Evaluation of non-radioactive in situ hybridisation method to detect *Chlamydia trachomatis* in cell culture

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SUMMARY A DNA probe combined with a non-radioactive stain was used to detect inclusions of *Chlamydia trachomatis* in cell culture. Of 39 positive cultures detected by monoclonal antibodies in combination with direct immunofluorescence, 35 were positive by the DNA hybridisation method, the sensitivity being 89.7%. Staining with iodine showed a sensitivity of 87.2%, corresponding to 34 positive cultures. The specificity of DNA hybridisation method was 100%, as all 162 cultures that were negative by the immunofluorescence method were also negative when assessed by the DNA hybridisation method.

In recent years several non-culture methods for the rapid detection of chlamydiae in clinical specimens have been developed.¹⁻³ These antigen detection tests are useful when cell culture facilities are not available and stringent transport conditions cannot be ensured. The method of choice for the laboratory identification of *Chlamydia trachomatis*, however, remains the isolation of the bacterium in cell cultures^{4,5} and the subsequent demonstration of characteristic intracytoplasmic chlamydial inclusions using stains such as Giemsa or iodine or by a direct immunofluorescence technique using specific antisera.

A new approach for detecting inclusions represents nucleic acid hybridisation.⁶ In the present study a DNA probe combined with a non-radioactive stain (Enzo Biochem, New York) was evaluated for visualising chlamydial inclusions in cell cultures. The results were compared with those obtained by a conventional iodine stain and a direct fluorescent monoclonal antibody reagent.

Patients, materials, and methods

STUDY POPULATION

Urogenital specimens were collected from 201 men and women attending a sexually transmitted diseases (STD) outpatient clinic.

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SPECIMEN COLLECTION

Urethral specimens were collected four hours after micturition by inserting an ENT swab (Mast Diagnostic) 2-4 cm into the urethra and rotating and withdrawing it. Endocervical specimens were obtained after removal of the cervical mucus by inserting an ENT swab into the cervical canal, rotating it several times and withdrawing it. The swab was placed into saccharose phosphate transport medium.

CELL CULTURE

McCoy cells were cultured on glass coverslips in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% fetal calf serum. After growth to monolayers, cell cultures were treated with cycloheximide at a final concentration of 1 µg/ml. Cultures were then inoculated with 0.2 ml transport medium, centrifuged at 3000 × g for one hour, and then incubated for up to 72 hours.

DETECTION OF INCLUSIONS BY IODINE STAIN AND IMMUNOFLOURESCENCE

To detect inclusions with iodine stain, cells were fixed in methanol for 10 minutes, Lugol's iodine was added, and after being washed and mounted the coverslip was examined by light microscopy.

To detect inclusions by direct immunofluorescence, cells were fixed in acetone for 10 minutes and 30 µl of fluorescein conjugated monoclonal antibody against the chlamydial group specific lipopolysaccharide (Progen Biotechnic, Heidelberg) was applied. After being incubated in a moist chamber for 15 minutes at 37°C, coverslips were rinsed with distilled water, air

Table Comparison of in situ hybridisation method and monoclonal antibody based immunofluorescence test to detect *Chlamydia trachomatis* inclusions in 201 cell cultures

Monoclonal antibody and immunofluorescence	DNA hybridisation		Iodine stain		Sensitivity	Specificity
	Positive	Negative	Positive	Negative		
Positive	35	4			89.7% (35/39)	
Negative	0	162				100% (162/162)
Positive			34	5	87.2% (34/39)	
Negative			0	162		100% (162/162)

dried, mounted, and examined with a Zeiss fluorescence microscope.

DETECTING INCLUSIONS BY DNA HYBRIDISATION
Cell culture samples on coverslips were fixed with acetone for five minutes. The fixative was tapped off, the cover slip was air dried, and 20 μ l biotinylated DNA probe (Enzo Biotech, New York) complementary to *C trachomatis* DNA was applied. The sample was covered with a glass coverslip, placed on a heating rack at 95°C for five minutes to denature the double stranded DNA, and incubated for 15 minutes at room temperature for the hybridisation procedure. The coverslip was then removed and the sample was immersed in washing solution (50% formamide in 0.1 mol/l phosphate buffered saline (PBS)) and rinsed with PBS. For visualisation, 60 μ l of a solution of preformed streptavidin-biotinylated horseradish complex was added, the sample was incubated for 20 minutes at room temperature and washed again with PBS, and 60 μ l substrate (N-amino-salicylic acid) was applied. The reaction was stopped by adding water under microscopy. Dark round or oval spots in the cells of the culture were judged as being inclusions.

Results

We investigated 201 urogenital specimens by the cell culture technique. Inclusion bodies were found in 39 when a monoclonal antibody in combination with direct immunofluorescence was used (table). The shape of the fluorescent spots and their intracytoplasmic location were characteristic, leaving no doubt that these cultures were positive. Staining with iodine showed chlamydial inclusions in only 34 cell cultures, which corresponded to a sensitivity of 87.2% taking the immunofluorescence test results as the standard.

With the DNA hybridisation test oval or round intracellular bodies were found in 35 cultures. These positive cultures were included in the 39 positive cultures assessed by the immunofluorescence test (table). Taking the immunofluorescence test as the standard, this corresponded to a sensitivity of 89.7%. The DNA hybridisation method failed to detect *C trachomatis* in four cases. In these cases fewer than five

inclusions were detected in the cultures by immunofluorescence.

Of the 162 specimens that, after investigation by cell culture, were negative when the immunofluorescence test was used as standard, none was found to be positive either by the DNA hybridisation method or with the iodine stain (table). Accordingly, the specificity of the DNA hybridisation method reached 100%.

Discussion

Recent advances in recombinant DNA technology have made it possible to produce large quantities of nucleic acid probes with defined sequences and permit the use of the DNA hybridisation technique in clinical diagnosis. The DNA hybridisation test evaluated in this study is easy to handle and shortens the time taken to detect chlamydial inclusions to one hour. This study shows that DNA hybridisation visualised by a non-radioactive label can be used effectively to detect *C trachomatis* inclusions in cell cultures.

Though there was some background staining, the specificity of the DNA hybridisation method was 100% when the criterion of intracytoplasmic oval or round shaped spots was strictly applied. Negative results were confirmed by staining with iodine and fluorescent monoclonal antibodies. This shows a high selectivity of the complementary DNA probe for chlamydial DNA sequences.

The sensitivity of the DNA hybridisation method was 89.7% when the immunofluorescence test was taken as standard. In the cases in which the DNA hybridisation method failed, the number of inclusions was low (as assessed by the immunofluorescence method). This suggests that the numbers of chlamydial DNA copies per cell limited detection by this method. This limitation can obviously not be counterbalanced by the streptavidin-biotin amplification system.

Higher sensitivities in detecting chlamydial inclusions have been reported of an amplification system based on a radioactive label.⁶ This system cannot, however, be performed as rapidly as the one we investigated and requires facilities that make it less applicable for diagnosis in the clinical setting.

In pilot studies the DNA hybridisation method based on radioactive label was also used for the direct detection of chlamydial elementary bodies in specimens. Sensitivities and specificities corresponding with those of direct immunofluorescence and enzyme immunoassay have been reported.^{6,7} In those studies false negative results due to failure of the DNA hybridisation method were also attributed to specimens with low numbers of chlamydial organisms and correspondingly low copy numbers of chlamydial DNA.⁸

According to our results, the most reliable method for detecting inclusions in cell culture remains the immunofluorescence test. Far lower sensitivity, roughly in the range of the conventional iodine stain, was found for the DNA hybridisation test, which is more laborious and expensive than the conventional test. To become a useful technique for clinical routine diagnosis, therefore, DNA hybridisation has to be improved, particularly regarding sensitivity. In identifying *C. trachomatis* in biopsy specimens the currently available test may be more advantageous than detection by immunofluorescence, which is impaired because of the procedure for fixing the tissue.

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