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Use of a *Chlamydia trachomatis* DNA Probe for Detection of Ocular Chlamydiae

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We examined the efficacy of a *Chlamydia trachomatis* DNA probe in detecting ocular chlamydiae by comparing it with tissue culture isolation, direct fluorescent-antibody cytology, and clinical eye exams. In a trachoma-endemic area of Nepal, 430 Nepalese villagers were examined according to the World Health Organization trachoma grading scale. Upper tarsal conjunctival specimens from each subject were obtained for DNA probing, tissue culture, and fluorescent-antibody screening. Moderate to severe intensity of inflammation was found in 85 (21%) of 430 people studied. An additional 25 (7.2%) of 345 people with low or no intensity of inflammation also had microbiologically proven infection, which may reflect asymptomatic carriage. Compared with culture, the DNA probe had a sensitivity of 86.9% and a specificity of 91%. For direct fluorescent antibody versus culture, the values were 47.8 and 96.9%, respectively. Results from this study indicate that the DNA probe for *C. trachomatis* might be considered a valuable epidemiologic tool in screening trachoma-endemic populations for ocular chlamydiae.

Trachoma is a chronic, potentially blinding eye infection afflicting over 500 million people worldwide. Approximately 100 million people have serious visual impairment, and over 7 million have been left blind by this disease (3). The causative agent of trachoma is *Chlamydia trachomatis*. The natural history of this disease has been well described in various reviews (2, 3, 17).

Three techniques have commonly been employed in field studies to detect *C. trachomatis* in ocular specimens: tissue culture, direct fluorescent-antibody cytology (DFA), and enzyme-linked immunoassays (1, 7, 10, 11, 18–20, 23). Tissue culture techniques for isolating chlamydiae from ocular specimens of patients with trachoma have been considered the "gold standard" diagnostic test. However, the ability to consistently isolate chlamydiae is not reliable; positive cultures usually occur in less than 50% of clinically active cases of trachoma (1, 7, 18). Additionally, culture techniques for chlamydiae are labor intensive, time-consuming, and expensive, and they require strict maintenance of a cold chain (less than −60°C during transport and storage) if there is a delay of more than 8 h in processing the samples. The DFA screening test has been considered an attractive alternative for confirmation of infection. This technique employs a fluorescent-labeled monoclonal antibody that detects the outer membrane protein of the infectious elementary bodies (EB) of chlamydiae in conjunctival samples (19, 20). The sensitivities of this test have varied, e.g., as low as 8% for clinically active cases (10, 23). Furthermore, technical expertise, a fluorescence microscope, and expensive reagents are required to perform DFA tests. Recently, an enzyme-linked immunoassay, IDEIA, was used in the Republic of the Gambia to detect chlamydial antigens in ocular specimens from patients with suspected trachoma (11). This test employs an enzyme-conjugated monoclonal antibody that binds to chlamydial glycolipids. In one study, the test was reported to have a sensitivity of 70.6% and a specificity of 90% for detecting chlamydiae in ocular specimens (11). However, there are discrepant sensitivity and specificity results for the detection of chlamydiae in active genital tract infections (6, 15, 21, 22; A. Freke, S. Wells, D. Sherwood, and S. Gatley, Letter, J. Clin. Microbiol. **25**:2032–2034, 1987). It is important to conduct further clinical trials with this reagent before considering it a valid field assay for trachoma. Unfortunately, these three tests, although currently used in some parts of the Third World, require considerable technical expertise and equipment and, therefore, are too difficult to reliably employ in the developing world. However, there is a need for a simple, reliable field assay to define the epidemiology of this infection, to assess the efficacy of control strategies, and to identify infected, asymptomatic carriers who would be important targets for health education and chemotherapeutic intervention.

A DNA hybridization probe has recently been developed that detects *C. trachomatis* in clinical specimens (13). This DNA probe employs a 7.0-kilobase cryptic plasmid from *C. trachomatis* which is ubiquitous and homologous for all 15 serovars of the organism. Additionally, there is no homology by DNA-DNA hybridization between *C. trachomatis* isolates and a 7.1-kilobase cryptic plasmid common to *Chlamydia psittaci*. The *C. trachomatis* plasmid has been found among 350 different clinical isolates from diverse sources (14). Horn et al. employed the probe to detect *C. trachomatis* in cervical scrapings from 200 young women in the San Francisco area (4, 5). Specimens were also obtained for isolation. Results indicated that all first-passage-positive cultures were also DNA probe positive. However, 2 (16%) of 12 culture-negative, first-passage specimens were also probe positive. This may reflect either a problem with the evaluation criteria used in reading probe results or an increased sensitivity of DNA hybridization over culture. When compared with culture, the DNA probe had a sensitivity of 93% and a specificity of 83%. From these data, the *C. trachomatis* DNA probe might have diagnostic relevance for ocular

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specimens. Additionally, since the probe can be used to screen hundreds of specimens at one time, is technically straightforward, requires no cold chain or elaborate equipment, and is relatively inexpensive compared with other diagnostic methods, we considered it a potentially excellent field test for detecting *C. trachomatis* in suspected trachoma cases. We report here the efficacy of this new DNA probe technique in detecting *C. trachomatis* in ocular specimens from individuals residing in a trachoma-endemic area of Western Nepal.

MATERIALS AND METHODS

Patients and specimen collection. A trachoma-endemic area of the Lumbini Zone in Western Nepal was chosen for conducting a field study on the efficacy of a *C. trachomatis* DNA probe. A demographic census was obtained from three rural villages by trained ophthalmic assistants. The census information included name, age, sex, relationship to other household members, and ethnic group of the individuals living within each household. All members of households with children less than or equal to 5 years of age were selected as study subjects. We examined 430 villagers comprising primarily Tharu and Chhetri ethnic groups. Ocular exams were performed by using a binocular (magnification, ×4) loupe and scored for trachoma according to the standard World Health Organization grading scale (3). The upper tarsus conjunctivae of both eyes were swabbed with a calginate swab (Puritan; Harwood Products Co., Guiford, Mass). Half the swab was rolled across an etched circle on a clean glass slide and allowed to air dry before fixing with acetone. The swab was then clipped from its metal shaft and placed into a vial containing 2 ml of chlamydia collection medium (see below). Within 6 h of collection, the vials which had been kept on ice were vortexed at high speed for approximately 1 min. The vials were kept at −70°C on dry ice before and during transport back to Stanford University within 1 week of collection. The samples were then maintained at −70°C in an ultrafreezer. A second upper tarsal specimen was taken from both eyes with a Dacron swab (American Scientific Products, McGaw Park, Ill.) which was then rolled across a 0.45-μm nitrocellulose filter (Schleicher & Schuell, Inc., Keene, N.H.). The filters were allowed to air dry and were stored in a cool, dry place. All diagnostic tests were performed at Stanford University.

Each villager seen in our field clinic was offered topical teramycin ophthalmic ointment (Pfizer Limited, Thane, India) with instructions for application. In addition, other ophthalmological and medical problems were identified and treated appropriately with available medical supplies.

DNA probe. The 7.0-kilobase cryptic plasmid of *C. trachomatis* serovar C strain TW3, which had been cloned as a *Bam*HI fragment into pBR322, was subsequently isolated from the recombinant plasmid pCHL4 (13). The cryptic chlamydia plasmid was separated from the vector sequences by electrophoresis, isolated from the gel, and extracted by phenol, chloroform, and ethanol precipitation (12). The purified DNA was then radiolabeled with [^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill.) by a nick translation kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) to a final specific activity of 5×10^7 to 5×10^8 cpm/μg. Each batch of nitrocellulose filters to be screened for *C. trachomatis* included a filter with a serovar L2 strain as a positive control and either normal yolk sac or blood as a negative control. The filters containing ocular specimens and controls were lysed and denatured in 0.5 M sodium hydroxide and then were neutralized in a 1 M ammonium acetate–0.02 M sodium hydroxide solution. DNA-DNA hybridization was performed and processed for autoradiography by standard techniques (4). Three exposure times for autoradiography were used: 1, 2, and 7 days. Each film was read at each time period without knowledge of clinical exam, culture, or DFA results. A grading scale of 0 to 3+ was established in comparison with known negative and positive controls for each film. Results of 2+ and 3+ were considered positive, providing that the positive control was clearly 2+ for the corresponding film.

Tissue culture. Patient specimens were transported in vials containing two sterile glass beads and 2 ml of standard chlamydia collection medium (8). The cold chain was maintained as described above. Sample numbers on each vial were entered into a data file and randomly recoded. Each vial, after thawing to room temperature, was vortexed at high speed for 1 min. A 0.5-ml portion of each sample was inoculated into two separate wells on two different 48-well tissue culture plates (Costar, Cambridge, Mass.), each containing a McCoy cell monolayer that had been washed with 0.1 M phosphate-buffered saline (pH 7.4). An L2 or ocular serovar of chlamydia was used as a positive control in each plate. The negative control consisted of isolation medium with phosphate-buffered saline. A 0.5-ml portion of standard chlamydia isolation medium was then added to each well. The plates were centrifuged at 2,000 × *g* for 1 h. The medium was aspirated, and 2 ml of fresh isolation medium with 0.5 μg of cyclohexamide per ml was added to each well. The plates were allowed to incubate at 35°C in 5% CO_2 for 3 days. At 72 h postinoculation, the monolayers were fixed with absolute methanol for 10 min and then stained with 30 μl of chlamydial tissue culture fluorescent-antibody reagent (California Integrated Diagnostics Inc., Berkeley, Calif.) as suggested by the supplier. The monolayers were assessed for chlamydial growth within 24 h of staining under a fluorescence microscope at magnifications of ×400 for screening and ×1,000 for morphologic confirmation. A positive result was defined as the presence of one or more cytoplasmic inclusions fluorescing on the same plane as the cell nucleus. All plates were read for at least 30 min without prior knowledge of the clinical status or other diagnostic test results. Two discrepancies were noted, and the plates were read a third time. A final decision was made by consensus. All culture specimens that were negative at initial inoculation were serially passaged. For serial passage, the monolayer of the duplicate plate on day 4 (at 96 h) was dislodged into the medium by scraping with a metal spatula. This suspension was then evenly divided, passed into one well on two different plates supporting a McCoy cell monolayer, and processed for culture as previously described.

DFA. All slides containing patient specimens were refixed with absolute methanol as suggested by the supplier, air dried, and stored at 0°C until they could be screened for chlamydiae. Specimen numbers were randomly recoded before processing. The slides were then fixed with fluorescent antibody (FA) (MicroTrak; Syva Inc., Palo Alto, Calif.) according to the standard DFA protocol (19). A positive control and a negative control, as provided by the supplier, were processed along with each set of specimens to ensure reliability of the reagents. The slides were read at a magnification of ×400 with a Zeiss opifluorescence microscope with a quartz halogen light source (12 V, 100 W, fluorescein isothiocyanate filter) for screening. Morphology for positive specimens was confirmed at a magnification of ×1,000. Specimens were considered positive only if a minimum of 10

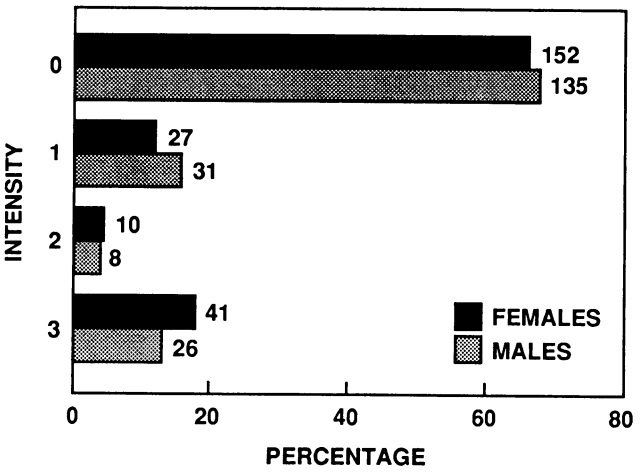

FIG. 1. Representation of combined intensity rates for males and females of Tharu and Chhetri ethnic groups. Intensity is classified according to the degree of inflammation as defined by the World Health Organization standard trachoma reading scale. Severe intensity is referred to as 3, moderate as 2, mild as 1, and no evidence of inflammation as 0. Rates for the Tharu and Chhetri ethnic groups are combined to give the represented percentage for each intensity category. The number of individuals is shown to the right of each bar.

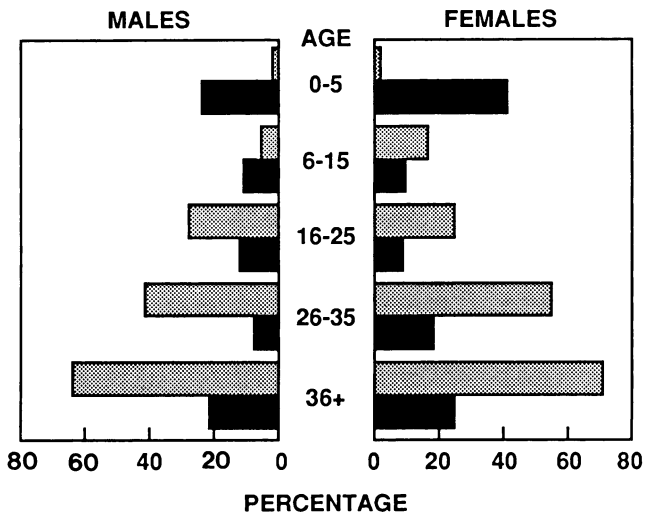

FIG. 2. Stratification of moderate to severe clinical intensity of inflammation (■) and conjunctival scarring (□) according to age and sex.

smooth EB were observed fluorescing a characteristic apple green on the same plane as the conjunctival cell nucleus. This is consistent with standards for reading ocular specimens as recommended by the supplier. All slides were screened for a minimum of 20 min. The total number of EB per slide was recorded without knowledge of clinical exam or diagnostic test results. All slides containing five or more EB and an additional randomly selected 94 negative slides were reviewed to confirm the initial results. This was done without knowledge of prior DFA results or other diagnostics, including clinical exam.

Statistical analyses. A total of 400 specimens out of the original 430 were available for statistical analyses. A total of seven culture vials were lost in transport, eight nitrocellulose filters were lost or destroyed in processing, and 15 slides could not be interpreted as they contained too few cells or excess cellular debris. Chi-square tests were performed on percent differences between males and females for intensity of disease and conjunctival scarring. Sensitivity, specificity, and positive predictive values were defined according to Lilienfeld and Lilienfeld (9). A true-positive specimen was defined as any specimen positive by culture alone or by the combination of positive DNA probe and DFA tests. A false-positive specimen was defined as any specimen positive by the DNA probe or DFA technique alone. The percent false-positive was defined as the total number of specimens false-positive by one test divided by the total number negative by the same technique plus the total number of false-positive specimens.

RESULTS

Clinical comparisons among Nepalese villagers. The intensity of inflammation and conjunctival scarring were recorded according to the World Health Organization trachoma grading scales (3). Combined intensity rates for Tharu and Chhetri study subjects are represented in Fig. 1. There were no statistically significant differences between these two ethnic groups. Moderate to severe intensity of inflammation was found in 85 (21%) of the 430 individuals examined. Figure 2 depicts the stratification of moderate to severe intensity of inflammation and conjunctival scarring according to age and sex. Most cases of this degree of intensity were in the 0-to-5 age group with a 41% prevalence for females, compared with 24% for males ($P = 0.08$). Older individuals also had evidence for severe inflammation; 9 cases (13.6%) were observed in the 26-to-35 age group and 15 (23%) were in the 36-or-older age group. The overall prevalence of moderate to severe conjunctival scarring was observed in 102 (23.7%) of 430 examined individuals. Only in the 26-to-35 age group was there statistical significance for excess scarring in a gender; 54% of women had moderate to severe scarring, compared with 41% of men ($P = 0.05$).

Correlations of diagnostic tests with intensity of disease. Positive results for DFA, DNA probe, and culture are compared with intensity of inflammation in Fig. 3. In reading

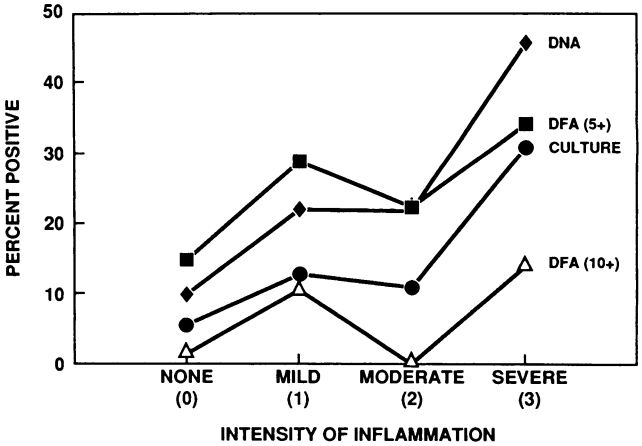

FIG. 3. Comparison of positive DNA probe, DFA (reported as five to nine [5+] or 10+ elementary bodies per slide), and culture test results with intensity of inflammation.

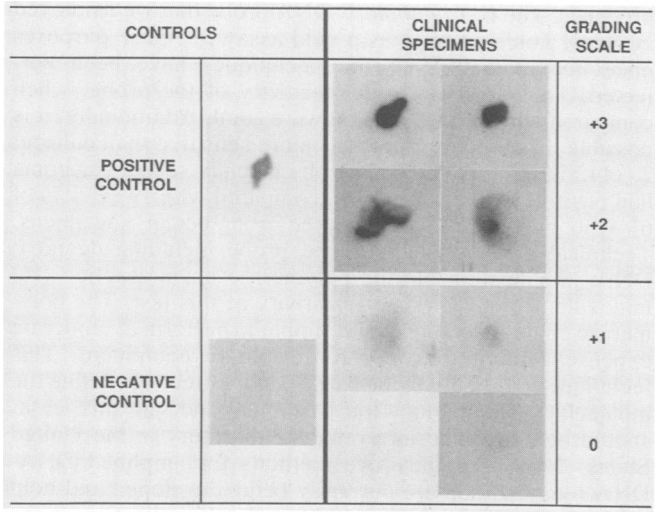

FIG. 4. The grading scale for DNA probe results recorded as 0 to 3+ and compared with the corresponding positive and negative controls from the same film. Results were considered positive if the dot blot was 2+ or 3+ and the positive control was 2+ for the appropriate film.

the DNA tests, a total of 10 equivocal results were noted out of the 400 specimens screened. These were classified as negative, as they fell within the 1+ reading category (Fig. 4). Readings for each exposure time were equivalent, with no further positive results noted on films from day 2 or 7. For culture specimens, 44 were initially positive; 356 negative samples were serially passaged. The number of inclusion bodies observed varied from 1 to 25 for all positive specimens. In reading the DFA tests, there were seven inconsistencies noted. However, these were confined to samples with less than nine EB per slide. In general, the number of positive culture and DNA probe tests increased as the severity of inflammation increased. However, there were few positives for any test among individuals with moderate intensity. In regards to DFA screening, correlations between positive results and intensity were inconsistent (i.e., the number of positive DFA tests did not always increase with increasing intensity of inflammation). Overall, the DNA probe had the highest rate of positive results for each intensity category except when considering a positive DFA test at 5+ EB per slide at the 0 or 1 intensity level.

Correlations of true- and false-positive test results for ocular chlamydia with intensity of disease. Table 1 is a comparison of five defined sets of true-positive tests with intensity of inflammation. A total of 46 (11.5%) positive results were identified among 400 clinical samples. Only 21 (26.5%) of 79 individuals with moderate to severe intensity of inflammation had microbiologically proven infection. These data suggest the diagnostic problems in confirming the presence of chlamydiae in clinically active trachoma. Culture failed to detect ocular chlamydiae in two individuals when both DFA and DNA probe tests indicated infection. Interestingly, these individuals were in the mild- or no-intensity categories. However, four true-positive test results by culture alone were missed by the DFA and DNA probe combination. These were also for individuals with no evidence of inflammation. The DNA probe test failed to indicate ocular chlamydiae in two individuals when both culture and DFA tests were positive. However, the DFA test failed to identify ocular chlamydiae in 20 cases (17 cases, each with a DFA cutoff of >5 EB per slide) when both culture and DNA tests were positive. Among these 20 cases, 10 of the individuals had moderate to severe intensity and 10 were associated with no or mild inflammation. It is unlikely that this would be explained by the failure of the DFA reagent to bind to ocular EB, since a similar antibody was able to bind to chlamydiae in cultured McCoy cells. A total of 25 (54%) of 46 true-positive specimens were from individuals with no clinical evidence for trachoma. This suggests that diagnostic tests with true-positive results identify a high percentage of asymptomatic carriers. Of these 25 asymptomatic individuals, 16 (64%) were female ($P = 0.12$). Of these 25, 15 (60%) were in their 20s or 30s; nine were female, and six were male.

The false-positive rates were 30 (9.1%) of 330 for the DNA probe and 9 (2.4%) of 369 for the DFA test. A total of 10 presumed false-positive DNA probe results were among cases with moderate to severe intensity, and the remainder were associated with asymptomatic individuals. All DFA false-positive results were from individuals with mild or no intensity of inflammation.

Sensitivity and specificity. Compared with culture, the DNA probe had a sensitivity of 86.9% and a specificity of 91% in detecting ocular chlamydiae (Table 2). For DFA results for 10+ EB versus culture, the findings were 47.8 and 96.9%, respectively. When considering DFA results positive at 5+, the sensitivity was 56.4% and the specificity was

TABLE 2. Comparison of DNA probe, DFA, and culture tests for sensitivity, specificity, and positive predictive value

Diagnostic test	No. positive/total no. tested (%) for:		
	Sensitivity	Specificity	Positive predictive value
Culture	44/46 (95.6)	356/356 (100)	44/44 (100)
DNA probe	40/46 (86.9)	324/356 (91)	40/70 (57)
DFA	22/46 (47.8)	345/356 (96.9)	22/33 (66)

TABLE 1. Comparison of DNA probe, FA, and culture in the diagnosis of trachoma according to intensity of disease

Intensity (no. of samples)	No. (%) of samples with true-positive results in the indicated set of tests[a]				
	DNA (+) FA[b] (+) Culture (+)	DNA (+) FA (+) Culture (−)	DNA (−) FA (−) Culture (+)	DNA (+) FA (−) Culture (+)	DNA (−) FA (+) Culture (+)
Severe (61)	10 (16)	0 (0)	0 (0)	9 (15)	0 (0)
Moderate (18)	0 (0)	0 (0)	0 (0)	1 (6)	1 (6)
Mild (54)	5 (9)	1 (2)	0 (0)	1 (2)	0 (0)
None (267)	3 (1)	1 (1)	4 (2)	9 (3)	1 (1)

[a] Results of each test are indicated as positive (+) or negative (−).
[b] Results were recorded as 10+ elementary bodies per slide.

94.3%. The positive predictive values were 57.1% for the DNA probe and 66 and 55% for the DFA criteria of 10+ and 5+ EB, respectively.

DISCUSSION

This study revealed two important findings from a public health standpoint. First, severe intensity of inflammation was found predominantly among females under age 5 and also in adult females over age 26. Older women may have a higher rate of severe inflammation from repeated exposure to infected children. However, it is unclear why female children should have a higher rate of infection than their male counterparts. These findings are consistent with previous observations in other developing countries (11, 19, 23). In addition, although we expected severe inflammation in younger individuals, we observed rates of 13.6% in the 26-to-35 age group and 22.7% in the 36-and-older group. Although the sample size is small, this is an unusual distribution. This may reflect a selection bias, since only members of households with children less than 5 years of age were enrolled. However, this may also represent an increase in the vertical transmission of chlamydiae among individuals in these households. This would reflect a cluster phenomenon. The second important finding is that 25 (7.2%) of 345 individuals with no or mild intensity of inflammation had microbiologically proven infection. These cases may represent early infection, latency, or chronic carriage. Mabey et al. noted that 5% of ocular specimens from individuals with no evidence of active inflammation were positive for chlamydiae by Chlamydiazyme (11). Therefore, it is possible that asymptomatic carriers may act as an unrecognized reservoir for chlamydiae within their community. This represents a potential epidemiologic problem in that any chemotherapeutic trial aimed at only those individuals with clinically active infection may not effectively control the transmission of *C. trachomatis*. A prospective study designed to evaluate the natural history of apparent asymptomatic ocular carriage of *C. trachomatis* appears to be needed before adequate trachoma control can be achieved. This type of study conducted within households and communities may also provide an important epidemiological perspective on the transmission patterns of this organism. From this information, rational interventional strategies could then be designed to reduce the high prevalence of trachoma among the Nepalese and other trachoma-endemic populations worldwide.

The ability to confirm trachoma microbiologically by a diagnostic test in the developing world is difficult for logistical reasons. These include the need for technical expertise, expensive equipment and reagents, and adequate electrical power. However, even under ideal conditions, chlamydiae can usually only be isolated from 50% of ocular specimens from individuals with active inflammation (1, 7, 11, 18). This rate drops when samples are frozen. Other authors have previously described a loss of infectivity when samples were shipped back to the United States in liquid nitrogen (1, 10, 18). Although our culture results were encouraging, only 21 (24.7%) of 85 specimens from villagers with active inflammation were culture positive. Additionally, the clinical exam was unreliable for predicting infection or carriage; 25 (7.2%) of 345 individuals with no signs of clinical inflammation had microbiologically proven infection. This emphasizes the need for a practical yet sensitive diagnostic field assay that can be used to define the epidemiology of this disease. This information is critical for designing rational interventional strategies to control trachoma while vaccine development proceeds. The *C. trachomatis* DNA probe that we employed in Nepal holds promise as a field assay for these purposes once nonradioactive labeling techniques have been perfected. The sensitivity and specificity of the probe, when compared with culture results, were good. Additionally, it is possible that the DNA probe is more sensitive than culture; 27 (44.2%) of 61 individuals with clinically severe trachoma had positive DNA probe tests, compared with 19 (31.1%) of 61 who had chlamydiae isolated from ocular specimens. Technical developments on the probe including de novo DNA amplification via the polymerase chain reaction (16) may enhance the intrinsic sensitivity of this test. The polymerase chain reaction is quite simple to perform at moderate expense without the need for elaborate equipment. This technique could initially be used to more precisely define the molecular epidemiology of trachoma and, in this case, should be performed in a reliable laboratory in the United States. New nonradioactive methods for amplified DNA-DNA hybridization are currently being developed and hold promise as epidemiologic tools for direct application in the field.

Our field study was designed to obtain a maximum of two swabbings per conjunctivae for the three required tests. This collection method may have introduced a sampling bias into our study; the use of a whole swab for DNA probing versus half a swab each for DFA and culture may have resulted in a higher rate of positive results for the DNA probe. However, the DNA probe may still be the better diagnostic test for the following reasons. (i) The initial swabbing should contain more EB than subsequent swabbings (as is the case with genital specimens cultured for gonococcus) and therefore result in a higher rate of positive culture and DFA results; Schachter et. al. found a sensitivity of 76% for isolation on the first swabbing of the conjunctivae versus 84% for a *C. trachomatis* DNA probe on swabbing 2, 3, or 4 times (J. Schachter, J. Moncada, C. R. Dawson, J. Sheppard, P. Courtright, M. E. Said, S. Zaki, and A. Lorrencz, Rev. Infect. Dis., in press). (ii) The patterns of positive results for each of the diagnostic tests in our study are not consistent with a sampling bias when considering the clinical status of individual patients. (iii) The observed difference in the sensitivity and specificity of culture versus those for DFA testing for trachoma in this study are consistent with the findings of others (10, 23). (iv) The DNA probe is the least affected by sample variation; swabs containing patient samples can be directly applied to filters which can then be stored indefinitely at room temperature until processed with the probe. (v) When the DNA probe and DFA test results were compared, the probe identified 20 samples that were also positive by culture yet negative by DFA (this number is reduced to 17 when using five EB as a cutoff which is not statistically significant when compared with >10 EB); the probe in this case has the advantage of detecting chlamydiae despite contamination with cellular debris or DNA from extraneous sources. (vi) Although the DFA test had a higher positive predictive value than the DNA probe, the probe picked up a total of 40 (86.9%) true-positive results, compared with 22 (47.8%) for DFA (or 25 [55%] for the >5 EB criteria). In screening populations in developing countries for trachoma, the sensitivity of a diagnostic test becomes paramount, since identifying and treating infected individuals and asymptomatic carriers is an important means for disease control. All three diagnostic techniques were comparable. However, culture and DFA have important limitations as defined above. With these considerations, we feel that the DNA probe has potential as a valuable epidemio-

logic tool for screening trachoma-endemic populations in the developing world for ocular chlamydial infections.

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