accuracy and positive and negative predictive values for assay performance were 99, 100, and 99%, respectively, for HIV-positive sera and 85, 86, and 93%, respectively, for HIV-negative sera.

TABLE 4. IgM EIA performance by HIV serology*  

<table>
<thead>
<tr>
<th>EIA result</th>
<th>HIV positive (sera)</th>
<th>HIV negative (sera)</th>
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</thead>
<tbody>
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<td></td>
<td>(n = 80)</td>
<td>sera</td>
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<tr>
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<td>46 (87%)</td>
</tr>
<tr>
<td>Negative</td>
<td>55 (69%)</td>
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</table>

* Accuracy and positive and negative predictive values for assay performance were 76, 65, and 89%, respectively, for HIV-positive sera and 69, 73, and 64%, respectively, for HIV-negative sera.

A potential recall bias of past GUD by the study subjects was minimized for standardization by using negative sera from clinic patients without any current or past GUD. Systematic limitations on the accuracy of standardization included the specificity of the antigen-antibody reaction and nonantigen binding of immunoglobulins, particularly in HIV-infected persons with nonspecifically elevated immunoglobulin levels. The fact that the assay measured antibody specific to H. ducreyi antigens is supported by the extinction of reactivity by the test antigen and the lack of extinction by an unrelated bacterial antigen. It has been suspected that HIV infection and associated immune system-related disease alter serologic reactions to vaccines (5, 6, 16, 25) and natural infections (18). The seroconversion of syphilis in HIV-positive populations has been carefully evaluated (17). In this study, we observed enhanced specificity of IgG and sensitivity of IgM EIA for sera from HIV-positive patients. There may be several explanations for this. A past GUD that was known to be associated with HIV positivity in this population (27) may have been underreported. Altered bacterial virulence in HIV-positive individuals (4) may modify the serologic reaction, resulting in increased EIA sensitivity of the IgG EIA in individuals with H. ducreyi-HIV coinfection. Since assay specificity for both the IgG and IgM EIA was preserved, HIV-related nonspecific hyperimmunoglobulinemia is a less likely explanation.

The performance of the EIA in settings other than the one described here needs further evaluation. Application of the EIA in settings with a high or a low prevalence of chancroid will alter the performance of this test with respect to positive and negative predictive values. The best application of this assay would be to identify and measure the prevalence of chancroid in individuals who may be or who are likely to be STD reservoirs (core groups), such as urban prostitutes and their clientele, or at STD treatment clinics. The application of this test would be valuable in identifying infected individuals and groups for targeting and evaluating public health chancroid control efforts, as part of larger interventions against STDs and the transmission of HIV.

ACKNOWLEDGMENTS

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REFERENCES

6. Collier, A. C., L. Corey, V. L. Murphy, and H. H. Handsfield. 1988. Antibody to human immunodeficiency virus (HIV) and