Laboratory Diagnosis and Identification of *Chlamydia trachomatis*

Shamala Moodley

Dept. of Biomedical Sciences, Mangosuthu University of Technology, KZN, South Africa

Chlamydiae are gram negative, obligate intracellular bacteria that grow and replicate in eukaryotic host cells. The developmental cycle is biphasic with morphologically distinct cell types. Cell types include the elementary bodies (Ebs), the infective particles, and the reticulate bodies (Rbs) which are the reproductive particles.

Successful isolation of the chlamydial organism in living cells makes it possible to study *C trachomatis* in depth. Serological and culture techniques will be reviewed followed by the nucleic acid amplification tests in identifying *C. trachomatis*. Present use of molecular techniques, using DNA technology has gained momentum resulting in development of the polymerase and ligase chain reactions tests for the rapid diagnosis of *C trachomatis.* The article will assess the evolution of assays for *C trachomatis* from culture techniques to the development of amplified DNA probes.

Keywords: *Chlamydia trachomatis*; replication; methodologies; cell culture; amplified DNA

1. Introduction

Historically the organism, *Chlamydia,* was referred to as *Bedsoniae, Miyagawanella* and Psittacosis, Lymphogranuloma venereum/trachoma and inclusion conjunctivitis agents. The organism was thereafter classified within the discrete classification *Chlamydiales,* the family, *Chlamydiaceae* and the genus *Chlamydia.* The genus *Chlamydia (C)* consists of three recognised species - *C.psittaci, C.pneumoniae* and *C.trachomatis,* all three cause human disease however, *C.psittaci* infections are zoonotic. A fourth species, *C.pecorum,* has also been described, but its role as a pathogen is unclear. *C.psittaci,* which is primarily an animal and avian pathogen, produces such diseases as bovine abortion, psittacosis, ornithosis and feline pneumonitis. [1, 2] *C.pneumoniae* has been identified as a cause of a variety of respiratory diseases. [3] The species *C.trachomatis,* which consists of 15 serovars, is associated with oculo-genital infection. As in Table 1, there are three serotypes (Ll-3) which cause Lymphogranuloma venereum (LGV) and four serotypes (A, B, Ba, C) which cause endemic trachoma. Eight serotypes (D through K) are associated with *C.trachomatis* in eye and genital infection (Table I). [4, 5]

It has been suggested that non-gonococcal ophthalmia might be due to infecting organisms acquired in the maternal genital tract. [6] In 1907 it was described as intra-epithelial inclusion in scrapings from the conjunctiva obtained from orangutans which had been inoculated with human trachomatous material.[7] Approximately two years later similar inclusions in smears taken from the conjunctiva from neonates with non-gonococcal ophthalmia was demonstrated.[8] Thereafter, a firm link was established between ocular and genital *C trachomatis* infections.[9] Explanation about the epidemiology of genital chlamydial infection and its function in NGU and ocular infections were reaffirmed . The trachoma agent was successfully isolated, by the use of yolk sacs of embryonated hens' eggs, for culture. [10]. Results were soon acknowledged by research teams in different regions internationally. The first isolate of the *C.trachomatis* organism was recovered from the cervix of the mother of an infant with ophthalmia neonatorum.[11] Sexual transmission of the organism and its role in NGU was firmly re-established when *C. trachomatis* was first isolated from the urethrae of men associated with conjunctivitis cases. [12]

The introduction of a tissue culture procedure, for the isolation of *C.trachomatis,* made large scale examination of specimens possible. [13]. Research workers began actively pursuing the study of chlamydial genital tract infection which led to the morphological and physical characterisation of the organism. [14; 15] Use of living cells instead of artificial media meant that cells needed to be propagated constantly. Certain restrictions such as the price of cell culture media, keeping cell culture viable and the turn - around time in diagnosis of the organism, motivated researchers for looking into rapid diagnostic techniques.

Numerous technological advances, in methodologies, have been made since the first cell culture isolation of Chlamydiae. The detection of chlamydial antigen by serological methods was introduced and the technique led to a large number of commercially available antigen-antibody detection kits being marketed. [16; 17] Present use of molecular techniques, using DNA technology has also gained momentum resulting in development of nucleic acid amplification tests (NAAT) for the rapid diagnosis of *C.trachomatis*. [18]

Table 1 Clinical spectrum of *Chlamydia trachomatis* infections associated with serovars*

Serotypes	Patients Infected	Disease	Complications
A, B, Ba, C	All	trachoma	blindness
L1-3	All	LGV	scarring, fibrosis
D-K	Males	NGU	epididymitis,
		PGV	Reiters syndrome
		conjunctivitis	not known
		proctitis	arthritis (SARA)
	Females	mucopurulent cervitis	salpingitis,perihepatitis
			infertility,
			ectopic pregnancy
		urethritis,	not known
		conjunctivitis,	not known
		proctitis	arthritis (SARA)
	Neonates	conjunctivitis	pneumonia

* indicates the common, but not exclusive associations of serotypes with disease.

2. Review of Diagnostic Methodologies

2.1 Life-cycle of *Clamydia trachomatis*

Chlamydiae are gram negative, obligate intracellular organisms which grow and replicate in eukaryotic host cells. Their survival is basically parasitic because of a lack of an electron transport system and the absence of cytochromes. These factors are essential for synthesis of adenosine triphosphate (ATP). Therefore, the organisms grow and replicate due to the host cell ATP and nutrients. The method of development and replication within the host cell is exclusive to Chlamydiae and consists of two morphologically distinct cell types, the elementary bodies (Ebs) which are the infective particles, and the reticulate bodies (Rbs) which are the reproductive particles. Elementary bodies, although relatively stable in the extra cellular environment, are not metabolically active. They never divide but attach to specific receptor sites on the surface of susceptible host cells and enter via phagocytic vesicles. Once within the cytoplasm of the cell, the elementary bodies develop into mature forms, the reticulate bodies, which are the metabolically active and dividing forms of Chlamydiae. The Rbs divide by binary fission, from approximately 8 hours post entry to 18 - 24 hours. The greatest amount of metabolic activity is displayed during this period. During this period the organisms are most sensitive to inhibitors of cell-wall synthesis and of bacterial metabolic activity. Beyond 24 hours the numbers of elementary bodies seem to predominate, although both forms of the organism are to be found. At some time between 48 - 72 hours the cell membrane ruptures and release a new generation of infective elementary bodies (Figure 1).

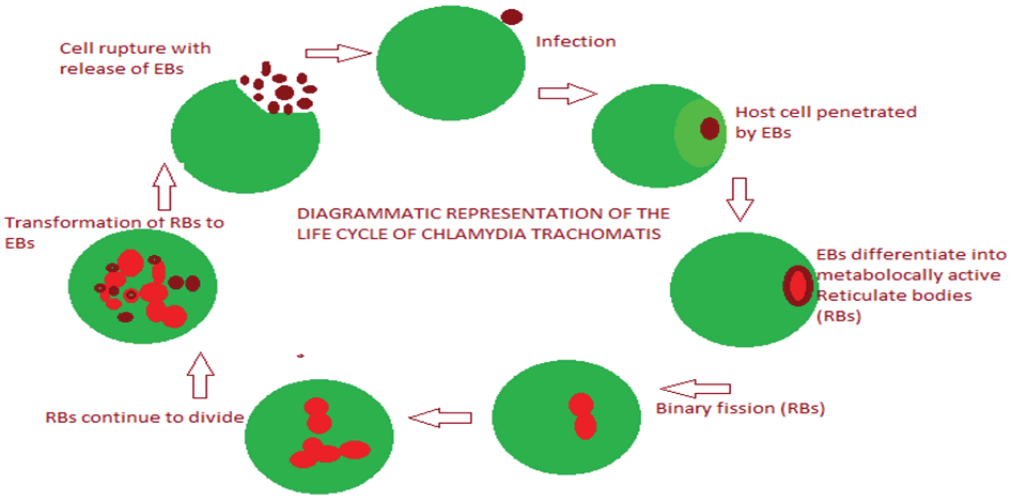

Fig. 1 Life cycle of Chlamydia trachomatis.

2.2 Laboratory Identification of *Chlamydia trachomatis*

There is no single laboratory test, appropriate for the identification of *C. trachomatis,* which would satisfactorily give a conclusive result. Various approaches are used to identify the presence of the organism such as visualisation of inclusion bodies in direct smears, culture of the organisms in cell lines, serological methods and application of

molecular biological techniques which use the presence of chlamydial DNA for identification of the organism. All these methods have limitations and advantages for their applicability in the laboratory.

2.3 Cytology

Cytological detection of *C.trachomatis* was achieved by applying scrapings evenly onto clean microscope slides and air dried. Thereafter, slides were fixed with alcohol or acetone, depending on the choice of staining method. Cytodiagnosis was used for chlamydial inclusion bodies in cases of ocular infection. The interfering debris is minimal with numerous inclusion bodies. However, the possibility of inclusion bodies in genital infections may be obscured, thus lowering the sensitivity of the technique. In *C.trachomatis* cytological techniques, the test depends on the identification of intracytoplasmic inclusion as: [19]

Stage 1: infected cells appear squamoid, often with slight nuclear alterations and diffuse, finely granular cytoplasm;
Stage 2: cells with single or multiple inclusion cells in a finely granular cytoplasm;
Stage 3: eosinophilic chlamydial particles and dense aggregates in the cytoplasm.

However it is generally accepted that cervical cytology is both a non-specific and insensitive indicator of chlamydial infection of the cervix. Under dark ground illumination the inclusions produce a yellow-green auto-fluorescence and under light microscopy the inclusions appear blue against the purple stain of the nuclei of the host cell. Iodine staining is a fairly rapid method and inclusions of *C.trachomatis* appear has a glycogen-like material staining dark brown on exposure to iodine. The supporting cellular matrix stains a pale yellow. However, glycogen, a product of the chlamydial growth cycle, is only present in the maturing chlamydial inclusion and this reduces the methods' sensitivity in low grade infections.

2.4 Antigen Detection Technique

Direct immunofluorescent staining techniques, which use polyclonal antibodies, proved more sensitive than cytological methods. Appropriate working dilutions of antisera are standardised against infected cell culture monolayers. Commercially available fluorescein-conjugated monoclonal antibodies made an alternate method, for *C.trachomatis* testing, possible on both cell culture and direct on clinical specimens.

2.4.1 Direct Fluorescent Antigen (DFA) Detection Technique

Monoclonal antibodies, labelled with fluorescein isothiocyanate, are prepared against the major outer membrane protein present in all 15 known human serotypes of *C.trachomatis.* Clinical specimens on direct staining specifically bind the antigen conjugate. Unbound antibodies are subsequently removed by a rinse step. When stained slides are viewed under a fluorescence microscope the presence of apple green elementary bodies and reticulate bodies, against a reddish-brown background of the cellular matrix, indicates a positive *C. trachomatis* result. Although, initially the procedure seemed less sensitive to cell culture it allowed for a more rapid screening of clinical specimens. To meet technological demands for a rapid screen test the technique was improved. The sensitivity and specificity of DFA is recorded at 81% to 98% and 82% to 100%, respectively. [20, 21] Therefore, it is the most widely used procedure for confirmation of the presence of *C.trachomatis* in a number of research studies. [22, 23, 24]. Performance characteristics of the test are useful for detecting chlamydial-infected cells from neonates in endemic areas of trachoma and high risk populations. A major advantage of DFA is that specimen adequacy can be evaluated by noting either the presence or absence of columnar epithelial cells in the slide preparation. [25, 26] In addition DFA also offers the advantage of a rapid processing time (15 min) and provides a cost effective test. The major limitation of DFA, is that slide evaluation requires a certain amount of expertise and technician fatigue may contribute to reading errors in laboratories with high test volumes.

2.4.2 Enzyme Immunoassay (EIA)

In the absence of cell culture facilities EIA has been employed as a large screening procedure. Basically, EIA relies on a colour change as an indicator of positivity rather than visualization of Ebs (immunofluorescence) or Rbs (cell culture). The procedures are standardised and numerous commercial kits are available to laboratories internationally.

The IDEIA (Indirect Enzyme Immunoassay) is based on a genus specific monoclonal antibody with a double enzyme amplification step. The presence of cell free lipopolysaccharides (LPS), available as a further amplification to the reaction reagents in the assay, allows a greater sensitivity. The Chlamydiazyme assay uses a polyclonal antibody which also reacts with LPS, however, a signal amplification step is not incorporated. Studies have suggested that the specificity problem in the assay may be due to cross-reactions with some other bacteria. [27, 28] To address the cross reactivity, the Chlamydiazyme kit has introduced a confirmatory neutralization step.

Most EIA commercial kits are accompanied with the appropriate type of swab, which reduces the detrimental effect on the signal of some enzyme immunoassays. The time taken between the specimen collection and EIA procedures is suggested at 2 hours, therefore, it is preferable to perform an EIA assay within this period. Some studies have demonstrated that an early morning first void urine sample is preferable, which is free of sampling errors and multiple

swab collections. [29] The specimen is suitable to achieve optimum evaluation of EIA in men. Although this technique is a non-invasive technique and generally facilitates the testing of *C.trachomatis* in asymptomatic males, this procedure is not applicable to the female population because endocervical carriage is more common than urethral. [29]

2.5 Antibody Detection Tests

Chlamydial infections induce antibodies which can be detected by a number of serological techniques, including complement fixation tests (CFT), radioimmuno- precipitation (RIP) and micro immunofluorescence (MIF).

2.5.1 Complement Fixation (CFT)

The technique is based on a genus specific antigen to all chlamydial organisms. CFT was found to be useful for the diagnosis of lymphogranuloma venereum (LGV), but was inappropriate for the diagnosis of chlamydial mucosal infections such as those found in oculo-genital infections.

2.5.2 Radio-Immunoprecipitation Test (RIP)

Like CFT, RIP test is group specific with greater sensitivity. The test utilises a ^{32}P labelled meningopneumonitis *(C. psittaci)* antigen. Studies indicated that RIP performed consistently better than CFT and was comparable with the micro immunofluorescence test [5, 30, 31]

2.5.3 Micro-immunofluorescent Technique (MIF)

The MIF technique uses all 14 serovars of *C.trachomatis* as antigens. The test is performed by placing microdot solutions of each of 14 serovars on a microscope slide, drying the solution and adding dilutions of patients' antisera, followed by fluorescein labelled anti-human antibodies. The microdots are, thereafter, viewed for specific fluorescence. Alternately, the 14 antigens can be replaced by 4 antigen pools representing trachoma (serotypes A, B, Ba, C), oculo-genital (serotypes E-I and K), LGV (serotypes L1-L3) and *C.psittaci* serotypes. The anti-human fluorescein conjugate can comprise of antisera reacting with IgM, IgA or IgG antibodies which allows detection of specific classes of antibodies. The highest dilution of serum to demonstrate fluorescence expressed as a reciprocal, is the titre of antibody present in the serum sample. However, the procedure for the preparation of the antigen is complicated therefore the test is not widely used. As an alternative to these complex procedures, a modified single antigen test was introduced which detected broadly cross-reacting chlamydial antibodies.

The role of serology is the most controversial issue associated with *C. trachomatis* infections. Due to the chronicity of many chlamydial infections, serum antibodies (IgG) may persist; therefore, serological tests are often positive when isolation is negative. This indicates limitations in its diagnostic value for predicting current infections. In practice, however, serological detection of chlamydial antibodies is useful in epidemiological studies and effective as a tool in gauging whether the patient has had previous exposure to *C. trachomatis* infection. [29] Serological techniques for the detection of chlamydial infections are convenient, as compared to culture, but the accuracy and reliability of these techniques are questionable. Specimens are obtained more easily, results are available more rapidly and viability of the chlamydial organism is not required for its detection. When using serological techniques, the basic limitations are that some populations, such as the sexually promiscuous, exhibit titres of antibodies in their system; this makes it difficult to assess whether there is current or previous exposure to chlamydial infection. Several serological techniques are available which demonstrate chlamydial infection; the test of choice varies between each laboratory and their diagnostic requirements.

2.6 Tissue Culture

Cell culture isolation techniques has variations in its application with six clearly defined common principles: collection of clinical specimens using swabs that are non-toxic to either the *Chlamydia* or cell culture monolayers; using the appropriate transport media with non-antichlamydial antibiotics, to inhibit growth of contaminating bacteria;transportation and storage of clinical specimens at the correct temperature; propagation of confluent cell monolayers for inoculation of clinical specimens; inoculation by centrifugation of clinical specimens and incubation of stationary cultures at 35-37°C for 24 - 72 hours; microscopic examination of stained cell cultures for typical inclusion bodies of *C. trachomatis.*

Two key stages, in the processing of specimens, for isolation in cell cultures is the centrifugation of the inoculum onto cell monolayers and the use of a stationary, non-dividing host cells. Centrifugation of the inoculum onto cell monolayers enhances the entry of elementary bodies into the host cells. The compound diethyl-aminoethyl dextran (DEAE dextran), a polycation, also enhances the entry of elementary bodies into host cells. [32] A number of cell lines such as Hela 229, Hep-2, baby hamster kidney cells (BHK 21) and McCoy cells, are susceptible to infection with *C. trachomatis.* The preference in most laboratories is pretreated McCoy cells. The cycloheximide method appears to be the method of choice when dealing with large numbers of clinical specimens as it is convenient to

include the drug with the inoculum. With cycloheximide, the host cells remain distinct from each other and the inclusions are generally larger and more numerous than other treatments. Various factors have been shown to affect the formation of intracytoplasmic inclusion bodies in cells. Among the most important are: the volume of the inoculum, the speed/force of centrifugation, the incubation temperature, the inhibitory effects of animal sera, the hydrogen ion concentration of media and buffer solutions and the addition of essential requirements, such as glucose and glutamine. [33, 34] The most commonly used transport medium is 2-Sucrose Phosphate buffer (2SP), whilst some have used a standard cell culture growth media such as Eagle's Minimum Essential Medium (EMEM). However, common to all transport media is the incorporation of suitable concentrations of antibiotics, to prevent spoilage of cell monolayers by other bacteria and fungi present in the clinical material. Streptomycin at concentrations between 50 - 200 µg/ml is used in combination with Vancomycin at 100 µg/ml and Nystatin at 25 µg/ml. Collection of adequate clinical specimens is a fundamental prerequisite for the successful recovery of *C. trachomatis.* Originally, techniques involved the scraping or curetting of the infected epithelium however; these methods have been replaced by less traumatic collection practices. [5] Studies have shown that cotton-tipped aluminum swabs are superior and less toxic to either cotton-tipped wood or calcium alginate tipped aluminum swabs [35, 36]

Staining of cell cultures is performed by either Giemsa, Iodine or Fluorescent stains. Giemsa stained slides are more sensitive than Iodine however, differentiation of inclusions may be difficult, time consuming and stringent quality control of reagents and stains are required. Iodine staining, although a rapid and easy useful screen for large numbers of specimen, has one drawback, in that, in McCoy cells the polysaccharide glycogen is present for a relatively short period of the *C.trachomatis* developmental cycle, thus the timing of iodine staining is critical. [37] A fluorescein-labelled monoclonal antibody stain for *C.trachomatis* has shown to be superior in sensitivity to the conventional stains. With this stain the chlamydial inclusions may be detected only 24 hours after the inoculation of the clinical specimen. [38] When iodine stained inclusions are not characteristic enough for diagnosis of chlamydial infection, McCoy cells can be de-stained by placing the coverslip in 95 % alcohol for 2 - 3 minutes and restained with the immunofluorescence reagent, to confirm the presence of *C. trachomatis* antigens. Cell culture is the preferred method for the isolation of chlamydial infections for specimens in which low numbers of organisms are anticipated, for example, in asymptomatic infection, because it encourages the growth of the organisms to detectable levels. Furthermore, culture allows the organism to be preserved for additional studies such as antimicrobial susceptibility testing. The difficulties which most microbiologists encounter are maintaining viability of the organism during collection and transportation and, the time required for isolation and identification before results are available.

2.7 Nucleic Acid Amplification Tests (NAATs)

Both polymerase chain reaction (PCR) and ligase chain reaction (LCR) techniques increase the number of specific target deoxyribose nucleic acid (DNA) sequences to increase sensitivity. First void urine (FVU), in both men and women, using the LCR techniques, recorded sensitivities of 96.4% and 82.2%. [39, 40] The indications varied that even expanded gold standards are not comparable because of differences in the results of different cell culture techniques and also because the measure to which the gold standard is extended in different sites of specimen collection and the different assays used in testing of specimens. Current use of the uncomfortable swab technique justifies the use of urine samples. However, as in PCR, LCR results need careful interpretation. These tests have shown to detect chlamydial DNA sequences after antimicrobial treatment while cultures remain negative [41, 42]

2.7.1 Polymerase Chain Reaction test

Polymerase chain reaction, was discovered and developed by scientists at the Cetus Corporation, during the 1980's. It is an *in vitro* method of nucleic acid synthesis by which a particular segment of DNA can be replicated to make millions of copies (amplification). By amplification, it is possible to study genetic material, which is present in such tiny amounts, which cannot be directly detected or analysed. The PCR reaction is a three step process, involving: Denaturation - DNA is heated at 94°C to separate the double stranded helix into single strands, to which oligonucleotide primers can bind. Annealing - Two primers (synthetic stretches of single stranded DNA, each about 20 -30 bases) attach to single strands and generate two short stretches of double stranded DNA; The process occurs at temperatures between 40°C - 60°C; Extension - Short stretches of DNA serve as starting blocks for the enzyme *Tag* polymerase starting at 3'end of the primers. *Taq* polymerase adds the nucleotide complementary to the template, at approximately 72°C, linking them together. The primers are extended in the direction of the target sequence, making a double strand out of two single strands whereby two new DNA double strands identical to the first strand are formed. The three step cycle can now be repeated as often as necessary, with the use of a thermocycler.

The commercially available Amplicor *Chlamydia trachomatis* PCR kit (Roche Diagnostics) is based on the above principle. Amplicor uses a cryptic plasmid, which is common to all serovars of *C. trachomatis,* as the target sequence to ensure the species specificity of the assay. Plasmid based PCR may have a higher sensitivity than the major outer membrane protein (MOMP) based PCR because of the presence of multiple copies of the plasmid in every chlamydial organism. [43] PCR may, therefore, detect *C trachomatis* at an earlier stage of the infection; however,

results require careful interpretation. The assay detects only a small part of the genome of a microorganism and is therefore not necessarily a measure of viability. [40]

2.7.2 Ligase Chain Reaction test

Chlamydial infections uses first void urine from both men and women. The LCR target, which is a short sequence (10 copies of EBs /RBs), is located within this plasmid. The LCx *Chlamydia trachomatis* assay (Abbott Diagnostics) uses the NAAT for LCR, to directly detect the presence of *C. trachomatis* plasmid DNA in clinical specimens. LCR uses four oligonucleotide probes which recognise and hybridize to a specific target sequence within the *C. trachomatis* plasmid DNA. These complementary probes, in the presence of the correct template, hybridize next to each other and ligate together. The ligated probes together with the original template serve as the template for the next cycle of hybridization and ligation. As subsequent cycles are performed, the amplification proceeds exponentially.

The DNA of *C. trachomatis* is released by disruption of the cells and separation of the DNA strands is accomplished by heating the specimen in resuspension buffer. The DNA is then made accessible to the enzymes and various components of the LCR reaction. Amplification is accomplished by adding the four oligonucleotide probes, thermostable ligase and polymerase, and individual nucleotides in buffer (LCR reagents). The four oligonucleotide probes hybridize to complementary single strand *C. trachomatis* target sequence. The hybridization of a pair of probes to the target sequence generates a gap of a few nucleotides between the probes. Polymerase fills in the gap and ligase covalently joins the pair of probes to form an amplification product that is complementary to the original target sequence and can itself serve as a target sequence in subsequent rounds of amplification, in a thermocycler. The amplified product can be detected by Microparticle Enzyme Immunoassay (MEIA).

2.7.3 GEN - PROBE PACE 2

The GEN-PROBE PACE 2 system (GEN-PROBE Incorporated) is a non- isotopic, DNA probe test which uses the technique of nucleic acid hybridization for the detection of *C. trachomatis.* The Hybridization Protection Assay (HPA) uses a chemiluminescence labelled, single-stranded DNA probe, which is complementary to the ribosomal RNA of the target organism. The ribosomal RNA combines with the labelled DNA probe to form a stable DNA: RNA hybrid. Thereafter the hybrid is separated from the non-hybridized probe and is measured in a luminometer (GEN-PROBE Inc). The results are calculated as the difference between the response of the specimen and the mean response of the negative reference and is expressed as Relative Light Units (RLU). The HPA format begins with Hybridization by adding acridinium ester-labelled probe to specimen and incubated for 10 minutes at 60°C. The Select process is adding differential hydrolysis reagent which is incubated for 5-10 minutes at 60°C. This is followed by Detection with the acridinium ester label associates with hybrid using the Gen-Probe Leader luminometer. The PACE 2 system for *C.trachomatis* can be used in conjunction with the PACE 2 *C.trachomatis* PROBE COMPETITION ASSAY (PCA) as a supplemental test to detect non-specific signal in endocervical and male urethral specimens. The total assay time is 2 hours with specificity at 88-95% and sensitivities between 96-98% [44, 45, 46].

3. Discussions

It is well recognised and accepted that there is no single test which when used on its own will conclusively diagnose chlamydial infections. In view of this a number of authorities recommend the use of an extended 'gold standard' or extended reference standard [42, 47]. Discrepant analysis attempts to identify the true positive patient that is not isolated by cell culture. The analysis is an attempt to provide estimates of sensitivity and specificity in what may be considered an imperfect 'gold standard'. Researchers have applied this technique to estimate the sensitivity and specificity of DNA amplification tests for the detection of *Chlamydia trachomatis.* [47] Apparent false positive samples (cell culture negative and DNA amplification positive) are subjected to further testing with DFA or alternatively, using DNA amplification which targets the major outer membrane protein. If any of these additional tests reveal a positive result then the original DNA amplification is considered a true positive and the original cell culture is considered a false negative.

4. Conclusion

Almost all published studies that use discrepant analysis to compare amplification tests against cell culture eventually reclassify 95 % of false positive samples as true positives after additional sequential testing. It was found that this method of calculating sensitivity and specificity was biased as retesting using different techniques result in overestimation of the performance of the new test because the decision rule in discrepant analysis invariably favours the new diagnostic test. [48]

Acknowledgements The support by Mangosuthu University of Technology and Global Laboratories is gratefully acknowledged.

References

[1] Treharne JD and Ballard RC. The expanding spectrum of the *Chlamydia*- a microbiological and clinical appraisal. Rev Med Micro. 1990; 1: 10-18.
[2] Storz J. *Chlamydia* and *Chlamydia* induced diseases. Charles C Thomas: Springfield Ill, 1971.
[3] Grayston JT. Infections caused by *Chlamydia pneumoniae* strain TWAR. Clin Inf Dis 1992; 15: 757-763.
[4] Mårdh PA, Paavonen J and Puolakkainen M. *Chlamydia.* 1 ed. Plenum Publishing Corporation, NY, 1989.
[5] Oriel JD and Ridgeway GL. Genital Infection by *Chlamydia trachomatis*: Current topics in infection series. London. Edward Arnold Ltd. 1982.
[6] Kroner T. Zur Aetiologie der Opthalmoblennorrhoea Neonatorum. Zentralblatt fur Gynakologie 1884; 8: 643.
[7] Halberstaedter L and Prowazek S. Uber zelleinschulsse Parasitarer natur beim Trachom. Albeiten aus dem Kaiserlichen Gesundheitsamke 1907; 26: 44-47.
[8] Heyman B. Uber die Fundorte der Powazetz'schen Korperchen. Berliner Klinische Wochenschrift 1910; 47: 663-666.
[9] Fritsh HO, Hofstatter A and Lindner K. Experimentelle Studie zur Trachomfrage. Graefe's Archiv fur Opthalmologie 1910; 76: 547.
[10] Tang FF, Chang HL, Huang YT and Wang KC. Studies on the aetiology of trachoma with special reference to isolation of the virus in chick embryo. Clin Med J 1957; 75: 429-447.
[11] Jones RB, collier LH and Smith CH. Isolation of virus from inclusion blennorrhoea. Lancet 1959; 1: 902-905.
[12] Dunlop EMC, Jones BR and Al- Hussaini MK. Genital infection in TRIC virus infection of the eye. Clinical and other findings. B J Ven Dis 1964; 40: 33-42.
[13] Gordon F and Quan AL. Isolation of Trachoma agent in cell culture. Proceedings of the Society of Experimental Biology (NY) 1965; 118: 354-359.
[14] Armstrong JA and Reed SE. Fine structure of LGV agent and the effect of penicillin and 5F-U. J Gen Micro 1967; 46: 435-444.
[15] Kramer MJ and Gordon FB. Ultrastructural analysis of the effects of penicillin and chlortetracycline on the development of a genital tract *Chlamydia*. Inf Imm 1971; 3: 333-341.
[16] Grayston JT and Wang SP. New knowledge of *Chlamydiae* and the disease they cause. J Inf Dis 1975; 132:87-105.
[17] Thomas BJ Feeve P and Oriel JD. Simplified serological tests for antibodies to *C trachomatis*. J Clin Micro 1976; 4:6-10.
[18] Schachter J Biology of *Chlamydia trachomatis*. In: Mandell, Douglas and Bennett, 4th ed. Principles and practice of Infectious Diseases. NY; Church Livingston 1995; 243-257.
[19] Gupta PK, Lee EF, Erozan YS, Frost JK Geddes ST and Donovan PA. Cytologic investigations of *Chlamydia* infection. Acta Cytol 1979; 23: 315-320.
[20] Clarke LM, Sierra MF, Daldone BJ, Lopez N, Corvino JM and McCormack WM. Comparison of Syva Microtrak enzyme immunoassay and Gen-Probe PACE 2 cell culture for diagnosis of cervical Chlamydia trachomatis infection in high prevalence female population. J Clin Micro 1993; 31: 968-971.
[21] Kellogg JA, Seiple JW and Stroll ES. Direct fluorescent antibody confirmation of chlamydial antigen below the detection threshold of the Chlamydiazyme enzyme linked immunosorbent assay. J Clin Micro 1993; 31: 1646-1647.
[22] Ostergaard L, and Møller JK. Use of PCR and direct immunofluoresecent microscopy for confirmation of results obtained by Syva MicroTrak Chlamydia enzyme immunoassay. J Clin Micro 1995; 33: 2620-2623.
[23] Sanders JW, Hook III EW, Welsh L, Shephard ME and Quinn TC. Evaluation of an enzyme immunoassay for the detection of *Chlamydia trachomatis* in urine of asymptomatic men. J Clin Micro 1994; 32: 24-27.
[24] Zeeberg B, Thelin I and Schalen C. *Chlamydia trachomatis* antigen detection by Chlamydiazyme blocking reagent verification. Int J Sex Trans Dis AIDS 1992; 3: 355-359.
[25] Barnes R. Laboratory diagnosis of human chlamydial infections. Clin Micro Rev 1989; 2: 119-136.
[26] Lombard and Pawel. *Chlamydia trachomatis* testing: an overview. Am Clin Prod Int Sci Comm 1989; 30 -39.
[27] Saikku P, Puolakkainem M, leinonen M, Nurminen M and Nissinen A. Cross reactivity between Chlamydiazyme and *Acinetobacter* strains. N Eng J Med 1986; 314: 922-923.
[28] Taylor-Robinson D, Thomas BJ and Osborn MF. Evaluation of enzyme immunoassay (Chlamydiazyme) for detecting *Chlamydia trachomatis* in genital tract specimens. J Clin Path 1987; 40: 194-199.
[29] Caul EO, Paul ID, Milne JD and Crowley T. Non – invasive sampling methods for detecting *Chlamydia trachomatis*. Lancet 1988; 2: 1246-1247.
[30] Dwyer R St, Treharne JD, Jones BR and Hering J. Results of micro immunofluorescent tests for the detection of type specific antibody in certain chlamydial infections. BJ Ven Dis 1972; 48: 452-459.
[31] Reeve P, Gerloff RK, Casper E, Phillip RN, Oriel JD and Powis P. Serological studies on the role of *Chlamydia* in aetiological of non-specific urethritis. BJ Ven Dis 1974; 50: 136-139.
[32] Rota T and Nichols R. Infection of cell culture by Trachoma agent: Enhancement by DEAE-Dextran. J Inf Dis 1971; 124: 419-420.
[33] Allan L and Pearce JH. Modulation by centrifugation of cell susceptibility to chlamydial infection. Gen Micro 1979; 111: 87-92.
[34] Darougar S and Treharne JD. Cell culture methods for the isolation of *C.Trachomatis*. In: Mårdh PA. Chlamydial infections, Amsterdam, Elsevier Biomedical Press 1982: 265-274.
[35] Mahony JB and Chernesky MA. Effect of swab type and storage temperature on the isolation of *Chlamydia trachomatis* from clinical specimens. J Clin Micro 1985; 22: 865-867.
[36] Stamm,WE. Diagnosis of *Chlamydia trachomatis* genitourinary infections. Ann Int Med 1988; 108: 710-717.

[37] Schachter J and Dawson CR. Human chlamydial infections. Washington DC, PSG publishing Co Inc, Littleton 1978: 122.
[38] Kuo CC, Wang SP, Wentworth BB and Grayston JT. Primary isolation of TRIC organism in HeLa 229 cells treated with DEAE-Dextran. J Inf Dis 1972; 125: 665-668.
[39] Chernesky M, Lee H and Schacter J. Diagnosis of *Chlamydia trachomatis* uretheral infection in symptomatic and asymptomatic men by testing first void urine in a ligase chain reaction. J Inf Dis 1994; 170:1308-1311.
[40] Van Doornum GJJ, Peerbooms PGJ, Buimer M, Henguet H and Burckardt HJ. Evaluation of Amplicor *Chlamydia trachomatis* for daily routine testing in screening women and men attending a STD clinic in comparison with Chlamydiazyme EIA. Sex Trans Dis 1994; 21(Suppl): 175.
[41] Radcliffe KW. Is a test of cure necessary following treatment of cervical infection with *Chlamydia trachomatis*? Genitourin Med 1990; 66: 444-446.
[42] Ossewaarde JM, Rieffe M, Rozenburg M, Ossenkoppele PM, Nawrocki RP and Van Loon AM. Development and clinical evaluation of a polymerase chain reaction test for detection of *Chlamydia trachomatis*. J Clin Micro 1992; 30: 2122-2128.
[43] Palmer L and Falkow S. A common plasmid in *Chlamydia trachomatis*. J Clin Micro 1986; 16: 52-62.
[44] Hipp S. Assessment of enzyme immunoassay and immunofluorescence tests for detection of *Chlamydia trachomatis*. J Clin Micro 1989; 25:938- 1943.
[45] Dean D. Use of *Chlamydia trachomatis* DNA probe for detection of ocular Chlamydiae. J Clin Micro 1989; 27: 1062-1067.
[46] Warren R, Dwyer B and Plackett M. Comparative evaluation of detection assays. J Clin Micro 1993; 31: 1663-1666.
[47] Schacter J, Stamm WE, Quinn TC, Andrew WW, Burczak JD and Lee HH. Ligase chain reaction to detect *Chlamydia trachomatis* infection of the cervix. J Clin Micro 1994; 32: 2540-2543.
[48] Hadgu A. The discrepancy in discrepant analysis. Lancet 1996; 23: 315-320.

