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George A Ndeta
Broderick Eribo, Howard University
James Gregory

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Determination of the Efficacy of the Antigen Extraction Technique in the Pathfinder-EIA Chlamydia Detection System

George A. Ndeta, Broderick E. Eribo, James Gregory
Department of Biology, Howard University, Washington, DC

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There is need for a fast and reliable method to detect Chlamydia trachomatis.

With new technology, a technologist can be processing newly arrived swab samples at the same time that an old batch is being assayed.

A technologist should be familiar with the bright apple-green fluorescence of the Chlamydia organism in the MicroTrak technique.

Two diagnostic tests for Chlamydia trachomatis (MicroTrak, Syva Co M, Palo Alto, CA; and TestPack Chlamydia, Abbott Laboratories M, Abbott Park, IL) were used to evaluate swab samples that had been processed through the antigen extraction phase of the Pathfinder technique (Kallestad Diagnostics M, Austin, TX). A total of 558 swab samples were tested using, first, the Pathfinder technique, and second, the MicroTrak technique. Using the TestPack Chlamydia technique, 25 swab samples that were all tested positive by the Pathfinder technique were filtered and tested for Chlamydia; this was followed by assay of 25 unfiltered swab samples. The MicroTrak technique had a 10.4% (57/558) positivity as compared to 9.3% (52/558) positivity for the Pathfinder technique. Agreement between the Pathfinder positives and MicroTrak positives was 91.2% (52/57), while agreement between the Pathfinder and MicroTrak negatives was 99.0% (501/506).

Also, agreement between the Pathfinder and TestPack Chlamydia assay methods was 84% (21/25).

Background
The name “trachoma” is derived from a Greek word, which means “rough”, and refers to the pebble-like appearance of cells of a tissue infected by Chlamydia trachomatis. The term was first used by a Sicilian physician, Pedanius Dioscorides in 60 AD, and a century later, an in-depth study of the disease was conducted by Galen. From the Middle East, chlamydial infection spread to Europe during the crusades. A fresh wave of infection called “Egyptian ophthalmia” would later be established after Napoleon’s Egyptian campaign.

Two researchers observed cytoplasmic inclusion bodies in epithelial scrapings that caused ‘inclusion blennorrhea’ (ophthalmia neonatorum). They found these bodies first in experimental studies involving apes and later in humans. They called the etiological finding “chlamydozoa” or “mantle bodies” because of the morphological characteristic exhibited with Giemsa stain. These findings were called into question by another researcher, who thought that the causative agent of neonatal conjunctivitis was Neisseria gonorrhoeae. In rebuttal, the authors of the first study reported they failed to find inclusions in urethral gonorrhea of women. With this finding, it was reported that inclusions were totally independent of gonococcal infection. This was confirmed by a study where genital secretions from a mother whose newborn had conjunctivitis were used to infect a baboon. Numerous inclusion bodies or ‘inclusion blennorrhea’ from the diseased conjunctiva of the baboon characterized the result of this infection. Tests of the infected materials from the newborn and the baboon was found to be Neisseria gonorrhoeae -free. It was concluded that the agent that causes urethritis and that which causes “inclusion blennorrhea” was Chlamydia trachomatis.

As a leading cause of sexually transmitted disease, there is need for a fast and reliable method to detect Chlamydia trachomatis. This study was to examine the efficacy of the antigen extraction technique in the Pathfinder System by using endo-cervical samples that were collected from patients that attended various clinics in the Washington DC metropolitan area. Sample collection was by using cotton-tip swabs. Positive samples in the Pathfinder technique were assayed by using the Testpack Chlamydia technique. The MicroTrak test served as the definitive test method in this study. The suitability of 3 techniques in the detection of Chlamydia trachomatis was determined by using the following parameters: sensitivity, specificity, cost, time it takes to perform each test, and reliability of methods. The observations made in this study are not an endorsement of one method over the others for patient testing, but rather the findings of the research study.

Materials and Methods
Endo-cervical swabs used had rounded cotton tips and shafts that were composed of plastic and stainless steel. Only one swab size was available for the cervix and urethra. Swabs were treated first with Pathfinder Chlamydia EIA Specimen Treatment Solution A (100 mL NaOH detergent solution with a pH indicator) and later, a Pathfinder Chlamydia EIA Specimen Treatment Solution B (a buffered neutralizing solution containing HCL) was added to extract elementary bodies from the swabs.
Extracted antigen was added to Pathfinder assay tube. Chlamydial antigens present in the specimen attached to the monoclonal antibody coating the walls of the plastic tube. Next, a horse-radish peroxidase-labeled polyclonal antibody directed against Chlamydia was added, which attached to the antigen captured by the antibody on the tube wall. When a peroxidase substrate was added to the tube, a blue color formed if Chlamydia was present in the sample. Other materials to complete the processing of samples were: disposable 12 x 75 mm glass test tubes, pipettes, vortex mixer, a timer, plastic film, disposable glass tube, an immunoassay washing system, and a spectrophotometer.

The TestPack Chlamydia test had 2 phases: an extraction and a reaction phase. Reagents A (NaOH-detergent solution with a pH indicator) and reagents B (buffered neutralizing solution containing HCl) were not needed for the extraction phase because this technique is similar to the Pathfinder. For the reaction phase, reagents C (phosphate buffered saline); D (0.2 µg/ml antibody to C. trachomatis made from rabbit); E (0.8 µg/ml anti-rabbit IgG in beta galactosidase); F (1.0M sodium chloride); and G (0.5% chromogen) were used.

If the results of the 3 techniques showed discrepancies during the first visit to the clinic, a new sample was requested and a second test performed. A gram stain for Neisseria gonorrhoeae located intracellularly and extracellularly was made to better distinguish inclusion bodies from non-inclusion bodies.

**Results**

A total of 558 swab samples were tested using the Pathfinder technique. Samples with spectrophotometric absorbance values of 0.500 nm and above were recognized as strongly positive, while swab sample values of 0.400 nm to the cut-off value were recognized as weakly positive. The cut-off value was calculated by adding a factor of 0.100 to the negative control value. The substrate blanks often read greater than 0.04.

Fifty-two of the 558 samples (9.3%) tested positive for Chlamydia by the Pathfinder (EIA) technique [T1]. The same samples used for the Pathfinder technique were assayed using the MicroTrak (DFA) technique. Of the 558 total swab samples, 57 swab samples (10.2%) were positive for Chlamydia by the fluorescent antibody technique [T1]. Of the 57 samples that tested positive by the MicroTrak technique, 5 swab samples were categorized as potentially negative. Only 2 of these potentially negative samples were also positive in the Pathfinder technique.

For the MicroTrak technique, the number of organisms counted in columnar epithelial cells ranged from greater than 10 elementary bodies to just a few. Eleven swab samples had columnar epithelial cells with greater than 10 elementary bodies despite the fact that the swabs were processed through the antigen extraction phase of the Pathfinder technique. One hundred and sixty-four slides had bacteria, yeast, and epithelial cells, while 78 slides had fungi with epithelial cells only.

Slides with greater than 10 elementary bodies took about 3 minutes to read, while slides with less than 5 elementary bodies took 6 minutes to read. Generally, samples categorized as potentially negative, took about 10 minutes to be evaluated since such slides had less than 3 organisms detected. In addition, slides with 1 organism had to be scrutinized using both low power and high power oil immersion lenses. The presence of artifacts [I1] on the slide increased the time required for interpretation. Only extracellular elementary bodies, visualized microscopically as discrete, evenly fluorescent, bright apple-green disks of about 300 nm in diameter or occasionally larger reticulate bodies were observed. No intact chlamydial inclusions were seen in smears.

The TestPack Chlamydia technique was used to test 25 randomly selected samples from the 52 swab samples that tested positive by the Pathfinder technique. Each of the 25 samples was initially filtered using the individual filter cups provided in the TestPack kit. After pouring 0.8 mL of each sample into 25 separate filter cups, a capped filter plunger was inserted into the sample cup and pushed down. Impurities were removed and samples collected in the receptacle at the base of the filter cup. All 25 samples tested negative following this

![Image](http://labmed.oxfordjournals.org/)
Sensitivity and Specificity of the EIA and the DFA Techniques

<table>
<thead>
<tr>
<th>Technique</th>
<th>Positivity</th>
<th>Sensitivity</th>
<th>Specificity</th>
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</thead>
<tbody>
<tr>
<td>Pathfinder Technique</td>
<td>(52 / 558)</td>
<td>9.3%</td>
<td>91.2%</td>
</tr>
<tr>
<td>Confirmed Assay</td>
<td>(52 / 57)</td>
<td>91.2%</td>
<td>99.6%</td>
</tr>
<tr>
<td>MicroTrak Technique</td>
<td>(504 / 506)</td>
<td>100.0%</td>
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A technologist can be processing newly arrived swab samples at the same time that an old batch is being assayed. This is not the case with the TestPack Chlamydia or the MicroTrak technique. These 2 procedures required the constant attention of the technologist during processing and assaying of samples. This favorable aspect of the Pathfinder technique may be compromised by the fact that suppression of chlamydial infection may conceivably occur during anti-microbial therapy without complete eradication of the organism. Perhaps this may explain why 0.9% of swab samples that were negative in the Pathfinder technique became positive in the MicroTrak technique as assessment of efficacy immediately after therapy may falsely indicate cure. Microbiologic evaluation at a longer interval after completion of therapy may better detect the presence of continued infection or complete treatment of the chlamydial infection. It is worth noting that to be cost-beneficial, microbiologic testing for Chlamydia should initially be used for persons who would not routinely get antibiotic therapy. This avoids the waste of a limited resource.

In patients who are in the early phase of infection or are victim of poor sample collection, the chances of false negative using the Pathfinder procedure are high. This is confirmed by a study in which the authors found that the majority of false-negatives for each rapid test occurred in cases in which very few manipulation. Another batch of 25 randomly selected swab samples that were independent of the first batch, and were positive by the Pathfinder technique were assayed by the TestPack Chlamydia technique without filtration. This time 21 of the 25 samples (84%) were positive. Of the 21 samples, 5 were weakly positive. These weakly positive samples took longer than 5 minutes for color development to take place.

The sensitivity rate for the Pathfinder technique was 91.2% as compared to 91.5% for the MicroTrak or the confirmed test [T2]. The specificity for the Pathfinder assay was 99.6% as compared to 100% [T2] for the confirmed test. The Pathfinder technique agreed with the MicroTrak technique in 501 negative swab samples. The 2 techniques also agreed in 54 positive swabs samples. This left three swab samples that were positive for the MicroTrak technique and negative for the Pathfinder assay technique. Two of 5 swab samples that were categorized as suspicious in the MicroTrak technique and were positive in the Pathfinder technique were used to calculate the confirmed assay values of positivity, sensitivity, and specificity.

When stained with fluorescein isothiocyanate that was specific for Chlamydia trachomatis, Staphylococcus aureus assumed a dull green color [I2]. When Streptococcus pyogenes was stained with the fluorescence reagent, the organism exhibited a red color [I3] just as epithelial cells or negative samples will do. Like S. pyogenes, Acinetobacter calcoaceticus also stained red [I4]. Trichomonas vaginalis, when stained with its own monoclonal antibody that was labeled with fluorescein isothiocyanate demonstrated a bright apple-green color [I5]. However, when this organism was stained with monoclonal antibody that was of Chlamydia trachomatis origin, there was failure to retain the stain [I6].

Discussion

Automation of the spectrophotometer and the washer in the Pathfinder technique makes this a fast and less expensive technique than the TestPack or

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[12] Staphylococcus aureus fixed with acetone and stained with reagent for Chlamydia trachomatis exhibits a dull green fluorescence. This indicates partial cross-reactivity with Chlamydia trachomatis

[13] Streptococcus pyogenes fixed with acetone and stained with reagent for Chlamydia trachomatis. The reddish color of organism demonstrates a lack of cross-reactivity between this species and Chlamydia trachomatis.

[14] Acinetobacter fixed on a slide using acetone was stained with reagent used for diagnosis of Chlamydia trachomatis. The red color of the bacteria as shown here demonstrates failure to ascertain cross-reactivity with Chlamydia trachomatis.
Chlamydia organisms were recovered from patients. In patients with organisms that are known to cross-react with C. trachomatis, the chance of false positive using the Pathfinder technique is equally high. Three swab samples that were negative using the Pathfinder technique were positive during assay using the MicroTrak technique.

The objective of the extraction phase in the Pathfinder technique was to garner a 100% extraction of the Chlamydia infected columnar epithelial cells from swab samples. However, 0.9% of the 558 samples that were negative by the Pathfinder technique gave a positive reaction by the MicroTrak technique. Also, 2% of the swab samples tested by the MicroTrak technique after undergoing extraction in the Pathfinder system were strongly positive, as if they never went through an extraction phase. Also with reports that Acinetobacter are known to cross-react with Chlamydia in the enzyme immunoassay technique, more studies of the antigen extraction phase are required. Acinetobacter strains are very common and different patient samples are often contaminated with these bacteria. This bacteria can cause urinary tract infections and have been shown to play a causative role in non-gonococcal urethritis similar to Chlamydia trachomatis.

The problem of cross-reactivity of Chlamydia with other organisms is demonstrated as in the case where a swab sample from the anal region of a 2-year-old boy who was thought to be a possible victim of sexual abuse because of the recent appearance of perianal warts. Test for C. trachomatis was strongly positive but when the smear was stained with fluorescein-labeled anti-Chlamydia antibody no elementary bodies could be recognized. Fluorescence, though extensive, was confined to larger cocci, which often grouped as tetrads.

Meanwhile, the fact that 4 of the 25 samples that tested positive by the Pathfinder technique tested negative by the TestPack Chlamydia technique further supports the need for re-examination of the extraction phase of the Pathfinder technique. False positives are less likely with the MicroTrak technique as an experienced technologist will be able to distinguish fluorescence due to presence of Chlamydia from fluorescence caused by contaminants. Also, the guidelines to accept or reject samples minimize the possibilities of false-positivity when using this technique.

The MicroTrak technique takes into account the fact that Chlamydia exists in 2 forms: elementary body (extracellular), and reticulate body (intracellular). One of the criteria used in the MicroTrak technique to reject a swab sample is the lack of columnar epithelial cells on a slide after staining with fluorescein isothiocyanate. This is to guard against positive being the result of contamination. The Pathfinder technique does not take these factors into consideration. Antigen extraction is based on recovering whatever substance is on the swab. A drawback to the MicroTrak technique is that slides that were categorized as potentially negative had to be read by a second person and the results confirmed or changed. This extra labor means extra cost to the patient. Since early diagnosis translates into early treatment of patients, many physicians will prefer the Pathfinder technique to the other 2 techniques.

Chlamydia trachomatis infections cause a wide range of clinical manifestations including trachoma, inclusion conjunctivitis, cervicitis, urethritis, epididymitis, proctitis, infantile pneumonia, and lymphogranuloma venereum. As one of the leading causes of sexually transmitted disease in the United States, C. trachomatis infections are of special concern to physicians and public health officials because nearly two-thirds of all Chlamydia infection in women are asymptomatic. In such asymptomatic patients, sub-optimal staining or marginal quantities of organisms could account for the discrepancies observed between the Pathfinder, TestPack, and the MicroTrak techniques. As long as the technologist is familiar with the bright apple-green fluorescence of the Chlamydia organism in the MicroTrack technique, this remains the most reliable, most sensitive, and most specific method for the diagnosis of Chlamydia trachomatis.


