Chlamydia trachomatis in the Clinical Laboratory: An Evaluation of Detection Methods and Review of the UCSF Experience

Anthony Nasr, PhD, MD
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C. trachomatis has received an enormous amount of attention over the last few years, attributable, of course, to its role as a sexually transmitted disease (STD), but recently due to the availability of several new, convenient detection methods. The possible role of these methods in the clinical laboratory will be considered in the context of the actual culture results at UCSF during the five months preceding the writing of this paper.

Chlamydia trachomatis

"Coccoid microorganisms whose obligately intracellular mode of multiplication within cytoplasmic vacuoles is characterized by change of small, rigid-walled infectious forms (elementary bodies) into larger, flexible-walled non-infectious forms (reticulate bodies) that divide by fission."¹. This terse description is the basis for Chlamydia forming its own order of microorganisms, Chlamydiales. The only genus, Chlamydia, is composed of two species, C. psittaci, the agent of human psittacosis, and C. trachomatis.

C. trachomatis is responsible for several discrete types of human infection. The chronic inflammation of the conjunctiva, from which the species derives its name (trachoma, Greek: rough swelling) continues to be the leading cause of preventable blindness in the world. This same organism is the most common STD in developed countries, with an estimated 4 million cases per year. This manifests itself as a chronic cervicitis in women and is a leading cause of non-gonococcal urethritis in men. While the immediate morbidity of these infections is not striking, the sequelae range from pelvic inflammatory disease and infertility to neonatal pneumonia. Unique biovars of the species are responsible for the bubonic disease, lymphogranuloma venereum. It has been estimated that infections due to chlamydiae are responsible for public health costs in excess of two billion dollars per year².

The unique life cycle of the organism begins with phagocytosis of the metabolically inactive elementary body by the host cell. This particle manages to avoid fusion with the primary lysosomes of the cell and within 6 to 8 hours has reorganised itself to the metabolically active reticulate bodies; active division of these can be seen at 8 to 10 hours post infection. The reticulate body,
while coding for many enzymes necessary for replication, is so biochemically
deficient that it depends on the host cell for most of its metabolic needs. The
reticulate bodies organise themselves into a focal area in the cell, visible at the
light microscopic level as an inclusion body. This structure is easily stained with
iodine by virtue of its glycogen content. About 18-24 hours post infection, the
reticulate bodies again undergo a reorganisation to infectious elementary
bodies which are released by the cell to complete the cycle.

Introduction to the Clinical Problem

The problem for the microbiology lab is, of course, to confirm or
refute the clinician's suspicion of chlamydial infection with a fair degree of
certainty. There are two general categories of approaching this problem: culture
and immunoassay. Culture has been considered the "gold standard" method,
but has a number of drawbacks. Immunoassay is undeniably faster and easier,
but is reported to have a lower sensitivity and specificity. Before comparing the
various techniques, the details of performing each will be presented.

Obtaining Clinical Material for the Evaluation of Chlamydia Infection.

Fresh material, minimally contaminated with bacteria, is key in
obtaining accurate data with any test method. In the case of cervical material,
this includes the avoidance of vaginal mucous. Schachter and his associates
have shown that an endocervical cytobrush, of the type routinely used for
Papanicolau smears, is the best device for obtaining culture material. The
cervix is first wiped free of mucous, then the cytobrush is inserted into the
endocervical canal and rotated gently. The brush is then removed, taking care
not to touch vaginal surfaces. A smear may be prepared at this point, or the
brush immediately placed in a transport medium appropriate for the assay to be
used. Specimens so prepared may be stored up to 72 hours at 4 degrees
centigrade. Specimen collection for other sites is similarly based on
obtaining viable, infected epithelial cells and minimizing bacterial or purulent
contamination; rectal specimens should be free of feces or pus.

Culture of Chlamydia trachomatis

A "shell vial" technique, of the type employed for virus culture, is
used for the culture of Chlamydia. A cylindrical vial holding a circular coverslip is
used as a culture vessel for McCoy cells, prepared to be nearly confluent on the
day of inoculation. The specimen, including the cell culture media (the "holding
media" supplied to the clinics) and collection swab or brush, is vigorously
vortexed for two minutes. The collection fluid is pipetted off and about 1 cc of
this fluid is placed into each of two freshly rinsed shell vials. The vials are
centrifuged at 3000g for one hour at room temperature and the supernatant
discarded. Fresh culture media, now containing cycloheximide, is added. The
optimal concentration of cycloheximide is best determined empirically, usually
0.5 to 1 mcg/ml. Both vials are cultured at 37 degrees. At three days, one of the
vials is examined for chlamydial growth and at four days the other vial is
passaged. Passage is accomplished by vortexing the vial, dividing the liquid into two fresh McCoy cell cultures and inoculating as before.

There are a number of ways a vial can be examined for growth. The easiest is to fix the coverslip for 10 minutes in methanol then stain with Gram's iodine. The entire coverslip is scanned at 20x, looking for the characteristic chlamydial inclusions which stain by virtue of their glycogen content. More sensitive fluorescent antibody staining may detect individual organisms at only two days, but at higher cost. Either method requires patience and a considerable degree of observer skill. In negative cases, the "second pass" culture is examined. This second culture is reported to identify an additional 3 to 10 percent of positive isolates6, but in actual practice the exact magnitude of this benefit is dependent on the effort expended on the first culture.

Direct fluorescent Antibody Detection of Chlamydia ("MicroTrac")

Detecting Chlamydia by this method begins at the time of specimen collection, with the preparation of an air-dried slide. In the laboratory, this slide is brought to room temperature and 30 mcl of direct fluorescent antibody is used to cover the specimen. Positive and negative control slides are prepared in parallel. After 15 minutes incubation the slide is rinsed in deionized water for 10 minutes. At that time it is ready to mount and examine. There must be a specified number of elementary bodies, usually 10, observed on the slide to identify the specimen as positive for Chlamydia. Inclusions are seldom observed in this direct preparation and confident identification of the elementary bodies does require time and skill comparable with that in the culture method.

Enzyme Immunoassay ("CHLAMYDIAZYME")

The basis of this test is that chlamydial LPS antigen, present in small amounts, should be detectable by a non-microscopic, antibody-linked enzyme assay of sufficient sensitivity. For this assay, the collection swab is placed in a "collection media", physically similar to the method used for culture, but in this case, the fluid is designed to maintain an antigen, not a viable, cultivable entity. For this reason, the specimens are somewhat more stable in storage before laboratory processing. The specimens are vortexed and the supernatant fluid pipetted into a well of a microtiter plate. A treated bead is added to each well; this binds chlamydial LPS and acts as the matrix for the subsequent immunoperoxidase fixation. Each well is sequentially treated with anti-C trachomatis antibody, rinsed and incubated with peroxidase enzyme conjugate. After thorough rinsing, substrate is added and the 492 nm absorbance of each well is determined. The techniques involved require a relatively low level of technical expertise and may be largely automated.

Comparison of Detection Methods

A number of recent reviews dealing with this subject are available7,8, one containing the prophetic statement that "...selection of test methods for C. trachomatis infections must be made with the understanding that
all current methods are imperfect. All of these reviews regard culture as the method of choice when available and when screening low-prevalence populations.

The advantage of culture as a detection method rests on its high sensitivity and specificity. However, since it is generally used as the "gold standard" for all other methods, an exact assessment of these values is difficult to come by. The main disadvantages of culture are (1) the long time investment (up to one week or more) before the results are known, (2) the necessity of having a qualified lab with well-trained personnel close enough to process the specimen promptly and (3) the problem of sample toxicity. Toxicity, or cytopathic effect (CPE) occurs in about 10% of specimens and refers to failure of the cell culture to thrive. The resulting death of the culture renders it useless for the evaluation of the presence of chlamydiae. Some of the factors influencing toxicity have been identified, and include poor collection technique and concomitant pathogens.

The advantages of the other two methods are based on their relative insensitivity to conditions of sample collection and handling, ease of sample preparation and rapidity of result. In fact, in populations where the prevalence of infection exceeds 12%, immunoassay may be cost-effective. These advantages must be weighed against the cost, both psychosocial and medical, of giving a false positive diagnosis. In the review of this subject by Barnes, over 20 independent studies show positive predictive values of the DFA test between 50 and 100 percent, depending to some extent on the prevalence of the disease in the population studied. Both bacterial and viral antigens have been shown to interfere with antibody-linked assays. In an effort to manipulate the specificity and sensitivity of the antibody-based assays, the cutoff points (absorbance in the case of the enzyme-linked assay or number of elementary bodies in the fluorescent test) may be changed. But this is discouraged by the manufacturers; one gains the benefits of one parameter only at the cost of the other.

Needless to say, this field is not static; there is enormous financial incentive to eliminate the problem of low specificity in the non-culture assay methods. One of the most promising approaches is to use a monoclonal antibody as a specific blocking agent. For EIA, this antibody, while being too low in affinity for practical use as direct marker, would be used in a confirmatory test as follows. Any specimen that appeared positive in the EIA would be repeated in the presence of the highly specific monoclonal antibody. If the antibody successfully blocked the reaction, the result could be regarded as a true positive. Initial tests of this technology suggest that the specificity may be as high as 99.9%. False-positives would thus be much less of a concern, although the method would still have the disadvantage of being 10-20 percent less sensitive than culture.

An Evaluation of the UCSF Experience

In an effort to elucidate some of the factors involved in the laboratory diagnosis of Chlamydia infection, a retrospective survey of the last 1086 specimens processed through this lab was undertaken. There is obviously
not enough information available at the laboratory level to assess many of the variables involved in specimen collection and handling, but it was felt that such a study might reveal sensitive points in the process of specimen evaluation. Specifically, a pattern to the submission of toxic (CPE) specimens was sought. Specimens were recorded with regard to patient unit number, submitting doctor, specimen site, clinic where the specimen was obtained, date of receipt in the microbiology lab and culture result.

Overall, the study showed a positivity rate of 4.2% with 6.6% of the cultures being reported as toxic.

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>Negative</th>
<th>Positive</th>
<th>TOTALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical</td>
<td>826</td>
<td>17</td>
<td>843</td>
</tr>
<tr>
<td>Nasopharynx</td>
<td>34</td>
<td>17</td>
<td>51</td>
</tr>
<tr>
<td>Eye or Conjunctiva</td>
<td>42</td>
<td>6</td>
<td>48</td>
</tr>
<tr>
<td>Tracheal</td>
<td>4P</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Urethral</td>
<td>48</td>
<td>9</td>
<td>57</td>
</tr>
<tr>
<td>Rectal</td>
<td>16</td>
<td>4</td>
<td>20</td>
</tr>
</tbody>
</table>

These cases demonstrated some interesting patterns in the result distribution. Allowing that the submission of one toxic specimen may represent chance contamination, the results of all physicians submitting two or more toxic specimens were evaluated. The percentage of toxic specimens submitted is compared with the experience of the submitting physician, defined as the total number of specimens submitted during the study period.

Doctor Experience

This group included physicians submitting between 3 and 110 specimens and showed toxicities of up to 66%. As can be appreciated from this graph, those doctors submitting only a few specimens during the study period had a far higher chance of submitting toxic ones. Similarly, those clinics with the highest...
percentage of toxic specimens were those which had submitted small numbers of specimens, as shown below.

<table>
<thead>
<tr>
<th>CLINIC</th>
<th>TOTAL #</th>
<th>#P</th>
<th>#T</th>
<th>#N</th>
<th>% toxic</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROC</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>100.00</td>
<td>0.00</td>
</tr>
<tr>
<td>NS15</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>50.00</td>
<td>0.00</td>
</tr>
<tr>
<td>SC15</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>50.00</td>
<td>25.00</td>
</tr>
<tr>
<td>L14</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>40.00</td>
<td>0.00</td>
</tr>
<tr>
<td>URO</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>40.00</td>
<td>0.00</td>
</tr>
<tr>
<td>GM1</td>
<td>7</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>28.57</td>
<td>0.00</td>
</tr>
<tr>
<td>C15</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>25.00</td>
<td>0.00</td>
</tr>
<tr>
<td>GM4</td>
<td>21</td>
<td>0</td>
<td>5</td>
<td>16</td>
<td>23.81</td>
<td>0.00</td>
</tr>
<tr>
<td>ENUN</td>
<td>10</td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>20.00</td>
<td>0.00</td>
</tr>
<tr>
<td>GM2</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>20.00</td>
<td>0.00</td>
</tr>
<tr>
<td>ER</td>
<td>43</td>
<td>4</td>
<td>8</td>
<td>31</td>
<td>18.60</td>
<td>9.30</td>
</tr>
<tr>
<td>ERP</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>12.50</td>
<td>12.50</td>
</tr>
<tr>
<td>PVT</td>
<td>41</td>
<td>1</td>
<td>3</td>
<td>37</td>
<td>7.32</td>
<td>2.44</td>
</tr>
<tr>
<td>GYN</td>
<td>210</td>
<td>7</td>
<td>12</td>
<td>191</td>
<td>5.71</td>
<td>3.33</td>
</tr>
<tr>
<td>PP2</td>
<td>124</td>
<td>10</td>
<td>7</td>
<td>107</td>
<td>5.65</td>
<td>8.06</td>
</tr>
<tr>
<td>OB</td>
<td>397</td>
<td>9</td>
<td>20</td>
<td>368</td>
<td>5.04</td>
<td>2.27</td>
</tr>
<tr>
<td>SAC</td>
<td>139</td>
<td>10</td>
<td>5</td>
<td>124</td>
<td>3.60</td>
<td>7.19</td>
</tr>
</tbody>
</table>

Each of the 4 clinics submitting 100 specimens or more had toxicity rates of between 3.6 and 5.7 percent; this group accounts for 80% of all specimens in this study. Those clinics submitting fewer than 50 specimens had the highest toxicity rates. Collectively, (that is, including those clinics submitting fewer than 50 specimens and no toxic ones) the rate of CPE in this group of clinics is 11.6% with some clinics going much higher. This probably reflects a number of variables, but may include availability of appropriate collection equipment and prompt transport mechanisms.

Rather surprisingly, the chance of a culture developing CPE was not independent of the day of the week when the lab received it. The following graph depicts positivity and toxicity rates as a function of the day of the week they were received.

![Graph](image)

The rate of positive cultures varied only slightly, from 3 to 4.8% over the days Monday - Friday. However, specimens received by the lab on Wednesday had
a nearly 3 fold increased chance of showing CPE. Following Wednesday, there is a steady decline in toxicity with Tuesday having the lowest rate, 2.7%.

Knowing that CPE rate may be affected by a specific doctor or clinic, the data was further evaluated by examining the submission dates from the physicians with the highest toxicity rates to determine if they made a disproportionate contribution to the specimen pool on Wednesday. No such pattern was observed, nor was there any recognizable connection with a particular clinic.

Of the 74 specimens initially reported as toxic, 18 were repeated. From this group, 16 of the subsequent cultures were negative and 2 were positive. The numbers are a bit small to extrapolate, but it is likely that a few (perhaps 6?) of the unrepeated toxics were in fact positive. Significantly, this data strongly suggests that the CPE “pool” of patients probably does not represent a group that is overwhelmingly positive for the organism.

Although the number of non-cervical specimens represents only 15% of the total contributed specimens, a few observations seem worthy of note. Of the 42 specimens submitted from the eye, most positives were described as being from the eyelid or a conjunctival scraping. Specimens submitted as “eye drainage” or “eye discharge” were uniformly negative. This is in keeping with advice found throughout the literature that, even though a chlamydial conjunctivitis may be purulent, this discharge is a poor source of organisms. Viabile, infected epithelial cells are necessary for diagnosis by either culture or DFA. Urethral specimens had about the same positivity and CPE rate as did other specimens. In investigating the clinical history of the 6 patients with toxic urethral cultures, one was on chemotherapy (velban) and another had a concomitant N gonorrhoea UTI. Of the 19 rectal specimens submitted for culture, none were positive. Three however were toxic, representing the highest rate for any site. It is not surprising that this site would generate such a high CPE rate and emphasizes the importance of avoiding heavy bacterial contamination.

In summary, problems relating to CPE of Chlamydia cultures are probably more dependent on clinician-related factors than inherent deficiencies of the culture system as a detection method. While immunoassay may be an appropriate alternative in high-prevalence settings or in areas where culture is unavailable, clinician education may be a more fruitful approach in optimizing culture results.


5 A mammalian cell culture medium which includes 10 mcg gentamycin, 100 mcg vancomycin and 10 U of mycostatin per cc. Less elaborate media may be used, but the use of penicillin should be avoided.


12 J. Schachter, personal communication

Within the group of 74 specimens, two are from the same patient, i.e. a toxic result was repeated, resulting in a second toxic specimen.