An Investigation into Swab and Urine Samples for the Detection of *Chlamydia trachomatis* Genital Infections

By

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Summary

Chlamydia trachomatis was first isolated in tissue culture in 1965 and urogenital swabbing followed by culture has remained the standard by which other, more recent, non-cultural methods are judged.

Viable *C.trachomatis* organisms do not survive well in urine. However, with the advent of non-cultural methods, where the presence of viable material is not a requirement, the potential of urine for the detection of chlamydial antigen is under scrutiny.

In order to remain competitive, manufacturers of chlamydia immunoassays and more recently DNA amplification tests, have sought validation of existing and new diagnostic methods, for use with urine samples, by hospital based clinical trials.

The aims of this study were to investigate the inhibitory effect that some urogenital samples have on non-cultural test outcome and to undertake a series of clinical trials. In addition a simplified culture method was evaluated.

In two investigations prior to the clinical trials, the little described "hook effect", observed in some enzyme immunoassays, was researched. It was found that an optical density parabola could be induced in all of the tests under scrutiny depending on the number of leucocytes present and the volume of urine centrifuged. Methods were developed to exclude the possibility of excess antigen causing a prozone effect. In the other investigation, undertaken at Unipath Limited Research and Development department, sample interference in the Clearview™ Chlamydia test, possibly caused by cations within the specimen and anionic groups or hydophobic components in Chlamydia lipopolysaccharide, were explored. Cationic interference was reduced by the addition of heparin to the test extraction reagent.

The eleven clinical trials resulted in successful validation of the methods for diagnostic use. The problems with discrepant results and sampling error were resolved by repeat testing or further analysis by alternative methods.

Key words: Chlamydia trachomatis, urine, culture, enzyme immunoassay, inhibitors

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Abbreviations

AU	Absorbence units
CT	Clinical Trial
cm	Centimetre
°C	Degrees centrigrade
D	Daltons
DNA	Deoxyribonucleic acid
DIF	Direct immunofluorescence
EBS	Elementary bodies
EIA	Enzyme immunoassay
EMU	Early morning urine
FDA	USA Food and Drugs Administration
FPU	First pass urine
g	acceleration due to gravity
GP	General practitioner
GUM	Genitourinary medicine
HVS	High vaginal swab
IDU	5-iodo-2-deoxyuridine
IF	Immunofluorescence
KDO	Keto-deoxy-D-manno-octulosonic acid
LCR	Ligase chain reaction
Leucs	Leucocytes
LGV	Lymphogranuloma venerum
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MEM	Minimum essential medium
MIF	Microimmunofluorescence test
Mwt	Molecular weight
µg	microgramme(s)
mg	milligramme(s)
µl	microlitre(s)
ml	millilitre(s)

mm	millimetre
MOMP	Major outer membrane protein
NGU	Non-gonococcal urethritis
nm	nanometre(s)
ng	nanogrammes(s)
NT	Not tested
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGU	Post gonococcal urethritis
PID	Pelvic inflammatory disease
P:N	Positive to negative ratio
PTFE	Polytetrafluoroethylene
rRNA	Ribosomal Ribonucleic acid
RBS	Reticulate bodies
rpm	revolutions per minute
VS	Versus

Clinical Trials

CT1 Phadebact Chlamydia EIA (Boule Diagnostics)
Urethral swab culture vs first pass urine EIA
107 male patients attending a GUM clinic.

CT2 Mastazyme Chlamydia EIA (Shield Diagnostics)
Urethral swab culture vs first pass urine EIA
304 male patients attending a GUM clinic.

CT3 Clearview™ Chlamydia test (Unipath Ltd)
Endocerval swab culture vs Clearview test
350 female patients in General Practice.

CT4 Amplicor Chlamydia Test (Roche)
Endocervical swab culture vs Amplicor PCR test
245 female patients attending a GUM clinic.

CT5 Clearview™ Chlamydia MF test (Unipath Ltd)
Endocervical swab culture vs Clearview test
320 female patients attending a GUM clinic.

CT6 Clearview™ Chlamydia MF test (Unipath Ltd)
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225 Symptomatic male patients attending a GUM clinic.

CT7 Clearview™ Chlamydia MF test (Unipath Ltd)
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CT8 Clearview™ Chlamydia MF test (Unipath Ltd)
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CT9 Clearview™ Chlamydia MF test (Unipath Ltd)
IDEIA™ Chlamydia EIA (Dako Diagnostics Ltd)
LCR™ *Chlamydia trachomatis* (Abbott Laboratories)
Urethral swab EIA vs first pass urine Clearview and LCR
70 male patients attending a GUM clinic.

CT10 IDEIA™ *PCE* (Dako Diagnostics)
Urethral swab IDEIA vs IDEIA *PCE*
159 male patients attending a GUM clinic.

CT11 IDEIA™ *PCE* Chlamydia EIA (Dako Diagnostics)
Clearview™ Chlamydia MF (Unipath Ltd)
LCR™ *Chlamydia trachomatis* (Abbott Laboratories)
Endocervical swab culture and Clearview vs first pass urine IDEIA *PCE* and LCR
161 female patients attending two Medical Centres.

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1. INTRODUCTION

1.1. Classification

1.1.1. *Chlamydia*

The term *Chlamydozoaceae* was first suggested by Halberstaedter and von Prowazek (1907) from the Greek word *chlamys* meaning a mantle or cloak to describe the inclusion matrix seen around the elementary bodies in cell cytoplasm. During the next 60 years confusion over whether these organisms were bacteria or viruses led to a proliferation of names until resolution by Page (1966) who proposed two species of the genus Chlamydia, sub-group A, *C.trachomatis* and sub-group B, *C.psittaci*. In recent years the psittaci sub group has been further sub-divided into two more species, *C.pneumoniae* (Grayston et al 1989) and *C.pecorum* (Fukushi and Hirai 1992) and is likely to be further sub-divided as judged by DNA homology studies (Herring, 1992)

1.1.2. *Chlamydia trachomatis*

The name trachoma was first used by a Sicilian physician in the first century from the Greek word meaning rough eye although the condition and its treatment had been described in the Ebers Papyrus in the nineteenth century BC (Thygeson, 1962 Duke Elder, 1965). Trachoma is still the single most common form of preventable blindness in the world. The genital strains of *C.trachomatis* may also cause eye infections particularly in the new born, by passage through the birth canal, although this condition was not linked to non-gonococcal urethritis in men and cervicitis in women until Fritsch et al (1910) utilised experimental inoculation of monkeys. It was with the advent of penicillin to reduce super infections, that Chlamydia was successfully cultivated in the yolk sac of fertile hens' eggs (T'ang et al 1957). Prior to this date diagnosis was by direct microscopic examination of cellular exudates using various staining procedures. It was not until Gordon and Quan (1965) described isolation of *C.trachomatis* in tissue culture that the possibility of examining large numbers of clinical specimens became a reality and caused an explosion of interest in this common organism.

1.1.3. *Microbiology and Morphology*

Because chlamydiae are small organisms, 300 to 1200 nm in diameter and unable to multiply in synthetic culture medium, they were originally thought to be viruses. (Photo 1). We now know that they are Gram negative coccoid bacteria which divide by binary fission, contain both DNA and RNA and are sensitive to antibiotics. However, as they are obligate intracellular parasites, cultivation is most often undertaken by virus laboratories using a variety of cell types and techniques. During the 48 hour life cycle the organism appears in two distinct forms. The smaller form or elementary body (EB) is phagocytosed by the host cell and lies within a cytoplasmic vacuole. In the first 10 hours incubation the EB enlarges into the initial or reticulate body (RB) and begins to multiply with active production of RNA. After a further 10 to 15 hours the RBS once again reduce in size to produce EBS and eventually after 36 to 48 hours incubation the host cell ruptures releasing infectious particles.

Photo 1. Photomicrograph of *Chlamydia trachomatis* elementary bodies in McCoy tissue culture cells stained by immunofluorescence

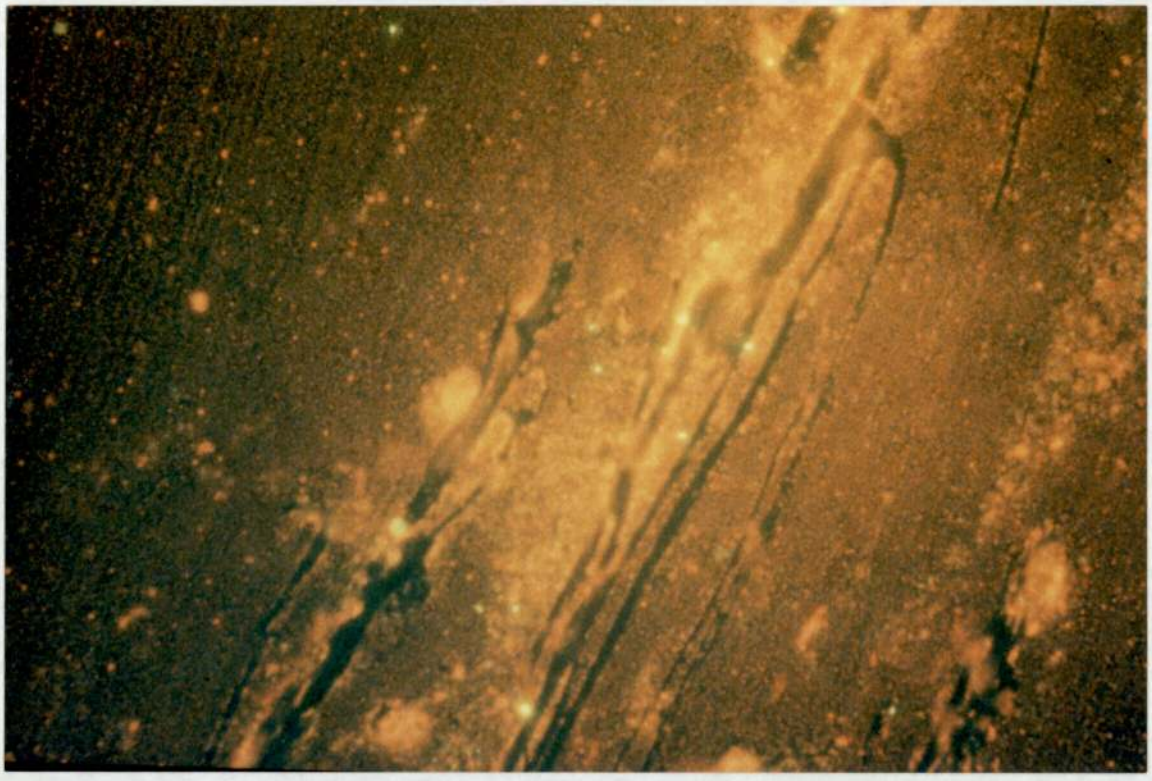
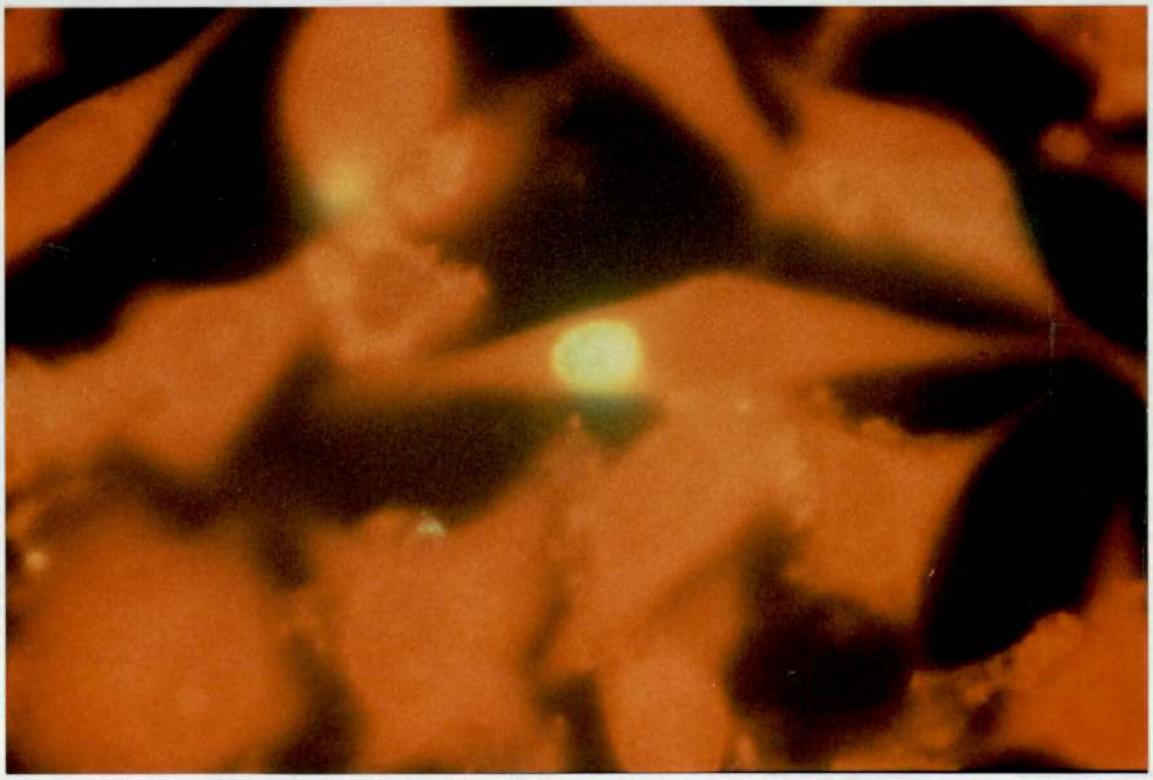


Photo 2. Photomicrograph of *Chlamydia trachomatis* inclusion body in McCoy tissue culture cells stained by immunofluorescence.



1.1.4. *C.trachomatis serotypes*

Chlamydia trachomatis shares a common genus specific complement fixing, lipopolysaccharide (LPS) antigen and a species specific major outer membrane protein (MOMP). Hence, both these epitopes are useful in the identification and differentiation of Chlamydia when using monoclonal antibodies (MAb).

The development of the microimmunofluorescence (MIF) test by Grayston (1975) demonstrated fifteen serovars, types A to C being endemic trachoma strains, D to K genital strains and L1 to L3 causing Lymphogranuloma venerum (LGV). This study is only concerned with *C.trachomatis* strains D to K.

1.2. *C.trachomatis* genital infections

1.2.1. *Genital infections in women*

C.trachomatis is commonly an asymptomatic infection in women and clinically difficult to recognise unless presenting as a classic cervicitis. Isolation rates of 12-31% have been reported (Oriel, 1982) although personal observation has shown a reduction in the higher limit in recent years. Because of its occult nature in the female genital tract, *C.trachomatis* can cause an ascending infection which left untreated leads to tubal damage and infertility. Recurrent infection will exacerbate this process and increase the risk of ectopic pregnancy (Paavonen, 1992). Other, though lesser, sites of infection in females include the urethra, rectum, pharynx and eye. Approximately 50% of babies born to patients with a *C.trachomatis* cervical infection will develop an ocular infection (Schachter, 1978).

1.2.2. *Genital infections of men*

In contrast to *C.trachomatis* infections in women, the symptoms in men are more readily recognised. Most will present with a urethral discharge which is easily differentiated from *Neisseria gonorrhoea* by Gram strain. Non-gonococcal urethritis (NGU) and post gonococcal urethritis (PGU) is caused by *C.trachomatis* in up to 50% of patients (Oriel, 1982). It is the commonest form of sexually transmitted disease in men and women it accounts for approximately 35,000 new cases reported annually in

the UK (Robinson & Ridgway, 1996). Complications in men can lead to epididymitis and reactive arthritis and as in women, it can also be recovered from the rectum, pharynx and eye.

1.3. Laboratory Diagnosis

1.3.1. *Isolation in tissue culture*

Within 10 years of the first isolation of *C.trachomatis* in tissue culture by Gordon and Quan (1965), many laboratories had established a diagnostic service based on culture using McCoy cells pre-treated with 5-iodo-2-deoxyuridine (IDU) or post treated with cycloheximide. The presence of typical intracytoplasmic *C.trachomatis* inclusions was confirmed by staining the coverslip culture monolayers with either Giemsa or iodine stain which has now been largely superseded by superior immunofluorescent methods (Photo 2). The culture method for *C.trachomatis* has remained the 'yard stick' by which all other antigen detection methods are measured and in clinical trials a 'blind' sub-culture of negative primary cultures is included. Whereas in other methods the specimen range is limited, in culture any specimen may be used for attempted isolation provided it is not toxic to the cell system. An exception to this acceptable culture range are urine samples which, although not toxic, have been shown to have low levels of viable organisms present (Smith and Weed, 1976; Matthews, et al 1988). However, culture is a tedious technique requiring a great deal of technical expertise and with the advent of MAb has shown a rapid decline in favour of alternative methods.

1.3.2. *Direct immunofluorescence*

In 1985 two commercially prepared fluorescein labelled Mab became available for the direct immunofluorescence (DIF) of *C.trachomatis*, one was a genus specific anti-LPS (Dako, Imagen) and the other a type specific anti-MOMP (Syva Micro Trak). Both of these MAbs were validated for use with urethral, cervical and ocular specimens and furthermore the anti-LPS MAb was validated for tissue culture confirmation use (Matthews, 1986). Problems incurred whilst using this method include inadequately collected smears and low numbers of detectable EBS and most laboratories set quality

standards, below which repeat specimens are requested. However, in expert hands, this technique has been used to great effect for many types of specimens and has been claimed to be as sensitive as molecular methods (Palmer, et al 1991).

1.3.3. *Enzyme immunoassays*

Shortly after the introduction of DIF a proliferation of enzyme immunoassays (EIA) were launched using polyclonal or monoclonal anti-LPS antisera. These tests revolutionised the detection of *C.trachomatis* and for the first time allowed large batch testing by less skilled operators. In view of the serious legal and social implications of diagnosing a sexually transmitted disease it is recommended that all positive EIA reactions are confirmed by IF on the remaining sample extract or by use of a *C.trachomatis* 'blocking reagent' in a repeat EIA test (Mumtaz, et al 1991). All EIAs are validated for use with urethral and cervical swabs whilst only some are validated for use with ocular and male urine samples and only one for use with naso-pharyngeal aspirates.

1.3.4. *Immunoassays*

In recent years several solid phase immunoassays have been devised for near patient testing. These simple devices take the format of a membrane supported in a plastic holder through which the antigen is passed. A positive result is indicated by a colour change on the membrane with an additional indicator to confirm the test has worked correctly. These devices are all validated for use with cervical swabs and some for use with male urethral swabs and male first pass urine samples. Although this type of test is unlikely to replace laboratory based techniques they may prove useful for rapid presumptive results in clinical settings (Arumainayagam, et al 1990)

1.3.5. *Molecular techniques*

Originally molecular techniques were developed 'in house' by research laboratories and this is still the case where a second, confirmatory test is required for validation of a primary method. There are three commercially prepared tests available. The DNA probe manufactured by Gene-Probe has recently been improved to include an amplification step but this test has been overshadowed by the polymerase chain

reaction (PCR) test produced by Roche and the ligase chain reaction (LCR™) test developed by Abbott Laboratories. In each of these methods primers are targeted at specific sequences of *C.trachomatis.* After amplification the resulting amplicon is detected by either enzyme immunoassay or chemiluminescence.

All of these tests are semi automated to reduce the chance of contamination by stray DNA and some manufacturer's claim to have minimised the problem by patented methodology. In theory, molecular techniques could be used for any type of sample, and to date, each has been validated for male and female genital swabs and female urine specimens. Beside the increased cost of these tests the major drawback is the confirmation of positive reactions. Visualisation of EBS by IF in the remaining amplicon is impossible following the extraction process. Therefore a stored aliquot of the original material has to be retained for further analysis. This necessarily increases the cost in any cost/benefit analysis.

1.3.6. The extended "Gold Standard"

The traditional gold standard for *C.trachomatis* diagnosis is growth in tissue culture, however, with the advent of molecular techniques, in theory capable of detecting one elementary body, there has been a reappraisal of the available methodology, (Schachter, 1997).

The exquisite sensitivity of molecular methods has highlighted the inadequacy of tissue culture and the need for an expanded standard of culture plus other non-cell culture methods to give optimal comparitive data. However, (Hadgu, 1996) has criticised the extended gold standard for being biased in favour of confirming discrepant positives at the expense of false negatives.

Nevertheless the expanded gold standard has become the norm in contemporary scientific publications when evaluating Chlamydia diagnostic tests. The problem of bias will not be overcome until either a single diagnostic method, such as LCR, becomes the new gold standard or unbiased samples are all treated in the same manner.

1.4. Genital Specimens

In all of the predescribed methods of chlamydia antigen detection the collection of male and female swab specimens remains the same, that is, any excess of pus or exudate is removed prior to the sample being taken. As the organism inhabits columnar epithelial cells it is essential that these cells are present in the sample. Serial or multiple swabbing has been shown to increase isolation rates (Dunlop, et al 1985).

1.4.1. *Female samples*

The use of a speculum is necessary for the successful collection of columnar epithelial cells and a simple high vaginal swab (HVS) will usually result in negative findings. The cervix is cleansed and a swab is inserted via the os into the endocervical canal one or two cm. A large or small swab may be used. An additional swab taken from the urethra and pooled with the cervical specimen will enhance detection rates (Manuel, et al 1987).

1.4.2. *Male samples*

Male patients attending genito urinary medicine (GUM) clinics are asked not to pass urine prior to their medical consultation. On examination any discharge is squeezed from the urethra and used for direct inoculation onto agar culture medium and microscopic slides for detection of *N.gonorrhoea*. For *C.trachomatis* a small wire shafted swab is passed two to four cm into the urethra and rotated prior to withdrawal. This is a painful process and often inhibits male patients from re-attendance.

1.4.3. *Urine samples*

The collection or urine samples from female patients for the detection of *C.trachomatis* by EIA and DIF has been described (Matthews, et al 1990) but the low levels of antigen present in the female urethra means this is not an ideal specimen for EIA (Sellors, et al 1991). However, the use of female urine samples for the Abbott LCR test is well established (Cherneskey, et al 1994, Ridgway, et al 1996) although the successful detection of antigen relies on either a female urethral infection, not normally the prime seat of infection, or contamination of urine with Chlamydia from the vaginal tract during micturition. In male patients the successful use of urine for

EIA and molecular methods is well documented (Matthews, et al 1988; Caul, et al 1988; Chernesky, et al 1994) and is universally popular as it is a non-invasive and painless process. To maximise antigen content, a first pass (FPU) or early morning urine (EMU) is recommended (Paul and Caul, 1990). Most researchers advocate 15-25 ml of FPU collected 1 to 3 hours since last void. EMU and urine passed in excess of 3 hours may be less sensitive (Matthews, et al 1993; Sellors, et al 1993). It would appear through personal observation, scientific discussion and correspondence with Dr Owen Caul (Bristol PHL) that there is a 'hook' effect with some urine samples in certain types of EIA tests. Although it can be shown that there is Chlamydia antigen present in these urine samples by DIF they give a negative or low OD reading by EIA. Whether the FPU is collected before or after urethral swabbing seems to make very little difference (Jawad, et al 1990). Markers in urine have also been used to indicate *C.trachomatis* infection, these include sterile pyuria, (Matthews, et al 1990) leucocyte esterase (White, et al 1989) and neopterin (Kelly, et al 1994).

1.5. Aims and Objectives

The aim and objectives of this study were threefold. Firstly, to validate for diagnostic purpose three Chlamydia antigen detection tests for use with FPU samples. Secondly, to investigate the so called 'hook' or inhibitory effect that some FPU samples and genital swabs have on EIA test outcome and thirdly to devise and evaluate a simplified chlamydia culture confirmation method as an alternative to established techniques.

1.5.1. *Validation of Antigen Detection Tests*

The validation of antigen detection tests for diagnostic use in order to comply with manufacturer's regulatory affairs, United States of America, Food and Drugs Administration (FDA) approval and acceptance of the scientific establishment by peer review through publication in learned journals, means that certain standards and methodology are adhered to rigorously. Deviations from these procedures are tolerated only if proved, by experiment and practice, to be equivalent to, or better than, tried and tested methods. Clinical trials 1 to 11 describe part of the validation process of the three tests now available commercially.

1.5.2. *The 'hook' effect*

There are few references to the 'hook' effect which is probably more correctly referred to as a prozone effect (Duncan, 1996). However, Wood and Wreghitt (1990) state, when referring to optimisation of EIA reagent concentrations, that "where the sample contains high concentrations of either antigen or antibody, the dilution curve may resemble a 'hook'". The optimum concentration of the capture antigen is the highest dilution that gives a positive result in relation to a negative result or the P:N ratio'. In a FPU sample showing a 'hook' effect the P:N ratio may not be with the largest volume centrifuged but with a smaller amount. Laboratories that routinely use FPU samples to diagnose Chlamydia infection by EIA would not necessarily know of those samples that might show a 'hook' effect and could report false negative results. One of the aims of this research was to investigate the 'hook' effect which might result either from reagent excess or else from inhibition of the assay. In Chapter 4, factors to reduce the inhibitory nature of genital swabs on the Clearview™ Chlamydia device were investigated in a joint study with Unipath Diagnostics Limited, Bedford, UK (Sheard et al, submitted for publication 1998).

1.5.3. *Simplified Chlamydia culture confirmation*

Since the introduction of Chlamydia EIA tests in 1987 the use of tissue culture in the United Kingdom for the detection of *C.trachomatis* has declined. The results of questionnaires by the National External Quality Assessment Scheme (1988, 1990) show that the number of responding laboratories offering culture has fallen from 36 in 1988 to 24 in 1990. Moreover, only two of the 24 offered culture alone and the other 22 also performed EIA and DIF. In the same period of time the number using EIA has risen from 105 to 135 and DIF from 141 to 154. The current estimate of laboratories that routinely culture for *C.trachomatis* in the UK is probably less than ten although there is an increasing demand from clinicians for the definitive result of isolation of the organism particularly in medical legal cases. One reason for the rapid decline in culture was the insensitivity of staining techniques to confirm Chlamydial inclusions before any attempt had been made to utilise FITC labelled MAb as a rapid accurate alternative. In this study a simple culture technique is described that

combines the excellence of the 'gold standard' coverslip method with a one step, cost effective alternative without loss of sensitivity and specificity.

2. CLINICAL SPECIMENS AND LABORATORY METHODS

Protocols for patient selection, type of specimens and laboratory methods were agreed between the laboratory, Genito Urinary Medicine (GUM) Clinic or General Practitioner and the manufacturer. Basically, patient selection and specimen types were common for each study. During the course of each male study, samples were collected from new and rebooked patients attending the GUM clinic at Whittal Street, Birmingham or Heartlands Hospital, Birmingham with signs or symptoms of a urethral infection. Swabs for culture were collected by passing a small wire shafted swab two to four cm into the urethra and then placing into 2 mls of culture transport medium. FPU samples (15 to 25 mls) were collected in sterile plastic universal containers and both specimens were stored at +4°C for no longer than 24 hours prior to delivery to the laboratory. A record was kept of the time since the patient had last voided urine. Those patients who had antimicrobial treatment within the preceding four to six weeks were excluded. The order of collection of samples was randomised, that is, in half the patients the swab was collected first followed by the FPU and in the other half this procedure was reversed. In female studies from General Practitioner Medical Centres, samples were collected and treated in a similar manner. If a second swab was required for testing by Clearview or EIA then this again was randomised and placed either into the EIA transport medium or transported in the Clearview swab tube.

In other clinical trials, not included in this study but taking place concurrently and occasionally referred to in the text, a likewise randomisation of samples took place. Detailed protocols were held by the physician, laboratory and the manufacturer. Where this investigation was part of a multicentre study similar protocols were agreed between all sites.

2.1. McCoy cell tissue culture

McCoy cells (Gibco Lifesciences, Irvine, Scotland) were passaged no more than forty times before being replaced from a stock supply stored in liquid nitrogen. The cells were cultured in Eagle minimum essential medium (MEM) supplemented with Earle's salts, vitamins, non-essential amino acids and 10% foetal calf serum in 75cm plastic

flasks at 37°C. The buffering system was sodium bicarbonate and gentamicin (50μg/ml) was added to reduce bacterial contamination without having effect on *C.trachomatic* viability.

McCoy cells were stripped from flasks using trypsin/versene and resuspended and seeded in culture medium at 100,000 cells per ml per vial. Flask and cell cultures were held at 37°C using the same culture medium until used.

Chlamydia culture transport medium was prepared from the same Eagle MEM as described above with the addition of 1% glucose (3M) and 2.5μg/ml fungizone and aliquoted in 2ml amounts in glass vials.

All tissue cultures and media underwent quality analysis for sterility and *C.trachomatic* growth promoting ability.

2.1.1. *Preparation of cell cultures pre inoculation*

Two sets of McCoy tissue culture cells were prepared for each sample. In the first set, for the gold standard method, untreated cells were cultured on 10mm round coverslips in screw top plastic tubes and in the second set cells were cultured in MEM growth medium containing 30μg/ml IDU on the flat base of screw top glass tubes. Both types of culture were prepared from the same stock of cells and seeded at 100,000 cells per 1ml of MEM growth medium and incubated at 37°C for a minimum of 24 hours and a maximum of 1 week prior to use.

2.1.2. *Inoculation of tissue culture*

Prior to inoculation the growth medium was removed from two coverslip cultures and replaced with 0.5 ml of medium containing double strength (0.2 μg) cycloheximide followed by 0.5 ml of the previously vortex mixed sample making a final cycloheximide concentration of 0.1 μg/ml. One IDU treated tube was inoculated after the medium was removed with 0.5 ml of the sample alone. All three tubes were centrifuged at 2500g for 1 hour and incubated at 35°C for 48-72 hours. Following the

first incubation, one of the cycloheximide treated cultures was blindly sub cultured to a similar coverslip culture, recentrifuged and incubated for a further 48-72 hours.

2.1.3. *Post inoculation treatment of cultures Gold Standard Method 1*

After incubation the coverslip cultures were fixed by decanting the culture medium and replacing with 1 ml of methanol for 10 minutes. After decanting the methanol the coverslips were removed from the plastic tubes by drilling through the base and then mounted cell side up onto a glass microscope slide with DPX mountant. After a few hours the coverslips were firmly attached and stained with 10µl of FITC labelled Chlamydia LPS MAb (Dako Imagen) and examined for typical intra-cytoplasmic inclusions. An estimation of inclusions per coverslip was record.

2.1.4. *Post inoculation treatment of cultures Scrape off Method 2*

The medium from the IDU treated cultures was removed and the cell sheet washed carefully by adding 1 ml of PBS to the tube, decanting the excess and scraping off the cells into the remaining residue. Using the same glass pasteur pipette, approximately 50% of the original cell population was deposited onto one 3mm well of a PTFE multislide allowed to air dry, fixed in acetone for 10 minutes and stained with 5µl of the same MAb as in Method 1.

2.2. Treatment of First Pass Urine and Urogenital Swab Samples

In three studies (CT1, CT2 and CT6) the urine was cultured for bacterial growth by inoculation onto blood agar using a standard 10µl loop and incubating aerobically at 37°C for 24 hours. In addition, in two of the studies, cell counts for leucocytes and red blood cells were included as were a test for antibacterial inhibitors and dipstick tests for urinary abnormalities. In two EIA studies (CT1 and CT2) the urines were warmed at 37°C to redissolved any cold precipitates and centrifuged at 2,500g for 20 minutes. In further studies involving FPU samples, (CT7, CT9 and CT11) no pre analysis of the urine was included. A summary of FPU treatment is shown in Table 1.

Table 1 - Treatment of First Pass Urine Samples

	CT2 Mastazyane (EIA)	CT1 Phadebact (EIA)	CT6 Unipath (Clearview)
Bacterial Growth	Yes	Yes	Yes
Cell Count	No	Yes	Yes
Antibacterial Agents	No	Yes	Yes
Dipstick Tests	Yes	Yes	No
Pre Warmed at 37°C	Yes	Yes	No
Volume Centrifuged	Max 15ml	2ml & 10ml	10ml + 10ml Dist H_2O
Centrifuge RCF	2500g 20 mins	2500g 20 mins	3000g 15 mins

2.2.1. *Treatment of male FPU samples for Clearview™ Chlamydia (CT6 and CT7)*

The samples were vortex mixed before removal of two 10ml amounts for the Clearview tests. If less than 20ml of FPU was received then the sample was divided equally down to a volume of 5mls in each and the reduced volume noted, 10mls of deionised water was added to each 10ml amount of FPU (or an equal volume pro rata) and left for two minutes before centrifuging at 3000g for 15 minutes. The supernatants were carefully discarded and one pellet tested in Clearview and the other stored at -70°C to aid analysis of discrepant results. The Clearview pellet was reconstituted by the addition of 0.6ml of extraction reagent, vortex mixed and transferred to an extraction cup prior to heating at 80°C for 10 minutes in a heating block. After cooling the attached combined filter and dropper top was positioned in the extraction cup and approximately 300µl (5 drops) was passed through the filter onto the sample window of the Clearview device. After 15 minutes any reaction line in the result window was recorded and a majority decision taken, two out of three observers, if the reaction line was weak.

2.2.2. *Treatment of male FPU samples tested by Clearview™ Chlamydia MF at 1 day and 5 days after collection (CT9)*

In this study the aim was to highlight any loss of antigen in FPU samples after the storage at +4°C for 5 days in comparison to the antigen level on day 1 or 2. Prior to the division of the FPU sample for the Clearview test, two 1ml aliquots were removed for testing by Abbott LCR Chlamydia. The remaining FPU sample was divided (as in section 2.2.1.) but only one 10ml aliquot was treated immediately, the other being retained at +4°C for treating and testing at 5 days.

2.2.3. *Male and Female FPU samples tested by Abbott LCR™ Chlamydia test (CT9 and CT11)*

Urine samples for the LCR test are recommended by Abbott to be held at +4°C for up to 24 hours prior to testing or -20°C for up to 60 days if the sample cannot be tested within the first 24 hours. To avoid possible contamination with stray DNA, two 1ml aliquots were removed from the original FPU sample, using dedicated automatic pipettes, and placed in eppendorf micro centrifuge tubes prior to any other procedure. One of the aliquots was processed immediately and the other stored at -20°C for resolution or confirmation of discordant results. The storage and prior freezing of the original FPU sample was amended as more experience and practice was gained by ourselves and others routinely using the Abbott LCR Chlamydia test, in short it was observed that pre freezing of all FPU samples gave a cleaner result with less inhibitory effect.

The 1ml aliquot of FPU for testing was centrifuged at 9000g for 15 minutes in a microcentrifuge and the supernatant, carefully and completely removed from the deposit within 15 minutes. The pelleted deposit was resuspended by vortex mixing in 1ml of resuspension buffer and placed in a dry heat block at 97°C for 15 minutes. After cooling for 15 minutes the eppendorf tube was pulse centrifuged for 10 to 15 seconds to remove any condensate from the cap and 100µl was pipetted into the factory prepared Chlamydia amplification vial and reagent and transferred to a thermal cycler together with negative and calibrator controls. The remaining extracted specimen was retained for further evaluation at -20°C.

The forty cycles in the thermal cycler were 93°C for 1 second, 59°C for 1 second and 62°C for 1 minute and 10 seconds. After amplification the vials were placed in the analyser together with the reagent pack and automatically processed utilising microparticle enzyme immunoassay instrumentation to detect a fluorescent reaction. A numerical value is given to the results in comparison to the controls. Positive results have values equal to or greater than 0.45 times the average of the two calibration samples for that run. Samples with numeric values falling within the 200 to 400 range were retested after first diluting 1:4 in case any inhibitors were present.

2.2.4. *Treatment of Endocervical Swabs for Roche Amplicor Chlamydia PCR (CT4)*

This study was undertaken in co-operation with the Microbiology Department, Queen Elizabeth Medical Centre, Edgbaston, Birmingham where the Roche Amplicor Chlamydia PCR was under evaluation.

Randomised endocervical swabs were taken in the usual manner and placed in the special specimen transport medium tube and agitated for 15 seconds. The liquid was expressed from the swab by pressing against the side of the tube, the swab was removed and discarded. The capped tube was held at +4°C and transported to the laboratory within 24 hours and tested within 10 days. Prior to testing 1ml of specimen diluent was added to the tube, vortex mixed for 5-10 seconds and left at room temperature for 10 minutes.

Together with appropriate controls, 50µl of Master Mix with AmpErase and 50µl of specimen was pipetted into each Microamp PCR tube and placed in the thermal cycler with one cycle of 95°C for 5 minutes and 60°C for 1 minute followed by 29 cycles of 95°C for 30 seconds and 60°C for 1 minute completed by 72°C indefinitely.

Once amplification had been completed the tests and controls were removed from the thermal cycler and 100µl of denaturation solution was added to each.

The detection procedure was in standard 96 well microwell format using the supplied foil packed 12 x 8 way strips. 100µl of hybridisation buffer was pipetted into each

test and control well followed by 25µl of the denatured amplicor and incubated at 37°C for 60 minutes.

The microtitre plates were washed five times with the wash buffer before 100µl of conjugate was added to each well and reincubated for a further 15 minutes at 37°C.

The plates were washed as before and 110µl of working substrate added to each well for 10 minutes at room temperate in the dark.

After adding 100µl of stopping reagent the plates were read at a wavelength of 450 in a spectrophotometer.

A clinical specimen with a reading of 0.25 is considered positive for the presence of *C.trachomatis*.

2.2.5. *Treatment of Female Swab Samples for Clearview™ (CT3, CT5, CT8 and CT11)*

If specimens were not tested by Clearview on the first day of collection they were held at +4°C for up to five days. A Clearview extraction cup was filled to the line with extraction reagent (0.6ml) for each swab to be tested. Swabs were immersed into the reagent, mixed by agitation and heat extracted for 10 minutes at 80°C in a heating block. After extraction and cooling the swab was removed from the cup, ensuring most of the reagent was expressed from the swab, by squeezing together the side walls of the tube (Photo 3). The combined filter and dropper top was attached and approximately 300µl (5 drops) was passed through the filter onto the sample window of the Clearview device (Photo 4).

Photo 3 Removal of swab from Clearview™ Chlamydia extraction tube

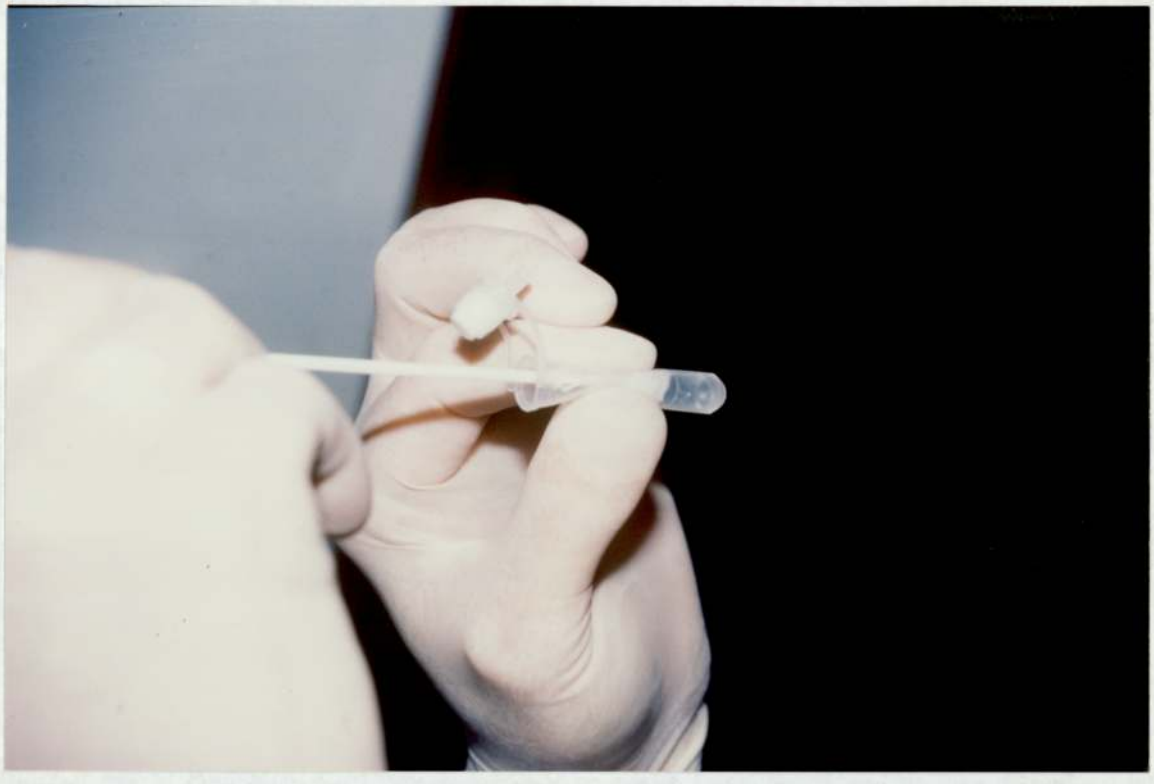


Photo 4 Clearview™ Chlamydia test sample addition

2.2.6. *Treatment of Male and Female Urogenital Swabs and FPU for EIA*

FPU samples were warmed and centrifuged as described at the beginning of this chapter. All the FPU centrifuged deposits were resuspended in the same volume of EIA transport medium as was recommended by the manufacturer for swab samples.

The specimens were vortex mixed for at least 15 seconds prior to the heat extraction step and again before testing. The volume of transport medium, extraction time and temperature were as detailed in Table 2.

Table 2 Treatment of Enzyme Immunoassay Samples

Test	Vol of Transport Medium	Extraction	Volume Tested	Clinical Trial
Phadebact EIA	0.5mls	10-15 min/boiling water	100µl	CT1
Mastazyme EIA	1.0ml	10mins/boiling waterbath	200µl	CT2
Dako IDEIA/PCE	1.0ml	20 mins/105°C dry heat block	200µl	CT10,11

After extraction the samples were allowed to cool before testing. Limitations on the length of time and temperature that specimens could be stored prior and post extraction were as recommended in the manufacturer's' protocols.

2.3. EIA Methods

Each of the three EIA tests require two or three negative controls, from which to calculate the optical density cut-off, a positive control to validate the reaction and an empty well to blank the spectrophotometer reader on air. A summary of the assay procedures is given in Table 3.

Table 3 Summary of EIA Assay Procedures

	Phadebact EIA CT1	Mastazyme EIA CT2	Dako IDEIA CT9	Dako IDEIA PCE CT10 - CT11
	-	25µl Conjugate	-	-
Control or Sample	100µl	200µl	200µl	200µl
	-	50 µl Antibody	-	50 µl Conjugate
Incubate	37°C/30 Mins	37°C/60 Mins	15-30°/120 Mins	15-30°C/90 Shaking
	50 µl Conjugate	-	50 µl Conjugate	-
Incubate	37°C/45 Mins	-	15-30°C/60 Mins	-
Wash	4 Cycles	5 Cycles	5 Cycles	5 Cycles
Substrate	100 µl	200 µl	100 µl	100 µl Amplifier
Incubate	37°C/60 Mins	22°C/20 Mins	15-30°C/40 Mins	15-30°C/30 Mins
	-	-	100µl/Amplifier 10 Mins	-
Stop	50 µl	50 µl	50 µl	50 µl
Reading Wavelength	405	450	492	492

All manufacturers emphasise the importance of the washing steps and in my experience it is difficult to overwash EIA wells. Inadequate or insufficient washing often results in high background O.D. readings which can invalidate results

Remaining swab samples and excess FPU were stored at -70°C or -20°C as were any remaining EIA or Clearview extracts and amplified products. In addition 200µl of each EIA extraction buffer was removed prior to the boiling step and retained as a stored aliquot for further evaluation, in the event of any discordant results. In some of the clinical trials the FPU centrifuged supernatant was retained and stored at -20°C for a concurrent Neopterin study (Kelly et al 1994).

2.4. Storage of Specimens

Remaining swab samples and excess FPU were stored at -70° or -20°C as were any remaining EIA or Clearview extracts and amplified products. In addition 200µl of each EIA extraction buffer was removed prior to the boiling step and retained as a stored aliquot for further evaluation, in the event of any discordant results. In some of the clinical trials the FPU centrifuged supernatant was retained and stored at -20°C for a concurrent Neopterin study (Kelly et al 1994).

2.5. Discordant results

In the event of any discrepant results, that is, culture or non-culture antigen tests giving contrary results then, if possible, all of the tests were repeated and in addition examined further by IF for evidence of *C.trachomatis* EB's.

2.6. Confirmation of Results

Results were confirmed by one of several methods depending on the type of test and remaining extraction fluid or stored original specimen.

2.6.1. *Confirmation by Immunofluorescence*

Confirmation of true positive and negative results by direct visualisation of Chlamydia elementary bodies by IF was possible on all remaining stored specimen types and extraction fluids with the exception of the denatured samples used for PCR and LCR.

Up to 1ml of extract of specimen was pipetted into a 1.5ml eppendorf centrifuged tube and centrifuged at 11,500 rpm for 10 minutes. The supernatant was removed and the deposit resuspended by vortex mixing in 1.5mls of PBS and centrifuged again as before. The resulting pellet was spread over a 6mm well on a Teflon coating microscope slide and allowed to air dry. It was important that the dried deposit was not too thick and the amount of the pellet spread on the well was adjusted depending on the size and consistency of the cellular deposit.

The air dried slide was fixed for 10 minutes in methanol and stained with 25µl of *C.trachomatis* anti-MOMP MAb FITC conjugate (Syva MicroTrak). Three or more typical, round apple green EBS were taken as the sample being positive. If necessary the process was repeated.

2.6.2. *Confirmation of Positive EIA reactions by blocking test*

In addition to IF, as described in 2.6.1., the manufacturers of the EIA tests evaluated in CT5, CT10, CT16 all supplied blocking antibody to confirm any positive results. The provision of blocking antibody for Chlamydia EIAs is required by the FDA for the use of those tests in that country.

Each manufacturer's blocking test had a similar format where a polyclonal rabbit anti-chlamydia serum and an equivalent concentration of normal rabbit serum was added to two wells of the repeat test after addition of the test samples. If a 50% or greater reduction of the optical density (OD) reading in the blocked well, as compared to the control well was recorded, then the test was confirmed as positive.

2.6.3. *Confirmation by Repeat Testing*

All specimen types could be repeat tested by the original method on stored samples. However, the viability of Chlamydia is reduced by freezing and attempting to culture a descrepant negative or reconfirm a weak positive may not yield the expected result, better to rely on IF on the remaining sample.

In some of the molecular tests where the number of EBS in the original sample could be very small and undetectable by other methods the only redress was to repeat the test to ensure reproductibility.

2.6.4. *Sampling Error*

Where a positive result was confirmed in one type of specimen and a negative result was confirmed from a second or different type of specimen taken at the same time from the same patient, then this was deemed to be sampling error. If the patient had a confirmed, true infection then the detection test used on the negative sample was

not at fault if no antigen was present in the specimen. This was taken into account in the resolution of the comparative results.

2.6.5. *Evaluation of the Data*

The diagnostic value of any novel test that is evaluated for clinical use is its ability to indicate the presence or absence of disease. The diagnostic value, or efficacy, of the tests that were evaluated in this study were calculated using the method in Table 4.

Table 4 - Method of Evaluation of Diagnostic Test Data

		Gold Standard reference test		
		Positive	Negative	Total
Test Under Evaluation	Positive	a	b	a + b
	Negative	c	d	c + d
	Total	a + c	b + d	a + b + c + d

Sensitivity a/(a+c) tests positive of true positives/true positives

Specificity d/(d+b) tests negative of true negatives/true negatives

Positive predictive value a/(a+b) true positives out of total test positives

Negative predictive value d/(d+c) true negatives out of total test negatives

Every attempt was made to eliminate bias from the selection of the patient groups, by randomisation of specimen collection and by resolution of the discrepant results (Ransohoff & Feinstein 1978) (Hadgu 1996).

3. PHADEBACT EIA PRE CLINICAL TRIAL EXPERIMENTS

3.1. Introduction

The Phadebact Chlamydia EIA test manufactured by Boule Diagnostics AB Sweden had been validated for the detection of *C.trachomatis* from both male and female urogenital samples (Halonen et al 1987) but has been shown to have poor performance with male FPU samples (Paul et al 1990) (Kok et al 1993).

It was noted in the studies by Paul and Kok that Boule Diagnostics had not validated the test for FPU samples and the investigators utilised similar volumes of FPU to the other EIA tests being evaluated (10mls in the former and 20mls in the latter).

With the approval of Boule Diagnostics and prior to the clinical trial subsequently supported by them, a series of evaluations to establish the optimum volume of FPU to centrifuge for the EIA test was investigated.

It had been observed at this laboratory that the *C.trachomatis* urethral infections were often associated with sterile pyuria and that DIF for Chlamydia was often superior to EIA in the centrifuged deposits of urine containing large amounts of leucocytes (Matthews et al 1990).

Proceeding on the premise that the number of leucocytes present in FPU samples had some relation to EIA test outcome, a series of experients were devised to investigate this prospect.

3.2. Results

3.2.1. *Using Single Patient known Chlamydia Positive Urines*

A panel of *C.trachomatis* DIF positive urine samples were collected and stored at -20°C for later test. Prior to testing, each sample was fully thawed and warmed at 37°C to redissolve any cold precipitant. A range of volumes from 10mls to 1ml were centrifuged as in Section 2.2. and the resulting deposits resuspended in 0.5mls of specimen diluent and transferred to 5ml glass screw top bijou bottles. If volumes of urine less than 1ml were examined then further dilutions of the 1ml resuspended

deposit were made with specimen diluent. All specimens were boiled for 10 to 15 minutes and cooled prior to testing as per the manufacturer's protocol or with slight modifications. The samples were tested by one or more of the following methods:

A. As per manufacturer's protocol.

B. Removal of the sample at 30 minutes and replacement of an equal volume of boiled diluent.

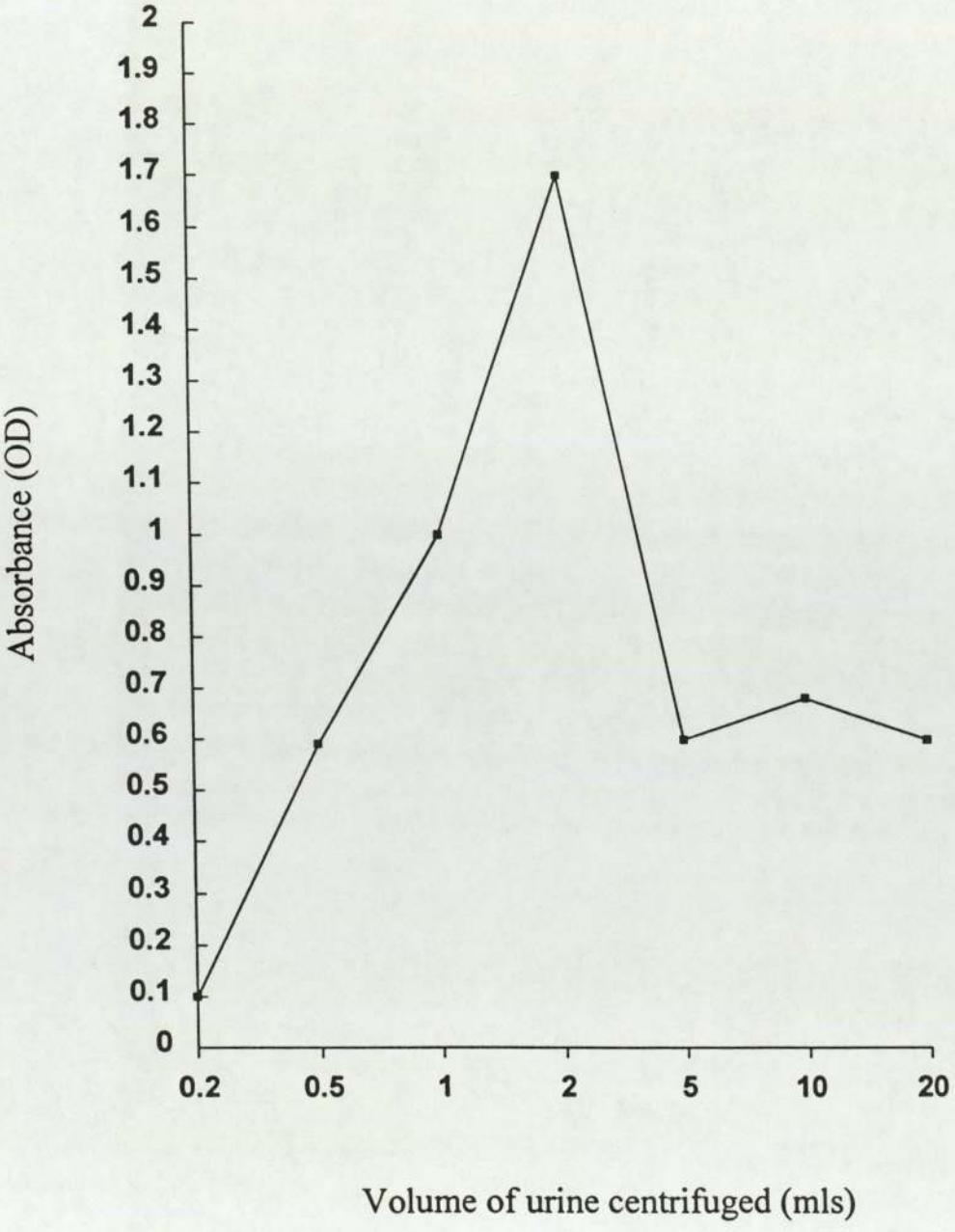
C. Removal of the sample at 30 minutes followed by a 4 x wash step and replacement with an equal volume of boiled diluent.

In all of the positive samples tested by the manufacturer's protocol the endpoint OD readings increased as the original volume of urine tested decreased with an optimum of 1 or 2mls demonstrating classical hook effect (figure 1). Whereas removal of the sample prior to the addition of the antibody only had an enhancing effect on those wells with samples from the higher end of the urine titration (5 to 10mls). Introduction of an extra washing step prior to addition of the antibody was discontinued as it did not enhance the sensitivity of the test.

The simple technology utilised in this type of EIA test relies upon the antigen absorbing to the well surface during the first incubation step. After 30 minutes an alkaline phosphatase labelled MAb is added and attaches to any Chlamydia antigen present. Following a wash step, to remove unbound antibody, a substrate is added and a yellow colour is produced in positive reactions.

After this first series of investigations it was surmised that the MAb attached more readily to the excess free antigen contained in the sample rather than to the absorbed antigen on the well surface and that during the wash step this free antigen MAb complex was washed away, resulting in a low endpoint signal. Hence, if there was less antigen present in the original sample then it would all be bound to the well surface, the MAb would only be able to bind at that point and a stronger reaction would result. In order to confirm that this phenomenon was a straightforward prozone effect and not a result of leucocyte excess a further set of experiments were devised.

Figure 1 - Phadebact Chlamydia EIA Test - the Hook Effect



3.2.2. *Pretreatment of EIA Microtitre Wells with Leucocytes*

A test was developed to evaluate the effect that leucocytes had on the EIA reaction and a comparison was made of untreated and pretreated EIA microtitre plate wells seeded with simulated leucocyte rich 3ml and 10ml urine samples (saline plus pus). After 30 minutes incubation the samples were removed and the wells further challenged with leucocyte free simulated *C.trachomatis* positive samples (10ml, 3mls, 1ml and 0.5ml of a standard saline and *C.trachomatis* mix) and then treated as per the manufacturer's protocol.

In this semi-chessboard experiment EIA wells that were pretreated with leucocytes in saline had an inhibitory effected when challenged with varying amounts of Chlamydia antigen. Untreated wells and those treated with saline diluent gave higher endpoint OD readings than those wells treated with leucocytes. Furthermore, it was shown that excess antigen did not cause a prozone effect, that is, the endpoint OD was not reduced in the untreated wells. The conclusion drawn from this set of experiments tended to discredit the straightforward excess antigen prozone effect and favoured the inhibitory effect of leucocytes on the EIA reaction. To substantiate these findings a further series of investigations using leucocyte containing, Chlamydia negative urines, clarified supernatant, saline and resuspended centrifuged deposits in saline were 'spiked' with a standard inoculum of *C.trachomatis*.

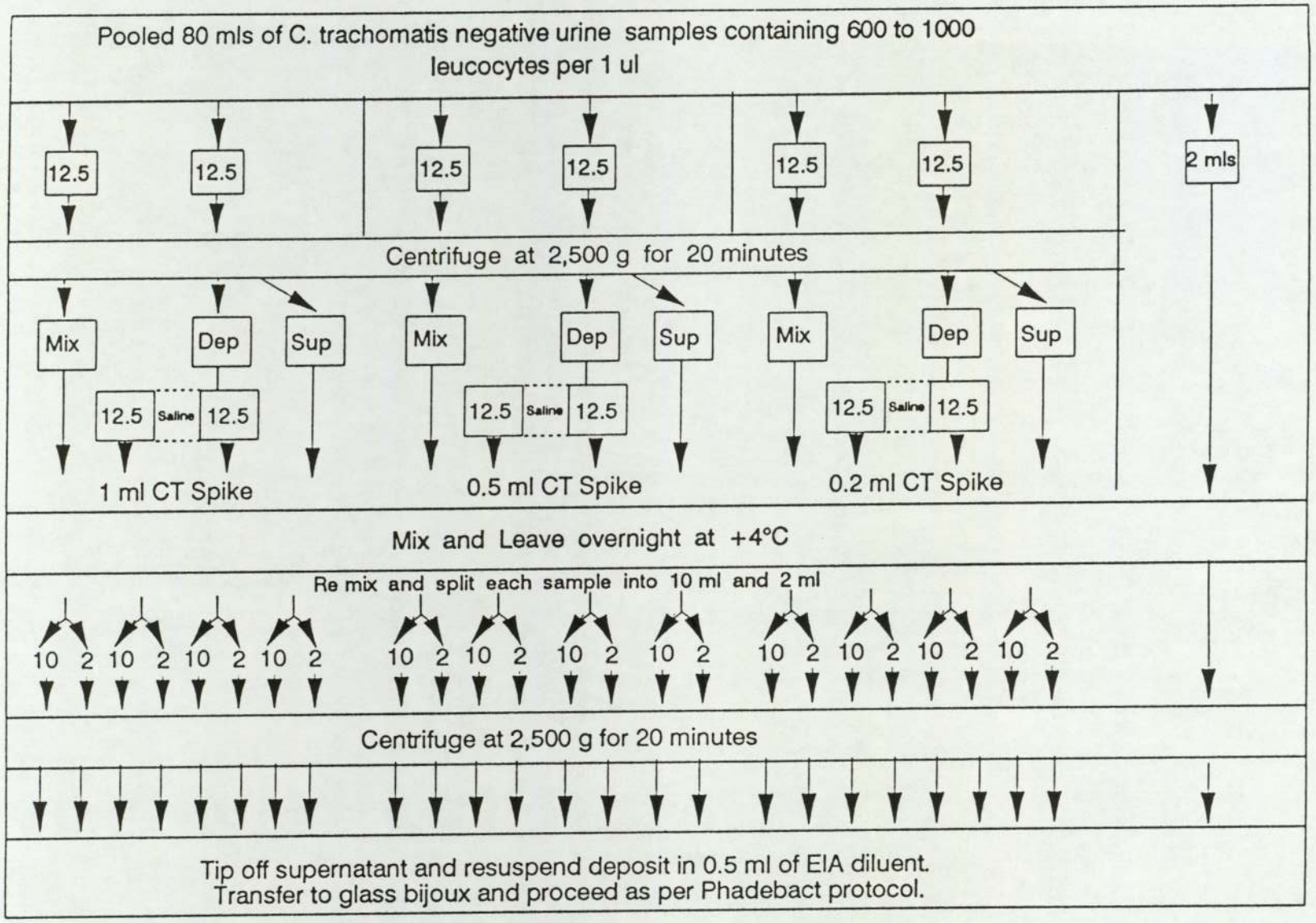
3.2.3. *'Spiking' of various components of Pooled Leucocyte Rich Urine Samples*

A series of EIA tests were set up as in 3.2.2. but with pooled leucocyte rich, *C.trachomatis* negative, urine samples compared with the same samples with the leucocytes removed. In addition the removed leucocytes were resuspended in an equal volume of saline and then all spiked with either 0.2, 0.5 or 1.0 ml of a 1:100 dilution of *C.trachomatis* stock antigen in saline. See flow diagram Figure 2.

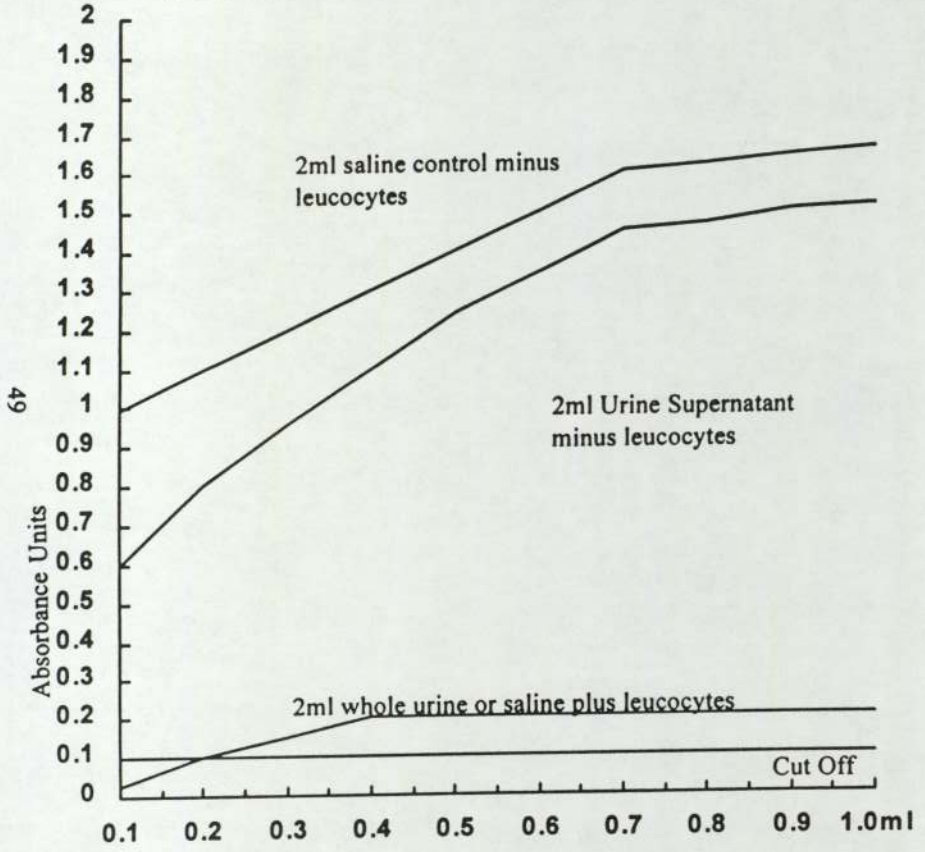
The whole urines and centrifuged deposits resuspended in saline gave a similar result as in the previous experiments, that is the OD readings rose as the volume of centrifuged urine was reduced, whereas in the clarified urine supernatant and saline controls the contrary was found, the highest OD readings were found in the largest

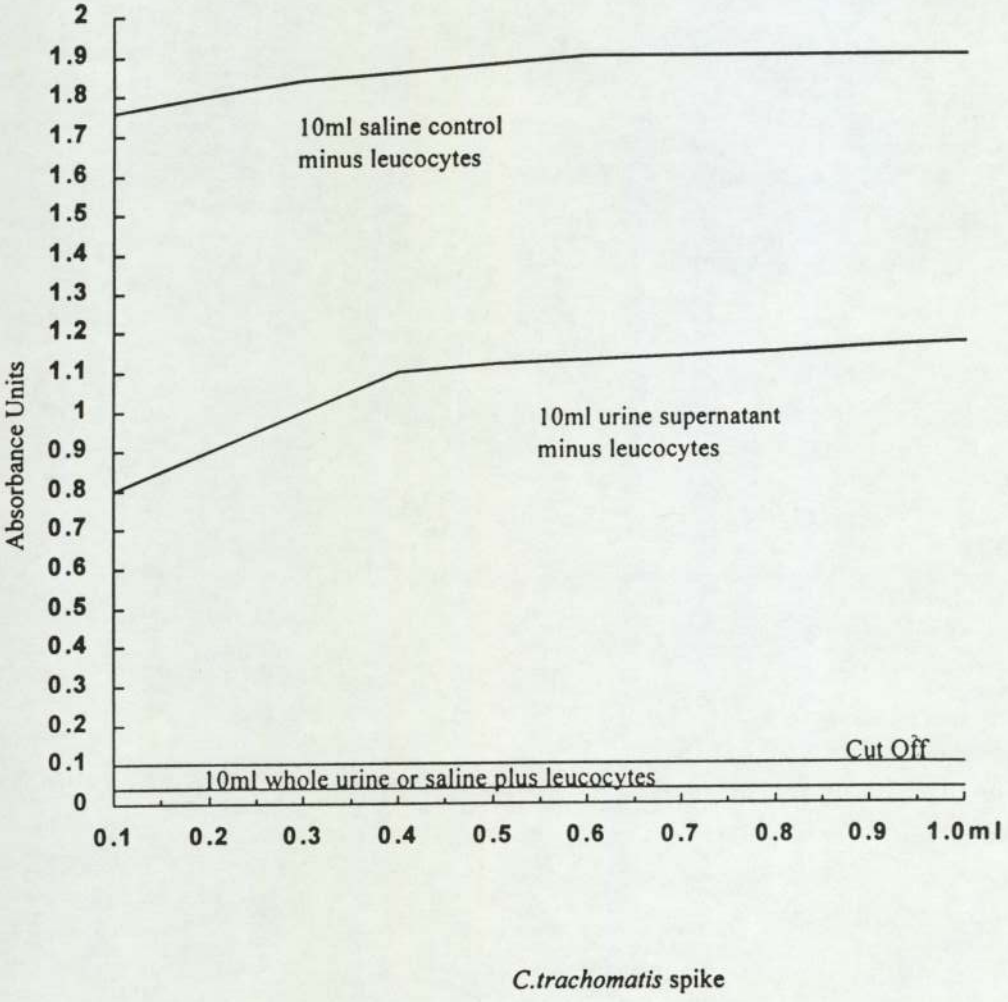
volumes centrifuged (Figures 3 and 4). These results confirmed that it was not an excess of *C.trachomatis* antigen or a factor within the supernatant that caused the prozone effect but rather the presence or absence of leucocytes.

Figure 2 Flow Chart of Experimental Method 3.2.3.



Figures 3 and 4 - Phadebact Chlamydia EIA Test: Urine and Saline, with and without Leucocytes spiked with *C.trachomatis*





3.2.4. *'Spiking' Filtered Urine*

A variation on experiment 3.2.3. was set up but including an extra line of post centrifuged, pre-spiked, filtered supernatant to exclude the possibility of soluble inhibitory effect on the EIA reaction. Part of the urine supernatant was further clarified through a 0.22 micron filter and compared with other results. Urine stored at -20°C was used in this investigation which was unsuitable for a leucocyte cell count. However, leucocyte esterase was reduced 45% by centrifugation and 88% by filtration. The positive OD result of the filtered urine was only increased when spiked with the largest volume of *C.trachomatis* used and this was only 0.40 absorbance units (AU) above that of the centrifuged urine (2.50 to 2.90 AU).

This indicated that the disrupted leucocyte small cellular fragments remaining in the centrifuged urine supernatant had a minimal effect on the OD outcome.

3.2.5. *Investigations using six differing Chlamydia Tests*

To evaluate whether the inhibitory effect of leucocytes on the Phadebact Chlamydia EIA test was unique to that particular test or not, other manufacturer's EIA tests were tested in parallel using the same samples. Positive Chlamydia DIF urines from males and females collected over several months were stored at -20°C and then grouped together in five pools by, gender, recorded leucocyte count and DIF positivity (Table 5).

Table 5 - Five pools of Chlamydia DIF positive urines

Pool	Gender	Recorded Leucs	Antigen Content	Leucocyte esterase
I	F	High	Low	++
II	F	Low	Low	-
III	F	High	High	+++
IV	M	High	Medium	++
V	F	Medium	Medium	+++

In the five EIA *C.trachomatis* tests and one immunoassay method included in this experiment some used pretreated wells with monoclonal antibodies specific to Chlamydia and some relied on antigen attachment to the solid phase without pretreatment. They also varied in the amount of diluent that centrifuged deposits were resuspended in and the amount of that diluent that was added to each test. A summary of each method is given in Table 6.

Table 6 - Six Manufacturers' EIA Methods

Key	Manufacturer	Volume Urine	Volume Diluent	Volume Tested	MAb Pretreated	Included in Project
A	Phadebact EIA	Under test	0.5mls	100µl	No	Yes (CT1)
B	Mastazyme EIA	15 to 25mls	1.0ml	200µl	Yes	Yes (CT2)
C	Dako EIA	20mls	1.0ml	200µl	Yes	Previously validated
E	Syva EIA	4mls	1.0ml	100µl	No	No
D	Abbott EIA	15 to 20mls	1.0ml	200µl	No	No
CV	Unipath (Immunoassay)	10mls	0.6ml	300µl	Yes	Yes (CT6)

In the evaluation, each of the five urine pools were split six ways and then further subdivided six times into volumes ranging from 1ml to 10mls resulting in 30 samples for each EIA kit tested. The 180 simulated urine samples were centrifuged and the deposits resuspended in EIA specimen diluent as described previously.

In the five EIA tests, the highest OD readings varied between manufacturer's and the amount of pooled urine centrifuged. Those companies that utilised pretreated EIA wells tended to favour larger volumes of centrifuged urine than those manufacturers with untreated EIA wells, albeit most optimum ODs were in volumes of less than 10mls. The Phadebact EIA test consistently required less urine than the other four

EIA tests, confirming previous findings. All of the EIA tests demonstrated at least in part, a 'Hook Effect'. (Figure 5).

Although the optimum volume of urine centrifuged may be less than 10ml or in the 1-10ml urine there is an inclination by manufacturer's to increase this volume so that urines containing low levels of antigen give signals above the OD cut off, even if this means a reduction in the optimum OD. Therefore the preferred volume of urine to centrifuge would be the maximum with any leucocyte count that remained above the OD cut off. In the majority of tests, 10 mls of more urine was acceptable. However, the Phadebact EIA test and to a lesser extent the Syva EIA test, would sometimes generate a negative result with 10 mls of urine but a positive result when smaller volumes were tested.

Whereas the leucocytes, in any concentration, had an effect on the OD outcome of both the Syva and Phadebact EIA tests they had a lesser influence on the other EIA tests. On the other hand the antigen content affected all of the results and, surprisingly, highlighted that the Abbott and Dako EIA tests were poor at detecting low levels of antigen (Figure 5 Pools I and II).

As the Clearview immunoassay only gives a positive or negative result, it could not be compared with numeric OD readings. However, the results followed closely those of the EIA tests with positive reactions from 1ml to 10mls on the pools III, IV and V. In pool I the Clearview test was negative at all dilutions and in pool II was positive with the 8ml volume only. As both of these latter pools contained the least antigen, the indication is that leucocyte content has little effect on Clearview test outcome.

Figure 5 **Six Chlamydia EIA Tests VS Five Chlamydia Positive Urines in Varying Centrifuged Volumes**

Urine I

mls	A	B	C	D	E	CV
1	+	+	-	-	+	-
2	⊞	+	+	-	+	-
4	+	+	+	+	⊞	-
6	-	+	+	+	+	-
8	-	⊞	+	+	+	-
10	-	+	⊞	⊞	+	-

Urine II

mls	A	B	C	D	E	CV
1	-	-	-	-	+	-
2	-	+	-	-	⊞	-
4	⊞	+	-	+	+	-
6	+	⊞	+	+	+	-
8	-	+	⊞	⊞	-	⊞
10	-	+	+	+	-	-

Urine III

mls	A	B	C	D	E	CV
1	⊞	+	+	+	⊞	+
2	-	⊞	+	+	+	+
4	-	+	+	+	+	+
6	-	+	⊞	+	-	+
8	-	+	+	+	-	⊞
10	-	+	+	⊞	-	+

Urine IV

mls	A	B	C	D	E	CV
1	⊞	+	+	+	+	-
2	+	+	+	+	+	+
4	+	⊞	+	+	⊞	+
6	+	+	+	+	+	⊞
8	-	+	⊞	+	+	+
10	-	+	+	⊞	+	+

Urine V

mls	A	B	C	D	E	CV
1	+	+	+	+	⊞	+
2	⊞	+	+	+	+	+
4	+	+	+	+	+	+
6	+	+	⊞	⊞	+	⊞
8	+	+	+	+	+	+
10	+	⊞	+	+	+	+

\+ Above O.D. cut off

⊞ Optimum centrifuged volume

\- Below O.D. cut off

Key A - Phadebact. B - Mastazyme, C - Dako IDEIA, D -Abbott Chlamydia zyme, E - Syva, CV - Clearview

3.2.6. *Phadebact EIA Test using other Manufacturer's EIA Diluent*

The Phadebact EIA test was evaluated using other manufacturer's sample diluents spiked with a constant volume of *C.trachomatis* antigen. Two variations of this test using spiked urine and known positive urine rather than diluent alone were also undertaken comparing Syva sample diluent at 100µl and 200µl in the Syva and Phadebact EIA tests.

The result of spiking one of two wells for each of the five diluents tested with 20µl of positive control showed that the Syva EIA diluent gave a higher OD than did the Phadebact manufacturer's own diluent. The Abbott EIA diluent was comparable to Phadebact, but the Dako EIA diluent and Mastazyme EIA diluent both gave negative reactions.

Although Syva EIA diluent spiked with Chlamydia antigen consistently out performed Phadebact EIA diluent in the Phadebact EIA test, if centrifuged urine deposit was added then the reverse was true. However, using pooled male urine with 50 leucocytes per 1µl a 6ml volume of urine in Syva EIA diluent gave the highest OD in both the Syva and Phadebact EIA tests. By contrast, using Phadebact EIA diluent, 8mls of centrifuged urine gave the highest OD, but this was lower than that of the Syva EIA diluent with 6mls.

3.2.7. *Simplification of the 'Spiking' Method*

To reduce the number of samples that were necessary to centrifuge and store when they were spiked with *C.trachomatis* antigen prior to fractionation and centrifugation, a series of tests were devised adding the antigen after centrifugation. It was found that by adding a small volume of antigen to the EIA test well, after addition of the sample, an accurate and reproducible result could be obtained free from 'sampling error', and at a cheaper cost.

Using examples of the worst and best EIA performers from previous experiments together with a simulated urine sample with an average leucocyte content (30 per 1µl), various volumes and dilutions of the stock antigen spike were tested. To give a range from below to above the detectable range for both low and high EIA test

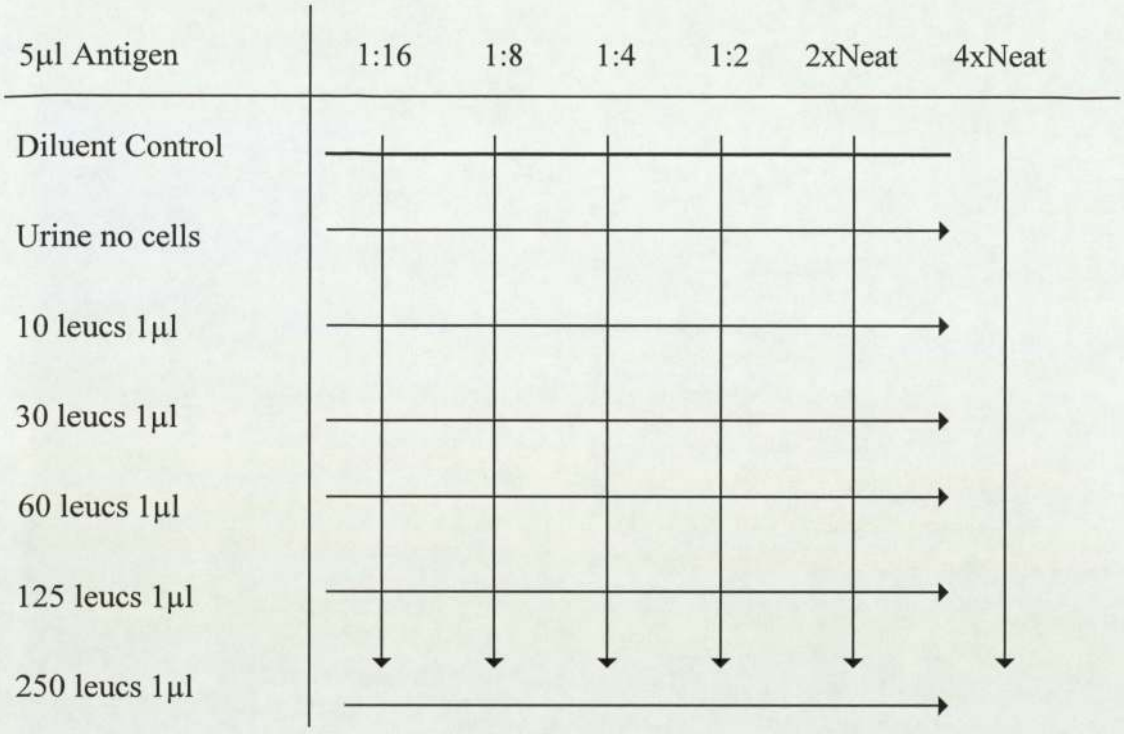
performers the chosen dilutions were 5µl of 1:16, 1:8, 1:4 and 1:2 and 10µl and 20µl of undiluted antigen spike.

Using the simplified *C.trachomatis* antigen spiking method two chessboard titrations for each EIA method was devised. Pus collected from a bacteriologically sterile abscess was added to normal male urine in amounts varying from 10 to 250 leucocytes per µl and left overnight at +4°C together with a plain urine control in 250ml amounts. After warming and remixing, each 250ml amount was divided into six 25ml amounts and centrifuged at 2,500g for 20 minutes. The resulting deposits were stored at -20°C until tested at a later date.

A pool of antigen was collected from positive *C.trachomatis* tissue cultures resuspended in normal saline, split into small aliquots and stored at -20°C. One of these aliquots was thawed, vortex mixed and titrated in two of the EIA tests in diluent and simulated infected urine containing 30 leucocytes per µl to ascertain the range required for the chessboard test from a low to a high positive OD. To ensure reproducibility for each chessboard, sufficient volume of each dilution was retained at -20°C.

To test each EIA, a set of the urine deposits were thawed and each resuspended in 1.2mls of sample diluent (equivalent to 1ml to 20mls of urine) and transferred to glass bijoux bottles if a boiling step was required. The samples were added to the EIA test wells laterally, followed by the *C.trachomatis* antigen vertically together with appropriate controls as in Figure 6.

Figure 6 **Format for each EIA Chessboard**



In all of the chessboards and in all of the varying amounts of antigen spike, with the exception of the Dako EIA test, the OD decreased as the leucocyte content increased (Tables 7 to 11, Photos 5 to 7).

In the opposite direction, all the ODs increased as the amount of spike increased regardless of the leucocyte content. The exception to this was the Syva EIA test, which was also the worst performer, and only gave positive results when the leucocyte count was 10 per 1µl or less. However if a cut off lower than that recommended by the manufacturer were employed, (for example 0.120) then this test would reflect results observed in other tests (Table 11, Photo 7).

Dako and Abbott were the only EIAs to show poor performance in the detection of low levels of antigen as was previously observed in Figure 5 and this is probably the reason why the manufacturer recommends centrifuging large (20mls) volumes of urine. Interestingly the leucocyte content in the Dako test appears to have generated with a maximum OD 'hook' effect at 60 leucocytes per 1µl (Table 9, Photo 5).

Phadebact, Mastazyme and Abbott all gave similar OD readings with negative results falling below the cut off level at about the same ratios of leucocytes to antigen spike (Figures 7, 8 and 10).

As described in Section 3.2.5. the Clearview immunoassay could not be compared with numeric readings, nevertheless a straightforward titration of leucocytes spiked with a constant volume of neat *C.trachomatis* antigen gave positive results at all cell counts but with visibly stronger reactions when there were less leucocytes present (Photograph 8).

Footnote: The sensitivity of Dako IDEIA™ Chlamydia test has recently been increased by a new improved version, IDEIA™ *PCE.* (Polymer Conjugate Enhanced). During the course of this study we have participated in the validation of this new version in comparison to the old (CT10).

Table 7 - Phadebact Chlamydia EIA Test Chessboard

Antigen spike	1:16	1:8	1:4	1:2	2 x Neat	4 x Neat
Diluent	0.253	0.530	0.935	1.512	2.446	
O cells	0.299	0.713	1.257	2.069	2.544	
10 leucs	0.077	0.181	0.384	0.841	1.285	1.273
30 leucs	0.044	0.059	0.184	0.402	0.803	0.808
60 leucs	0.023	0.053	0.127	0.283	0.539	0.577
130 leucs	0.009	0.029	0.056	0.146	0.326	0.337
250 leucs	0.006	0.000	0.009	0.021	0.056	0.087

Cut off = 0.070 absorbance units, indicated by bold line.

Table 8 - Mastazyme Chlamydia EIA Test Chessboard

Antigen Spike	1:16	1:8	1:4	1:2	2 x Neat	4 x Neat
Diluent	0.670	1.523	>2.0	>2.0	>2.0	>2.0
O Cells	0.722	1.594	>2.0	>2.0	>2.0	>2.0
10 leucs	0.841	1.485	>2.0	>2.0	>2.0	>2.0
30 leucs	0.258	0.753	1.670	1.767	1.472	1.297
60 leucs	0.044	0.111	0.724	1.740	1.392	1.433
130 leucs	0.027	0.031	0.033	0.036	0.427	0.847
250 leucs	0.024	0.032	0.036	0.027	0.069	0.170

Cut off = 0.16 absorbance units, indicated by bold line

Table 9 - Dako Chlamydia IDEIA™ Test Chessboard

Antigen Spike	1:16	1:8	1:4	1:2	2 x Neat	4 x Neat
Diluent	0.108	0.135	0.195	0.268	0.751	1.335
O Cells	0.088	0.098	0.093	0.152	0.574	0.962
19 leucs	0.111	0.129	0.168	0.365	1.343	2.392
30 leucs	0.118	0.180	0.272	0.417	1.862	2.533
60 leucs	0.140	0.189	0.321	0.532	2.428	3.055
130 leucs	0.105	0.126	0.245	0.412	1.843	2.952
250 leucs	0.109	0.130	0.213	0.470	1.812	2.822

Cut off = 0.15 absorbance units, indicated by bold line

Photograph 5 Dako Chlamydia IDEIA™ Test Chessboard

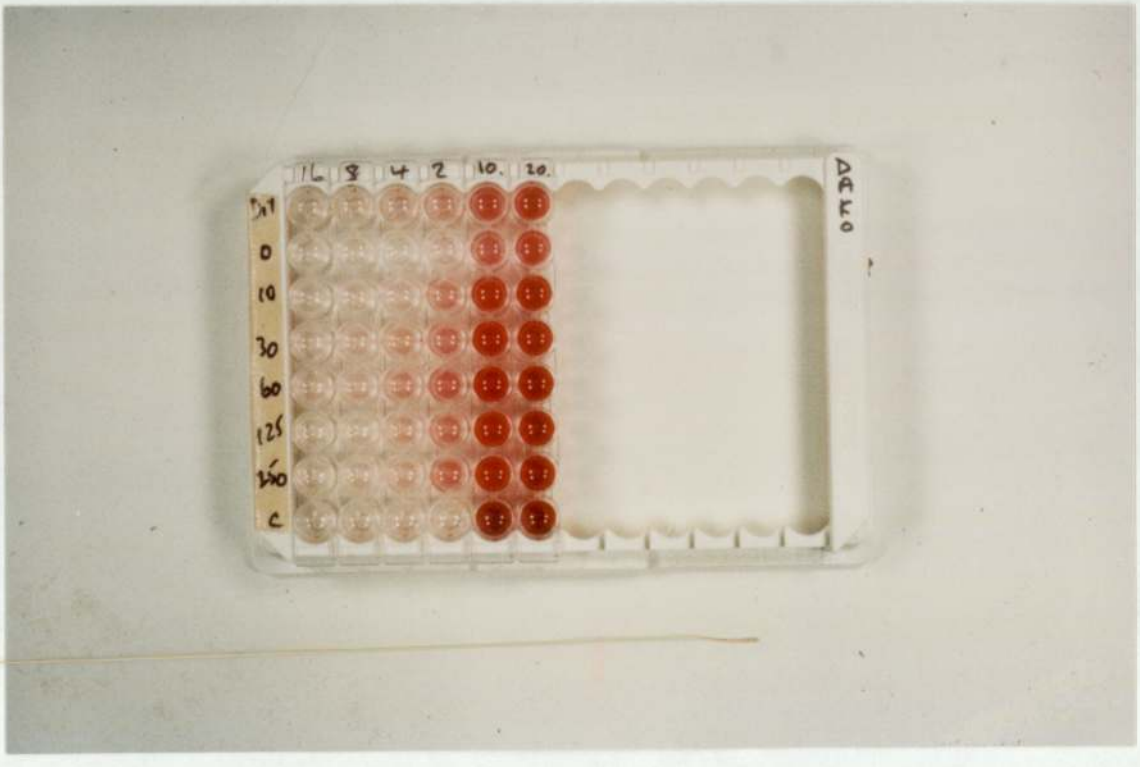


Table 10 - Abbott Chlamydiazyme EIA Test Chessboard

Antigen Spike	1:16	1:8	1:4	1:2	2 x Neat	4 x Neat
Diluent	0.093	0.110	0.359	0.992	>2.0	>2.0
O Cells	0.091	0.107	0.320	1.101	>2.0	>2.0
10 leucs	0.061	0.066	0.211	0.702	>2.0	>2.0
30 leucs	0.034	0.040	0.083	0.336	1.593	1.876
60 leucs	0.016	0.021	0.031	0.139	0.759	1.308
130 leucs	0.009	0.009	0.029	0.062	0.337	0.533
250 leucs	0.010	0.007	0.010	0.020	0.057	0.117

Cut off = 0.10 absorbance units, indicated by bold line

Photograph 6 Abbott Chlamydiazyme EIA Test Chessboard

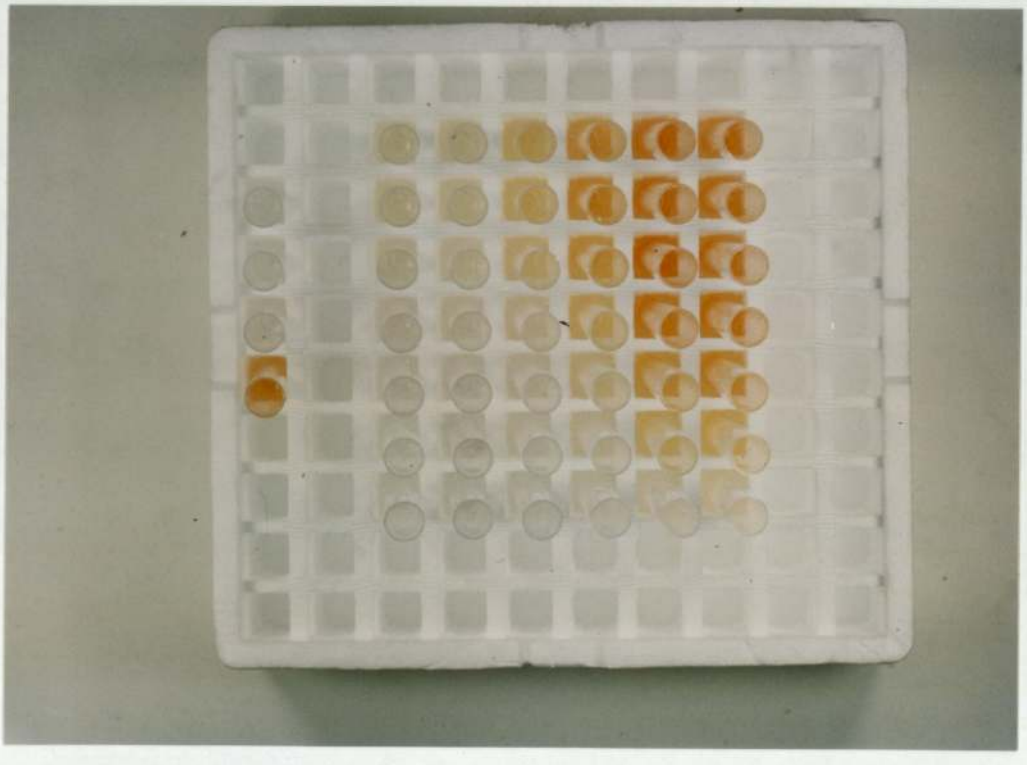
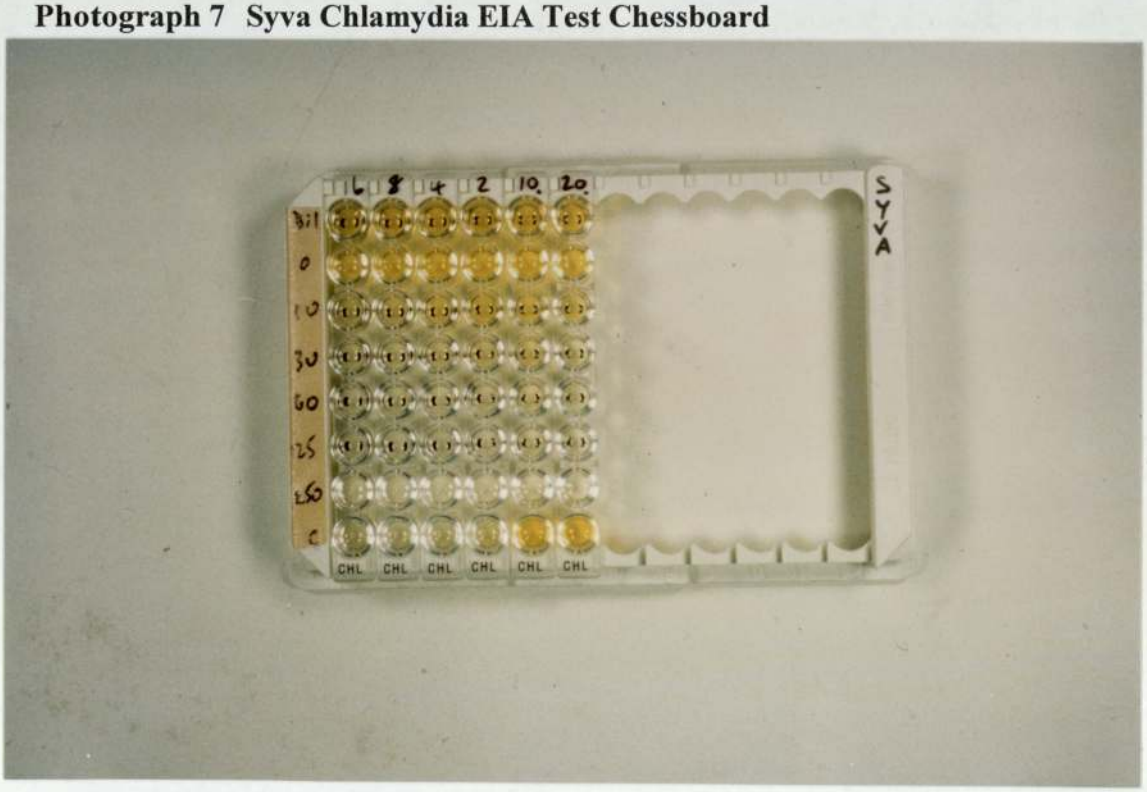


Table 11 - Syva Chlamydia EIA Test Chessboard

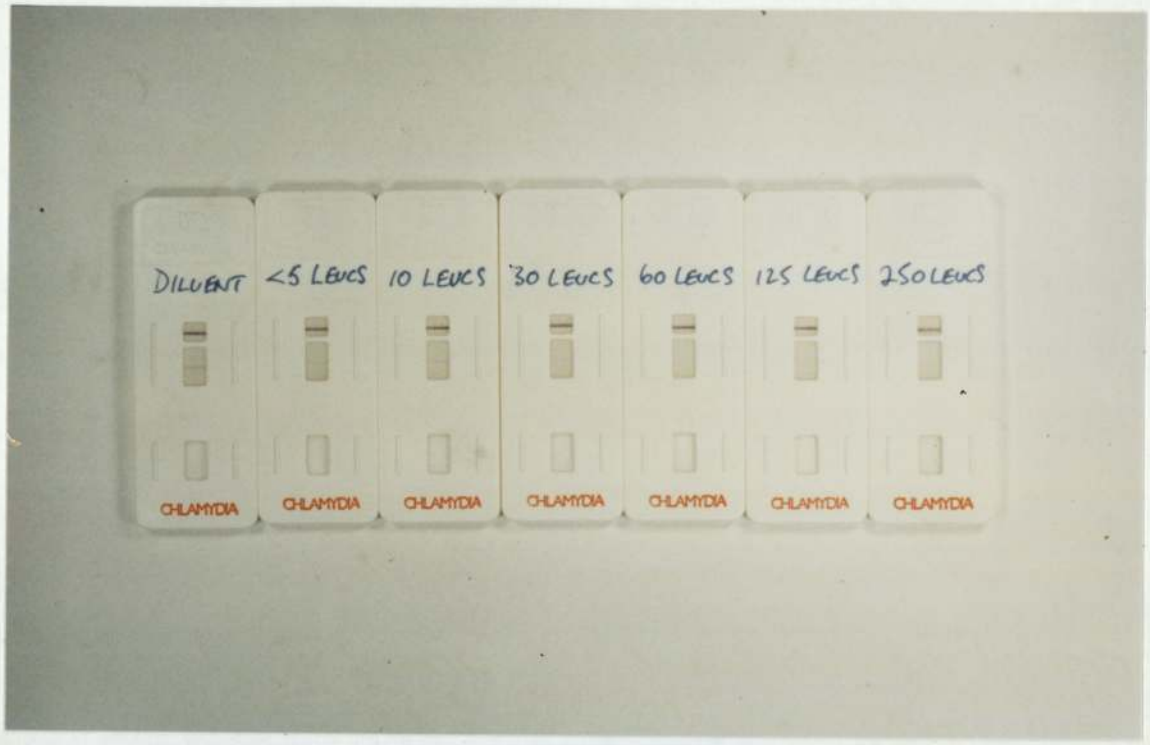
Antigen Spike	1:16	1:8	1:4	1:2	2 x Neat	4 x Neat
Diluent	1.374	1.916	2.397	2.776	2.564	1.836
O Cells	1.092	1.871	2.283	2.604	2.440	1.691
10 leucs	0.328	0.382	0.562	0.939	0.641	0.577
30 leucs	0.119	0.134	0.200	0.253	0.310	0.229
60 leucs	0.083	0.112	0.097	0.123	0.209	0.150
130 leucs	0.082	0.088	0.077	0.085	0.124	0.105
250 leucs	0.071	0.074	0.074	0.070	0.070	0.083

Cut off = 0.33 absorbance untis, indicated by bold line

Photograph 7 Syva Chlamydia EIA Test Chessboard



Photograph 8 Clearview™ Test Positive Result



3.3. Discussion

As suspected in the original premise, the more leucocytes that were present in the urine sampes the lower the OD was in the Phadebact Chlamydia EIA test.

The question arose as to whether this was a hook effect caused by excess Chlamydia antigen or some other component. As far as immunoassays are concerned there are no differences between prozones and hook effects. Any antibody antigen system in which a dilution of one of the components, be it sample, conjugate, indicator, etc., gives an increased signal, relative to the undiluted material, it is said to prozone. In extreme examples the signal may go to zero (Duncan 1996).

The Phadebact Chlamydia EIA is a simultaneous antigen sandwich format where the sample (antigen) is added to the well first followed by the antibody, there is no intervening was step (See Table 3). This type of assay often tends to generate prozones. However, introducing a wash step not only removed the offending leucocytes but also all of the antigen and was abandoned as a quick solution to the problem. Removal of the sample after 30 minutes and replacing with an equal

volume of diluent prior to the addition of the MAb, did have some positive effect but only when 5mls or 10mls of urine were centrifuged.

When this experiment was repeated using a suspension of leucocytes and saline to pre-challenge the EIA wells followed by titration of Chlamydia spike, similar results were obtained, but more importantly there was no prozone in the control wells using saline or urine supernatant as the matrix.

Armed with the results of these initial experiments several other more complex investigations all confirmed and consolidated the early observations.

Surprisingly when these challenges were made against other assays there were similar patterns of inhibition, but not to the same extent as the Phadebact EIA, with the exception of Syva Chlamydia EIA. In some aspects the Syva test appeared to have a greater problem than Phadebact, of which the manufacturer is presumably aware.

The fall of the OD to below the cut off was very steep and was negative, no matter how high the antigen spike, once the leucocyte count was more than 10 per cm (Table 11). To allow fair comparison for the Syva test the volume of urine centrifuged, in the last chessboard experiments, was equivalent to 20ml per test for each EIA manufacturer. In this way each EIA was treated in a similar manner including Phadebact which had already been established to perform best when 2mls of urine was centrifuged. However, the recommended volume of FPU to centrifuge in the Syva protocol was 4mls resuspended in 1ml of diluent and 100µl added per well rather than the usual 200µl used by other manufacturer's (See Table 6). This may explain why the results were so poor and indicates that the manufacturer probably had encountered problems in validating the test for use with FPU.

In comparison the Phadebact EIA protocol also uses 100µl of diluent for extraction but only 50µl is added per well. In proportion, the volumes used in the tests are the same (Syva: 4mls FPU-1ml diluent-100µl test). This is equivalent to 20% of the actual volume of original FPU that other manufacturer's utilise and if apportioned to

leucocytes in the chessboards would have slightly improved the Phadebact EIA but would have had little effect on the overall outcome of the Syva test.

These preclinical trial studies of the Phadebact Chlamydia EIA test indicated that 2mls of FPU gave the optimum OD photometric readings. There is, however, a conflict in that the lower the volume of urine centrifuged, then it follows that, less Chlamydia antigen would be concentrated. A point can be reached when low levels of antigen cannot be detected unless the sensitivity of the test or the volume of the sample is increased. In transferring this knowledge to the clinical trial, two volumes of FPU were utilised, 2mls and 10mls.

4. CLEARVIEW™ CHLAMYDIA MF PRE-CLINICAL TRIAL EXPERIMENTS

4.1. Introduction

The rapid immunoassay test for *C.trachomatis*, Clearview™ Chlamydia (Unipath Limited), was first introduced in 1990 and has been subject to both favourable and unfavourable scientific publications on its clinical performance. In original validation studies a poor sensitivity was achieved for specimens taken from the male urethra and the manufacturer decided to seek validation of the test for female endocervical swabs alone (Arumainayagam et al 1990). However, there has been commercial pressure on Unipath to improve the performance of the test to include male urethral swabs or FPU samples.

In a study with the Research and Development Department of Unipath Limited, Bedford, UK, culminating in a multicentre clinical trial of the new improved version of Clearview™ Chlamydia, a reason was sought for the previous poor performance, although it had been recognised that there was some inhibiting factor within the clinical sample (Paul Sheard, Unipath Limited, personal communication). Experience in the pre-clinical trial evaluation of the Phadebact EIA had shown that leucocytes had little effect on the Clearview™ Chlamydia test result outcome, albeit the positive reactions became weaker as the leucocyte count increased (see figure 5 and photograph 10). The new Clearview™ Chlamydia test was called 'Clearview™ Chlamydia MF' where the MF indicates its use for both male and female samples. The development of the Clearview™ Chlamydia MF was undertaken by Paul Sheard of the Research and Development Department of Unipath Limited, Bedford, UK.

The test is based on an immunochromatography method using a coloured latex particle coated with a genus-specific MAb as the mobile phase. Five drops (300µl) of heat extracted Chlamydia LPS is applied to an absorbent pad in the sample window where antigen combines with the antibody-coated latex to produce a mobile complex (Figure 7). The complex is carried on a membrane strip to a band of immobilised genus specific MAb, which captures the complex to form a sandwich,

resulting in a clearly visible blue line within 15 minutes. Excess antibody-coated latex or complex, in both negative and positive reactions, is carried past the result window to a second, antimouse polyclonal antibody line, which confirms that the device has worked correctly by producing a second visible control line. Failure to produce this control line invalidates the test (Figure 8 Photograph 8).

Figure 7 - Clearview™ Chlamydia Rapid Assay Format.

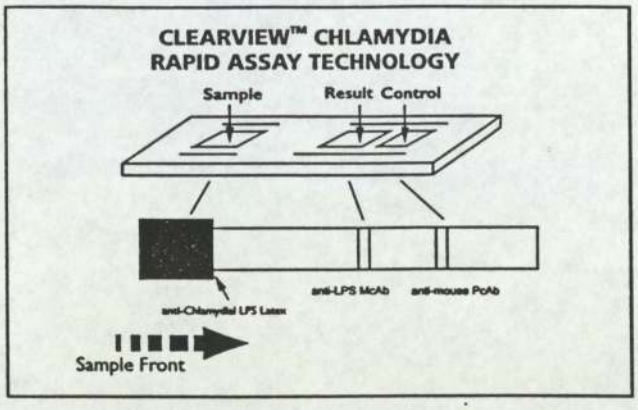
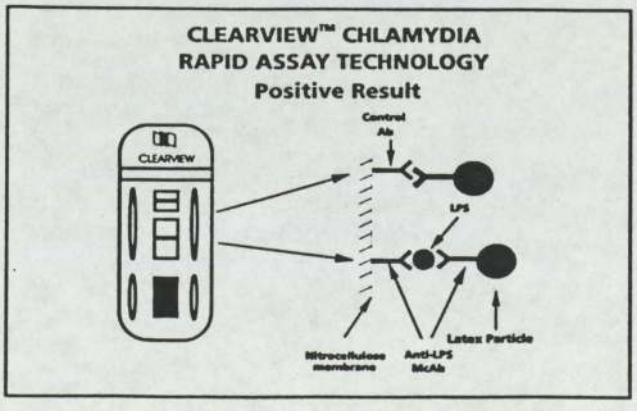


Figure 8 - Clearview™ Chlamydia Rapid Assay Positive Result.



The process of antigen extraction from the patient sample must not only be specific for *C.trachomatis* but also ensure that the analyte remains in a reactive form for subsequent detection.

Chlamydial LPS is comprised of a tri-saccharide antigen (keto-deoxy-D-manno-octulosonic acid [KDO]) and a lipid region (lipid A), linked to the KDO through the acylated glucosamine di-saccharide (Rietschel et al 1997). In the cell membrane, LPS is stabilised by interactions between anionic groups (carboxylate) on the KDO through the acylated glucosamine antigen and divalent cations, and by hydrophobic interactions between neighbouring fatty acid groups (Nikado and Vaara 1985). Therefore, interactions between cations present in the specimen and the anionic groups on KDO or, alternatively, hydrophobic components and the fatty acids present in the lipid A region, could give rise to interference within an immunoassay.

Assuming that it was the sample itself, as a whole, and not the swab or extraction process that caused the interference, a method was devised to investigate and understand the mechanisms involved.

The result of investigations into the cause of sample interference in immunoassays for the detection of LPS have been applied to the continued development and improvement of the Clearview™ Chlamydia test by Unipath Research and Development Department.

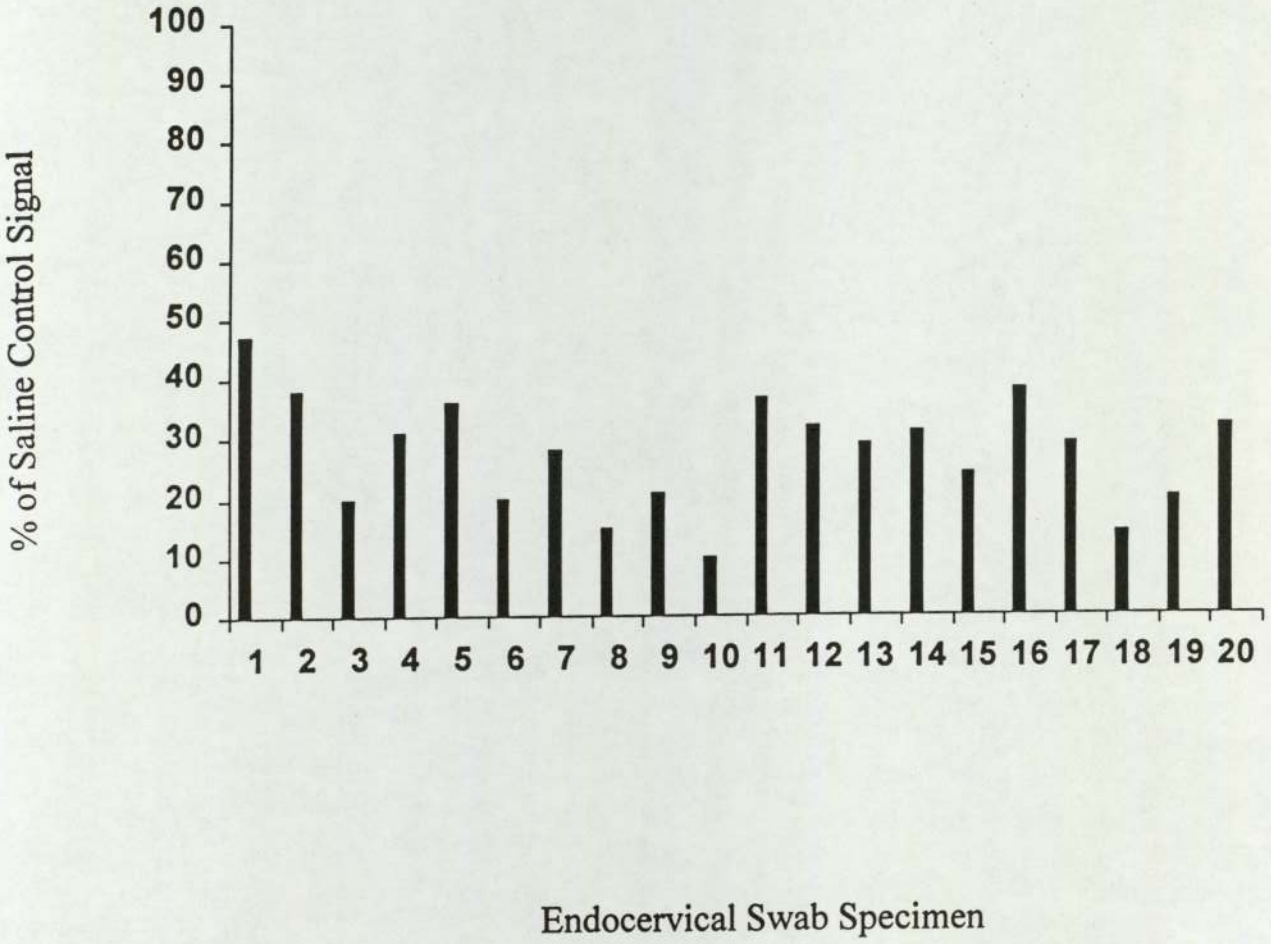
4.2. Results

4.2.1. *Demonstration of Interference in Clinical Specimens*

To measure the effect of sample extract on LPS antigen, approximately 10^4 EBS, (*C.trachomatis* serovar LGV2-UP8, Birmingham University, UK) in a 50µl volume of saline, was added to each of twenty extraction tubes following by 0.6ml of extraction reagent. Endocervical swabs from Chlamydia negative patients screened by DIF (Section 1.3.2.) were added to each of the tubes excluding one control tube. The swabs and EBS were heat extracted and tested by Clearview™ Chlamydia as previously described. The intensity of the positive result line was measured using an in-house reflectance instrument as compared to the signal of the positive control.

All of the 20 endocervical swabs reduced the signal in comparison to the control by at least 50%. Four of the swabs by 80% to 100% 15 by 60% to 80% and one sample by less than 60%, (Figure 9).

Figure 9 - Clearview Chlamydia, Effect of endocervical swab specimens on assay signal



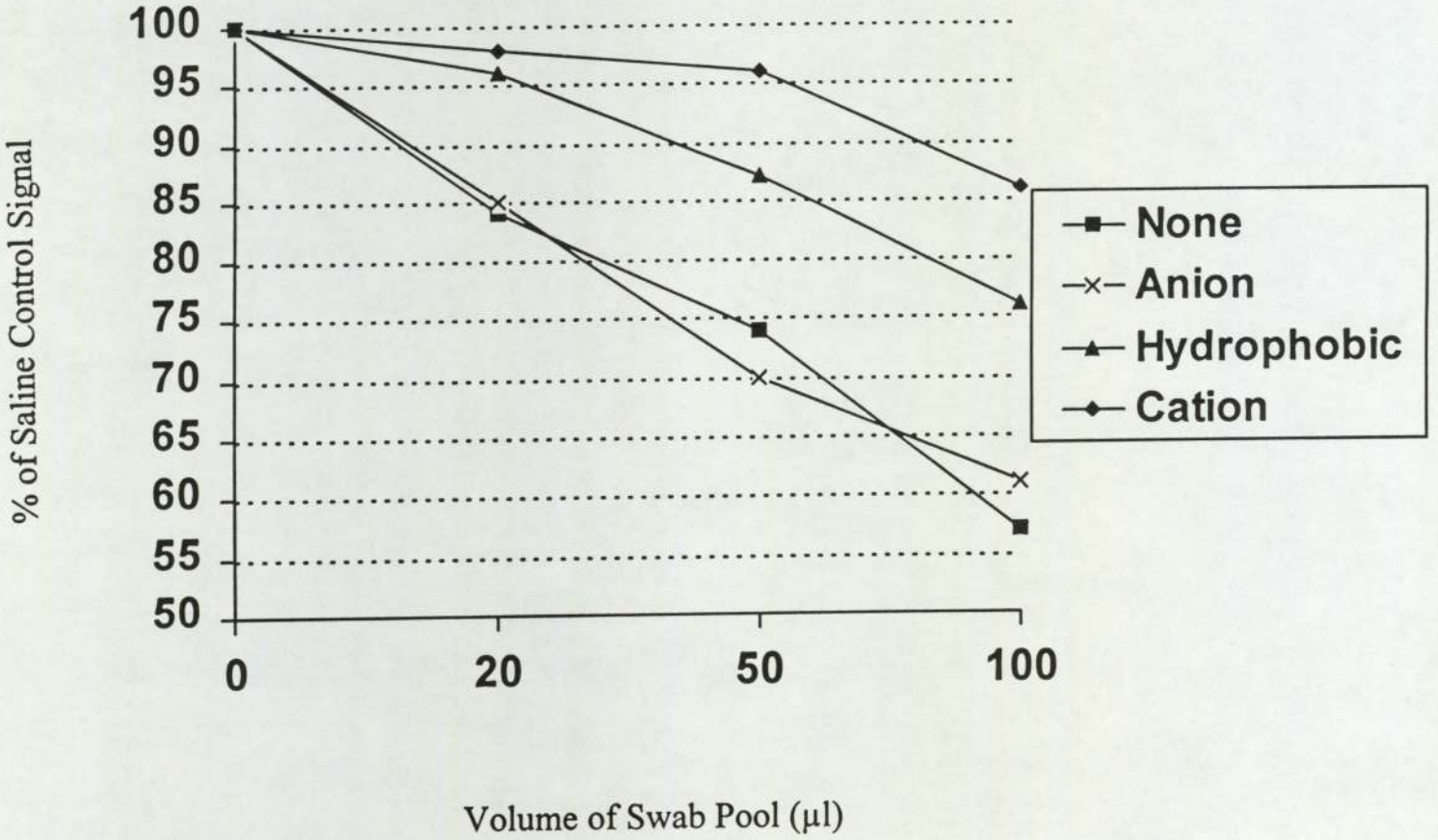
4.2.2. *Removal of Interfering Components by Adsorption*

To investigate the effect of adsorbing components from swab extracts, a pool of extract was prepared from 20 DIF negative endocervical swabs by adding 0.5ml of saline to each swab tip in a 1.5ml eppendorf tube and vortexing for 30 seconds to release clinical material. The tubes were centrifuged at 13,500 rpm to recover the maximum volume of diluent from the swabs and then each tested by the method described in Section 4.2.1. for their ability to interfere with the Chlamydia assay. Those extracts showing the highest interference were pooled.

Aliquots of the same pool were treated with either anion (quaternary ammonium), cation (propyl sulphonate) or hydrophobic (Octyl) exchange resins (Pierce Chemical Company). Duplicate 0.3ml amounts of pooled extract or control saline were added to previously washed resins and mixed for 15 minutes prior to centrifugation. A 20, 50 or 100µl volume of each supernatant was tested by Clearview™ Chlamydia together with untreated swab pools and saline controls.

The signal of the 10^4 EBS control was used as a 100% reference standard against which all the other results were measured. The signal was reduced depending on the volume of the swab extract added. For 20, 50 and 100µl volumes the assay signal was reduced by 16, 26 and 43% respectively. After contact with either cationic, anionic or hydrophobic resins the 100µl volume of swab extract was reduced by 14, 39 or 24% respectively in comparison to 43% in the untreated control. The largest reversal of inhibitory effect was recorded after contact with the cationic resin, followed by the hydrophobic resin, whereas the anionic resin had no effect, (Figure 10).

Figure 10 - Effect of Adsorbing a Swab Pool with various exchange resins prior to Clearview Chlamydia test

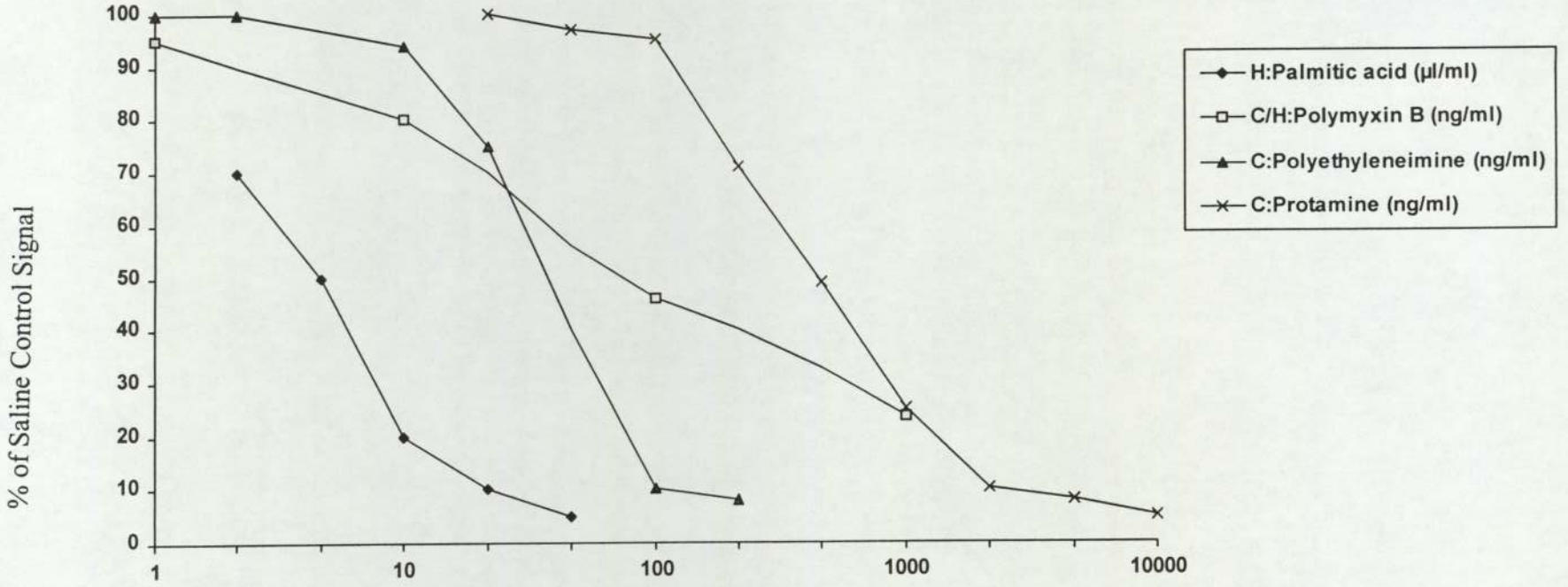


4.2.3. *Simulating Clinical Interference*

Reagents expressing either cationic (eg protamine; Mwt = 4,700D or polyethyleneimine; Mwt = 50,000D) or hydrophobic activity (eg palmitic acid; Mwt = 256D) or both (eg polymyxin B; Mwt = 1400D) were selected and tested for their ability to interfere with LPS detection in Clearview™. A range of concentrations (protamine, 25ng/ml to 10μg/ml; polyethyleneimine, 0.1ng/ml to 1μg/ml; polymyxin B, 1ng/ml to 1μg/ml; palmitic acid, 1μg/ml to 300μg/ml) of each reagent was separately added to the extraction reagent, followed by a known amount of chlamydia suspension (10^4 elementary bodies per tube). The sample was then extracted according to the pack insert, followed by measurement of line intensity in the Result Window using the reflectance instrument.

The effect of the addition of reagents with either cationic (protamine or polyethyleneimine), hydrophobic (palmitic acid) or both cationic and hydrophobic (polymyxin B) activity on assay signal is shown in Figure 11. Polyethyleneimine had the highest inhibitory effect, followed by polymyxin B, protamine and palmitic acid. 50% assay inhibition was observed with concentrations of 42ng/ml, 200ng/ml, 400ng/ml and 5.7μg/ml, respectively.

Figure 11 - Effect of Cationic (C) and Hydrophobic (H) Reagents on Clearview Chlamydia tests



Concentration of Reagent (See Key)

4.2.4. *Preventing Sample Interference*

The effect of modifying the extraction procedure to reduce interference against LPS and antibody binding was then evaluated. Preliminary investigations confirmed that interference could be reduced by competition with complex anions of similar structure to the KDO region on LPS (e.g. porcine heparin). Further investigations were performed as follows. Heparin was added to the extraction reagent at a concentration range of 1µg/ml to 1mg/ml. The ability of the modified extraction reagent to remove interference in the presence of either interfering components (polyethyleneimine [100ng/ml] or protamine [300ng/ml]) or clinical specimens was determined.

The effect of the addition of heparin to the extraction reagent containing 10^4 EBS and either polyethyleneimine (100ng/ml) or protamine (300ng/ml) is shown in Figure 12. The presence of heparin in the extraction reagent at a concentration range of 3µl/ml up to the highest concentration tested, (1mg/ml), removed interference caused by either cation. Heparin did not affect the signal observed for 10^4 elementary bodies with any of the concentrations tested. The effect of extracting six swab pools containing 10^4 elementary bodies in the presence of 1mg/ml heparin is shown in Figure 13. The presence of heparin completely removed the effect of inhibitory components in pool 1 and partially removed the effect in pools 2, 3, 4, 5 and 6.

Figure 12 - Effect of Heparin on inhibition of the Clearview Chlamydia test

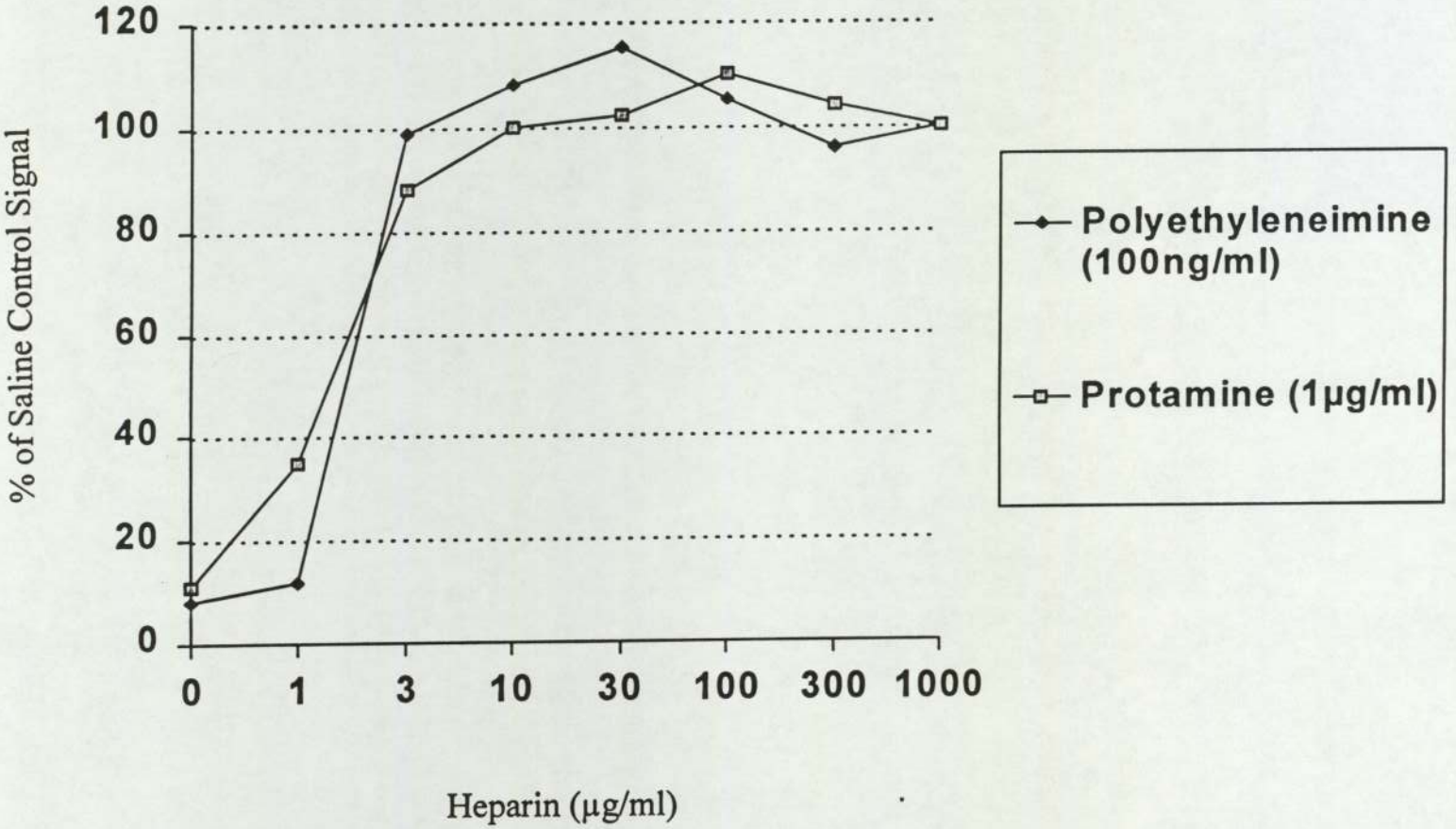
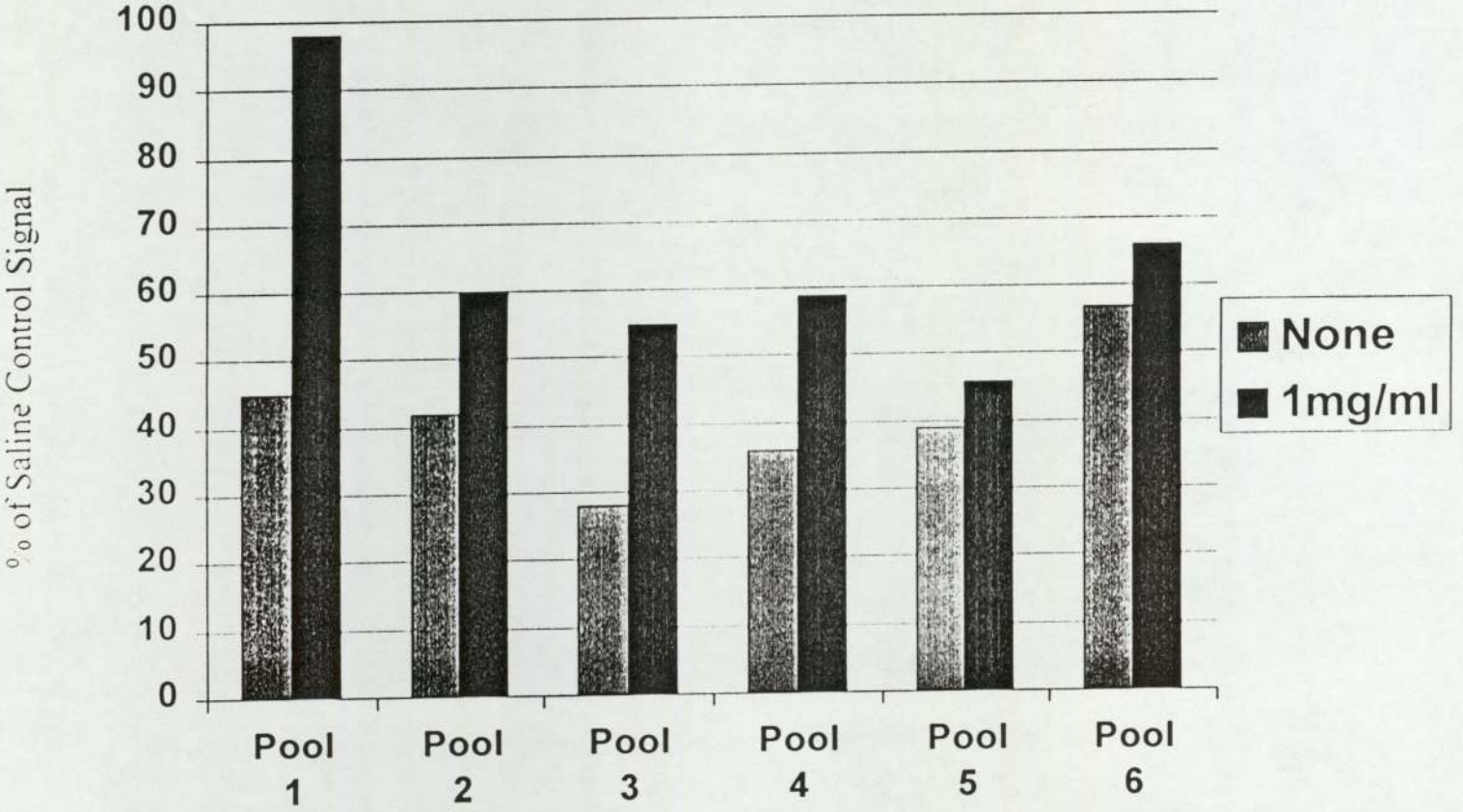


Figure 13 - Effect of Heparin on inhibition of the Clearview Chlamydia test in the presence of added *C.trachomatis*



4.3. Discussion

In this study sample interference with the performance of the Clearview™ Chlamydia test was demonstrated by the addition of Chlamydia negative swab samples to known positive controls.

Simulating pools of swab extracts allowed a standardised approach for further investigations and a mechanism to evaluate other manufacturer's immunoassays with similar material. The inhibitory effect could be demonstrated in all the Chlamydia immunoassays tested (data not shown). Comparative evaluations of Chlamydia tests that only contain EBS in saline without a sample of matrix, could, therefore overestimate the sensitivity of the tests evaluated (Thomas et al 1993).

Removing either cationic or hydrophobic components from the clinical sample using affinity resins significantly reduced the amount of interference to the test which suggested that they were the main cause of interference. The route was followed of simulating interference through deducing, from the structural of LPS, the likely interference mechanisms occurring.

Chemical components that were likely to interact with LPS were added to the extraction process. There were two main causes of interference between components present in the clinical sample and Chlamydia LPS. First, an interaction between cations present in the specimen and the anionic groups on KDO preventing detection of the LPS by the test antibody and second, an interaction between hydrophobic components and the fatty acids present in the lipid A region. For the cationic additives tested, the number of cationic sites present per ml for the concentration giving 50% inhibition was calculated. All additives had very similar levels of cationic sites, with polyethyleneimine containing 9×10^{11} sites, polymyxin B containing 1×10^{12} and protamine containing 4×10^{11} sites.

The rationale for removing or reducing sample interference was based on the hypothesis that competition with a similar anionic group to that on LPS, or addition of small monovalent cations to LPS during the extraction step, might protect exposed anionic groups on LPS from components in the specimen. Attempts to remove

cationic interference by either the addition of simple cations or anions to either compete with or block interfering were unsuccessful. Consequently, it was necessary to evaluate more complex anions with a similar structure to the carbohydrate structure present in chlamydial LPS. The most successful method was to use a polysaccharide with a repeating anionic carboxylate group, similar to that found on chlamydial LPS.

Of the treatments tested, the most convenient additive found was heparin. Concentrations of heparin in excess of 1mg/ml were tested but were found to be inhibitory (data not shown). The maximum concentration of heparin that could be used without compromising sensitivity of the test was 1mg/ml. This successfully reduced interference caused by either complex cations (eg protamine and polyethyleneimine) or components present in the clinical specimen.

The addition of 1mg/ml heparin to the kit extraction reagent removed interference caused by soluble components present in the clinical specimen. In the performance evaluation, this improvement to the test was found to increase the sensitivity of the kit to 96.7% for a high prevalence group (Shear et al 1998) in comparison with 79.4%, reported in a previous evaluation (Ridgway et al 1991).

5. CLINICAL TRIAL 1 - MALE URETHRAL SWAB CULTURE VS FPU PHADEBACT CHLAMYDIA EIA

5.1. Introduction

It was established in the pre-clinical trial evaluation that 2mls of centrifuged FPU gave the highest EIA OD readings. However, in order to compare this clinical trial with the previous studies by Paul et al (1990) and Kok et al (1993), a 10ml volume of FPU was also tested.

Randomised FPU samples and urethral swabs were collected from 110 males attending the GUM Clinic. Three patients were excluded as no FPU samples were received. Of the 107 remaining patients, two had evidence of antimicrobial agents in their urine but as one of these was positive by culture, albeit with reduced numbers of inclusions, but not discrepant, both were included in the data.

5.2. Results

5.2.1. *Bacterial Culture and Microscopy of FPU*

The majority of urines yielded no growth or scanty skin flora with only one showing a significant finding of >1000 leucocytes and heavy growth of *Escherichia coli*.

As expected in males attending a GUM Clinic with signs or symptoms of a urethral infection, many had leucocytes or leucocyte esterase present in their urine. The significance of leucocytes in relation to EIA outcome is detailed in Chapter 3.

5.2.2. *Chlamydia Coverslip Culture Method 1 VS Scrape Off Method 2*

There were no discrepant results between the gold standard coverslip method 1 and the simplified method 2 both detecting the 21 patients positive by urethral swab culture. The sensitivity of tissue culture was 95.5% (21/22) as compared to the overall detection rate. The cumulative results of the modified culture technique are discussed in Chapter 16.

5.2.3. *Comparisong of 2ml and 10ml volume of FPU EIA Results*

Of the 107 patients, 22 (20.6%) were positive by any method and 21 (19.6%) by culture alone. The 2ml volume of centrifuged FPU detected 68.2% (15/22) of the positives as did the 10ml volume, although not the same 15 of the 22. All of the EIA positives were confirmed positive by the blocking test. A summary of the results is given in Table 12.

Table 12 - Summary of results 5.2.3.

Urethral Swab Culture	+	+	+	-	+	-		
FPU EIA 2ml Centrifuged	+	+	-	+	-	-		
FPU EIA 10ml Centrifuged	+	-	+	+	-	-		
	10	4	4	1	3*	85	=	107

*One of these was positive in a recentrifuged 2ml stored sample (4796).

The combined result of the 2ml and 10ml volumes detected 86.4% (19/22) of the positive which compares favourably with other manufacturer's claims for FPU (Dako 84.7%, Abbott 84.7%, Syva 84%). However, in this evaluation, two volumes of FPU were tested, which would be unacceptable to most users, and a more detailed examination of the results was undertaken.

The four positives that were missed by the 2ml volume had low leucocyte counts (10, 10, <5 and <5) and low Chlamydia inclusion counts on first culture (200, 200, 100 and 73 respectively) (sample numbers 2886, 3271, 3805, 5397).

The four positives that were missed by the 10ml volume had higher leucocyte counts (60, 250, 150 and 400) and with the the exception of 4792, which had evidence of antibiotic treatment, had higher Chlamydia culture inclusion counts (>1000, 500, 1 and 750) (2984, 4533, 4792, 5394 respectively).

The three patients that were urethral swab culture positive and FPU EIA negative in both the 2ml and 10ml volume, all had high leucocyte counts, (80, 400 and 750) with

low culture inclusion counts. (29, positive but toxic and 100 respectively) and all had high levels of leucocyte esterase present (4627, 4796, 5174). One of them (4796) was positive with a recentrifuged 2ml sample of stored whole urine (OD 0.122). A full summary of the positive results including which volume of FPU centrifuge gave the highest EIA OD reading is detailed in Figure 14.

5.3. Discussion

It was noted that, as in the pre-clinical evaluation, the number of leucocytes present in the FPU sample influenced which volume centrifuged gave the highest EIA OD reading. With the exception of two of the 22 positive urines (4307 and 5503) the highest ODs were in 2ml volumes if the leucocyte count was more then 50 per µl and in 10ml volumes if the count was less then 50 per µl . Furthermore, in the 8 FPUs that were positive in only one of the centrifuged volumes, the positive predictive value of this formula was 100%. This was also true of the additional FPU that was positive on the 2ml recentrifuged sample where the leucocyte count was 400 per µl (4796).

By applying this formula all of the FPU positives could have been detected in utilising one EIA well for each sample instead of two.

Figure 14 - Phadebact Chlamydia EIA : Male FPU vs Urethral Swab Culture

Analysis of Positive Results

Urine	Urethral Swab Culture Inclusions	Centrifuge Urine EIA		
Leucs/per ml		2mls	Highest	10mls
70	negative	+	←	+
10	200	-	→	+
100	>1000	+	←	+
60	>1000	+	←	-
10	200	-	→	+
250	>1000	+	←	+
<5	>1000	+	→	+
<5	100	-	→	+
40	>1000	+	→	+
90	>1000	+	→	+
250	500	+	←	-
80	29	-		-
150	1	+	←	-
400	+ toxic	-		-
300	>1000	+	←	+
250	>1000	+	←	+
20	>1000	+	→	+
750	100	-		-
400	750	+	←	-
<5	73	-	→	+
80	41	+	→	+
20	>1000	+	→	+

Key: + Positive, - Negative, → arrow indicates which volume gave the highest OD.

The use of two FPU volumes for each test is unacceptable on cost grounds to most users, consequently the Phadebact Chlamydia EIA test is still not validated for use with urine samples, nevertheless Boule Diagnostics AB and the UK supplier (Launch Diagnostics) recommend the 2ml and 10ml formula to users who enquire about its use with urine samples. Boule Diagnostics AB is researching a new extraction process where 250µl of digitonin is added to 5mls of whole urine, left for 5 minutes and then centrifuged as before. Early results indicate that some ODs have increased four of five times and that the number of leucocytes present has little effect on test outcome.

The results of this clinical trial confirms the original observation that the early studies by Paul et al (1990) and Kok et al (1993) were wrong to assume that the volumes of FPU recommended by other manufacturer's for use in their EIA tests could also be applied to an unknown and untested method. The best results were obtained from a 2 ml volume of FPU rather than the 10 or 20 ml volumes previously utilised. Although the results of this study did not have a satisfactory conclusion they highlighted the unpredictable nature of individual detection methods which became apparent in later studies with other immunoassays and molecular methods.

6. CLINICAL TRIAL 2 - MALE URETHRAL SWAB CULTURE VS FPU MASTAZYME CHLAMYDIA EIA

6.1. Introduction

FPU samples from 304 males were tested by the Mastazyme Chlamydia EIA test manufactured by Shield Diagnostics in comparison to conventional tissue culture on urethral swabs.

6.2. Results

6.2.1. *Bacterial Cultures and Leucocyte Esterase of FPU*

Although a wide variation in results were recorded from the blood agar culture inoculated with a standard 10µl loop, the majority were of 'no growth' or 'scanty skin flora'. There were no specimens that were EIA positive and chlamydia culture negative that could not be confirmed as true positives and none of those samples with bacterial growth caused any significant rise in the recorded optical density (OD). A leucocyte cell count was not included in CT10 but an estimate of leucocyte esterase was made using a dipstick method. It may be significant that the majority of swab cultures with low inclusion counts and with negative FPU EIA reactions also had high leucocyte esterase levels.

6.2.1. *Chlamydia Coverslip Culture Method 1 vs Scrape Off Method 2*

The results of method 1, the acknowledged 'Gold Standard', were used for statistical purposes when comparing with the results of the FPU EIA test.

The overall isolation rate was 33.2% (101/304) with 99 positives detected by both methods and two detected by the coverslip method 1 alone. The sensitivity of the simplified method 2 was 98% (99/101) with a specificity of 100% (203/203) and positive and negative predictive values of 100% (99/99) and 99% (203/205) respectively. If no 'blind passage' had been included, as is the more usual procedure in routine testing, then the simplified method 2 would have been as sensitive as method 1 since two of the positives were only detected after sub culture. The cumulative results of the modified culture technique are discussed in Chapter 16.

6.2.3. *Comparison of Urethral Swab Culture and FPU EIA Results*

There were seven swab samples that caused very slight toxicity in the first monolayers of both negative and positive cultures, but as none of these was thought toxic enough to warrant exclusion from the trial all of the collected samples were included.

In the first group of 151 patients where the swab was taken first, the overall detection rate by both EIA and culture was 28.4% (43/151) and by tissue culture alone 27.1% (41/151). In the second group of 153, where the urine was collected first the detection rates were 40.5% (62/153) and 39.2% (60/153) respectively. The order of specimen collection made no significant difference to the FPU EIA sensitivity compared with positive urethral cultures, being 82.0% (34/41) in the first group and 82.7% (49/60) in the second. In contrast to the previous findings by Jawad et al (1990) and Hay et al (1991) this study suggests a higher overall detection rate when urine is collected prior to the swab. However, since the two methods employed different cohorts of patients the higher detection rate may simply reflect a higher incidence of infection. A summary of the results is given in Table 13.

Table 13 - Summary of Results 6.2.3.

Urethral Culture	+	+	-	-		
Urine EIA	+	-	+	-		
	83	18	4	199	=	304

6.2.4.. *Comparison of Positive Results using Blocking Antibody*

Of the 89 specimens that were retested using the blocking reagent 86 were confirmed as positive by blocking and in one there was insufficient sample remaining. In the other two specimens that did not block, one was culture positive, but with no visible EBS in the stored aliquot, and the other was culture negative, but had EBS in the stored aliquot and remaining swab sample. All three had OD readings within the 'grey zone' (the threshold plus 0.05 absorbance units) but, with reproducibility being a signal of positivity, coupled with a finite amount of specimen remaining, they were

classified as EIA blocking test negative according to the manufacturer's protocol, even though they were proved positive by the other means. It was noted that in those samples that gave original high OD readings and were diluted prior to retesting, the strength of the new OD signal was not always reduced in proportion to the dilution and in a few cases actually increased.

6.2.5. *Discordant Results*

Besides analysing the discordant results, EIA positive and culture negative samples, it was considered that all the culture positive and EIA negative sample should also be examined. There were four of the former (Table 14) and 18 of the latter (Table 15).

Table 14 - Four EIA Positive/Culture Negative Samples

			Detected		
			EBS		
Lab No	**FPU EIA**	**EIA Blocked**	**Stored Aliquot**	**EIA**	**Swab**
2810	+	No	Yes	N.T.	Yes
2963	+	Yes	Yes	N.T.	Yes
4041	+	Yes	No	Yes	Yes
4568	+	Yes	Yes	N.T.	No

N.T. Not Tested + Positive - Negative

Table 15 - Eighteen EIA Negative/Culture Positive Samples

			EBS Detected	
Number of Samples	**Swab Culture**	**FPU EIA**	**Stored Aliquot**	**Swab**
8	+	-	No	Yes
10	+	-	Yes	Yes

Of the 22 samples that initially gave negative EIA results with positive cultures four became EIA positive when repeated and all proved to be true positives by either or both the blocking test or IF on the stored aliquot.

6.3. Discussion

As there was no significant difference between the first and second groups of patients the overall detection rate by both methods was 34.5% (105/304) with 28.6% (87/304) by FPU EIA and 33.2% (101/304) by urethral swab culture. The sensitivity of the FPU EIA as compared with the urethral swab culture was 82.2% (83/101) with a specificity of 98% (199/203). The positive and negative predictive values were 95.4% (83/87) and 91.7% (199/217) respectively with overall agreement of 92.8% (282/304).

After resolution of the discrepant results the sensitivity of the FPU EIA rose slightly to 82.8% (87/105) as compared with the overall detection rate by both culture and EIA. If this new figure is used for comparison of the EIA sensitivity then the adjusted results are 79.8% (82.9%) in the first group and 78.6% (81.7%) in the second (original results in brackets). This lower sensitivity represents a more accurate figure and should be considered when evaluating differing methods of detection. EIA is probably only 85 - 95% as sensitive as culture which, in turn is thought to detect only 80 - 90% of actual infections (Caul 1991). More recently the sensitivity of Chlamydia culture in comparison to molecular methods has been shown to be in the region of 75 - 80% (Schachter 1997).

The Mastazyme Chlamydia EIA test gave good positive and negative predictive values (95.4% and 91.7% respectively) on a highly selective group of males attending a GUM Clinic; however, caution should be taken when extrapolating these findings to a lower prevalence population where a slight variation in discordant results can vastly change positive predictive values. For this reason it would be useful to evaluate this EIA test and accompanying blocking reagents on a low prevalence group of patients to see if these results could be maintained. Taking this into account and if EIA is the only method available for the detection of *C.trachomatis* from male urogenital specimens, then there is little to be gained from taking urethral swabs when FPU samples will give the same, or slightly reduced, sensitivity.

7. CLINICAL TRIAL 3 - ENDOCERVICAL SWAB CULTURE VS CLEARVIEW™ CHLAMYDIA TEST

7.1. Introduction

This study, using the original Clearview ™ Chlamydia test, was on female patients attending an inner city general practice. Of the 468 patients enrolled in the study, 117 refused or were excluded leaving 351. Two randomised endocervical swabs were taken for Chlamydia culture and Clearview testing and in addition all patients had bacterial swabs collected and some had urine and urethral swabs taken.

About one third (131/351) of the patients attended their GP because they had signs or symptoms of a genital infection or thought they may have a sexually transmitted disease (STD). The other two thirds (220/351) were attending for a routine reason, cervical smear or family planning.

7.2. Results

Of the 131 patients with symptoms, 16% (21/131) had an STD of which 12 were *C.trachomatis* alone or in combination with *Trichomonas vaginalis, Herpes simplex or N.gonorrhoea.*

In the 220 patients with no symptoms 15.9% (35/220) had a STD of which 18 were *C.trachomatis* alone or in combination with *Trichomonas vaginalis*, genital warts or pubic lice.

7.2.1. *Comparison of Endocervical Swab Culture and Clearview Results*

A total of 24 patients were *C.trachomatis* positive by both methods and a further 6 were positive by culture alone. Three patients gave false positive Clearview reactions. A summary of the results is given in Table 16.

Table 16 - Summary of Results 7.2.1.

Endocervical Clearview	+	-	+	-		
Endocervical Culture	+	+	-	-		
	24	6	3*	318	=	351

*All negative by IF on the remaining Clearview extracts and culture swab samples.

The detection rate of *C.trachomatis* in the patients without symptoms was 8.2% (18/220) and in those with symptoms 9.2% (12/131) which mirrored proportionally the evidence of all STDs in both groups (15.9% to 16% respectively) and was a surprising result from the symptomless group.

The sensitivity of Clearview to culture was 80% (24/30) and specificity 99% (318/321) with a positive predictive value of 88.8% (24/27) and negative predictive value of 98.1% (318/324).

7.2.2. *Chlamydia Coverslip Culture Method vs Scrape Off Method 2*

There was one extra positive detected by the gold standard culture method 1 as compared to the scrape off method 2. However, one that was culture positive by both methods was only positive on sub culture in method 1. The cumulative results of the modified culture technique are discussed in Chapter 16.

7.2.3. *Investigation of Six Culture Positive and Clearview Negative Swabs*

All of the six culture positive and Clearview negative patients had low levels of viable *C.trachomatis* detected by culture. A summary of the results is detailed in Table 17.

Table 17 - Summary of Six Culture Positive Patients

Patient	Culture Result	Cleaview Result	IF on Extract
4337	5 Inclusions	Negative (Repeat swab positive)	Negative
6157	12 Inclusions	Negative	Negative
7626	<10 Inclusions	Negative	Not Tested
1750	2 Inclusions	Negative	Negative
5393	35 Inclusions	Negative	Positive
9118	3 Inclusions	Negative	Positive

At least three of the patients had no detectable EBS in the remaining Clearview extraction fluid and were therefore classified as sampling error (See Section 2.6.4.) which is not surprising considering the low number of inclusions amplified by culture from the corresponding swabs.

An interesting example of sampling error is detailed in Table 18. This patient (5440) was excluded from the study as she was believed to be not sexually active and had an unusual set of results.

Table 18 - Summary of Results of Patient 5440

	Culture	Clearview	Immunofluorescence
Cervical Swab	Negative	Negative	Negative
Urethral Swab	Positive >1000 Inclusions	Not Tested	Positive
Urine Sample	Not Tested	Not Tested	Positive

As it was difficult to take an endocervical swab, with only the cervix being swabbed, a urethral swab was collected for Chlamydia culture together with a urine sample. No urethral swab was collected for Clearview. This 23 year old ethnic Chinese patient was considered to have contracted a *C.trachomatis* urethral infection by intimate contact without penile penetration.

7.3. Discussion

In this blinded study, which took two years to complete, the Clearview test was undertaken by the practice nurse in the clinical setting with the remaining swab extracts being forwarded to the laboratory for further analysis of discrepant results. The Clearview™ Chlamydia test is designed for near patient or small batch testing, however, most of the validation studies for this type of test are undertaken by laboratories on high prevalence populations and there is a danger when relating these figures to low prevalence groups tested in the Physician's office. Unlike laboratory based EIAs, the Clearview™ test is read visually and is therefore subjective and adequate controls must be included to ensure quality assurance. Nevertheless this was an invaluable study on the prevalence of *C.trachomatis* and other STDs in General Practice and highlighted the pool of undiagnosed infection present in the population.

The practice nurse commented that the plastic-shafted dacron tipped swab supplied by Unipath for the Clearview test was often difficult to insert into the OS particularly when there was cervical scarring following diathermy. The culture swabs most often used for endocervical and urethral samples are small and wire shafted. This may have had some influence on result outcome although there have been no comments from other investigators in subsequent clinical trials. The nurse also observed 12 reactions where there was a very faint line in the result window of the Clearview test but recorded them as negative before the culture negative result was reported. This is an unusual observation as, in the author's experience, any reaction in the Clearview result window is indicative of a positive result and may reflect the performance of tests when undertaken by non laboratory personnel.

The results of this study indicated that the Clearview test was fairly accurate as a negative predicator (98.1%) with a specificity of 99%, however, its use is not recommended for use in low-prevalence groups but may be useful as a rapid method when immediate results are required for patient management (Black, 1997) Positive or negative results should be considered as presumptive and additional samples taken for laboratory based tests performed by laboratory trained staff.

8. CLINICAL TRIAL 4 - ENDOCERVICAL SWAB CULTURE VS ROCHE

AMPLICOR CHLAMYDIA TEST

8.1. Introduction

This study was in co-operation with the Microbiology Department, Queen Elizabeth Medical Centre, Birmingham where the Amplicor Chlamydia polymerase chain reaction (PCR) was undertaken.

Two randomised endocervical swabs were taken from 245 patients attending a GUM Clinic for infection screening. One swab was for conventional Chlamydia cell culture and the other for PCR. Both swabs were placed into relevant transport medium and forwarded to each laboratory site within 24 hours of collection (See 2.2.4.). This was a double blind study where neither laboratory knew the initial result outcome. If the results were discrepant then the remaining culture swab was examining for EBS by IF and the PCR was repeated.

8.2. Results

8.2.1. *Comparison of Endocervical Swab Culture and Amplicor PCR Results*

The combined *C.trachomatis* detection rate by culture and PCR was 10.2% (25/245) and 9.4% (23/245). A summary of the results is detailed in Table 19.

Table 19 - Summary of Results (8.2.1.)

Endocervical Swab PCR	+	+	-	-		
Endocervical Swab Culture	+	-	+	-		
	18	2	5	220	=	245

Further analysis of the results showed that 7.3% (18/245) of the patients were positive for *C.trachomatis* by both PCR and culture. Seven samples had initially

discrepant results: 2 (0.8%) were positive for PCR and negative for culture of which one was positive by IF on the remaining culture swab. 5 (2%) were positive for culture and negative for PCR, giving PCR an initial sensitivity of 78.3% (18/23) but repeat PCR showed 3 of these to be positive improving the sensitivity of 91.3% (21/23). The specificity of the PCR was 97.8% (220/225) and 99.1% (220/222) after resolution of the discrepancies.

8.2.2. *Chlamydia Coverslip Culture Method 1 VS Scrape Off Method 2*

In the study of the gold standard Chlamydia culture method 1 in comparison to the simplified scrape off method 2, there was one additional isolate detected by the gold standard method after sub culture. The cumulative results of the modified culture technique are discussed in Chapter 16.

8.3. Discussion

Amplification of DNA by molecular techniques is slowly replacing conventional methods, particularly when antigen detection is technically difficult. The transition from the research to routine laboratory has not been easy. It was assumed that there would be a problem with false positive results due to contamination with stray DNA but in reality the converse was true.

PCR appears to be very sensitive in detecting *C.trachomatis* antigen in male urethral swabs and FPU samples but less sensitive in endocervical swabs than female FPU (Schachter 1997). There are apparent naturally occurring substances found in clinical specimens that are inhibitory to PCR. Several causes and solutions have been suggested for this phenomenon. Incomplete neutralisation of the detergent or lysis of the sample in the transport medium during extraction or the presence of thermolabile inhibitors are identified as possible causes. Freezing and thawing of samples, and further diluting have been suggested for removal of inhibitors (Ossewaarde et al 1997). Recent studies have shown alternative transport medium or dry swabs improve results (Black, 1997). In this study three out of five initially PCR negative swabs became positive on repeat testing of undiluted extracts after freezing and thawing. These five samples were repeated because they were discordant to the

culture result. The duplicate testing of the other 220 negative specimens would probably have detected further false negatives but would have been an expensive procedure and certainly not cost effective.

There is a danger that although PCR shows great potential in maximising the detection of *C.trachomatis* from clinical samples it must not be discredited by inadequate evaluation of which samples to take from where and how they should be treated. The high cost of the Roche Amplicor Chlamydia PCR test coupled with the problems of false negative reactions will prevent routine diagnostic laboratories from introducing this technology in the near future, however, there is an increasing demand from GUM Physicians for molecular detection methods which will ultimately hasten their introduction.

9. CLINICAL TRIAL 5 - ENDOCERVICAL SWAB CULTURE VS CLEARVIEW™ CHLAMYDIA MF

9.1. Introduction

This study was carried out to evaluate the improved version of Clearview ™ Chlamydia by comparing 320 randomised endocervical swabs by conventional Chlamydia culture to Clearview™ Chlamydia MF. Specimens were collected from patients attending a GUM Clinic for infection screening.

9.2. Results

9.2.1. *Comparison of Endocervical Swab Culture and Clearview MF Results*

Of the 320 specimens tested 10.6% (34/320) were positive by cell culture and 10.0% (31/320) of these were also positive by Clearview. EBS were seen in the remaining swab sample transport medium for all 3 Clearview false-negative specimens. These samples had less than 100 inclusion bodies on primary cell culture. No EBS were seen in the remaining swab sample transport medium or Clearview extract for the one Clearview false positive specimen. A summary of the results is detailed in Table 20.

Table 20 - Summary of Result (9.2.1.)

Endocervical Clearview MF	+	+	-	-		
Endocervical Culture	+	-	+	-		
	31	1	3	285	=	320

Clearview had a sensitivity of 91.2% (31/34) and specificity of 99.7% (285/286) and positive and negative predictive values of 96.9% (31/32) and 98.9% (285/288) respectively.

9.2.2. *Chlamydia Coverslip Culture Method 1 VS Scrape Off Method 2*

There were two extra Chlamydia culture positives detected by the gold standard coverslip method as compared to the simplified scrape off method. Both of these had

low numbers of inclusions present on the first coverslip (13 in each). A summary of the numbers of inclusions in the first coverslip culture in relation to the Clearview result is detailed in Table 21. The cumulative results of the modified culture method are discussed in Chapter 16.

Table 21 - Summary of Culture Inclusion Counts (9.2.2.)

	Culture Result (Inclusions)				
	>1000	500-1000	100-500	<100	Negative
Clearview Positive	21	1	3	6	1
Clearview Negative	0	0	0	3	285

9.3. Discussion

This clinical trial was the first in which the new Clearview™ Chlamydia MF was evaluated following the improved method for removal of clinical interference (see Chapter 4). The results were encouraging with a difference in sensitivity of little more than a half of one percent when compared to culture. The majority of the positive patients had viable chlamydia inclusion counts of more than 1000 per coverslip all of which were also positive by Clearview. The three patients that were culture positive and Clearview negative were amongst the six patients that had less than 100 inclusions per coverslip and demonstrates the lower end of the positive threshold range of the Clearview test. The cumulative results of the Clearview™ Chlamydia MF test are more fully discussed in Chapter 15.

10. CLINICAL TRIAL 6 - SYMPTOMATIC MALE URETHRAL SWAB CULTURE VS FPU CLEARVIEW™ CHLAMYDIA MF

10.1. Introduction

This study validated the improved version of Clearview™ Chlamydia for use with male FPU samples. Urethral swabs for culture were collected together with FPU samples from 225 symptomatic males attending a GUM clinic for infection screening. The order of sample collection was randomised.

10.2. Results

10.2.1. *Comparison of Urethral Swab Culture and FPU Clearview MF Results*

Of the 225 specimens tested, 27.1% (61/225) were positive by cell culture, 22.2% (50/225) of these were also positive by Clearview and a further 2 out of 5 additional Clearview positives were found positive by IF on remaining extraction fluid after resolution of the discrepant results. Of the 11 false negative specimens on Clearview, 5 had <100 inclusion bodies on corresponding primary cell culture (Table 23) and 6 had no detectable EBS in remaining Clearview extraction fluid (Sampling error). A summary of the results is detailed in Table 22.

Table 22 - Summary of results (10.2.1.)

Male FPU Clearview MF	+	+	-	-		
Male Urethral Swab Culture	+	-	+	-		
	50	5	11	159	=	225

Clearview had a sensitivity of 82.0% (50/61) and specificity of 96.9% (159/164) with positive and negative predictive values of 90.9% (55/55) and 93.5% (159/170). After resolution of the discrepant results the sensitivity rose to 82.5% (52/63), specificity to 98.1% (159/162) and positive predictive value to 94.5% (52/55).

10.2.2. *Chlamydia Coverslip Culture Method 1 VS Scrape Off Method 2*

There were three extra Chlamydia culture positive detected by the conventional two coverslip method as compared with the scrape off method. One of these was only detected in the second coverslip culture and both of the others had less than 10 inclusions in the first coverslip. A summary of the numbers of inclusions in the coverslip cultures as compared to the Clearview result is detailed in Table 23. The cumulative results of the modified culture method are discussed in Chapter 16.

Table 23 - Summary of Culture Inclusion Counts (10.2.2.)

	>1000	500-1000	100-500	<100	Negative
Clearview Positive	29	4	6	11	5 (2 IF positive)
Clearview Negative	1	2	3	5*	159

*One was positive on the second coverslip only.

10.3. Discussion

This study was the first evaluation of new Clearview™ Chlamydia MF test utilising male FPU for Clamydia detection as compared to conventional urethral swab culture. There were discrepant results across the whole range of urethral swab culture inclusion counts from one negative Clearview in the 30 patients with counts of more than 1000 inclusions per coverslip to 11 in the 16 with less than 100 inclusions. Of the five Clearview positive and culture negative patients, two were found to be true positives by detection of EBS in the remaining FPU sample but not in the remaining swab sample. When two different samples are used to evaluate detection methods, whether or not they are the same or differing detection methods, there is the added complication of sampling error, (see Section 2.6.4.). The influence of sampling error and the cumulative results of the Clearview™ Chlamydia MF test are discussed in Chapter 15.

11. CLINICAL TRIAL 7 - ASYMPTOMATIC MALE URETHRAL SWAB CULTURE VS FPU CLEARVIEW™ CHLAMYDIA MF TEST

11.1. Introduction

This study was included in the validation of the new rapid Clearview™ Chlamydia MF assay for use with FPU samples from asymptomatic males attending a GUM Clinic. Randomised FPU samples for Clearview testing and urethral swabs for cell culture were collected over a six week period.

11.2. Result

11.2.1. *Comparison of Urethral Swab Culture and Clearview MF Results*

Of the 79 patients enrolled in the study 2 were excluded for incomplete sample sets and of the remaining 77 patients, 18.2% (14/77) were positive for *C.trachomatis* by culture and of these 16.8% (13/77) were also positive by Clearview. There were three additional apparent positives by Clearview, one of which was confirmed to be a true positive by IF on the remaining stored swab culture transport medium and Clearview extraction fluid. A summary of the results is detailed in Table 24.

Table 24 - Summary of Results (11.2.1.)

Male FPU Clearview MF	+	+	-	-		
Male Urethral Swab Culture	+	-	+	-		
	13	3	1	60	=	77

Clearview had a sensitivity of 92.8% (13/14) in this group of patients with a specificity of 95.2% (60/63) and negative and positive predictive values of 81.2% (13/16) and 98.4% (60/61) respectively.

After resolution of the results the sensitivity rose to 93.3% (14/15) specificity to 96.8% (60/62) and positive value of 87.5% (14/16).

11.3. Discussion

The asymptomatic males studied in this otherwise similar study to that described in Chapter 10, resulted in a better sensitivity than that of the symptomatic males, 92.5% as compared to 82.0%, albeit with a smaller group studied (77 patients and 225 patients respectively), which was probably too small to be of statistical value. The cumulatively results of the Clearview™ Chlamydia MF test are more fully discussed in Chapter 15.

12. CLINICAL TRIAL 8 - ENDOCERVICAL SWAB CULTURE VS CLEARVIEW™ CHLAMYDIA MF TEST

12.1. Introduction

This study was rapidly completed over a three week period with endocervical swab samples being taken at two Birmingham GUM Clinics from patients attending for infection screening. The clinical trial was part of the validation process of Clearview™ Chlamydia MF prior to commercial release.

12.2. Results

12.2.1. *Comparison of Endocervical Swab Culture and Clearview MF Results*

Of the 280 patients enrolled in this study, 7 were excluded due to incomplete sets of samples. The isolation rate of *C.trachomatis* by cell culture was 13.5% (37/273) of which 31 were also detected by Clearview. In the six that Clearview failed to detect, two had EBS detected in the remaining swab extracts. A summary of the results is detailed in Table 25.

Table 25 - Summary of Results (12.2.1.)

Endocervical Clearview MF	+	+	-	-		
Endocervical Culture	+	-	+	-		
	31	0	6	236	=	273

The sensitivity of Clearview was 83.8% (31/37) and specificity of 100% (236/236) with positive and negative predictive values of 100% (31/31) and 97.5% (236/242) respectively.

12.3. Discussion

The 273 female patients included in this study were from a similar group as the 320 described in Chapter 9 but were collected over a short three week period rather than the six months it took to complete CT5. This study was undertaken one year after the first.

The isolation rates from the endocervical swabs were 10.6% (34/320) from the first study and 13.5% (37/273) from the second, with the sensitivity of Clearview being 91.2% (31/34) and 83.8% (31/37) respectively. After resolution of the discrepant results, antigen detection from Clearview swabs in the latter study rose to 89.2% (33/37) even though the two extra positives were only detected by IF and were below the positive threshold of the Clearview test. This new percentage of antigen detection indicated that both sets of results were within a similar range, taking into account other factors such as seasonal variation.

The results of the Clearview™ Chlamydia MF clinical trials are more fully discussed in Chapter 15.

13. CLINICAL TRIAL 9 - URETHRAL SWAB EIA (DAKO DIAGNOSTICS IDEIA™) VS FPU CLEARVIEW™ CHLAMYDIA MF TEST AT ONE DAY AND FIVE DAYS, AND FPU CHLAMYDIA LCR™ (ABBOTT LABORATORIES)

13.1. Introduction

Originally this study was to evaluate the stability of FPU samples stored over a five day period at +4°C for use with the new Clearview Chlamydia MF test. The opportunity was taken to extend this study to include an evaluation of Abbott Laboratories Chlamydia LCR test on the same samples as compared to conventional urethral swab EIA. Randomised samples were collected from 70 male patients attending a GUM Clinic for infection screening. FPU samples were tested by Clearview and LCR on day one and again by Clearview on day five.

13.2. Results

13.2.1. *Comparison of Urethral Swab EIA and FPU Clearview MF and LCR Results*

The *C.trachomatis* detection rate by urethral swab EIA was 15% (9/60). Clearview detected six of the positives on day one and four of the positives on day five. One extra weak positive Clearview, detected by majority decision on day one only, was shown to be a false positive by IF on remaining extract fluids.

FPU LCR detected seven out of eight swab EIA positives and was not tested on the ninth. The one EIA positive that was missed by LCR was positive on the repeat stored FPU aliquot. A summary of the results is shown in Table 26.

Table 26 - Summary of Results 13.2.1.

FPU Clearview Day 1	+	+	+	-	+	-	
FPU Clearview Day 5	+	-	+	-	-	-	
Urethral Swab EIA	+	+	+	+	-	-	
FPU LCR	+	+	-*	+	-	-	
	2	2	2	3	1	60	= 70

*One FPU was not tested by LCR and the other was positive in repeat.

The sensitivity of Clearview on day one as compared to urethral swab EIA was 66.6% (6/9) and specificity of 98.4% (60/61) with positive and negative predictive values of 85.7% (6/7) and 95.2% (60/63). Storage of FPU for as long as five days was unacceptable for testing by Clearview. The sensitivity of Abbott Chlamydia LCR on FPU as compared to urethral swab EIA after exclusion of the untested sample was 87.5% (7/8) with a specificity of 100% (61/61) and positive and negative predicative values of 100% (7/7) and 98.4% (61/62) respectively. After repeat testing of the discrepant, negative LCR FPU sample, which was positive on the stored aliquot, the sensitivity as compared to EIA was 100% (8/8).

13.3. Discussion

In this study of 70 male patients from a high prevalence group attending a GUM clinic with signs or symptoms of a urethral infection, the prime objective was to establish whether Chlamydia antigen could be repeatedly detected in FPU by Clearview™ Chlamydia MF test after five days storage at +4°C as compared to one day.

Clearview detected 6 out of 9 (66.6%) positives by EIA on day one which reduced to 4 out of 9 by day five. The information which was required by the manufacturer for submission for FDA approval confirmed the original protocol method requiring testing of FPU within 24 hours of collection. In practice as long as the FPU is processed within 24 hours to the stage where the centrifuged deposit could be stored

at -70°C (section 2.2.1.) rather than remaining as whole urine at +4°C, then later batch testing is possible. The storage of FPU for five days at +4°C appears to have a detrimental affect on the stability of the Chlamydia LPS antigen and is not recommended by any of the manufacturer's either for EIA or molecular testing.

The testing of the FPU samples by Abbott Laboratories Chlamydia LCR gave the same results as the urethral swab EIA with the exception of one sample which was initially LCR negative but positive on repeat testing of the -20°C stored FPU aliquot. This was a disappointing result and was reminiscent of Clinical Trial 4 where similar findings were observed with the Roche Amplicor Chlamydia PCR test (Chapter 8).

The Abbott Laboratories Chlamydia LCR test and Clearview™ Chlamydia MF test are more fully discussed in Chapter 15.

14. CLINICAL TRIAL 10 - MALE URETHRAL SWAB EIA DAKO DIAGNOSTICS IDEIA™ CHLAMYDIA VS IDEIA™ *PCE* CHLAMYDIA

14.1. Introduction

The aim of this study was to evaluate the performance of a new improved Chlamydia EIA, IDEIA™ *PCE* (Polymer Conjugate Enhanced) Chlamydia test for the detection of Chlamydia specific lipopolysaccharide antigen in male urethral swabs. Urethral swabs were collected prospectively from both symptomatic and asymptomatic males attending a GUM Clinic. The swabs were placed in 1ml of a new antifoam EIA transport medium supplied by the manufacturer and suitable for use with both the new and existing formats of the IDEIA™ Chlamydia Test. The polymer conjugate utilised in this modification to the original Chlamydia IDEIA™ test, consists of a dextran backbone to which the alkaline phosphatase is bound. Chlamydia antigen is actively captured onto the solid phase microtitre plate well by Chlamydia MAb. In theory, if only a small amount of antigen is captured by the MAb a primary amplification takes place when the long strand of polymer adheres to the site with multiple enzyme copies available for the secondary amplification. To further enhance the effect of the polymer conjugate and shorten the incubation period, the test is best performed in a shaking incubator. This study was part of the validation process of Dako Chlamydia IDEIA™ *PCE* test prior to commercial use.

14.2. Results

14.2.1. *Comparison of Urethal Swab IDEIA Chlamydia and IDEIA PCE Result*

Of the 159 males entered into the study 20 were positive for Chlamydia by both methods and there were five discrepant results. After confirmation of the positives by blocking test and resolution of the discordant results by IF the overall detection rate of the new IDEIA™ *PCE* Chlamydia test was 13.8% (22/159) as compared to the existing IDEIA™ Chlamydia test of 12.6% (20/159). A summary of the results is detailed in Table 27.

Table 27 - Summary of Results 14.2.1.

IDEIA™ Chlamydia Test	+	+	-	-		
IDEIA™ *PCE* Chlamydia Test	+	-	+	-		
	20	2	3	134	=	159

The two IDEIA™ apparent positives were negative on repeat and one of the three positives by IDEIA™ *PCE* was within 10% (below) the OD cut off and negative by IF. In this study the new test was an improvement of the reference test and therefore the increase in resolved detection was the only data of value. In similar studies at Bristol and Sheffield Public Health Laboratories the increase in detection rate was 9.3% to 11.2% in endocervical swabs from high risk patients but with no increase utilising male FPU (Caul et al 1996) and 5.3% to 6.5% in endocervical swabs from mainly low risk patients (Kudesia et al 1996).

14.3. Discussion

The new IDEIA™ *PCE* Chlamydia test has been designed to increase the user friendliness of the existing EIA. The antifoam transport medium and liquid format of the reagents in dropper bottles make the test suitable for use both by hand and by automated sample processor. In this study the sensitivity of the test was enhanced by the novel polymer conjugate which not only increased the optical density readings in all of the positive results but also revealed two additional positives that were photometrically undetected by the old test. The IDEIA™ Chlamydia test has been validated for use since 1985 and is the method of choice by many laboratories. The new IDEIA™ *PCE* Chlamydia test is a considerable improvement on the existing test and has shown an increased sensitivity in male patients from a high risk group.

15. CLINICAL TRIAL 11 - ENDOCERVICAL SWAB CULTURE AND CLEARVIEW™ CHLAMYDIA MF TEST VS FPU IDEIA™ *PCE* CHLAMYDIA AND ABBOTT CHLAMYDIA LCR™ TEST

15.1. Introduction

This study was from a low prevalence group of patients attending two inner city medical centres. Two randomised endocervical swabs were collected, one for Chlamydia culture and one for Clearview testing, together with a FPU sample for Chlamydia EIA and LCR on centrifuged deposit. Of the 187 patients enrolled in the study, 26 were excluded due to incomplete or insufficient sample sets, however, all had culture swabs taken, and three overgrew with *T.vaginalis*.

15.2. Results

15.2.1. *Comparison of Swab Culture and Clearview Test vs FPU EIA and LCR Test Results*

The overall detection rate by culture was 5.9% (11/187) although one of the positives was in the 26 excluded patients as no FPU was received. The resolved detection rate by culture was 6.2% (10/161). Five patients were positive by all four tests but one of these was only positive by LCR on repeat testing. A further three were positive by culture, Clearview and LCR, two by culture alone and two by LCR. A summary of the results is detailed in Table 28.

Table 28 - Summary of Results 15.2.1.

Endocervical Swab Culture	+	+	+	+	+	-	-	
Endocervical Swab Clearview MF	+	+	+	-*	+	-	-	
FPU Dako IDEIA *PCE*	+	+	NT	-	-*	-*	-	
FPU Abbott LCR	+	-	NT	-	+	+	-	
	4	1[a]	(1)	2[b]	3[c]	2	149	= 161

(1) Excluded from study

a LCR positive on repeat testing.

b One swab positive on sub culture only and the other <10 inclusions.

c One swab positive on sub culture only and the others <200 inclusions.

* EBS detected by IF in one each of these remaining stored extracts.

It will be noted that where there were small numbers of inclusions in cultures from the endocervical swabs then the FPU EIA or LCR results were either negative or showed low levels of antigen (Table 28 b and c). Furthermore, in the two patients that were FPU LCR positive alone, there were no EBS detected in remaining endocervical swab samples. There were large numbers (>1000) of inclusion bodies in the endocervical swab culture of the one patient that was initially negative by FPU LCR but positive on repeat testing and is further evidence of demonstrable inhibition in some FPU samples also observed in the Roche Chlamydia PCR study, CT4. (see Chapter 8).

The sensitivity of Clearview swabs to culture was 80% (8/10) or 82.1% (9/11) if the positive patient without the FPU sample is included, with specificity of 100% (151/151) and positive and negative predictive values of 100% (8/8) and 98/7% (151/153).

The sensitivity of FPU LCR to swab culture was 70% (7/10) with specificity of 98.7% (149/151) and positive and negative predictive values of 77.8% (7/9) and 98% (149/152) respectively. Conversely if LCR was regarded as the 'gold standard' then the sensitivity of swab culture to FPU LCR was 77.8% (7/9) with sensitivity of 98.0% (149/152) and positive and negative predictive values of 70% (7/10) and 98.7% (149/152) respectively.

The sensitivity and positive predictive values of both swab culture and FPU LCR were poor when each was assumed to be the "gold standard" method of antigen detection.

Extending the gold standard swab cell culture to include the two positive by FPU LCR raised the detection rate of *C.trachomatis* in this low prevalence group of patients, from 6.2% (10/161) to 7.4% (12/161).

15.3. Discussion

In this study from a low prevalence group of female patients, one endocervical swab was collected for Chlamydia culture and another swab collected for Clearview™ Chlamydia MF testing. A FPU sample was collected for Abbott Chlamydia LCR testing and the same sample was tested by Dako IDEIA *PCE*.

The detection of Chlamydia antigen in female FPU samples by EIA is not validated for diagnostic use and was included in this study from anecdotal evidence that it might be a useful test and of interest to the clinician, manufacturer and investigator. As expected, the detection rate by FPU EIA was poor when compared to swab culture; 50% (5/10).

However, the Dako IDEIA *PCE* may be useful on female FPU as a positive predictor when no other sample is available but it would not be suitable as a negative predictor where either a more sensitive test such as PCR or LCR should be used or an endocervical swab recommended.

The Abbott Laboratories LCR test in this study and in the earlier clinical trial (CT9) failed to initially detect one true positive in each. Both were subsequently found positive by repeat testing on stored aliquots. In addition, there were two further discrepant results, swab culture positive and FPU LCR negative, which were repeatably LCR negative. The two latter results were examples of sampling error where the female FPU had no detectable antigen present and reflected the low inclusion counts from the corresponding cervical swab cultures (<10 in each).

In the earlier study (CT9), where male patients attending a GUM Clinic were evaluated, there were no examples of low levels of antigen in the FPU samples and indeed the one false negative FPU LCR had a large amount of detectable antigen present as did the other false negative FPU LCR in the current study. Both of these

false negative FPU samples gave positive reactions in the other, less sensitive methods under evaluation, Clearview in the former and IDEIA *PCE* in the latter.

There is little doubt that there are inhibitors present in some urine samples that interfere with the amplification process of molecular methods, these have been previously described in Chapter 8 and many investigators are now attempting to understand what mechanisms cause this interference.

The second part of this study evaluated the use of the Clearview™ Chlamydia MF test as a detection method for *C.trachomatis* in low prevalence patients.

The sensitivity as compared to culture was 82.1% (9/11) and in the earlier clinical trials from high prevalence female patients (CT5 and CT8) was 91.2% (31/34) and 83.8% (31/37) respectively. There were no false positive Clearview reactions in the present study and only one in the whole of the 754 female patients included in the three clinical trials.

The Clearview test failed to give positive reactions in the same two patients that were also negative by FPU LCR, although EBS were subsequently detected by IF in one of the remaining swab extracts and was indicative of a lack of sensitivity rather than sampling error.

The sensitivity and specificity of the improved Clearview™ Chlamydia MF test in a low prevalence female group were marginally better than in the original Clearview™ Chlamydia test described in Chapter 7. The sensitivity as compared to culture has risen from 80% (24/30) to 82.1% (9/11) and specificity from 99% (318/321) to 100% (151/151).

Although Unipath Limited have improved the overall performance of the Clearview™ Chlamydia test the main objective was to extend its use for the diagnosis of *C.trachomatis* in male patients.

Preliminary investigations on utilising male urethral swabs gave poor results compared to FPU (unpublished data).

In the two clinical trials evaluating male FPU in comparison to conventional urethral swab culture (CT6 and CT7) the sensitivities were 82.0% (50/61) and 92.8% (13/14) and specificity's 96.9% (159/164) and 95.2% (60/63) respectively.

The new extraction procedure developed by Unipath Limited has reduced the interference caused by soluble components within the sample without any loss of detectable Chlamydia LPS. In this low prevalence study (CT11) and the other evaluation studies (CT5 to CT8) the average positive predictive value was 93.8% with two studies predicting 100% (CT8 and CT11). The high positive predictive value and specificity range 95.2% to 100% reduces the risk of false positive reactions which is essential if the test used within a clinical setting.

The performance of this immunoassay in a low prevalence general practice population is encouraging and provided that positive reactions are confirmed by the laboratory, may prove to be a useful addition to infection screening programmes for genital Chlamydia infections.

16. GOLD STANDARD COVERSLIP CULTURE METHOD 1 VS SIMPLIFIED CULTURE SCRAPE OFF METHOD 2

16.1. Introduction

In the clinical trials (CT1, CT2, CT3, CT4, CT5 and CT6) two types of culture system were inoculated in parallel with each other as described in Section 2.1. The patients included in this study were both male and female from low and high prevalence groups with and without symptoms.

16.2. Results

16.2.1. *Results of the Gold Standard Cell Culture Method 1 vs the Simplified Scrape Off Method 2*

From the 1552 patients included in this study, *C.trachomatis* was isolated from 17.4% (270/1552) by any method. A summary of the results for each group is detailed in Tables 29 to 37.

Table 29 - Culture Results CT2 Symptomatic Males

Scrape Off Method	+	-	+	-	-		
First Coverslip Culture	+	+	-	-	-		
Second Coverslip Culture	+	+	+	+	-		
	97	2	2	0	203	=	304

Table 30 - Culture Results CT1 Symptomatic and Asymptomatic Males

Scrape Off Method	+	-	+	-	-		
First Coverslip Culture	+	+	-	-	-		
Second Coverslip Culture	+	+	+	+	-		
	21	0	0	0	86	=	107

Table 31 - Culture Results CT6 Symptomatic Males

Scrape Off Method	+	-	+	-	-		
First Coverslip Culture	+	+	-	-	-		
Second Coverslip Culture	+	+	+	+	-		
	58	2	0	1	164	=	225

Table 32 - Culture Results CT3 Low Prevalence Females

Scrape Off Method	+	-	+	-	-		
First Coverslip Culture	+	+	-	-	-		
Second Coverslip Culture	+	+	+	+	-		
	28	1	1	0	321	=	351

Table 33 - Culture Results CT4 Symptomatic Females

Scrape Off Method	+	-	+	-	-		
First Coverslip Culture	+	+	-	-	-		
Second Coverslip Culture	+	+	+	+	-		
	22	0	0	1	222	=	245

Table 34 - Culture Results CT5 Symptomatic Females

Scrape Off Method	+	-	+	-	-		
First Coverslip Culture	+	+	-	-	-		
Second Coverslip Culture	+	+	+	+	-		
	32	2	0	0	286	=	320

Table 35 - Culture Results All Females

Scrape Off Method	+	-	+	-	-		
First Coverslip Culture	+	+	-	-	-		
Second Coverslip Culture	+	+	+	+	-		
	82	3	1	1	829	=	916

Table 36 - Culture Results All Males

Scrape Off Method	+	-	+	-	-		
First Coverslip Culture	+	+	-	-	-		
Second Coverslip Culture	+	+	+	+	-		
	176	4	2	1	453	=	636

Table 37 - Culture Results All Patients

Scrape Off Method	+	-	+	-	-		
First Coverslip Culture	+	+	-	-	-		
Second Coverslip Culture	+	+	+	+	-		
	258	7	3	2	1282	=	1552

Overall, the number of additional infections that were identified by the gold standard method was 9 (0.58%). There were no additional positives detected by the scrape off method, however, five of the gold standard isolates were only detected on the sub cultured second coverslip, and three of these were detected by the scrape off method. The sensitivity of the scrape off method as compared to two coverslip cultures was 96.7% (261/270) with specificity of 100% (1282/1282) and positive and negative predictive values of 100 (261/261) and 99.3% (1282/1291). If only one coverslip was used, as is more usual in routine cultures, then the sensitivity of the scrape off method rose to 97.3% (258/265) with specificity of 99.8% (1284/1287) and positive

and negative predictive values of 98.8% (258/261) and 99.4% (1284/1291) respectively. A summary of the detection rates by each method on male and female cell culture swabs is detailed in Table 38.

Table 38 - Summary of Detection rate of Gender and Method

	Females	Males	Combined Total
Scrape off Method	9.1% (83.916)	28.0% (178/636)	16.8% (261/1552)
One Coverslip Culture	9.3% (85/916)	28.3% (180/636)	17.1% (265/1552)
Two Coverslip Cultures	9.5% (87/916)	28.8% (183/636)	17.4% (270/1552)

16.3. Discussion

Many methods have been used to simplify and speed up the routine culture of *C.trachomatis,* including the use of 96 well microtitre plates and novel staining techniques, but most have been abandoned in favour of DIF, EIA or molecular techniques. However, the coverslip method of culture, with one blind passage and confirmation by immunofluurescence is still recognised as the reference method when evaluating new detection methods or undertaking clinical trials. The simplified culture scrape off method utilised in the clinical trials CT1, CT2, CT3, CT4, CT5 and CT6 is a rapid and simple technique that encompasses the excellence of the gold standard coverslip method in a cost effective way without compromising any of the accuracy.

The main advantages of the simplified method are the reduction of monoclonal antisera utilised, (5µl rather than 10 µl), and remaining unused cells can be salvaged for sub-culture whereas in the comparative coverslip method all the material is fixed.

Other advantages include the speed at which multiwell slides can be examined and the low cost of flat bottomed glass culture tubes as compared to coverslip cultures in the initial culture stage.

It could be argued that examining approximately 50% of the original cell population would, in theory, result in low numbers of positive inclusions (<2 per culture) being

overlooked but, in practice, the contrary is true. This can be explained by the smaller, more concentrated area that is examined and the high visibility of even poor inclusions caused by the expression of Chlamydia lipopolysaccharide following cell disruption, air drying and fixation in acetone. Furthermore, if the results are inconclusive, or retention of a positive culture is required, there will be remaining material available.

In this study 0.58% (9/1545) additional positives were isolated by the gold standard two coverslip methods. However, if only one coverslip culture had been used then this figure would fall to 0.45% (7/1547).

As the sensitivity of Chlamydia culture is superior to EIA in the region of 5 to 15% (Section 6.4.) then a reduction in the sensitivity of culture by less than 0.5% would seem an acceptable loss.

The two diagnostic laboratories associated with this study have adopted this simplified method (whilst retaining the conventional two coverslip method for scientific studies) without any noticeable loss of sensitivity. The "scrape off" technique offers a cheap, reliable and sensitive method for the confirmation of Chlamydial inclusions for those laboratories wishing to retain cultural systems.

17. CONCLUSIONS

The present reference standard for the detection of *Chlamydia trachomatis* from urogenital samples is by tissue culture. Although this is by no means 100% sensitive it is still the "benchmark" by which other methods are assessed. However, when utilising FPU samples as an antigen source, tissue culture is of little value and DIF, EIA or molecular techniques are the methods of choice. A difficulty arises when investigating FPU as an *alternative* to urogenital swabs as to which samples and methods are employed to compare results.

The use of FPU has become universally popular with both patients and clinicians as a non-invasive and therefore painless method of sample collection. In addition, FPU offers a non-biased specimen for comparative clinical trials without the problems of sampling error.

However, FPU for Chlamydia detection requires a significant amount of antigen and/or DNA to be present and although this is usually possible in male patients where the urethra is the prime seat of infection, this is not usually the case in female patients where the endocervix is the primary antigen source. In addition, when the urethra, in both male and females, is infected with *C.trachomatis* then it follows that the patient will have, to some degree, a urethritis with resultant inflammation, cellular damage and a discharge of leucocytes and epithelial cells.

In male patients the discharge from the urethra is removed prior to taking a urethral swab for Chlamydia testing, and yet when a urine sample is collected a *first pass* sample is recommended, taken at least one hour after last void to maximise antigen content and hence includes cellular exudate. This would also be true in female patients.

In the series of eleven clinical trials and the pre-trial investigations described in this study, an answer has been sought to some of the problems associated with the use of urogenital samples, especially FPU, for the diagnosis of genital *Chlamydia trachomatis* infections.

With the exception of two of the clinical trials, Chlamydia swab culture was used as the reference test against which the methods under evaluation were judged, and the simplified culture method was shown to be a practical alternative to the conventional method. This is particularly important as culture is still, for all its failings, superior to most non-amplified antigen detection methods.

On occasions there were discrepant results which were either resolved by repeat or alternative testing or shown to be sampling error. Where two test methods were evaluated using one FPU specimen then sampling error could be ignored and a more accurate outcome described.

A recurring observation throughout this study was that some samples, be they FPU or swabs tested by culture or by other methods, had large amounts of detectable antigen by one method which was not detectable by another. This was particularly highlighted in the DNA amplification tests where a high degree of sensitivity was expected and yet, on several occasions, repeat testing was required to resolve primary false negative results.

This poses the question that if resources allowed repeat testing of all of the negative PCR or LCR samples, would more positives have been detected? As Hadgu (1996) pointed out in his review article on the resolution of discrepant results utilised by Abbott Laboratories in the validation of the Chlamydia LCR test; "82 of 84 samples initially interpreted as false positives were eventually declared as true positives by discrepant analysis". Thus bias towards the new test invariably favours that method and must be borne in mind when interpreting what is now called the expanded gold standard.

Since this study commenced, and, as discussed in Chapter 8, it has been recognised that there are naturally occurring substances found in clinical specimens that are also inhibitory to molecular methods. That this phenomenon should occur in this study in DNA amplification tests when compared to non-amplified methods showing large amounts of antigen, and consequently excessive cellular exudate, may be a coincidence, but it is worthy of further investigation in the future.

The problem of inhibitors or sample interference was explored in the two pre-clinical trial investigations and the presence of leucocytes, whether or not the cause or a marker in FPU, was indicative that some inhibition would be caused, to a greater or lesser extent, in all of the EIA methods evaluated. Of particular importance is the efficiency of the sample extraction procedure to release the target antigen and reduce the sample interference without affecting the avidity and strength of the resultant signal. A new extraction process was described for male FPU and female endocervical swabs tested by the Clearview™ Chlamydia MF test that has now been released for commercial use. Although this sample test does not suit the large batch testing common in UK laboratories it is popular in smaller clinic settings often found in other European countries and North America.

The three EIA clinical trials to validate the use of FPU in two of them and the revalidation of urethral swabs in the improved version of the third, were all successful. Although the first evaluation, described in Chapter 5, required two volumes of FPU to obtain a valid result, following this research the company have recently overcome the problem with a new extraction process. The other two EIA tests are both available for diagnostic use.

A recent report by the Government's Chief Medical Officer's Expert Advisory Group *(Chlamydia trachomatis 1998)* has advised that action is required to reduce the prevalence and morbidity associated with Chlamydial infection. The group concluded that there would be significant benefit to the health of the nation and particularly to women under the age of 25 years if a national screening programme is introduced.

Chlamydia trachomatis is the most common, treatable, STD in the UK today. There were 32,000 new cases reported in 1995 with up to 70% of women and 50% of men being asymptomatic. With such a high percentage of patients being asymptomatic it follows that there is an even larger pool of underlying infection and a screening programme will have to be opportunistic.

In my opinion we should separate those patients or contacts with signs or symptoms of infection from those included in a screening programme. Antigen detection tests appropriate for one group of patients may not necessarily be appropriate for the other.

Utilising current technology I think that urethral swabs from males and endocervical swabs from females, tested by the best of the Chlamydia EIA tests, would have a level of sensitivity and specificity sufficient to detect all but a few of these suffering with infection. Furthermore, symptomatic patients and contacts would also undergo a routine medical examination which would facilitate the taking of swab samples. In contrast, it may be difficult to persuade the mass population to have swabs taken when there is little or no evidence of genital infection. Hence, a more appropriate sample in this group may be urine which is then tested by DNA amplification methods.

Further experimental work utilising radical and novel approaches to this problem may find an answer. It is my understanding that there are groups of investigators re-examining the use of HVS or self taken vulval swabs for both EIA and DNA techniques and yet other workers are evaluating the possibility of pooling FPU with self taken swabs.

Whatever the outcome of these studies, it needs to be remembered that the Scandinavian countries have significantly reduced the incidence of Chlamydia infection and its sequealae by using conventional immunoassay techniques and that a comprehensive cost benefit analysis of the use of FPU and molecular methods will be the first priority of the UK scheme.

Having said that, and assuming that the government will provide the necessary funding, if the opportunistic screening programme is to commence in the near future then the use of FPU as the sample and amplified DNA as the test, is the only option.

It is comforting to know that in 1988 at the First European Society for Chlamydia Research in Bologna I presented one of only two submitted papers on the use of FPU for Chlamydia diagnosis (Matthews et al. 1988). If I was about to commence this period of study in 1998 my title would be *"Chlamydia trachomatis* genital infections,

what sample, which test?" With the work undertaken in this study there is a good indication of both answers.

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