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Standardization of an Enzyme Immunoassay for Human Antibody to *Haemophilus ducreyi*

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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% CO_2 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% CO_2 for 24 h. Antigen was prepared in the same manner as described above for *H. ducreyi*. The protein concentra-

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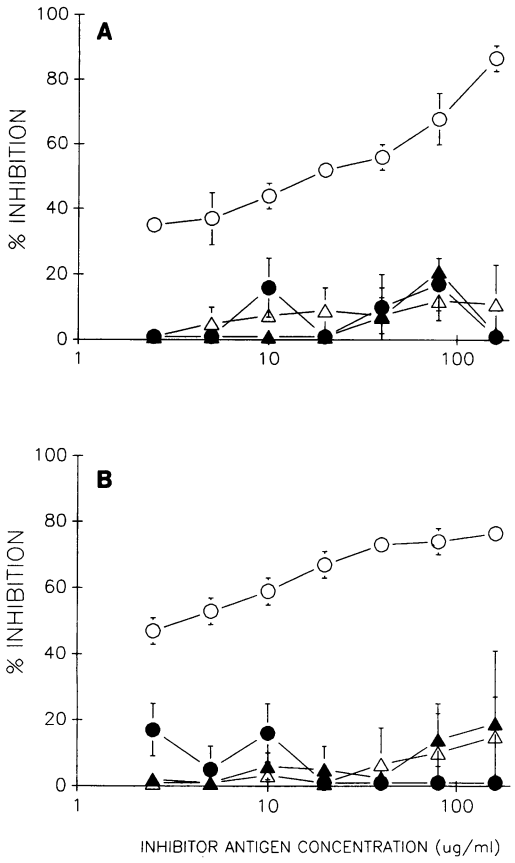

FIG. 1. Inhibition of reactivity by the IgG (A) and IgM (B) EIAs. Positive (circles) and negative (triangles) control samples were adsorbed with serial twofold dilutions of soluble *H. ducreyi* antigen extract (open symbols) or *E. coli* O55:B5 lipopolysaccharide (closed symbols) and tested for inhibition of reactivity by soluble antigen. Substantial inhibition by soluble antigen alone confirmed the specific antigen-antibody reaction in the EIA.

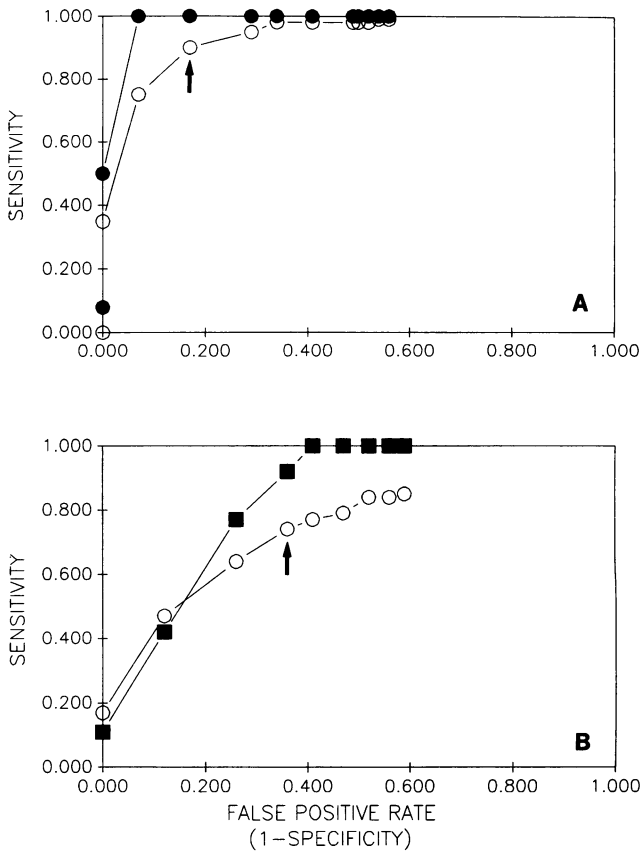

FIG. 2. ROC curve for the IgG (A) and IgM (B) EIAs. Curves for sera at 4 weeks from the time of disease onset (closed circles) and all sera (open circles) for the IgG EIA and at 3 weeks from the time of disease onset (closed squares) and all sera (open squares) for the IgM EIA were compared. Optimally performing thresholds, indicated by the arrows, were 10 standard deviations above the value for the negative plate control for the IgG EIA and 2 standard deviations above the value for the negative plate control for the IgM EIA.

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 (Polysorb 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-ethylbenthizoline-6-sulfonate) (Boehringer Mannheim) and 0.03% H_2O_2 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, Beerse, 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 chancroid (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 chancroid, 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 chancroid, 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 over 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 sensitivity 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 chancroid with respect to the time of phlebotomy from the time of onset of acute chancroid 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 highest 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).

TABLE 1. IgG EIA performance with respect to time from onset of genital ulceration[a]

EIA result	No. (%) of serum samples with the following microbiologic and clinical diagnosis:		
	STD-naive sera (n = 128)	Chancroid-positive sera (n = 134)	Sera positive ≥4 wk (n = 43)
Positive	21	126 (94)[b]	43 (100)[c]
Negative	107 (84)[d]	8	0

[a] Accuracy and positive and negative predictive values for assay performance were 89, 86, and 93%, respectively, for IgG.
[b] Sensitivity of EIA for all *H. ducreyi* positive-chancroid sera.
[c] Sensitivity of EIA at 4 weeks from ulceration (P was not significant compared with the sera described in footnote *b*).
[d] IgG EIA specificity.

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

TABLE 2. IgM EIA performance with respect to time from onset of genital ulceration[a]

EIA result	No. (%) of serum samples with the following microbiologic and clinical diagnosis:		
	STD-naive sera (n = 128)	Chancroid-positive sera (n = 171)	Sera positive 3 wk from ulceration (n = 26)
Positive	57	126 (74)[b]	24 (92)[c]
Negative	81 (64)[d]	45	2

[a] Accuracy and positive and negative predictive values for assay performance were 69, 73, and 64%, respectively, for IgM.
[b] Sensitivity of EIA for all *H. ducreyi* positive chancroid sera.
[c] Sensitivity of EIA at 3 weeks from ulceration ($P < 0.01$ compared with the sera described in footnote *b*).
[d] IgM EIA specificity.

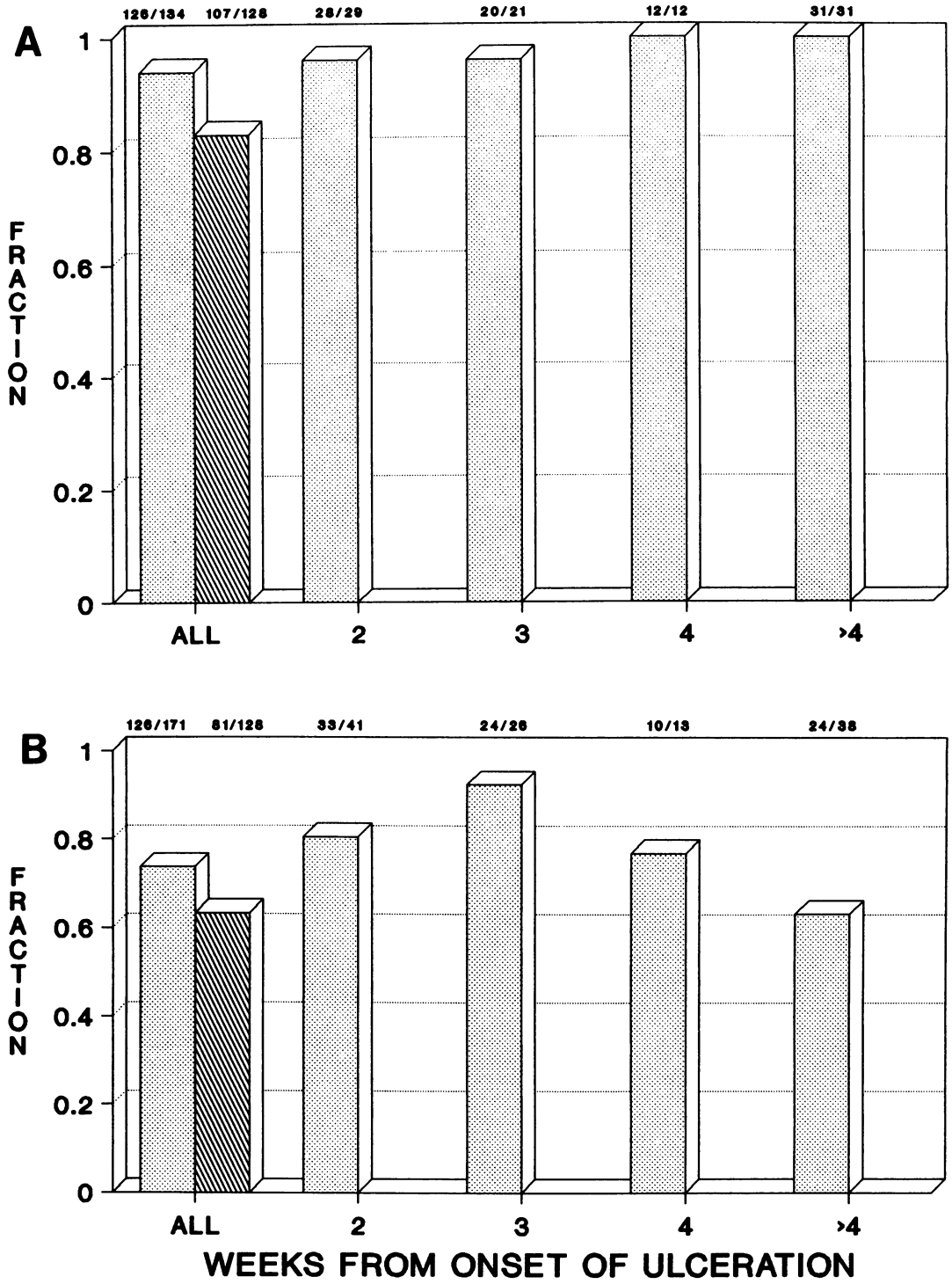

FIG. 3. Specificity (dashed boxes) and sensitivity (shaded boxes) of the IgG (A) and IgM (B) EIAs overall and at weekly intervals from the time of onset of genital ulcers to phlebotomy. Fractions refer to the number of case serum samples that were found to be positive at weekly intervals. The sensitivity 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[a]

EIA result	No. (%) of serum samples			
	HIV positive		HIV negative	
	STD-naive sera (n = 80)	Chancroid-positive sera (n = 53)	STD-naive sera (n = 128)	Chancroid-positive sera (n = 134)
Positive	0	52 (98%)[b]	21	126 (94)[b]
Negative	80 (100)[c]	2	107 (84)[c]	8

[a] Accuracy and positive and negative predictive values for assay performance were 99, 100, and 99%, respectively, for HIV-positive sera and 89, 86, and 93%, respectively, for HIV-negative sera.

[b] Assay sensitivity was not significantly affected by HIV seropositivity (P = 0.4).

[c] Assay specificity was significantly increased in the presence of HIV seropositivity ($P < 0.001$).

performance in the corresponding ROC curve (data not shown).

DISCUSSION

The performance of an *H. ducreyi* EIA was evaluated by using ROC curves. ROC curve analysis permitted the selection of a threshold for obtaining maximum assay performance for epidemiologic application. In the past, estimation of the prevalence or presence of chancroid in a population has been limited by the absence of a well-characterized, practical, sensitive screening assay.

An EIA has been described by Museyi et al. (22). A seropositivity rate of 89% was reported in a similar population of chancroid cases, with a specificity of 95% with sera from unrelated sources. Differences in performance between that serological assay and the one described here are probably due to the different antigen preparations and our preadsorption of test sera with sorbent from other *Haemophilus* species to reduce nonspecific reactivity. The antigen preparation described by Museyi et al. (22) consisted of extraction of the insoluble (pellet) fractions of *H. ducreyi*, whereas we used the soluble fraction (supernatant) to coat the 96-well round-bottom plates (22).

The performance of the EIA estimated by the standardization technique described here depends on biological and systematic limitations. Biological limitations include the quality of information used to characterize the serum specimens. Sera defined to be positive or negative were chosen by using several concurrent clinical and microbiologic parameters that indicate an acute-phase primary *H. ducreyi* infection or that *H. ducreyi* is absent, respectively. There is a potential recall bias of past GUD by the study subjects. This bias was minimized for standardization by using negative sera from clinic patients without any current or past GUD.

TABLE 4. IgM EIA performance by HIV serology[a]

EIA result	No. (%) of serum samples			
	HIV positive		HIV negative	
	STD-naive sera (n = 80)	Chancroid-positive sera (n = 53)	STD-naive sera (n = 128)	Chancroid-positive sera (n = 171)
Positive	25	46 (87)[b]	47	126 (74)[b]
Negative	55 (69)[c]	7	81 (64)[c]	45

[a] 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.

[b] Assay sensitivity was significantly increased in the presence of HIV seropositivity ($P < 0.01$).

[c] Assay specificity was not significantly affected by HIV seropositivity.

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 serodiagnosis of syphilis in HIV-positive populations has been carefully evaluated (17). In this study, we observed enhanced specificity of IgG and sensitivity of IgM EIAs 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 IgM EIA in individuals with *H. ducreyi*-HIV coinfection. Since assay specificity for both the IgG and IgM EIAs 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.

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