Sensitive Immune Dot Blot Test for Diagnosis of *Chlamydia trachomatis* Infection

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The sensitivity and specificity of an immune dot blot test (IDBT), which relies on a ^{125}I-labeled genus-specific monoclonal antibody to detect the *Chlamydia* lipopolysaccharide (LPS) antigen, were improved by pretreatment of specimens with proteinase K. This enzyme destroys protein A and therefore eliminates false-positive reactions caused by the presence of *Staphylococcus aureus*. Proteinase K treatment also improved the ability of the assay to detect the *Chlamydia* LPS antigen. When the improved IDBT was compared with culture for detection of *C. trachomatis* in 1,394 urogenital specimens obtained from a genitourinary medicine clinic, the overall sensitivity was 96%, and LPS antigen was detected in 76 of 83 (92%) specimens that yielded less than 10 inclusions in culture. The specificity and positive and negative predictive values of the test were 97, 81.5, and 99%, respectively. Of 123 conjunctival swabs, 7 were positive by both tests and 4 swabs were positive only by IDBT. This improved IDBT provides a simple, reliable alternative to culture for the detection of *C. trachomatis* in urogenital and conjunctival specimens.

Immunological techniques which detect chlamydial antigens are attractive alternatives to culture for the diagnosis of *Chlamydia trachomatis* infection, since such tests are rapid and controllable and do not require viable organisms. Moreover, unlike both culture and diagnosis by direct examination of smears with fluorescein-conjugated monoclonal antibodies, microscopy is avoided. However, considerable reservations have been expressed recently about the sensitivity of such assays, with suggestions that their true sensitivity may be as low as 65% (7, 9, 11).

We recently described an immune dot blot technique for the detection of the *Chlamydia* genus antigen which compared favorably with a rigorous cell culture technique for the diagnosis of genital chlamydial infections (8). In this test, the genus-specific lipopolysaccharide (LPS) antigen was detected with ^{125}I-labeled monoclonal antibody by autoradiography of the nitrocellulose membrane (NCM). When 950 urogenital specimens were assessed by this test, 92% of all culture-positive specimens and 62% of those specimens that yielded less than 10 inclusions in culture were detected. However, maximum sensitivity was only obtained when autoradiographs were exposed for 72 h, and a false-positive reaction caused by protein A was experienced in eye swabs from a neonate with *Staphylococcus aureus* colonization of the conjunctiva.

We describe here modifications to this test which have improved its speed, sensitivity, and specificity.

MATERIALS AND METHODS

Clinical specimens. Specimens submitted to the North Manchester Regional Virus Laboratory for routine diagnosis of *C. trachomatis* infection were examined by both culture and the immune dot blot test (IDBT). The majority of these were urogenital specimens (1,394 specimens; endocervical or urethral swabs) obtained from patients who attended the Genito-Urinary Medicine Clinic at the Manchester Royal Infirmary. This clinic serves a patient population in which the chlamydial isolation rate in new attenders is about 20%. The remaining 123 specimens were conjunctival swabs ob-

tained from patients suspected of ocular infection caused by genital *C. trachomatis* strains. All swabs were collected in 1.5 ml of sucrose phosphate transport medium which contained antibiotics but no serum (5). After collection, the majority of specimens were held at 4°C and cultured within 24 h. When this was not possible, specimens were stored at −160°C in liquid nitrogen after collection until they were tested.

An additional 20 selected urogenital specimens (16 of which yielded *C. trachomatis* in culture) were used to assess the effect of proteinase K treatment of specimens on the sensitivity of the IDBT.

Culture technique. Isolation of *C. trachomatis* was attempted in cycloheximide-treated McCoy cells by a standard technique. Each specimen was inoculated into one culture vial only, and blind passage was not carried out. Inclusions were recognized by indirect immunofluorescence with a hyperimmune serum sample obtained by repeated immunization of a rabbit with yolk sac-propagated *C. trachomatis* serotype L2. Two positive controls were included in each batch of specimens tested, one of which yielded less than 10 inclusions per monolayer (8). The monolayer from any specimen which was positive by IDBT but initially found to be negative by culture was carefully reexamined for inclusions before the specimen was scored as culture negative.

Monoclonal antibody. The genus-specific monoclonal antibody J12, which reacts with a chlamydia-specific epitope on the *Chlamydia* LPS antigen (10), was used for all comparisons between culture and IDBT. This was radiolabeled with ^{125}I as described previously (8) and stored at 4°C.

Protein A. *S. aureus* Cowan 1 was used as the source of protein A (4). A broth culture, which was stored in liquid nitrogen, was diluted 10% (vol/vol) in phosphate-buffered saline (PBS; pH 7.2) and then diluted further in twofold steps in PBS. These dilutions were tested in the IDBT with and without prior treatment with proteinase K (P-0390; Sigma Chemical Co., St. Louis, Mo.). This enzyme was dissolved in water (25 mg ml^{-1}) and stored in aliquots at −20°C. Immediately before use it was thawed, diluted 10-fold in water, and then added to the *S. aureus* dilutions to give a final concentration of 250 μg ml^{-1}. The suspensions were
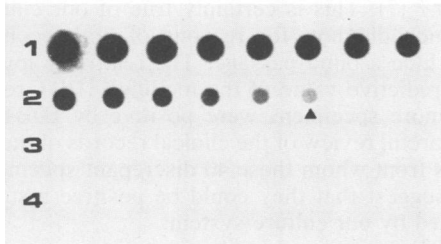
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FIG. 1. Effect of proteinase K on reactivity of protein A in IDBT. Twofold dilutions of a crude cell wall preparation of *S. aureus* Cowan 1 were tested from a starting dilution of 10% (vol/vol). Lanes 1 and 2, Untreated; lanes 3 and 4, proteinase K treated. The final two wells of lanes 2 and 4 were blank. The highest untreated dilution tested (0.001%; arrowhead) was clearly reactive in the IDBT, but all reactivity at each dilution was destroyed by proteinase K treatment.
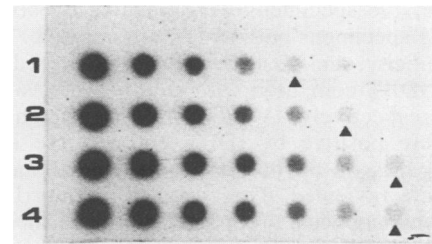

FIG. 2. Effect of proteinase K on reactivity of *C. trachomatis* serotype L2 in IDBT. Cell-grown stocks of L2 were treated with proteinase K, and then twofold dilutions were tested by IDBT. Lane 1, Untreated; lanes 2 through 4, proteinase K treated. The concentration of proteinase K was 500 μg ml^{-1} (lane 2), 250 μg ml^{-1} (lane 3), and 125 μg ml^{-1} (lane 4). The endpoints of each titration are indicated by arrowheads.

held at 56°C for 30 min and then steam treated for 15 min to inactivate the proteinase K before they were tested in the IDBT.

IDBT. The previously described IDBT (8) was modified in two ways. (i) Specimens were pretreated with proteinase K, since experiments with protein A showed that this enzyme destroys its reactivity in the IDBT (see below), and (ii) a more sensitive film (X-Omat AR; Eastman Kodak Co., Rochester, N.Y.) was used for autoradiography.

On arrival in the laboratory, 0.4 ml of each specimen was removed for culture of *C. trachomatis*. Proteinase K was added to the remainder of the specimen, to give a final concentration of about 250 μg ml^{-1}. Specimens were vortexed for 10 s and held at 56°C for 30 min, and then the enzyme was inactivated by steam treatment in a pressure cooker for 15 min. Specimens were then processed as described previously (8). Briefly, a 0.4-ml volume of specimens was added to the wells of a 96-well microfiltration apparatus (Bio-Dot; Bio-Rad Laboratories Ltd., Watford, United Kingdom) that was assembled with NCM moistened in PBS (one well per specimen). Specimens were drawn through the NCM by suction, and any residue which remained in a well was removed by aspiration. The membrane was blocked in PBS which contained 5% (wt/vol) powdered skim milk at 37°C for a minimum of 30 min. It was washed once in PBS with 0.05% (vol/vol) Tween 20 (PBS-T) and then incubated overnight at 4°C in 40 ml of PBS-T with 5% milk to which ^{125}I-labeled monoclonal antibody (2 × 10^6 to 3 × 10^6 cpm) was added. After thorough washing in PBS-T for 30 min, the NCM was dried and then autoradiographed with preflashed X-Omat AR film (Kodak) and Super-rapid screens (Kodak). The exposure time was usually 24 h, and it never exceeded 48 h. The developed film was examined on a light box, and a specimen was considered positive when a solid dot was seen in a position which corresponded to one of the specimen wells on the NCM.

Positive controls were included on each NCM. These consisted of a twofold titration of a cell culture stock of *C. trachomatis* serotype L2. Dilutions were adjusted to yield a range of dots of various intensities, with the endpoint being at about the fifth dilution.

RESULTS

Effect of proteinase K. Protein A produced a strong reaction in the IDBT in the absence of any chlamydial antigen, and it was still clearly reactive at a dilution of 0.001% (vol/

vol) of the original *S. aureus* suspension (Fig. 1). This presumably occurred because protein A attached to the NCM and then bound the monoclonal antibody J12, which belongs to the immunoglobulin G2a subclass (10). The reaction was completely destroyed by treatment of protein A with proteinase K before it was applied to the NCM (Fig. 1). Proteinase K treatment of clinical specimens and titrations of cell-cultured stocks of *C. trachomatis* serotype L2 were then assessed to determine whether the enzyme interfered with the ability of the monoclonal antibody to detect chlamydial LPS. The sensitivity of the test was increased rather than decreased by this treatment. *C. trachomatis* serotype L2 could be detected at a lower dilution after treatment (Fig. 2), and IDBT-positive clinical specimens were sometimes more reactive after treatment (Fig. 3). Clinical material was solubilized by the proteinase K, so that it passed through the membrane more readily and low-speed centrifugation of specimens before they were added to the wells of the microfiltration apparatus (8) was no longer necessary. Thus, proteinase K treatment not only destroyed protein A, it also improved and simplified the test. Pretreatment of specimens with this enzyme was therefore incorporated into our routine IDBT protocol.

Comparison of IDBT and culture: urogenital tract specimens. There was an overall 97% concordance between IDBT
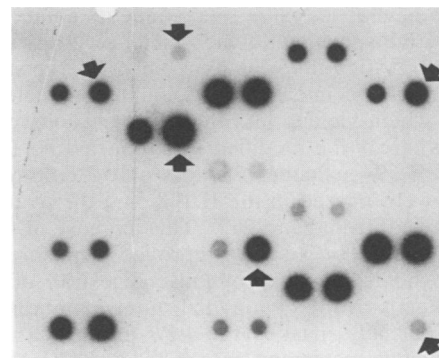

FIG. 3. Effect of proteinase K on reactivity of 20 selected urogenital tract specimens in the IDBT. Each specimen was tested twice, before (left-hand well) and after (right-hand well) proteinase K treatment. There is a pair of blank wells between each specimen pair. Of 15 specimens that were positive by IDBT, 6 were more reactive after treatment (arrows). Direct sensitivity comparisons between culture and IDBT were not possible, as a number of specimens had to be diluted after culture to allow duplicate IDBTs.

and culture; 176 specimens were positive by both assays, and in 1,171 specimens both tests were negative. The sensitivity, specificity, and positive and negative predictive values of the IDBT compared with culture were 96, 97, 81.5, and 99%, respectively. A total of 40 specimens from 36 patients were positive by IDBT but negative in culture. Clinical records on 32 of these patients (13 men and 19 women) were reviewed. Three men had gonorrhea, seven men had nongonococcal urethritis, and the partner of one of the remaining three men had proven *C. trachomatis* infection. Six women had gonorrhea and seven women were consorts of men with nongonococcal urethritis, three of whom had proven chlamydial infections. Seven culture-positive specimens were negative by IDBT; each of these specimens yielded less than 10 inclusions in culture, as did 76 of the specimens which were positive in both tests. The IDBT therefore detected 92% of all these weak culture-positive specimens.

Comparison of IDBT and culture: eye specimens. A total of 7 culture-positive conjunctival swabs obtained from 6 patients were also positive by IDBT, 4 were positive by IDBT only, and the remaining 112 specimens were negative by both tests. Two of the four patients whose specimens were positive by IDBT only were 7-day-old neonates with ophthalmia; the other two were young adults (a 22-year-old man and a 20-year-old woman) with follicular conjunctivitis of several weeks duration. Two of these four patients were known to have had topical antibiotic therapy before collection of the swab. *S. aureus* was not recovered from the three patients for whom results of the bacteriological examination were known, and a serum specimen that was available from the 22-year-old patient had an immunofluorescent-chlamydial-antibody titer of 1:640.

DISCUSSION

The modified IDBT described here is simpler, quicker, and more sensitive than our original test (8). The X-Omat AR film (Kodak) used in the modified test is more sensitive than the S1596 film (Kodak) used previously, so the autoradiography exposure time could be reduced to 24 h. In addition, pretreatment of specimens with proteinase K, which was introduced in order to prevent interference by protein A in the test (3), had the unexpected benefit of improving its sensitivity. A stringent way to assess sensitivity of a new *Chlamydia* diagnostic test is to examine its ability to detect culture-positive specimens which yield very few inclusions in culture (8). When this was done on two large samples of urogenital tract specimens, the original test detected 62% of specimens which yielded less than 10 inclusions per monolayer (8), whereas the modified test reported here detected 92%; of the 1,394 specimens tested, only 7 culture-positive specimens were missed in the IDBT, and the overall sensitivity improved, from 92 to 96%. This increase in sensitivity was presumably due to the improved exposure of LPS antigen to the NCM by proteinase digestion of adjacent protein antigens in the chlamydial outer membrane, which may have partially masked the LPS antigen. The greater ease with which specimens passed through the NCM after enzyme treatment may also have contributed to the increased sensitivity.

Culture is currently regarded as the "gold standard" against which new *Chlamydia* diagnostic tests are assessed, when the assumption is made that culture is 100% sensitive (6). However, the true sensitivity of culture, even when carried out under optimum conditions, is probably well below 100% (7). This is certainly true of our culture technique, which did not, for reasons of both economy and speed, include a blind passage. The relatively low (81.5%) positive predictive value of the modified IDBT reflects the fact that more specimens were positive by IDBT than by culture. Careful review of the clinical records of the majority of patients from whom these 40 discrepant specimens were obtained suggest that they could be positive results which were missed by our culture system.

The IDBT also appeared to be more sensitive than culture for the recognition of *C. trachomatis* in eye specimens. This may be because patients with ocular chlamydial infection were frequently treated with topical antibiotics such as chloramphenicol before conjunctival swabs were obtained. Such treatment does not eliminate the infection but makes recovery of viable organisms more difficult. In this series of 123 eye specimens, all 7 culture-positive specimens contained low numbers of inclusions. The clinical presentations in the four patients whose eye swabs were positive only by IDBT were all suggestive of chlamydial infection; two patients were known to have had topical antibiotics before eye swabs were taken, and there was no evidence of false-positive results caused by the presence of *S. aureus*. However, continued critical review of IDBT results on eye specimens is being carried out, to gain further experience with the performance of the test on this type of clinical material.

The same monoclonal antibody (J12) was used to compare the IDBT with culture as was used in the original study (8), since it was important to assess the effect of modifications to the technique without altering other parameters of the test. However, experience with a genus-specific monoclonal antibody made in our laboratory (1), which appears to react against the same epitope on the LPS antigen as J12, suggests that it performs as well as J12 (unpublished data). The success of the IDBT therefore does not rely solely on one particularly avid monoclonal antibody. Results of a preliminary report indicated that a species-specific monoclonal antibody could also be used to detect *C. trachomatis* in an immune assay on NCM (J. D. Patel, J. M. Joseph, and W. A. Falker, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, C23, p. 131). However, it would not be possible to use proteinase K in such an assay, which relies on the detection of a protein antigen, and unlike tests based on the recognition of the *Chlamydia* genus-specific LPS antigen, it would not detect infection caused by *Chlamydia psittaci*. The IDBT has the potential to recognize such infections, both in veterinary medicine and in humans with respiratory tract infections caused by the TWAR strain of *C. psittaci* (2), although it has not yet been assessed in such situations.

Our laboratory receives between 250 and 300 specimens each week for diagnostic *Chlamydia* tests. These specimens generally arrive in the laboratory at midday, and results of the IDBT are reported to clinicians 48 h later. Although it is not as quick as an enzyme-linked immunosorbent assay, the IDBT is at least 24 h quicker than our routine culture technique because of the time saved by elimination of all microscopy. The test is also simpler to perform than culture. In this laboratory, therefore, the IDBT is proving a simple and reliable alternative to culture for the diagnosis of genital *C. trachomatis* infections.

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