# ASPECTS OF HUMAN CHLAMYDIAL INFECTIONS

Aspekten van humane chlamydia-infekties

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CHAPTER 1

# INTRODUCTION

Urethritis in the male accompanied by discharge and dysuria has long been related to promiscuity. After Neisser's discovery of the gonococcus and the introduction of the Gram stain in 1880 differentiation between gonococcal and non-gonococcal urethritis (NGU) became possible. At the same time ophtalmia neonatorum -then a common affection- could be divided into a gonococcal and a non-gonococcal form. Clues for an aetiologic agent of these sexually transmitted non-gonococcal infections were found three decades later.
In 1907 Halberstaedter and Von Prowazek observed typical inclusions in conjunctival smears from monkeys infected with trachomatous material (16). The eye disease trachoma, which finally leads to cicatrisation of the cornea, had long been known. Similar structures were observed in conjunctival smears from neonates with non-gonococcal ophtalmia neonatorum. A few years later inclusions were observed in cervical and urethral cells from the parents of a child with ophtalmia neonatorum. Moreover, the typical inclusions were seen in urethral smears from men suffering from non-gonococcal urethritis (NGU). Experimental infections of simian conjunctivae with material obtained from neonates with ophtalmia, with cervical smears from their mothers and with discharge from patients with NGU revealed the relationship between ocular and genital infections on the one hand, and on the other hand that they were sexually transmissible. Moreover, the aetiological agent was identified. Further research into this micro-organism, Chlamydia trachomatis, was seriously impeded by the lack of a suitable culture technique. It was not until 1957 that T'ang succeeded in culturing Chlamydia on the yolk-sacs of incubated hen's eggs. This lead to renewed epidemiological research, the results of which were not essentially different from those obtained at the begin of the century. The introduction of Chlamydia cultures on continuous cell lines by Gordon and Quan (13) was a great advance in epidemiological and biological Chlamydia-research.
The genus Chlamydia is taxonomically assigned to a separate family, the Chlamydiaceae, order Chlamydiales. The genus includes two species: Chlamydia psittaci and Chlamydia trachomatis. So far, 15 serovars of C. trachomatis have been identified: A, B, Ba, C, D through K, L1, L2 and L3. Since Chlamydia

are micro-organisms growing only intracellularly, they were initially classified among the viruses. However, Chlamydia contain ribosomes, multiply by binary fission and are sensitive to antibiotics such as tetracycline, erythromycin and rifampicin. They are therefore bacteria, which require a eukaryotic cell for growth. Moulder established that they were above all "energy parasites", dependent on the host cell for ATP, among other things (29). The organism can exist in two forms. One measures 0.3 $\mu$m and is metabolically inactive but infectious. This form, known as Elementary Body (EB), invades the eukaryote by an as yet unexplained mechanism described as "parasite-induced phagocytosis" (5). The phagosome that contains the EB does not fuse with the host lysosomes (10, 12). Within the phagosome the EB transforms into the larger (1 $\mu$m), vegetative but non-infectious form known as the Reticulate Body (RB). After a large number of fissions the phagosome, containing numerous EBs and RBs, is microscopically visible as an inclusion vacuole or inclusion. After the transformation of the RB into the EB rupture of the host cell occurs and the infectious particles are released. In this way the infection becomes manifest. The entire cycle takes about 48 hours.

It is now certain that Chlamydia trachomatis is the aetiologic agent of trachoma, some of the sexually transmitted non-gonococcal oculogenital infections, and Nicholas-Favre's lymphogranuloma venereum. The three groups of diseases are associated with different serovars of Chlamydia: A, B, Ba and C are found in trachoma, D through K in the oculogenital infections, and L1 through L3 in lymphogranuloma venereum. Many epidemiological studies have reported the presence of Chlamydia trachomatis in oculogenital infections and related diseases: non-gonococcal urethritis (8, 31, 35, 39, 41), acute epidydimitis (3, 8), and Reiter's disease (19, 25) in males, and cervicitis (8, 31, 35, 41, 42), urethral syndrome (4, 38), bartholinitis (9), peri-hepatitis (8, 30, 32, 45) and salpingitis or pelvic inflammatory disease (PID) in females (8, 11, 18, 21-24, 40), proctitis (8, 31, 33), paratrachoma and neonatal conjunctivitis or pneumonitis (2, 8, 31, 35, 37, 41). The infectiousness of Chlamydia was demonstrated in contact-tracing studies (42, 43) and in a prospective study of Chlamydia-positive pregnant women with regard to neonatal infections in their children (1, 17, 36). Serological studies pointed out the role of Chlamydia in the pathogenesis of tubal infertility (6, 8, 27). Chlamydia and gonococcal infections are often found to be entirely analogous as to clinical symptoms and sequelae, and in most cases this is obvious.

However, apart from numerous publications indicating a high prevalence of Chlamydia in various oculogenital infections there are reports raising doubts

about the pathogenetic significance of the presence of Chlamydia in the urogenital tract. For example: strikingly high rates (25%) of asymptomatic chlamydial infections in males (39). The incubation period of a chlamydial infection is uncertain but for convenience held to be ten days or more. However, the organism has been found in NGU patients without known recent sexual contacts. Several contact-tracing studies have revealed large percentages of asymptomatic patients (42, 43). A study by McCormack showed that Chlamydia may remain in the cervix for months or perhaps even years without giving rise to symptoms (24). Although Swedish and French studies strongly indicate the relationship between salpingitis and Chlamydia (18, 22-24), the organism is rarely found in the salpinx in the USA (11, 40). Yet various screening studies in Europe and in the USA reveal similar chlamydia-prevalences in diverse populations. The differences found in salpingitis are explained by differences in sampling and culturing techniques, but proof for this assumption has so far been lacking. As regards the pathogenicity of Chlamydia it is the large percentage of subclinical infections that raise more questions than are answered, particularly since on the other hand serious conditions such as salpingitis and subsequent tubal infertility are being related to Chlamydia. Richmond (34) suggested that so-called latent chlamydial infections may be activated by several external stimuli, e.g. the presence of other micro-organisms. The prevalence of Chlamydia is higher in longstanding cases of NGU than in recent cases. Latent chlamydial infections -i.e. presence without growth- have already been demonstrated and studied in tissue cultures (28). Darougar (7) demonstrated in an animal model that combined chlamydial and streptococcal infections took a more severe course than infections caused by the separate micro-organisms. Unfortunately, no analogous experiments with regard to genital infections have so far been performed.
The mechanism by which the organism damages the host is likewise very uncertain. Mårdh (24) already noted that higher antichlamydial antibody titres reduced the chance of actually isolating the organism. Moreover, higher antibody titres were associated with more serious clinical symptoms, e.g. in the case of salpingitis (44). Although only one case report on Reiter's disease mentions isolation of Chlamydia from the synovial fluid, the immunological respons of the host to a (recurrent) chlamydial infection seems a more likely cause of the synovial damage (25). Grayston and Wang (14, 15) also hold that chronic trachoma characterized by pannus formation and cicatrisation is a result of the immunological response to chlamydial reinfection. It seems as if Chlamydia themselves can cause mild but clinically evident infections in some

cases. For serious damage to the host or complicating infections, however, an additional factor is believed to be required. At present one can only speculate whether an additional microbial infection or a unique immunological response of the host to chlamydial antigen is involved in the pathogenesis of the more serious forms of infection.

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# CHAPTER 2

## PROBLEM DEFINITION

This thesis takes a closer look at three aspects of human chlamydial infections. With regard to diagnosis the influence of logistics on the sensitivity of the culture method is discussed, along with optimalization of the culture itself and an evaluation of new diagnostic methods. Next, epidemiological data are discussed with regard to the prevalence and role of *Chlamydia*, on the one hand in asymptomatic persons from low-risk groups and on the other hand in women with postinflammatory tubal infertility. Finally the therapeutic problems are considered with reference to measurements of the in-vitro sensitivity of various chlamydial strains to tetracycline and some recently developed chemotherapeutic agents.
The significance of *Chlamydia* for some of the sexually transmitted non-gonococcal oculogenital infections was not generally recognised until relatively recently. One of the reasons for this was undoubtedly the lack of a culture method to isolate the organism for study of its biological characteristics. Even after the introduction of a technique to culture *Chlamydia* on cell lines, large scale epidemiological research was impeded by lack of adequately equipped laboratories where the complicated method might be employed. The manner of collecting patient material for culture, transport from outpatient clinic to laboratory, and the culture method employed are critical parameters which determine the ultimate result. A disadvantage of the culture method is the relatively long time required (6 days). Rapid diagnosis and if necessary treatment, especially of asymptomatic persons, is of great epidemiological importance. Chlamydial antigen-detecting methods have recently been developed which can present results within a few hours. They are based on the immunofluorescence (IF) technique and on the enzyme immunoassay (EIA). Both techniques employ monoclonal antibodies against genus-specific epitopes of the Major Outer Membrane Protein (IF technique) or of the lipopolysaccharide (EIA). The chlamydiae in patient material need no longer be alive for analysis, and transport to the laboratory therefore no longer influences the sensitivity of detection.
Regarding diagnosis, chapters 3 through 5 attempt to answer the following questions.

1) How does transportation of the patient material affect the sensitivity of the culture,
2) How can the traditional culture method using vials, subpassage and Giemsa stain be optimalized, and
3) What is the value of chlamydial antigen-detecting techniques.
Chapters 6 and 7 present studies of the prevalence of chlamydial infections in populations who in symptomatological terms are at the extremes of the pathogenetic spectrum. The experience obtained with the rapid, sensitive microtitre culture method was used to investigate the presence of *Chlamydia* in semen samples destined for artificial insemination by donor (AI). Screening of the donors by AI institutes is usually based exclusively on the donor's history and assessment of the fertilising potency of the semen. The donors are selected from so-called low-risk groups and are asked before they give semen whether they have symptoms of a urogenital infection, e.g. dysuria or discharge. The semen samples were tested not only for *Chlamydia* but also for other microorganisms associated with sexually transmitted diseases (STD).
In addition to this study of the prevalence of chlamydial infections in asymptomatic males who on the basis of anamnestic data are not considered to be at risk, the role of *Chlamydia* was studied in relation to the most serious complication that may be caused by an ascent of the infection from the lower genital tract in females: postinflammatory tubal infertility. For this purpose infertile women were screened for serological evidence of a history of chlamydial infection. In most of these women tubal pathology could be established, suggesting an inflammatory process in the past. Especially Swedish investigators are convinced of a strong association between salpingitis and *Chlamydia*. A prospective study by Weström has shown that the risk of tubal infertility following salpingitis is 11%. This risk increases to more than 60% in women with a history of three or more episodes of salpingitis (2). To investigate the role of *Chlamydia* in the pathogenesis of tubal infertility, a prospective study would be required of the course of events following cervical contamination of women with *Chlamydia*. Since this is neither ethical nor feasible, a transverse study of women with tubal infertility was started. They were serologically tested for the presence of antichlamydial antibodies with the aid of the micro-immunofluorescence test developed by Wang (1). Although the presence of antichlamydial antibodies is not conclusive of a chlamydial aetiology of tubal lesions, such a finding can support the postulate of the role of *Chlamydia* in tubal infertility. Because gonococci and *Mycoplasma hominis* may play a role in salpingitis beside

Chlamydia, antibodies against these bacteria were likewise searched for. So far, tetracycline and erythromycin have proved effective against chlamydial infections. As with gonococci, however, the possible development of resistant strains will have to be taken into account. It is therefore necessary to develop methods by which the Minimal Inhibitory and Bactericidal Concentration (MIC and MBC) of isolated strains for these and newer antibiotics can be determined simply and effectively. To answer the question whether the resistance problem reported for gonococci also arises with regard to Chlamydia, the MIC and MBC values of 22 chlamydial strains for tetracycline and some recently developed chemotherapeutics such as temocillin and the 4-fluoroquinolones were determined. A rapid microtitre method was used for 12 strains.

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CHAPTER 3

# SURVIVAL OF CHLAMYDIA TRACHOMATIS IN DIFFERENT TRANSPORT MEDIA AND AT DIFFERENT TEMPERATURES: DIAGNOSTIC IMPLICATIONS*

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We compared the survival of a laboratory strain of Chlamydia trachomatis serovar L-2 in different media and at different temperatures (room temperature, 4°C, and -70°C). At these temperatures the best storage medium was 2SP (0.2 mol/l sucrose in 0.02 mol/l phosphate buffer supplemented with 10% fetal calf serum). We used material obtained from patients to study the sensitivity of the culture method as a function of sample storage time and temperature. Compared with results on direct inoculation, material stored in 2SP for 48 hours gave 11% fewer positive cultures at 4°C and 14% fewer at room temperature. Of samples which gave negative results on direct inoculation, 4% were positive after storage at 4°C for 48 hours and 2% after storage at -70°C for a week. As expected, the number of inclusion forming units in the original material proved to be important for the percentage of positive cultures among the stored samples.

## INTRODUCTION

Culture of the organism from patient material on a suitable cell line is still the most important method of diagnosing chlamydial infection. Many studies have aimed at improvement of the culture method. The sampling of material (1, 2) and the cell line (3, 4) have all been subject to investigation. Few data are available, however, on the survival of C. trachomatis in the most widely used transport medium, 2SP (0.2 mol/l sucrose in 0.02 mol/l phosphate buffer), and the diagnostic implications of survival rates. For such studies it is important to use patient material that, unlike clean laboratory strains, is rich in many substances and cells (such as leucocytes, antichlamydial anti-

*Br J Vener Dis 1984;60:92-4.

bodies, bacteria, and sperm constituents) which can influence the growth of chlamydiae on the monolayer. Reeve found a 17% decrease in positive cultures after storage of the material at -70°C for one week (5), whereas Ngeow observed no loss after freezing the material in liquid nitrogen (6). We studied a) the survival of a laboratory strain of C. trachomatis, serovar L-2, in three different media and b) how storage of patient material under different conditions influenced the sensitivity of the culture method.

## MATERIALS AND METHODS

### Culture procedure

A 100 μl sample of material to be tested for the presence of chlamydiae was inoculated on an HeLa 229 monolayer formed on a round coverslip in a flat bottomed tube. Except when the inoculum was the laboratory strain L-2, the monolayer was washed twice with 30 μg/ml diethylaminoethanol (DEAE) dextran in Hank's balanced salt solution before inoculation. After centrifuging the inoculated monolayers at 3700 g for one hour we added 1 ml growth medium which consisted of Eagle's modified minimal essential medium which contained 5% fetal calf serum, 4.5 mg/ml glucose, 25 U/ml nystatin, 25 μg/ml vancomycin and 25 μg/ml streptomycin (called CMGA). After three days the monolayers were stained by Giemsa's method and evaluated with dark field illumination at 250 x magnification. The number of inclusions per 30 microscopic fields or per coverslip was counted, and counts were made in triplicate.

### Survival of C. trachomatis serovar L-2 in three media at three temperatures

A 100 μl sample of chlamydia suspension in SPG (75 g sucrose, 0.52 g potassium phosphate, 1.22 g disodium acid phosphate, and 0.72 g L-glutamine, dissolved in 1 l water) was added to 0.9 ml of the test medium. The three media used were Earle's balanced salt solution, CMGA, and 2SP, and all contained 10% fetal calf serum. Portions of the medium containing chlamydiae were incubated at 4°C and at room temperature and frozen at -70°C. Samples were obtained at intervals and the number of inclusion forming units (ifu) in them was measured.

### Processing of patient material

An aluminium swabstick (ENT swab, Boehringer, Mannheim, FRG) was used to obtain a urethral sample from men attending the outpatient clinic for sexual-

ly transmitted diseases (STD) of the Rotterdam University Hospital. The material was suspended in 2SP and divided into three portions; one was stored at 4°C, the second at -70°C, and of the third portion a fraction was inoculated immediately while another fraction was stored at room temperature. After 24 and 48 hours the samples stored at 4°C and at room temperature were inoculated in triplicate. After one week the samples stored at -70°C were thawed and inoculated. The 2SP medium also contained 25 U/ml nystatin, 20 $\mu$g/ml vancomycin, and 25 $\mu$g/ml streptomycin.

Table I Comparison of survival of C. trachomatis serovar L-2 after suspension in three media at three temperatures for varying times

| Storage temperature and time | Mean No (%) inclusions per 30 microscope fields (x 250 magnification) of three coverslips produced by $10^{-3}$ dilution of the suspensions on: | | |
|---|---|---|---|
| | CMGA | EBS | 2SP |
| Room temperature | | | |
| 4 hours | 263 (100) | 358 (100) | 396 (100) |
| 8 hours | 213 (81) | 344 (96) | 293 (74) |
| 25 hours | 33 (13) | 41 (11) | 179 (45) |
| 50 hours | 3 ($<1$) | 3 ($<1$) | 22 (6) |
| 4°C | | | |
| 4 hours | 396 (100) | 446 (100) | 571 (100) |
| 50 hours | 10 (3) | 15 (3) | 30 ($>5$)* |
| 80 hours | 8 (2) | 15 (3) | 30 ($>5$)* |
| 120 hours | 1 (1) | 1 (1) | 30 ($>5$)* |
| -70°C | | | |
| 2 days | 223 (100) | 205 (100) | 284 (100) |
| 6 days | 16 (7) | 16 (8) | 57 (20)# |
| 1 week | 12 (5) | 13 (6) | 50 (18)# |
| 2 weeks | 26 (12) | 22 (11) | 62 (22)# |

CMGA = Eagle's modified minimal essential medium; EBS = Earle's balanced salt solution; 2SP = 0.2 mol/l sucrose in 0.02 mol/l phosphate buffer.

*At 50, 80, and 120 hours $>100$ inclusion were counted in a $10^{-1}$ dilution on 2SP.

#2SP proved to be significantly ($p<0.05$) (Student's t test) superior to the other media.

RESULTS

Our comparison of survival in different culture media showed that chlamydiae survived best in 2SP at all temperatures studied. Table I lists the number of inclusions obtained by inoculating a $10^{-3}$ dilution of the suspensions in phosphate buffered saline (PBS). Undiluted 2SP suspensions produced 158 and 96 inclusions after storage for 150 and 174 hours respectively at room temperature. Clinical conditions were probably best mimicked by diluted suspensions. In general these showed substantial reduction of inclusions after storage for 25 hours at room temperature, 50 hours at 4°C, and six days at -70°C.

Table II Number of positive cultures obtained from patient material after immediate inoculation compared with results after storage

| | No of positive cultures after: | |
|---|---|---|
| Storage temperature and time | Immediate inoculation | Storage (%) |
| Room temperature | | |
| 24 hours | 46 | 39 (85) |
| 48 hours | 44* | 38 (86) |
| 4°C | | |
| 24 hours | 46 | 41 (89) |
| 48 hours | 45* | 40 (89) |
| -70°C | | |
| 1 week | 44* | 39 (89) |

*Some samples were not tested under all storage conditions.

Table II shows the results of our investigation into the effect of storing material from patients on chlamydial survival. Storing reduced the number of positive cultures by 11-15% depending on the storage temperature. Storing patient material also unmistakably reduced the number of inclusions per coverslip. Table III shows that this reduction depended on storage times and temperatures and amounted to 40-70% of the number of inclusions formed after inoculation of the material immediately after sampling. Eleven positive specimens became negative under one or several storage conditions and averaged seven inclusions per coverslip, while 27 remained positive under all storage conditions and averaged 98 inclusions per coverslip. Table IV shows that in a group of patient samples which gave negative results after immediate inocula-

tion, 4% (7 of 178) gave positive results after storage for 48 hours at 4°C, and 2% (4 of 162) after 1 week at -70°C.

Table III Effect on number of inclusions by storing patient material at three temperatures for different times

| Storage temperature | Percentage of inclusions on immediate inoculation after storage for: | | |
|---|---|---|---|
| | 24 hrs | 48 hrs | 1 week |
| Room temperature | 50 | 30 | |
| 4°C | 60 | 60 | |
| -70°C | | | 55 |

Significant (Student's t test; $p < 0.05$) difference between number of inclusions after 48 hours at room temperature and one week at -70°C, and between 48 hours at 4°C and 48 hours at room temperature.

Table IV Number of cultures of patient material which were negative after immediate inoculation and positive after postponed inoculation

| No of negative cultures after immediate inoculation | No (%) of positive cultures after: | |
|---|---|---|
| | 48 hrs at 4°C | 1 week at -70°C |
| 178 | 7 (4) | |
| 162* | | 4 (2) |

*No material was stored at -70°C from 16 patients.

## DISCUSSION

The experiments with our laboratory strain L-2 show that 2SP is a better storage and transport medium than Earle's balanced salt solution and CMGA. We do not know why survival is better in 2SP, but the fact that sucrose is cryoprotective may play a part (7).
As might be expected, the sensitivity of the culture diminishes when the patient material is not immediately inoculated on the monolayer. The number of inclusions decreases noticeably, but the influence of this decrease on the culture is of course limited. After all the demonstration of only one inclu-

sion is enough to give a positive result. A correlation exists, however, between the number of inclusion forming units in the initial material and the reversion of culture results after storage, with fewer inclusions increasing the risk of reversion. Apart from the difficulty of finding a single inclusion in a specimen, the degeneration of chlamydiae constitutes a real problem in this context.

Immediate freezing, the procedure routinely followed for patient material, produces a loss of 11% inclusions. When the unfrozen material is in transit for one or two days this loss increases to 25%. The loss is compensated for only partly by positive results in material found to be negative after immediate inoculation. A possible explanation for this finding lies in the intracellular localisation of chlamydiae. Freezing or storage can cause the eukaryotic host cells to break open. Brief sonication of the patient material immediately before inoculation would therefore seem to be rational. The disintegration of toxic material during storage might also play a part (6).

Facilities for culturing chlamydiae are restricted to a few centres. Proper processing of the materials can therefore encounter logistical problems which unfavourably affect the sensitivity of the culture method. Ridgway inoculated patient material immediately and after transport to a central reference laboratory, which entailed loss of 22.6% (8).

This study stresses the necessity to process patient material as quickly as possible. One has to deal with an unacceptable loss in sensitivity by transporting material for chlamydial culture, unless there are laboratory facilities to inoculate specimens directly.

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CHAPTER 4

# EVALUATION OF THE DIRECT FLUORESCENT ANTIBODY TEST FOR DIAGNOSIS OF CHLAMYDIAL INFECTIONS*

K.H. Tjiam, R.V.W. van Eijk, B.Y.M. van Heijst, G.J. Tideman, Th. van Joost, E. Stolz, M.F. Michel

The direct fluorescent antibody test and two culture methods were compared for accurate diagnosis of chlamydial infections. Using the same samples, 109 were found to be positive in the microtitre method with the direct confirmation test without subpassage, whereas 66 were positive in the vial method with Giemsa staining and subpassage. The direct test was evaluated for accuracy using cervical and male urethral specimens. Specimens for culture were obtained prior to sampling for the direct test. For cervical samples the sensitivity of the direct test, with the vial method taken as reference, appeared to be 72.2% with a specificity of 93.5%. With the microtitre method as standard, these values were 55.9% and 91.3%, respectively for females, and for male patients 49% and 95.6%, respectively. For cervical samples, in which sampling for the direct test was carried out prior to sampling for culture, these values were 46.3% and 93.2% respectively. Both culture method and study population influenced the sensitivity of the direct test. According to our findings, the direct test cannot replace the culture method for diagnosis of chlamydial infections.

## INTRODUCTION

*Chlamydia trachomatis* is an obligate, intracellular microorganism, which has been increasingly acknowledged to cause sexually transmitted non-gonococcal genital infections. An accurate means of diagnosing chlamydial infections is mandatory in view of the complications possible in infected women. Culture testing is considered the most reliable method for detection of *Chlamydia*, although the required transportation of specimens and culturing facilities pose problems. Recently it has become possible to detect chlamydial antigens

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in specimens with either an enzyme immunoassay (1) or immunofluorescence techniques (2, 3).
The aim of this study was to evaluate the direct fluorescent antibody test (DT) by comparing its accuracy with the major culture methods: the vial method with Giemsa stain and the microtitre method with immunofluorescence staining for detecting inclusions. We also determine the value of simple laboratory techniques such as Gram stain and urine sediment for predicting culture results, which could potentially be used to screen high-risk populations.

## MATERIALS AND METHODS

### Study Population

The study population consisted of unselected male and female patients who attended the venereal disease outpatient clinic of the University Hospital Rotterdam, The Netherlands. The males formed three groups: urethritis patients (n= 95) with more than ten leucocytes in the resuspended urine sediment of 20-40 ml of first-voided urine (250x); patients (n= 35) complaining of dysuria and/or discharge but showing no abnormalities in the urine sediment; and asymptomatic males (n= 92) with neither complaints nor sediment abnormalities. The women (n= 416) were assigned a semiquantitative score of 0-4 based on the number of leucocytes in the cervical Gram stain (800x): 0 = no leucocytes, 1 = 1-5/field, 2 = 6-10/field, 3 = 11-20/field, 4 = $>$20/field.
Male and female patients with gonorrhoea were excluded from the study.

### Sampling Procedure

In the males with discharge, specimens were first obtained for the Gram stain. Next, a metal cotton-tipped swab was moistened in transport medium and then inserted 4-5 cm into the urethra and rotated gently before withdrawal. After this specimen was suspended in the vial containing transport medium, the swab was inserted again, rolled over a microscopic slide for the direct test, and placed in the transport vial. Finally, 20-40 ml of first-voided urine was collected for the two-glass test, after material was sampled for gonococcal culture. In the females, specimens from both urethra and cervix were obtained for Gram stain and gonococcal cultures. The sampling procedure described above was used for chlamydial detection. In a second part of our study, only material from the cervix was sampled with a dry cotton-tipped swab. The order of obtaining specimens was also reversed, specimens for the direct test being

taken first. In the latter group chlamydial cultures were carried out by the microtitre method.

Transport Medium

The transport medium (2SP) consisted of 1.5 ml of 0.2 M sucrose in 0.02 M phosphate buffer, supplemented with 5% foetal calf serum (FCS), 25 μg/ml gentamicin and 2.5 μg/ml amphotericin B. Within 6 h of suspending the sample in the medium, the tubes were stored at -70°C.

Culture Method

For the vial culture method 200 μl specimen were inoculated in duplicate on a HeLa 229 monolayer, grown on a 12-mm round glass coverslip in a flat-bottom tube. Prior to inoculation the monolayer was rinsed with 30 μg/ml DEAE-dextran in Hank's balanced salt solution. After centrifugation of the vials at 3000 g for 1 h, the inoculum was replaced by growth medium consisting of Eagle's Minimal Essential Medium, modified with 4.5 mM glucose, 5% FCS, 25 μg/ml gentamicin, 25 μg/ml vancomycin, and 2.5 μg/ml amphotericin B. After incubation for 72 h at 36°C the monolayer was fixed, stained with Giemsa and screened for the presence of inclusions by darkfield illumination (250x). The duplicate vial was sonicated for 10 s prior to inoculation on a fresh monolayer for subpassage.

A modification of the method described elsewhere (4) was used in the microtitre plates. Specimens were inoculated in two wells (200 μl/well) of a 96-well microtitre plate (Falcon No. 3070) on a HeLa 229 monolayer pretreated with DEAE dextran. After centrifugation at 3000 g for 1 h, the inoculum was replaced by 100μl growth medium, and the plate incubated at 36°C in a $CO_2$ incubator for 48 h. Next the monolayer was fixed and subsequently stained with fluorescein-conjugated monoclonal antibodies (MicroTrak Culture Confirmation Test, Syva Inc. Munster IN). The plates were examined inverted under a Leitz fluorescence microscope, using a lens with long focal length (320x). Duplicate microtitre plates were used for subpassage. The monolayer was disrupted by a freeze-thaw procedure.

Direct Test

Patient specimen was rolled over a circular area (8 mm diameter) etched on the slide. The smear was dried, fixed for 1 min in acetone and stored at -20°C until staining. Staining was done with the MicroTrak Direct Specimen Test (Syva Inc.). Slides were scored positive if ten or more chlamydial particles

were observed, according to the manufacturer's recommendations. Only slides with epithelial cells present were considered. The slides and cultures were examined without prior knowledge of the result of the other test.

### Statistical Method

Fisher's exact test was used to determine the level of significance.

## RESULTS

When the vial method was tested without subpassage, 50 of 1116 male urethral and cervical samples were positive; with subpassage 80 were positive, signifying an increase of 60%. Subpassage proved less important for the microtitre method. Of 926 male urethral and cervical specimens cultured with the microtitre method 110 were positive without subpassage and 123 with, an increase of only 11.8%. A comparison of the accuracy of the vial method with subpassage (V) and the microtitre method without subpassage (MT) using 932 male urethral and cervical samples (Table 1) showed more positive cultures with MT (109) than with V (66).

Table 1 Comparison of the microtitre (MT) and the vial method (V) for detection of Chlamydia from the cervix and male urethra in 932 specimens.

| | | No. of test reactions | | | |
|---|---|---|---|---|---|
| | V | + | + | - | - |
| Specimen origin | MT | + | - | + | - |
| Male urethra | | 23 | 7 | 27 | 262 |
| Cervix | | 26 | 10 | 33 | 544 |
| Total | | 49 | 17 | 60 | 806 |

The results of the direct test were then compared with the MT and V methods (Table 2). Thirty-four of 301 (11.3%) cervical, and 25 of 296 (8.4%) female urethral specimens, and 49 of 252 (19.4%) male urethral specimens were culture positive with the MT method. With the V method the respective percentages were 7.3, 2.7, and 9.5, and with the direct test they were 11.3, 7.4, and 13.1, respectively.

Table 2 Comparison of the results of the microtitre method, direct test and vial methods.

| | Pattern of reaction | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H |
| Microtitre method | + | + | + | – | + | – | – | – |
| Vialmethod | + | + | – | + | – | + | – | – |
| Direct Test | + | – | + | + | – | – | + | – |

| | No. of patients with reaction pattern | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H |
| Cervix leucocyte score 3-4 | 8 | 3 | 2 | 1 | 2 | 1 | 5 | 66 |
| Cervix leucocyte score 0-2 | 4 | 2 | 1 | 1 | 8 | – | 5 | 152 |
| Cervix total[a] | 13 | 5 | 6 | 3 | 10 | 1 | 12 | 251 |
| Female urethra | 2 | 3 | 7 | 1 | 13 | 2 | 12 | 256 |
| Males with urethritis | 8 | 4 | 6 | 3 | 8 | – | 3 | 63 |
| Males without urethritis | 3 | 1 | 3 | – | 8 | – | 3 | 109 |
| Male urethra total[a] | 15 | 6 | 9 | 3 | 19 | – | 6 | 194 |

[a]Leucocyte scores and sediment abnormalities were not determined in all patients.

Table 3 Sensitivity and specificity of the direct test (DT) using either the microtitre (MT) or vial method (V) as standards. Values in percent.

| | Sensitivity with reference to | | Specificity with reference to | |
|---|---|---|---|---|
| Patient subgroups | MT | V | MT | V |
| Cervix leucocyte score 3-4 | 66.7 | 71.4 | 91.8 | 90.7 |
| Cervic leucocyte score 0-2 | 33.3 | 71.4 | 95.6 | 95.8 |
| Cervic total[a] | 55.9 | 72.7 | 94.4 | 93.5 |
| Female urethra | 36 | 37.5 | 95.2 | 93.4 |
| Males with urethritis | 53.8 | 73.3 | 91.3 | 88.7 |
| Males without urethritis | 40 | 75 | 97.3 | 95.1 |
| Male urethra total[a] | 49 | 75 | 95.6 | 93.4 |

[a]Leucocyte scores and sediment abnormalities were not determined in all patients.

The MT method was positive for 14 of 48 (29.2%) chlamydia-infected women solely on the basis of urethral samples. The sensitivities and specificities of DT using either culture method as standard were also analysed by subgroups (Table 3). The overall sensitivity of the DT with the MT as standard in cervical and male urethral specimens was 55.9% and 49% respectively. Higher values, 72.7% and 75% respectively, were calculated if V was taken as standard. No difference was observed in sensitivities of the DT using either standard with female urethral specimens. The sensitivity of DT compared to that of MT appeared to be dependent on the subpopulation under consideration: In the presence of a high leucocyte score of 3-4 a higher sensitivity (66.7%) was found than in the subgroup with low leucocyte scores of 0-2 (33.3%). In male patients, higher sensitivites were also detected in those with urethritis. The order of obtaining specimens was reversed in a subsequent study in which only cervical specimens were taken into account. DT specimens were sampled prior to culture; 41 of 188 (21.8%) were chlamydia-positive in the MT. The overall sensitivity and specificity were 46.3 and 93.2% respectively. The sensitivity of the DT was significantly higher in the group with leucocyte scores of 3-4 than in the group with scores of 0-2 (76.5% vs 15.8%, $p < 0.05$). Specificities were 92.7% and 93.6% respectively. The prevalences of positive cultures were 29.3% in the former group and 19.6% in the latter.
There was a correlation between leucocyte score and percentage of positive cultures in cervical samples. In the groups with leucocyte score of 0, 1, 2, 3, and 4, the percentages chlamydia-positive women were 7.1, 8, 20.9, 21.6, and 29.2, respectively. Thus, a leucocyte score of 2-4 had a predictive value for a positive and a negative culture of 18.1% and 92.9%, respectively. In 95 of 222 (42.8%) males, a diagnosis of urethritis was established on the basis of the urine sediment in the two-glass test. Of these 95 males, 26 (27.3%) were chlamydia-positive. Sensitivity, specificity, and predictive values for a positive and a negative culture result were in this group 63.4%, 61.9%, 27.4%, and 88.2%, respectively. Neither signs of urethritis nor complaints of discharge and/or dysuria were apparent in 11 of the 41 chlamydia-positive males (26.8%).

## DISCUSSION

Our results show the superiority of the microtitre method with immunofluorescence detection of inclusions over the more traditional vial method with

Giemsa staining. The two methods differ from each other in inoculum size per monolayer surface, incubation conditions and detection method. As our study was undertaken to compare the accuracy of the two culture methods with that of the nonculture method, it was not possible to determine the contribution of each parameter to the greater sensitivity of the MT.

The advantages of fluorescein-conjugated monoclonal antibodies for the detection of chlamydial inclusions have been described by other investigators (4, 5). In their studies subpassage appeared necessary in both vial and microtitre systems for detection by Giemsa or iodine staining. They also found a 55% increase in positive cultures, agreeing with our results. However, we found that immunofluorescence without subpassage gave better results than Giemsa staining and subpassage (Table 1).

Yoder et al. (6) also compared the microtitre method with the vial technique, using iodine to detect inclusions. The two methods did not clearly differ in sensitivity, although the inoculum varied. Moreover, in the microtitre wells, only a 100 $\mu l$ sample was used in contrast to a 200 $\mu l$ sample in the vials. Centrifugal force of 1100 g was applied in the MT method as compared with 2400 g in the V method. However, by increasing the force from 1000 g to 3000 g, the number of positive specimens rose nearly threefold (7, 8). The superiority of the MT method might therefore have been more apparent if Yoder et al. (6) had used larger inocula and a greater centrifugal force. This might explain out 65% increase in positive cultures with the microtitre method with immunofluorescence detection without subpassage (Table 1).

We were not able to confirm the very good results with the direct test described elsewhere (2, 3, 9). Although our twofold sampling technique and modified culture method might have negatively influenced the sensitivity of the DT, neither the use of an insensitive culture method (V) nor the classification of subgroups with a high chlamydial prevalence yielded the high sensitivities of 93-100% described elsewhere, Maximal sensitivity was obtained in the female subgroup with a high leucocyte score of 3-4, when specimens for DT were sampled prior to culture (76.5%). In particular, the results with female urethral specimens were disappointing. Unfortunately, such specimens have not been evaluated by others. Nevertheless, the female urethra proved to be an important sampling site: in 29.2% of all chlamydia-infected women, the organism could be isolated only from the urethra. The sensitivity for male urethral specimens was also lower than that reported elsewhere, but we took specimens for the culture only prior to sampling for the DT.

Our results illustrate the influence of both culture technique and study

population on the sensitivity of the DT. There was also a distinct correlation between the sensitivity of the DT and the number of leucocytes at the sampling site. Both the percentages of chlamydia-positives and DT sensitivity increased at higher leucocyte scores. The correlation between higher inflammatory response and presence of more infectious particles was also apparent from the study of Brunham et al. (10). Twenty of 40 women (50%) with more than ten leucocytes in the cervical Gram stain (1000x) were chlamydia-positive compared to only two of 60 women without this finding.

Theoretically the low sensitivity of the direct test might be increased by considering less than ten chlamydial particles a positive result. However, Uyeda (3) was not able to detect any change in sensitivity when he altered the cutoff value from two to 10 particles.

The occurrence of culture-negative, but DT-positive results might be due to artefacts, insensitive culture techniques or non-viable organisms. Which of these factors is involved in our study remains to be investigated.

To evaluate a new test like the DT, an optimal culture method should be used. The technique, sampling procedure and study population must also be exactly described. Our findings indicate that the direct test cannot replace the culture method for diagnosing chlamydial infections.

The expense of these methods for detecting chlamydia recommend more selective screening of women. Since our results indicated a predictive value of 92.9% for a negative culture result in women with a score $<2$, as was the case in 156 of 401 women studied (38.9%), the screening could be restricted to women with a score $\geq 2$.

Unfortunately, there does not appear to be a comparable group of male patients. Adger et al. (11) reported that a positive culture gave a predictive value of 91% in the case of sediment abnormalities in first-voided urine. We were unable to confirm this result, finding a predictive value of only 27.4%. The same result was obtained when the study population was restricted to those with more than 20 (instead of 10) leucocytes in the sediment of a first-voided urine sample. In Rotterdam, however, such patients always receive therapy for a chlamydial infection: 2 g tetracycline orally per day for 7 days. More important are those chlamydia-positive men without complaints or signs of urethritis. They amounted in this study to 11 of 41 chlamydia-positive men (26.8%), which is in agreement with findings reported previously (12). Since such patients are able to transmit the infection if not treated, culture screening of these asymptomatic men appears warranted in high-risk populations.

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CHAPTER 5

# EVALUATION OF AN ENZYME IMMUNOASSAY FOR THE DIAGNOSIS OF CHLAMYDIAL INFECTIONS IN UROGENITAL SPECIMENS*

K. Hong Tjiam, B. Yvonne M. van Heijst, Anneke van Zuuren, Johan H.T. Wagenvoort, Theo van Joost, Ernst Stolz, Marc F. Michel

A total of 194 male urethral and 402 cervical specimens were obtained from patients at the venereal disease outpatient clinic of University Hospital, Rotterdam, The Netherlands, to evaluate the IDEIA test (Boots Celltech) for the detection of chlamydial infections. The prevalences of culture-positive males and females were 17.5 and 8.2%, respectively. The respective overall sensitivities and specificities found were 67.6 and 93.7% for the males and 63.6 and 93.8% for the females. The highest sensitivity (83.3%) was found in male patients with more than 20 leukocytes per field in the sediment of the first-voided urine (magnification, x250) and in women with more than 10 leukocytes per field in a cervical Gram stain (magnification, x800). However, in men without urethritis and in women with fewer than 10 leukocytes per field in the Gram stain, sensitivities of 44.4 and 40%, respectively, were found.
Culture-positive, IDEIA-negative results were predominantly observed in samples with few inclusions in the culture.

## INTRODUCTION

The diagnosis of chlamydial infections is based mainly on the culture of the organism on HeLa 229 or McCoy cells, a procedure which takes 2 to 6 days, depending on the method used. The culture requires cell culture facilities, and the transport and storage of the patient's material may influence the reliability of the results considerably (6, 9).
Recently, antigen-detecting techniques have been developed and tested for the purpose of overcoming these disadvantages. They are based either on an enzyme

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immunoassay (2, 3, 11) or on an immunofluorescence technique (4, 8, 10). These methods take only a few hours to perform and eliminate the need for viable organisms.

In view of the high prevalence of chlamydial infections, thorough evaluation of these quick diagnostic tests in several centers seems indicated. In this study, cervical and male urethral samples were obtained from patients at a venereal disease outpatient clinic to evaluate the performance of the IDEIA test (Boots Celltech) in the detection of chlamydial infections. In this test, antigen is detected with a genus-specific monoclonal antibody in an amplified enzyme immunoassay.

## MATERIALS AND METHODS

**Study population.** Visitors to the venereal disease outpatient clinic of the University Hospital in Rotterdam, The Netherlands, were screened for, among other things, chlamydial infections. A total of 402 cervical and 194 male urethral samples were investigated. Patients suffering from gonorrhoea were excluded from the study. In the males, a Gram stain of a sample from the urethra was made first. Next, endourethral samplings for the IDEIA, chlamydial culture, and gonococcal culture were carried out consecutively. A diagnosis of urethritis was made if more than 10 leukocytes per field were found in the sediment of 30 to 40 ml of the first-voided urine (magnification, x250).

In the women, cervical material was sampled for Gram stain, gonococcal culture, IDEIA, and chlamydial culture, in that order. The number of leukocytes in the Gram stain was semiquantitatively determined, with scores of 0 to 4 as follows: 0, no leukocytes; 1, 1 to 5 leukocytes; 2, 6 to 10 leukocytes; 3, 11 to 20 leukocytes, and 4, more than 20 leukocytes per field (magnification, x800).

**Sampling procedure.** A metal cotton-tipped swab was used both for the IDEIA and for the culture. The swab for the IDEIA was suspended in 1 ml of transport medium (Boots Celltech) after endourethral or endocervical insertion and rotation. The sample, which was contained in a 2.5-ml polypropylene tube, was stored at 4°C and processed within 3 days. When this treatment was not possible, it was stored at -70°C and processed within 1 week. This procedure did not influence the test results.

Patient's material on the swab for culture was suspended in 1.5 ml of 2SP (0.2 M sucrose in 0.02 M phosphate buffer with 10% fetal calf serum, 25 $\mu$g of

gentamicin per ml, and 25 U of nystatin per ml). The same swab was introduced endourethrally or endocervically once again, rotated, and stored within 6 hours in 2SP, at -70°C.

Table 1 Prevalence of chlamydial infection and discordant and concordant IDEIA results among groups of patients

| Type of patient | No. of patients with microtiter culture/IDEIA results: | | | |
|---|---|---|---|---|
| | +/+ | +/- | -/+ | -/- |
| Male | | | | |
| With nongonococcal urethritis (>20)[a] | 10 | 2 | 2 | 29 |
| With nongonococcal urethritis (10-20)[a] | 9 | 4 | 5 | 30 |
| Without urethritis | 4 | 5 | 3 | 91 |
| Female with score of: | | | | |
| 4 | 4 | | | 22 |
| 3 | 11 | 3 | 13 | 85 |
| 2 | 5 | 6 | 8 | 124 |
| 1 | | 2 | 2 | 70 |
| 0 | 1 | 1 | | 45 |

[a]Number of leukocytes in sediment of first-voided urine.

**Culture method.** A modified culture method as described by Stamm et al. (7) was used. A sample (200 $\mu l$) was inoculated on a HeLa 229 monolayer, which was grown in a 96-well microtiter cell culture plate (Falcon no. 3070). The monolayer had been rinsed with 30 $\mu g$ of DEAE-dextran in Hank's balanced salt solution per ml prior to inoculation. Two wells per sample were used. After centrifugation for 1 h at 3,000 x g, the sample was replaced by 100 $\mu l$ of growth medium (minimal essential medium; Eagle's medium modified with 5% fetal calf serum, 4.5 mM glucose, 25 $\mu g$ of vancomycin per ml, 25 $\mu g$ of gentamicin per ml, and 25 U of nystatin per ml). After 48 h of incubation at 37°C, the monolayer was fixed and stained with the MicroTrak Confirmation Test (Syva Inc.). The microtiter plates were turned upside down and screened for the presence of inclusions with a fluorescence microscope equipped with a Leitz long-distance objective (32x).

**IDEIA method.** For the IDEIA method, samples were placed in a boiling-water bath for 15 min. A cooled specimen (200 $\mu l$) was added to duplicate wells on

Table 2 Sensitivity, specificity, and predictive values for positive and negative test results of the IDEIA, with the culture method as the golden standard among groups of patients

| Type of patient | n | Results (%) of IDEIA[a] | | | |
|---|---|---|---|---|---|
| | | Sensitivity | Specificity | PV(+) | PV(-) |
| Male | | | | | |
| With nongonococcal urethritis (>20)[b] | 43 | 83.3 | 93.5 | 83.3 | 93.5 |
| With nongonococcal urethritis (10-20)[b] | 48 | 69.2 | 85.7 | 64.3 | 88.2 |
| Without urethritis | 103 | 44.2 | 96.8 | 57.1 | 94.8 |
| Overall result | | 67.6 | 93.7 | 69.7 | 93.2 |
| Female with leukocyte score of: | | | | | |
| 3-4 | 138 | 83.3 | 89.2 | 53.6 | 97.3 |
| 0-2 | 264 | 40[c] | 95.8 | 37.5 | 96.4 |
| Overall result | | 63.6 | 93.8 | 47.7 | 96.6 |

[a] PV(+), Predictive value for positive test result; PV(-), predictive value for negative test result.

[b] Number of leukocytes in sediment of first-voided urine.

[c] $p < 0.05$ with regard to sensitivity in women with a leukocyte score of 3 to 4.

a 96-well microtiter plate, which was coated with genus-specific monoclonal antibodies. These antibodies react both to elementary bodies and to group-specific lipopolysaccharide antigen. A total of 46 samples could be tested in one plate. In every plate, four wells were used for two positive and two negative controls.

After incubation for 2 h at room temperature, 50 $\mu$l of alkaline phosphatase-conjugated monoclonal antibody was added to each well. Next, after incubation and a washing procedure, this solution was replaced by 100 $\mu$l of NADP. The addition of 200 $\mu$l of amplifier caused the development of a red-coloured formazan derivative in the case of a positive result. The $A_{492}$ was read with a Titertek Multiskan spectrophotometer. A value greater than 0.2 optical density units above the mean absorbance reading of the negative control was regarded as a positive result.

**Definitions of sensitivity, specificity, and predictive value for positive and negative results.** Definitions of terms are as follows: sensitivity (%) = (number of culture-positive persons with a positive IDEIA test/number of culture-positive persons tested) x 100; specificity (%) = (number of culture-negative persons with a negative IDEIA test/number of culture-negative persons tested) x 100; predictive value for positive test result = (number of culture-positive persons with positive IDEIA test/number of persons with positive IDEIA test) x 100; predictive value for negative test result = (number of culture-negative persons with negative IDEIA test/number of persons with negative IDEIA test) x 100.
**Statistical methods.** The Fisher exact test was used to determine statistical significance.

RESULTS

Of 194 male patients, 34 (17.5%) were culture positive, as were 33 (8.2%) of 402 women. Urethritis patients were divided into a group with more than 20 leukocytes in the urine sediment (n = 43) and a group with 10 to 20 leukocytes in the sediment (n = 48). Women were classified by the semiquantitative leukocyte score (Table 1). The highest prevalences of culture-positive chlamydial infections were seen in the urethritis patients (27.5%) and in women with leukocyte scores of 3 and 4 (12.5 and 15.4%, respectively). In males without urethritis (n = 103) and in women with leukocyte scores of 0, 1, and 2 the prevalences were 8.7, 4.3, 2.7, and 7.7%, respectively.
From the discordant and concordant results, it was possible to calculate the sensitivity and specificity of the test for different patient groups. The culture results were taken as the "golden standard" (Table 2). In male patients, the sensitivity decreased from 83.3% in those with more than 20 leukocytes in the urine sediment to 44.4% in those without urethritis. The overall sensitivity and specificity were 67.6 and 93.7%, respectively.
In women with a leukocyte score of 3 to 4, the sensitivity was 83.3%, compared with 40% in those with a leukocyte score of 0 to 2 ($p < 0.05$). The overall sensitivity and specificity were 63.6 and 93.8%, respectively.
To evaluate the discrepant culture-positive, IDEIA-negative results, the number of inclusions was determined in 47 culture-positive samples. Culture samples were always inoculated in duplicate wells (see Materials and Methods). Culture-positive, IDEIA-negative samples were found to be positive

in only one well significantly more often than were culture-positive, IDEIA-positive samples, which were predominantly positive in both wells ($p < 0.05$; Table 3).

In previous experiments, a significant relationship was found between a high number of inclusions in either of two wells inoculated with the same specimen and a concordant culture result in both wells. Positive samples which gave more than 20 inclusions in one or both wells appeared to be more often positive in both wells ($p < 0.01$; Table 4).

Table 3 Relationship between culture-positive, IDEIA-negative specimens and the culture result in two wells inoculated with the same sample

| Culture results in two wells | No. of specimens with culture/ IDEIA results: | |
|---|---|---|
| | +/- | +/+ |
| Concordant | 13 | 37 |
| Discordant | 12 | 8[a] |

[a]$p < 0.05$

Table 4 Relationship between culture result in two wells inoculated with the same sample and the number of inclusions in either of the monolayers

| Culture results in two wells | No. of patients with inclusions | |
|---|---|---|
| | $\leqslant 20$ | $> 20$ |
| Concordant | 11 | 15 |
| Discordant | 18 | 3[a] |

[a]$p < 0.01$

## DISCUSSION

Our results show that the sensitivity of the IDEIA is strongly dependent on the study population (Table 2). A higher sensitivity was observed in patients

with urethritis or with a leukocyte score of 3 to 4 than in those with a leukocyte score of 0 to 2 or without urethritis. A relationship was found to exist between a high number of leukocytes and a high prevalence of chlamydial infections (Table 1). This observation is in agreement with the findings of Brunham et al. (1), who were able to diagnose a chlamydial infection in 50% of women with mucopurulent cervicitis but in only 2 of 40 women without cervicitis. Van Ulsen et al. (11) also found an enzyme immunoassay test to have a higher sensitivity in men with urethritis than in men without urethritis. Moreover, culture-positive, IDEIA-negative results were predominantly observed in samples with few inclusions in the culture (Tables 3 and 4). These observations suggest that there is a positive correlation between the strength of the inflammatory response and the number of infectious chlamydial organisms. From these findings it may be inferred that the sensitivity of antigen-detecting systems is dependent on the antigen level available for analysis.

The culture method used is superior to the more traditional vial method with subpassage and Giemsa stain. In our system, two wells per sample were used, and the centrifugal force was increased from 1,100 x g (7) to 3,000 x g. According to Reeve et al. (5), this increase could mean a 30% augmentation of positive culture results. Besides, sampling of culture specimens occurred by double swabbing, which might improve the yield. However, we did not carry out studies to confirm this issue. The use of a more sensitive culture technique as the standard will obviously lead to a lower sensitivity of the test under investigation.

Caul and Paul (2) reported a sensitivity and specificity of 95.5 and 99%, respectively, with the IDEIA. However, they did not describe in detail the study population and the culture method used. The results of another chlamydial antigen-detecting enzyme immunoassay, performed in different laboratories, also revealed discrepancies (3, 11). From our study it seems likely that discrepancies in test results may be attributable in part to differences in study populations and culture techniques.

Unfortunately, the IDEIA has a low sensitivity in patients lacking inflammatory symptoms, such as men without urethritis and women without cervicitis. These patients in particular will not be treated in the absence of adequate diagnostic facilities and are therefore able to transmit the infection inadvertently.

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CHAPTER 6

# THE PRESENCE OF SEXUALLY COMMUNICABLE MICRO-ORGANISMS IN HUMAN SEMEN SAMPLES, TO BE USED FOR ARTIFICIAL INSEMINATION BY DONOR*

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Twohundred thirty-seven semen samples from 10 institutions for artificial insemination by donor (AI) in Belgium and the Netherlands were tested for the presence of Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma hominis, Ureaplasma urealyticum, herpes simplex virus and cytomegalovirus. The prevalence of these micro-organisms in the semen samples was 0%, 6.3%, 4.6%, 35.9%, 0% and 0.4% respectively. Forty percent of all samples were found to be infected with one or more of these micro-organisms. As the ejaculates from which the samples had been taken had already been or would be used for AID purposes, it seems indicated to exclude microbiological contamination with sexually communicable micro-organisms prior to insemination.

## INTRODUCTION

Artificial insemination by donor (AI) is used thousands of times a year in the Netherlands and Belgium in cases of male infertility. In general, microbiological examination of the semen samples prior to insemination is incomplete, especially as use is often made of fresh semen. In view of the epidemic incidence of sexually communicable diseases it would seem to be important that semen samples intended for AI should be subjected to such tests as are necessary to prevent iatrogenic infections in women. For this reason, a study was set up in Rotterdam, the Netherlands, to investigate the incidence of Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma hominis, Ureaplasma urealyticum, herpes simplex virus and cytomegalovirus in frozen semen samples which had been or would be used for AI.

*Accepted in Genitourinary Medicine

MATERIALS AND METHODS

## Semen samples

Twohundred thirty-seven semen samples frozen at -196°C, from ten AI institutes in the Netherlands and Belgium, were supplied in 0.25 ml or 0.5 ml straws. The semen was given voluntarily by the donors, who received a small sum for expenses. The samples were all diluted 2:1 or 1:1 with a cryoprotective liquid containing glycerol and sometimes also egg yolk or bovine serum albumin. Fifty samples contained ampicillin (380 μg/ml) and 36 contained kanamycin (5 μg/ml). Forty samples came from an institution which used only fresh semen. The semen was diluted 1:1 with Eagle's modified Minimal Essential Medium (EMEM) with 15% foetal calf serum and frozen to -196°C. 120-200 μl of sample were diluted with 2 ml of EMEM for the culture of N. gonorrhoeae, C. trachomatis, herpes simplex virus and cytomegalovirus. 60-200 μl of sample were suspended in 2 ml of U-9 medium (1) for the culture of M. hominis and U. urealyticum.

## Culture methods

### Culture for Neisseria gonorrhoeae (Ng)

The sample diluted with EMEM was inoculated with a loop on a medium consisting of GC mediumbase (Difco), haemoglobin (Difco) and Isovitalex (BBL) and incubated for 48 hours.

### Culture for C. trachomatis (Ct).

200 μl of the sample diluted with EMEM were again diluted 1:1 with EMEM. Of each dilution, 200 μl per well were inoculated in duplo on a HeLa 229 monolayer which had been pre-rinsed with 30 μg/ml DEAE dextran and grown in a 96-well microtiter plate (Falcon 3070). After centrifugation for 1 h at 3000 g the inoculum was replaced by 100 μl of EMEM with 4.5 mM glucose, 5% foetal calf serum, 25 U/ml nystatin, 25 μg/ml gentamicin, 25 μg/ml vancomycin. After incubation for 48 h at 37°C the monolayers were screened for the presence of inclusions after colouring with fluorescent monoclonal antibodies (Syva Inc.).

### Culture for herpes simplex virus (HSV) and cytomegalovirus (CMV)

200 μl of the sample diluted with EMEM were put on a leighton tube in which a HEL cell monolayer had been grown. Inoculation was carried out in duplo. After incubation for 1 h at 37°C the inoculum was replaced by 1.5 ml of Dulbecco MEM with 2% foetal calf serum. The medium was renewed every week. After 3 weeks the cells were transferred to new tubes. The cell culture was maintained for

6 weeks. If a cytopathogenic effect was present the monolayer was coloured with virus-specific fluorescent monoclonal antibodies (M.A. Bioproducts).

Culture for M. hominis (Mh) en U. urealyticum (Uu).
The medium of Chanock (2) was used for Mycoplasma cultures. Yeast extract was prepared as described by Hers (3) and inactivated horse serum was applied. U-9 and A-7 media (1, 4) were used for the Ureaplasma cultures. Ureaplasma was detected by the phenol-red colouring in the U-9 medium and the typical colony form revealed by microscopic examination of the A-7 plate. Mycoplasma was recognized by the colony form on Chanock's medium, and further identified with the indirect immunofluorescencetest of unfixed colonies (5).

## RESULTS

Neither gonococci nor HSV were cultivated from any of the 237 samples. The prevalence of C. trachomatis, M. hominis, U. urealyticum and CMV was 6.3%, 4.6%, 35.9% and 0.4% respectively (Table 1). Forty percent of the samples were found to be infected with one or more of the micro-organisms studied. Fifteen of the 237 samples (6.3%) were infected with two or three micro-organisms. The microbiological screening of the semen samples carried out by the institutes and the upper limit of leukocytes accepted varied widely. Six institutes did not perform any microbiological tests on their samples. None of the institutes carried out a culture for HSV or CMV. Though two institutes examined the samples for Ureaplasma and Mycoplasma, some of them were nonetheless found to be infected with these micro-organisms. Two of 50 ampicillin-containing samples were chlamydia-positive. The culture of Mycoplasma and/or Ureaplasma were positive in 11 out of 25 kanamycin-containing specimens. Two of the 10 institutes carried out a urine sediment examination of the donor.

## DISCUSSION

Micro-organisms were found to be present in 40% of the 237 semen samples intended for AI. This percentage is probably a minimum value. The quantity of sample which could be extracted from the straws varied fairly widely (120-200 $\mu$l). Moreover, the samples had to be diluted in order to avoid a cytotoxic effect on the cell lines used. On the other hand, however, a

Table 1 Culture results in 237 semen samples[1)]

| Micro-organism[2] | No. of positive culture results |
|---|---|
| C. trachomatis | 15 ( 6.3) |
| M. hominis | 11 ( 4.6) |
| U. urealyticum | 85 (35.9) |
| Cytomegalovirus | 1 ( 0.4) |
| None | 142 (59.9) |

1) The origin of one sample is unknown; the culture results were negative.

2) All cultures of N. gonorrhoeae and herpes simplex virus were negative. Percentages are given in parenthesis.

decrease in the concentration of semen components which have a growth-inhibiting effect might enhance the chlamydial cultures (6). The presence of kanamycin and ampicillin in a number of specimens tested will inhibit the growth of gonococci, Chlamydia, Mycoplasma and Ureaplasma.
The examinations carried out by the institutes are not sufficient to rule out the presence of these micro-organisms in the semen they use. The only way for the institutions to ensure that the samples they obtain are free of pathogens is for them to carry out the necessary examinations of the donor and the semen sample. The presence or absence of micro-organisms does not seem to be related to other properties of semen, such as motility, abnormal morphology and leucospermia (7, 8). Nor do physical and simple laboratory examinations of the donor provide conclusive evidence, as carriership of micro-organisms causing sexually communicable diseases, is frequently asymptomatic.
In view of the large number of inseminations carried out annually, it is notable that little mention is made in the literature of genital infections in the recipients caused by AI. Gonococcal and Ureaplasma infections transmitted through AI have, however, been described (9, 10). Moreover, gonococci, Chlamydia and other bacteria, including beta-haemolytic streptococci, have been reported to survive a freezing procedure (11, 12, 13, 14). The incidence of these other bacteria was not investigated in this study, nor was the incidence of hepatitis B and HTLV-III virus. However, the occurrence of HTLV-III virus infections through AI has recently been described (15) and serological examination of the donor for both of these viruses is therefore certainly indicated.

It is not known whether the contaminated semen samples have caused infections in the recipients. It is not clear either whether the presence of the cultivated micro-organisms is of pathogenic significance. This will be determined by the number and serotype of the micro-organism in question and by the condition of the recipient's defense mechanisms.
However, the importance of C. trachomatis as a pathogenic agent in urethritis, cervicitis, salpingitis, neonatal conjunctivitis and pneumonitis has been well documented (16).
M. hominis is associated with salpingitis, pyelonephritis and postpartum fever, while U. urealyticum is linked with chorioamnionitis and possibly low birth weight (17). One of the causes of the "TORCHES" syndrome in neonates is a congenital CMV infection.
The results of this screening therefore point to the need to examine both the donor and the semen prior to insemination in order to avoid contamination with pathogenic micro-organisms. This implies that only frozen semen samples should be used for AI purposes.

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CHAPTER 7

# PREVALENCE OF ANTIBODIES TO CHLAMYDIA TRACHOMATIS, NEISSERIA GONORRHOEAE, AND MYCOPLASMA HOMINIS IN INFERTILE WOMEN*

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SUMMARY

A total of 57 infertile women who had been referred for in vitro fertilisation or for diagnostic laparoscopy, were tested for the presence of antibodies to *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Mycoplasma hominis*. Four were excluded from the study. Of the remaining 53, 33 had laparoscopically obvious tubal disorders, such as adhesions, distal occlusions and strictures, and 20 did not. Antibodies to *C. trachomatis* were found in 7/33 (21.2%) v 0/20, antibodies to *N. gonorrhoeae* in 20/38 (60.6%) v 5/20 (25%), and antibodies to *M. hominis* in 18/24 (75%) women with tubal disorders v 13/19 (68.4%) of those with no disorder. Antibodies to *C. trachomatis* and *N. gonorrhoeae* were significantly ($p < 0.05$) more common in women with tubal disorders. The high prevalence of antibodies to *N. gonorrhoeae* in infertile women without tubal disorders suggests that ciliated tubal epithelium is damaged after inflammation without this being laparoscopically visible. Our results confirm the important role of *N. gonorrhoeae* and *C. trachomatis* in the aetiology of infertility after tubal inflammation.

INTRODUCTION

Salpingitis may lead to tubal infertility, the relative risk of which, after one or more episodes of salpingitis, varies between 12.8% and 75% (1, 2). Moreover women who have had salpingitis have a sevenfold to tenfold risk of ectopic pregnancy. Tubal damage after infection is estimated to be an aetiolo-

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gical factor in 30% to 40% of infertile women (2). Most cases of salpingitis are caused by the ascending spread of infection from the lower genital tract. Acute salpingitis has a polymicrobial aetiology. Anaerobic micro-organisms such as Peptostreptococcus, Peptococcus, Clostridium and Bacteroides species have been isolated, either individually or in combination with N. gonorrhoeae, M. hominis, or C. trachomatis (3). The relative importance of these organisms as causative agents has not been agreed. In the United States gonococci and anaerobes are more commonly associated with salpingitis than in Sweden (4) and France (5), where most isolates yield C. trachomatis. These differences in the prevalence of causative organisms may be attributed to epidemiological factors, the length of time after infection, and the methods used for taking specimens for culture. Moore et al. showed antibodies to C. trachomatis in 73% of infertile women with distal tubal occlusion, but antibodies could not be detected in women with normal tubes (6). Punnonen et al. (7) and Conway et al. (8) made similar observations. These studies did not, however, evaluate antibodies to M. hominis or N. gonorrhoeae.

We undertook the study reported here to assess the relative importance of N. gonorrhoeae, M. hominis and C. trachomatis as organisms causing tubal inflammation leading to infertility by measuring the prevalence of antibodies to these organisms in a group of infertile women.

## PATIENTS AND METHODS

### Study population

We tested serum from 57 women who had been infertile for at least 18 months and had been referred by their doctors to the gynaecological outpatient clinic of the University Hospital Rotterdam, the Netherlands for in vitro fertilisation or diagnostic laparascopy.

### Classification of disorders of the fallopian tubes

We regarded the presence of peritubal adhesions, strictures, tubal occlusions, or a combination of these disorders as a macroscopic feature of tubal disease. This was the case in 33 women, two of whom showed only peritubal adhesions. The 20 women who did not show such abnormalities were considered to be infertile for unknown reasons. Four women had well documented causes of tubal disorders: one had had endometriosis, two had had tuberculous salpingitis, and one had had peritonitis after a perforated appendix. These four women were

Table I Demographic, historical, and serological findings in relation to the condition of fallopian tubes as seen at laparoscopy

| | Tubal disorder | No tubal disorder |
|---|---|---|
| Mean (SEM) age (years) | 32.0(0.7) (n=33) | 31.4(0.9) (n=20) |
| No (%) with secondary infertility | 17/33 (51.5%) | 9/20 (45%) |
| Mean (SEM) duration of infertility (years) | 7.4(0.8) (n=31) | 5.2(0.7) (n=18) |
| No (%) with history of salpingitis | 14/20 (70%) | 1/16 (6.3%)* |
| No (%) with chlamydial antibodies | 7/33 (21.2%) | 0/20 (0%)** |
| No (%) with gonococcal antibodies | 20/33 (60.6%) | 5/20 (25%)** |
| No (%) with chlamydial or gonococcal antibodies, or both | 24/33 (72.7%) | 5/20 (25%)* |
| No (%) with mycoplasmal antibodies | 18/24 (75%) | 13/19 (68.4%) |

*$p < 0.001$; **$0.01 < p < 0.05$.

Table II Prevalence of chlamydial, gonococcal and mycoplasmal antibodies in infertile women related to historical data

| | No (%) with chlamydial antibodies/No with or with no history | | No (%) with gonococcal antibodies/No with or with no history | | No (%) with chlamydial or gonococcal antibodies, or both/No with or with no history | | No (%) with mycoplasmal antibodies/No with or with no history | |
|---|---|---|---|---|---|---|---|---|
| | With history | No history | With history | No history | With history | No history | With history | No history |
| History of: | | | | | | | | |
| Salpingitis | 5/14(35.7) | 1/23 (4.3)* | 9/15(60) | 9/23(39.1) | 12/15(80) | 9/23(39.1)* | 9/14(64.3) | 13/12(61.9) |
| Secondary infertility | 4/29(13.8) | 2/27 (7.4) | 12/29(41.4) | 14/29(51.9) | 14/29(48.3) | 15/27(55.6) | 21/27(77.8) | 12/24(50) |
| Ectopic pregnancy | 1/9 (11.1) | 6/48(12.5) | 4/9 (44.4) | 22/48(45.8) | 4/9 (44.4) | 26/48(54.2) | 5/8 (62.5) | 28/44(63.6) |

*$0.01 < p < 0.05$

excluded from evaluation.

Investigations of infertility

We recorded each patient's age and history of pregnancy, salpingitis, pelvic surgery, and the use of an intrauterine device (IUD). Infertility investigations consisted of semen analysis, basal temperature curve, postcoital test, hysterosalpingography, and laparoscopy with intrauterine injection of methylene blue to assess tubal patency.

Serology tests

We tested serum samples for the presence of antibodies to C. trachomatis by the microimmunofluorescence technique of Wang et al. (9). Six pooled antigens were used: A to C, DE, FG, I, J, and K. A titre of $>1/8$ was consisted to be positive. Antibodies against gonococcal pili antigens were shown by an enzyme linked immunosorbent assay (ELISA), as described by Oranje et al. (10). An extinction of $>1.15$ was considered to be positive. Anti-human immunoglobulin conjugate was used in both tests.
Antibodies to M. hominis were shown by the indirect immunofluorescence method of Rosendal et al. (11). A strain of M. hominis that had been isolated from a patient served as antigen. Positive control serum was kindly supplied by Dr. K. Lind (Statens Serum Institut, Copenhagen, Denmark). Serum dilutions of 1/40 and 1/80 were prepared for the test. Anti-human IgG conjugate was used. Serum samples, from 25 healthy women blood donors aged 22 to 34 were tested for the presence of the above mentioned antibodies.

Statistical methods

The $\chi^2$ method for independent samples was used to assess the level of significance.

RESULTS

Table I shows that significantly more ($0.01 < p < 0.05$) women with macroscopically visible tubal disorders had antibodies to C. trachomatis and to gonococcal pili in their serum than those without such disorders. Positive serology test results for chlamydiae were found in seven (13.2%) out of 53 women. In these seven women the most common serogroup was DE (in five women) followed by A-C (in four) and FG (in four), I (in three) and J (in two.). In

three out of these seven women only one antigen pool could be found. Antibodies to gonococcal pili antigen were present in 25 (47.2%) out of 53 women. Antibodies to M. hominis were present in 31 (72%) out of 43 women, of whom 18 had tubal disorders and 13 did not. Of the 25 women blood donors, none had antibodies to C. trachomatis, one had antibodies to gonococcal pili, and four had antibodies to M. hominis.

Table II shows that antibodies to C. trachomatis were significantly ($0.01 < p < 0.05$) more common in women with a history of salpingitis. Antibodies to gonococcal pili were also more common in such women, although not significantly so. Antibodies to M. hominis were found more commonly in women with secondary infertility than in those with primary infertility. The number of women (nine) with a history of ectopic pregnancy was too small to allow statistical analysis. As the historical data on the use of IUDs were incomplete, these were not evaluated.

Table III shows that the predictive value for the presence of mascroscopically visible tubal disorders in serum containing chlamydial or gonococcal antibodies, or both, ranged from 64.2% to 94.4%.

Table III Predictive value of positive serology test results for tubal disorders, as seen at laparoscopy

| Serology test results | Predictive value within 95% confidence limits |
|---|---|
| Chlamydial antibodies | 59-100 |
| Gonococcal antibodies | 59.3-93.2 |
| Chlamydial or gonococcal antibodies, or both | 64.2-94.1 |

## DISCUSSION

The prevalence of antibodies to C. trachomatis in our patients was low (13.2%) compared with that (about 30%) observed by Moore et al. (6). Epidemiological differences in the prevalence of chlamydial infections in both populations might offer an explanation for this discrepancy. Moreover, the mean duration

of infertility in women in our study with tubal disorders was 7 years and 4 months, and in women without such disorders it was 5 years and 2 months. The length of time after infection that antibodies to chlamydiae were demonstrable in man remains to be assessed. Mårdh reported the disappearence of IgG and IgM antibodies after 1 year and 5 months despite initial high titres (12). The long duration of infertility may have affected our results. Moore et al. found a mean duration of raised titres of four years, which was considerably less time than we found in our study.

The high prevalence of antibodies to gonococcal pili both in the total study population (47.2%) and in the women with tubal disorders (60.6%) was unexpected. Mårdh detected gonococcal antibodies in less than 18% of 60 women with laparoscopically confirmed salpingitis (4). Eschenbach et al., however, found positive serology test results in 84% for 38 patients with salpingitis and gonococcal cervicitis and in 36% of 35 patients without current gonorrhoea (3).

Our findings suggest a higher prevalence of gonococcal than chlamydial infections at the time of salpingitis. In 248 women, who attended the venereal diseases outpatient clinic in Utrecht, the Netherlands in 1979-80, N. gonorrhoeae was isolated in 34%, C. trachomatis in 15%, and M. hominis in 63% (13).

Gonococcal antibodies were found in 25% of infertile women without laparoscopically obvious tubal disorders. This figure was considerably higher than that for gonococcal antibodies in our blood donors, which may indicate that gross examination by laparoscopy does not detect all tubal abnormalities in patients with tubal infertility. In such patients, dysfunction of ciliated tubal epithelium after inflammation may only be detectable by histopathological investigations (14, 15).

Antibodies to M. hominis were shown in 72% of our study population. The prevalence, however, was similar in women with and without tubal disorders. (Table I). The role of M. hominis as a causative organism of salpingitis needs to be established. Animal studies suggest that it has a role in the aetiology of parametritis, rather than salpingitis (17).

The difference in prevalence of chlamydial antibodies in women with and without a history of salpingitis was striking (Table II). Antibodies to gonococcal pili were also more common in women with a history of salpingitis than in those with no such history, but the difference was not significant. In general, salpingitis associated with chlamydiae has a clinically milder course than that caused by gonococci or anaerobes, which seldom remains unnoticed. We

cannot explain our results, which contradict this fact.
Our study clearly suggests a role for sexually transmitted organisms in the aetiology of infertility after tubal inflammation. Prospective studies of women with salpingitis, well documented by laparoscopic and bacteriological data, are necessary to gain further insight into the role of several micro-organisms in the development of tubal infertility. The high predictive value of the presence of antibodies to chlamydiae or gonococcal pili, or both, for the presence of tubal pathology is worth mentioning.
Positive serological test results may also be valuable in explaining the aetiology of infertility in women when this is not known. Testing for these antibodies should therefore form a part of every routine infertility investigation.

We thank Mrs P Landsmeer for her technical help.

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CHAPTER 8

# IN VITRO ACTIVITY OF TEMOCILLIN (BRL 17421) AGAINST CHLAMYDIA TRACHOMATIS*

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Sir,

The susceptibility of Chlamydia trachomatis to different antibiotics is not tested routinely. The great efficacy of tetracycline in eradicating non-gonococcal infections of the urogenital tract has probably obviated to a large extent cumbersome in vitro testing of the activity of different antibiotics against clinically isolated Chlamydia strains. However, some resistance to erythromycin has been detected by Mourad (1). In view of this finding and because in recent years new antibiotics have been developed whose anti-chlamydial activity had not been investigated, the present study was undertaken.

Isolates of Chlamydia trachomatis examined in this study comprised ten strains from men suffering from non-gonococcal urethritis who attended the venereal disease out-patient clinic of the University Hospital, Rotterdam, the Netherlands. The strains were passed eight to nine times on HeLa 229 cells in order to increase the inoculum size. Antibiotics evaluated were cefuroxime (Glaxo, England), cefotaxime (Roussel, France), tetracycline (Nogepha, the Netherlands), and temocillin (BRL 17421, Beecham, the Netherlands). The latter is a semisynthetic $\beta$-lactam antibiotic which is resistant to $\beta$-lactamase produced by Neisseria gonorrhoeae.

HeLa 229 cells were maintained and passed on an antibiotic-free medium (henceforth called "growth medium"), consisting of Eagle's Minimal Essential Medium modified with 5% fetal calf serum and 4.5 mM glucose. Experiments were carried out in flat bottom-tubes containing the HeLa 229 monolayer on a glass coverslip. The monolayer was inoculated with 100 $\mu$l of a chlamydial suspension. A proper dilution in phosphate buffered saline was prepared to obtain two to nine inclusions per microscope field (magnification x250). After centrifugation of the infected monolayers at 3,800 x g 1 h, 1 ml of growth

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medium containing the various antibiotics in the desired concentration was added. For each antibiotic dilution, four infected monolayers and one control (non-infected) monolayer were incubated at 36°C for 60 h. The control monolayer was used to detect any cytopathic effect of the antibiotic.
After incubation, two of the monolayers were fixed with methanol and stained with Giemsa to determine, by darkfield illumination, the presence of the chlamydial inclusions. In the remaining chlamydia-inoculated monolayers, the growth medium was replaced by 1 ml fresh antibiotic-free medium. The cultures were frozen at -70°C, thawed twice, and subsequently put on a new monolayer. After centrifugation, the medium was replaced by a fresh antibiotic-free one. The next passages were carried out in the same way, with the exception that sonication for 15 s was employed to disrupt the monolayer instead of the freeze-thaw procedure. At this stage, growth medium containing nystatin 25 U/ml, vancomycin 25 μg/ml and gentamicin 20 μg/ml was used.
Serial two-fold dilutions of antibiotics in growth medium were then carried out. The lowest concentration at which monolayers in duplicate vials were free from inclusions was considered to be the MIC. The lowest concentration at which no inclusion could be seen after three passages was considered to be the MBC. In the case of three strains, six passages were carried out.
Both cefuroxime and cefotaxime showed MIC values $\geqslant$32 μg/ml, i.e. at the highest concentration used. As could be expected from previous investigations (2, 3, 4), the cephalosporins tested lacked activity against Chlamydia trachomatis. In our out-patient clinic both drugs are commonly given as single "one-shot" therapy against gonorrhoea. Hence, this regimen does not eradicate a concomittant chlamydial infection.
The results of sensitivity testing of temocillin and tetracycline are shown in Table 1. Micro-organisms are considered to be susceptible to temocillin if MBC values are $\leqslant$16 μg/ml. According to our in vitro results, temocillin might be an effective agent against Chlamydia trachomatis. However, strains 6, 9 and 10 were isolated from men who developed post-gonococcal urethritis after treatment with 1g temocillin i.m. for gonococcal urethritis. Furthermore, strain 6 showed an MBC value of $\geqslant$80 μg/ml after 6 passages. This illustrates the importance of repeated passages in determining an MBC.
MBC values for tetracycline are approximately the same as those reported elsewhere (1, 3); the average blood level of the drug during therapy is supposed to be 2-3 μg/ml. This finding suggests that tetracycline is effective against chlamydial infections. However, the in vitro studies presented here are based on a single growth cycle of the organism, while chlamydial infections require

Table 1 In vitro activity of temocillin and tetracycline against ten Chlamydia trachomatis strains. MIC and MBC values are expressed in µg/ml.
MBC values after six passages are given in parentheses. The serial dilutions employed for tetracycline were 0.025 - 0.05 - 0.125 - 0.250 - 0.500 µg/ml.

| Strain no. | Temocillin | | Tetracycline | |
|---|---|---|---|---|
| | MIC | MBC | MIC | MBC |
| 1-3 | 5 | 5 | 0.05 | 0.125 |
| 4-5 | 10 | 10 | 0.05 | 0.125 |
| 6 | 10 | 10 (≥80) | 0.125 | 0.125 (0.125) |
| 7 | 10 | 10 (10) | 0.05 | 0.125 (0.25) |
| 8 | 5 | 5 (5) | 0.125 | 0.125 (0.25) |
| 9 | 10 | 20 | 0.05 | 0.125 |
| 10 | 10 | 10 | <0.125 | 0.125 |

long-term treatment. Hence no absolute significance may be attributed to MIC and MBC values in predicting the clinical efficacy of a drug.
In view of the high prevalence of chlamydial infections and the frequent choice of tetracycline for the treatment of these infections, conducting surveys regularly to determine the susceptibility of clinical isolates seem appropriate.

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# IN VITRO ACTIVITY OF THE TWO NEW 4-QUINOLONES OF A56619 AND A56620 AGAINST NEISSERIA GONORRHOEAE, CHLAMYDIA TRACHOMATIS, MYCOPLASMA HOMINIS, UREAPLASMA UREALYTICUM AND GARDNERELLA VAGINALIS*

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The in vitro activity of tetracycline, ciprofloxacin and two recently developed 1-aryl-fluoroquinolones, A56619 and A56620, was tested against 65 beta-lactamase-negative and 35 beta-lactamase-positive *Neisseria gonorrhoeae* strains, 12 *Chlamydia trachomatis*, 50 *Mycoplasma hominis*, 28 *Ureaplasma urealyticum* and 50 *Gardnerella vaginalis*. In the case of *Chlamydia trachomatis* and *Mycoplasma hominis* both the MIC and the MBC were determined. The MIC90 of ciprofloxacin for *Neisseria gonorrhoeae* was 0.008 μg/ml and of A56619 and A56620 $\leqslant$0.03 μg/ml. No difference was observed between the activity against beta-lactamase-negative and beta-lactamase-positive strains. The MIC90 of ciprofloxacin and A56620 for *Chlamydia trachomatis*, *Mycoplasma hominis* and *Ureaplasma urealyticum* were identical, the values being 2 μg/ml, 1 μg/ml and 4 μg/ml respectively. The MIC90 of A56619 for *Chlamydia trachomatis* and *Ureaplasma urealyticum* was 0.5 μg/ml and 1 μg/ml respectively. The MBC90 of the three quinolones for *Chlamydia trachomatis* and *Mycoplasma hominis* were $\leqslant$ 2 μg/ml. The activity of the quinolones against *Gardnerella vaginalis* was rather low, the MIC90 being $\geqslant$4 μg/ml. It is concluded that A56619 and A56620 might be useful for single-dose therapy of gonococcal infections.

## INTRODUCTION

Ciprofloxacin, a recently developed 4-quinolone, has been reported to have a very high activity against *Neisseria gonorrhoeae* and a moderate activity against *Chlamydia trachomatis*, *Mycoplasma hominis* and *Ureaplasma urealyticum* (1, 2, 3). Since the quinolones might be of value in the chemotherapy of sexually transmitted diseases, the in vitro activity of ciprofloxacin, two new 1-aryl-fluorquinolones, A56619 and A56620, and tetracycline was tested against

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clinical isolates of Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma hominis, Ureaplasma urealyticum and Gardnerella vaginalis. In the case of Chlamydia trachomatis and Mycoplasma hominis both MIC and MBC were determined.

## MATERIALS AND METHODS

Antibiotics. Ciprofloxacin was obtained from Bayer, FRG, A56619 and A56620 from Abbott, USA, and tetracycline.HCl from Nogepha, The Netherlands.
Organisms. The 35 beta-lactamase-positive and 65 beta-lactamase-negative Neisseria gonorrhoeae strains, 12 Chlamydia trachomatis, 50 Mycoplasma hominis and 28 Ureaplasma urealyticum strains used had recently been isolated from specimens obtained from patients attending the venereological outpatient department of the University Hospital, Rotterdam, The Netherlands. Fifty clinical isolates of Gardnerella vaginalis were obtained from the Institute of Tropical Medicine, Antwerp, Belgium.
MIC and MBC Determinations.
Neisseria gonorrhoeae. The activity of penicillin G (Gist-Brocades, The Netherlands) and cefuroxime (Glaxo, UK) against this organism was also tested. Twofold dilutions of antibiotics in water were added to a medium consisting of GC medium base (Difco, USA), hemoglobin (Difco, USA) and Isovitalex (BBL, USA). An antibiotic-free control medium was included in each series. The organisms to be tested were cultured overnight. Colonies were freshly suspended in trypticase soy broth (BBL) until an optical density of 0.1 was reached, resulting in an inoculum of $10^6$ CFU/ml. These suspensions were inoculated with a Steers multipoint replicator onto the plate series, resulting in spot inocula of $10^4$ CFU. All strains were tested for beta-lactamase production by the chromogenic cephalosporin test (Nitrocephin, BBL, USA). After incubation for 18-20 h in a $CO_2$ incubator (5%) at 37°C the MIC was determined by observing the lowest concentration of antibiotics in which bacterial growth was completely or almost completely inhibited, as judged by the naked eye. A haze of growth or a single colony was disregarded.
Chlamydia trachomatis. Clinical isolates were passed on a HeLa 229 monolayer seven to nine times in order to increase the inoculum. Hundred $\mu$l of a test strain, containing two to nine inclusions per field (magnification x 320), was grown in a 96-well microtiter tissue culture plate (Falcon 3070, Becton Dickinson, USA). After centrifugation at 3000 x g for 1 h the inoculum was replaced by 100 $\mu$l of growth medium consisting of Eagle's modified Minimal

Essential Medium (Flow, UK) with 10% fetal calf serum and 4.5 mM glucose. Twofold dilutions of antibiotics were prepared in growth medium. After 48 h of incubation the monolayers were fixed and subsequently stained with fluorescing monoclonal antibodies (Syva, USA). The plate was turned upside down and the monolayers were screened for the presence of inclusions with a Leitz fluorescence microscope equipped with a long distance objective (32x). One row of wells contained growth medium with the maximal concentration of each antibiotic but no chlamydiae in order to detect a cytotoxic effect. No antibiotic was found to be cytotoxic in the maximal concentration tested. Another row of wells contained Chlamydia without antibiotics. Every dilution was tested in duplicate. The lowest concentration of antibiotics in which no inclusions could be observed was defined as the MIC. A duplicate plate was used for the subpassage. The monolayer was disrupted by a freeze-thaw procedure. After the second subpassage growth medium was applied with 25 $\mu$g/ml gentamicin, 25 $\mu$g/ml vancomycin and 25 U/ml nystatin. Six subpassages were carried out to determine the MBC. The MBC was defined as the lowest concentration of antibiotics in which no inclusions could be observed after the sixth subpassage.

Mycoplasma hominis. The MIC determinations were carried out in 12 x 8 well microtiter plates. The growth medium consisted of PPLO broth (Difco, USA) as described by Chanock (4), containing 0.05% thallium acetate and 0.2% arginine HCl. Twofold dilutions of antibiotics were prepared in growth medium. One well contained 200 $\mu$l of growth medium and 50 $\mu$l of Mycoplasma hominis suspension, resulting in an inoculum size of $3 \times 10^4$ to $1 \times 10^5$ CFU/ml. After inoculation the trays were carefully shaken for 30 min in a Titertek mixer and subsequently incubated for three days in a $CO_2$ incubator (5%) at 35°C. For the MBC determination 1 $\mu$l from each well was inoculated on PPLO agar of the same composition as described above but without thallium acetate. MICs were read after adding one to three drops of 0.0025% phenol red solution to each well. The lowest concentration in which no red-purple colour was developed was defined as the MIC. The MBC was defined as the lowest concentration in which no surviving organism could be detected, i.e. the lowest concentration causing at least 99% reduction of the inoculum in three days.

Ureaplasma urealyticum. Clinical isolates of Ureaplasma urealyticum were cultured on medium 1 consisting of 23.25 g trypic soy broth (Oxoid, UK) 20 ml of 1% $MnSO_4.4H_2O$, 110 ml yeast extract as described by Hers (5), 200 ml inactivated horse serum, 5 ml of 20% urea, 2.5 ml of 4% L-cysteine, 16 g agar and 700 ml distilled water. Isolates were transferred to medium 2 consisting

of 22.5 g tryptic soy broth, 12.5 ml of 0.2% phenol red, 30 ml yeast extract, 200 ml inactivated horse serum, 50 ml of 20% of urea and 750 ml distilled water. The isolates were kept alive by daily transfer in medium 2. As inocula for the MIC determinations, undiluted 18 h cultures in medium 2 were used. Twofold dilutions of the antibiotics were prepared in medium 1 and poured into petri dishes. Multipoint inoculation was carried out using a Steers replicator. Growth was visible on microscopic examination as tiny black colonies after 48 h of incubation under anaerobic conditions (95% $N_2$ + 5% $CO_2$) at 35°C. MICs were also determined on medium 2 with 8.5 g of agar. Growth was indicated by a colour change from orange to purple after 30 h of incubation at 35°C. The MICs determined by the two methods were virtually identical. The maximal difference were at most one dilution step.
*Gardnerella vaginalis*. The methods used were similar to those for *Neisseria gonorrhoeae*. Twofold dilutions of antibiotics were prepared in a medium consisting of Mueller-Hinton agar No. 2 (BBL, USA) with 5% fetal calf serum. The plates were incubated at 35°C in a $CO_2$ incubator (10%).

## RESULTS

No difference was observed between beta-lactamase-positive and beta-lactamase-negative *Neisseria gonorrhoeae* strains as regards susceptibility to the three quinolones tested. Ciprofloxacin displayed the highest activity, with a MIC90 of 0.008 μg/ml. The two new quinolones, A56619 and A56620, showed similar activity, with MIC90 values $\leq$ 0.03 μg/ml. For beta-lactamase-negative gonococci the MIC50 and MIC90 of penicillin G was 0.08 μg/ml and 1.28 μg/ml respectively. For 2 of 35 beta-lactamase-positive strains and 10 out of 65 beta-lactamase-negative strains the MIC of tetracycline was $\geq$ 4 μg/ml (Table 1). *Chlamydia trachomatis* appeared to be less susceptible to the quinolones than gonococci, MIC90 values being $\leq$ 1 μg/ml.
The lowest MIC90 value of the three quinolones tested, 0.5 μg/ml, was found in the case of A56619. The MBC90 values of the three quinolones were identical (2 μg/ml, Table 2). For almost all *Mycoplasma hominis* strains the MIC and MBC values were $\leq$1μg/ml and $\leq$ 2 μg/ml respectively. The MIC and MBC of ciprofloxacin for one strain was 2 μg/ml. For two strains the MBC of A56620 was $>$ 2 μg/ml, and for two others ciprofloxacin had the same value (Table 2). For 9 of 49 strains the MBC of tetracycline was $>$2 μg/ml. The MIC of tetracycline for two strains and the MBC for another strain was not determined.

The MIC50 and MIC90 values of all the agents tested except A56619 were two to four times higher for Ureaplasma urealyticum than for Mycoplasma hominis. The MIC of tetracycline was not evaluated for two Ureaplasma urealyticum strains.

In general, Gardnerella vaginalis demonstrated lower susceptibility than the other micro-organisms to the four agents tested. The MIC90 values of tetracycline, ciprofloxacin, A56619 and A56620 were $\geqslant$32, 4, 8 and 4 µg/ml respectively (Table 1).

DISCUSSION

Our study confirms a previous report in which a high activity of ciprofloxacin against both beta-lactamase-positive and beta-lactamase-negative gonococci was found (2). In that study an MIC90 of 0.002 µg/ml was reported, which is lower than our value of 0.008 µg/ml. The activity of penicillin G against the strains tested was similar. These findings suggest that ciprofloxacin could be of benefit in the therapy of gonococcal infections (6, 7, 8). This was recently confirmed in a study in 57 men who were cured of gonorrhoea after a single oral dose of 250 mg ciprofloxacin (9). In the present study the new 1-aryl-fluoroquinolones, A56619 and A56620, were also found to display high activity against Neisseria gonorrhoeae in vitro. Although human pharmacokinetic data on these quinolones have not yet been published, preliminary reports suggest that susceptible micro-organisms should include those for which MIC values are $\leqslant$ 4 µg/ml for A56619 and $\leqslant$ 2 µg/ml for A56620 (10). In such cases effective therapy of gonococcal infections might be expected.

The activity of ciprofloxacin against Chlamydia trachomatis, Mycoplasma hominis and Ureaplasma urealyticum was evaluated in previous studies and MIC values of $\leqslant$ 2 µg/ml, $\leqslant$ 0.5 µg/ml, and 2 µg/ml respectively were reported (1, 3). Our study showed similar results. A56619 was found to be more active against Ureaplasma urealyticum and Chlamydia trachomatis than the other quinolones.

Chlamydia trachomatis was susceptible to tetracycline, mean serum levels after current therapeutical dosages being 2-3 µg/ml. However, a relatively high MBC (2 µg/ml) was noted for one isolate. This may have been a slow-growing strain which was inhibited incompletely during the short incubation period (48 h) in a medium containing tetracycline, although the possibility of cross contamination cannot definitely be ruled out. For 9 of 49 Mycoplasma hominis strains

Table 1 MIC values of several antibiotics against Neisseria gonorrhoeae, Ureaplasma urealyticum and Gardnerella vaginalis (range of MIC values and MIC values required to inhibit up to 50% and 90% of the strains respectively).

| | | MIC (µg/ml) | | |
|---|---|---|---|---|
| Organism (n) | Agent | Range | MIC50 | MIC90 |
| Beta-lactamase- | tetracycline | 0.5 -4 | 1 | 2 |
| positive | cefuroxime | ≤0.015-1 | 0.12 | 0.5 |
| Neisseria | ciprofloxacin | 0.002-0.015 | 0.004 | 0.008 |
| gonorrhoeae | A56619 | 0.004-0.06 | 0.008 | 0.015 |
| (35) | A56620 | 0.004-0.015 | 0.004 | 0.015 |
| Beta-lactamase- | tetracycline | 0.12 -4 | 0.5 | 2 |
| negative | cefuroxime | ≤0.01 -1 | 0.03 | 0.25 |
| Neisseria | penicillin G | 0.01 -2.56 | 0.08 | 1.28 |
| gonorrhoeae | ciprofloxacin | 0.002-0.015 | 0.004 | 0.008 |
| (65) | A56619 | 0.004-0.06 | 0.015 | 0.03 |
| | A56620 | ≤0.001-0.03 | 0.008 | 0.015 |
| Ureaplasma | tetracycline | 0.25-≥16 | 1 | 2 |
| urealyticum | ciprofloxacin | 1 -4 | 2 | 4 |
| (28) | A56619 | 0.5 -1 | 1 | 1 |
| | A56620 | 1 -8 | 4 | 4 |
| Gardnerella | tetracycline | 0.25-≥32 | 8 | ≥32 |
| vaginalis | ciprofloxacin | 1 - 4 | 4 | 4 |
| (12) | A56619 | 1 - 8 | 8 | 8 |
| | A56620 | 1 - 8 | 4 | 4 |

the MBC of > 2 µg/ml. Tetracycline resistance of Mycoplasma hominis has been reported previously. A tetracycline MIC ≥ 16 µg/ml was observed in 27 (34%) of 79 isolates (11).

The clinical efficacy of the quinolones in therapy of genital infections caused by Chlamydia trachomatis, Mycoplasma hominis and Ureaplasma urealyticum has still to be determined. A single oral dose of 250 mg of ciprofloxacin has been shown to be insufficient in therapy of Chlamydia trachomatis infections, however, single dose therapy has never proved effective in eliminating this organism (9). Serum levels ≥2 µg/ml after well-tolerated oral ciprofloxacin dosages have been reported, but less is known about the

Table 2 MIC and MBC values of four antibiotics against _Chlamydia trachomatis_ and _Mycoplasma hominis_. (range of MBC or MIC values required to inhibit up to 50% and 90% of the strains respectively).

| Organism (n) | Agent | MIC (μg/ml) | | | MBC (μg/ml) | | |
|---|---|---|---|---|---|---|---|
| | | range | MIC50 | MIC90 | range | MBC50 | MIC90 |
| _C. trachomatis_ | tetracycline | 0.03 -0.25 | 0.06 | 0.25 | 0.03 - 2 | 0.125 | 0.25 |
| (12) | ciprofloxacin | 0.125-2 | 0.25 | 1 | 0.25 - 4 | 1 | 2 |
| | A56619 | 0.06 -0.5 | 0.25 | 0.5 | 0.125 - 2 | 0.5 | 2 |
| | A56620 | 0.125-2 | 0.5 | 1 | 0.125 - 2 | 0.5 | 2 |
| _M. hominis_ | tetracycline | 0.25-1 | 0.5 | 1 | 0.5 ->4 | 1 | >4 |
| (50) | ciprofloxacin | 0.5 -1 | 1 | 1 | 1 ->2 | 2 | 2 |
| | A56619 | 0.25-1 | 0.5 | 1 | 0.5 - 1 | 1 | 1 |
| | A56620 | 0.5 -1 | 1 | 1 | 1 ->2 | 2 | 2 |

tissue distribution of the drug in the urogenital tract (6, 7). Moreover, the interaction of the bacteria in the genital tract remains to be elucidated. After tetracycline therapy _Mycoplasma hominis_ but not _Ureaplasma urealyticum_ appeared to be eradicated, despite similarly low MICs of tetracycline (12). The low activity of the quinolones against _Gardnerella vaginalis_ does not necessarily mean poor activity in treatment of bacterial vaginosis, as this condition is mainly the result of anaerobic overgrowth (13). Metronidazole, which is moderately active against _Gardnerella vaginalis_ in vitro, is very useful in treating cases of vaginosis.

In summary, our study shows that A56619 and A56620 have high in vitro activity against gonococci. A beneficial therapeutic effect might thus be expected after a single oral dose in the case of gonococcal infections. As the in vitro activity of these agents against _Chlamydia trachomatis_, _Mycoplasma hominis_ and _Ureaplasma urealyticum_ is lower, higher dosages and prolonged therapy might be required in infections with these organisms.

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CHAPTER 9

## GENERAL DISCUSSION

The study of Chlamydia survival during transportation of patient material has shown an unacceptable decrease in yield of positive samples. If material is frozen for subsequent analysis a day after transportation at room temperature, the loss increases to 25%. Immediate processing of patient material is therefore of crucial importance for the ultimate sensitivity of culture results; in actual practice, however, this is rarely possible.
In view of this antigen-detecting methods seems an asset. Live Chlamydia are no longer required and consequently the transportation problem is avoided. Unfortunately, the rapid tests so far evaluated have been insufficiently sensitive. As might be expected, their sensitivity depends on the culture technique used as "golden standard". The microtitre technique without subpassage was considerably more sensitive than the traditional vial technique with subpassage. For an effective comparison of the test results from various research centres the use of the most sensitive culture technique, e.g. the microtitre method described, is a prerequisite. Nevertheless the use of these antigen-detecting methods may be of some value when logistics for handling patient material are poor and the culture technique used is only moderate. The result, moreover, is available within a few hours, and this compares favourably with the 48 hours required for the microtitre culture method. However, the sensitivity of the tests is remarkably low in asymptomatic cases, but precisely in these cases rapid, adequate diagnosis is needed to prevent dissemination of the infection. Research in the asymptomatic population should be part of the evaluation of these tests. The antigen-detecting techniques would be useful in centres where a good culture technique is available if they might be used as screening tests for chlamydial infections like the Gram stain is used in gonorrhoea.
The percentage of patients without signs of urethritis among chlamydia-positive men amounted to 27%. Moreover, the percentage chlamydial carriers among men without signs of urethritis appeared to be 12%. All these men belonged to so-called at risk groups. The by no means negligible presence of Chlamydia in semen samples to be used in AI (6%) was an unexpected finding. The institutes recruit donors mostly from low-risk groups, and the donors in

question had had no complaints suggestive of a urogenital infection before donating semen. Since the chlamydial culture from semen is of limited sensitivity, the 6% detected probably is a minimum value. The findings show that Chlamydia is capable of surviving a freezing procedure as used at AI institutes. In addition to Chlamydia, other micro-organisms associated with sexually transmitted diseases were found: M. hominis, U. urealyticum and cytomegalovirus. Although it is not known whether the presence of these micro-organisms did produce clinical symptoms in the recipient, it is justifiable to recommend that semen to be used in AI be tested more thoroughly for the presence of pathogens (3, 4).

After screening asymptomatic persons for the presence of Chlamydia, women with postinflammatory tubal infertility were studied. This condition can be regarded as the most serious complication of an ascending chlamydial infection. On the basis of the serology gonococci play a more prominent role in the aetiology of tubal pathology than Chlamydia. Antichlamydial antibodies were much more frequently found in women with tubal infertility studied elsewhere (1, 2, 5). Epidemiological differences in Chlamydia prevalence between the Netherlands and the USA might be of importance in this respect. A remarkably large percentage (30%) of women were unaware of having salpingitis in spite their tubal damage. Kosseim (2) reported an even larger percentage (60%). This is consistent with Weström's personal communication that only 15% of his patients with salpingitis showed the "classical" symptoms. A practical point that can be deduced from the infertility studies is the high predictive value of a positive gonococcal and/or chlamydial serology for the presence of tubal lesions.

Determinations of the MIC and MBC of tetracycline for C. trachomatis strains did not point to the existence of resistance to this widely used antibiotic. However, regular sensitivity determinations seems advisable, partly also in view of the frequent use of tetracycline in and outside outpatient clinics for venereal diseases. The newer 4-fluoroquinolones with a high tissue penetration might in future be important as monotherapy for mixed gonococcal-chlamydial infections.

Studies done so far show that methods to detect Chlamydia are far from optimal. The sensitive but time-consuming culture technique remains dependent on an adequate supply of live Chlamydia in patient material; on the other hand, the very rapid antigen-detecting techniques score poorly in the category most in need of good diagnostics: asymptomatic patients.

As culture methods are expensive, one may raise doubts about the necessity of screening every visitor of a venereal diseases outpatient clinic for chlamydial infections. The possibility of treating persons on clinical or epidemiological grounds only should also be considered, whereas screening may be restricted to asymptomatics (7). Cost-benefit studies are not in favour of screening every visitor (6).

Among Chlamydia carriers a large proportion of the patients is without signs of infection. Likewise, screening studies in both high- and low-risk groups reveal considerable percentages of chlamydial infections among men without signs of urethritis. Although Chlamydia does not give the impression of being aggressive the organism has proved capable of playing a role in the pathogenesis of postinflammatory tubal infertility.

Tetracycline remains the antibiotic of choice in the treatment of chlamydial infections; resistance against this agent has not so far been demonstrated in vitro.

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## SUMMARY

Cultures of patient material on continuous cell lines are regarded as the most sensitive aid in the diagnosis of chlamydial infections. Sampling technique, transport of material from ward to laboratory and the culture technique as such largely determine the ultimate quality. Chapter 3 shows that transfer of patient material under suboptimal conditions may cause a 25% loss of positive scores. Chapters 4 and 5 describe an improved culture technique on microtitre plates which is more sensitive than the traditional technique using vials and Giemsa stain. Two antigen-detecting methods for *Chlamydia* are evaluated in these two chapters. Live material is no longer required, and the problem of transporting patient material to the laboratory is thus avoided. These diagnostic tests, based either on the immunofluorescence technique or on the enzyme immunoassay, score very poorly in asymptomatic patients. Precisely for this category, however, there is an urgent need for a reliable, rapid diagnostic test to prevent epidemiological spreading of the infection.
The precentage of men without signs of urethritis among *Chlamydia* carriers amounts to 27%. The proportion of chlamydial infections among men without signs of urethritis appears to be 12%. These high percentages are also shown in a study described in chapter 6, in which *Chlamydia* were found in 6% of the semen samples to be used in AI. Unlike the population discussed in chapters 4 and 5, the donors were recruited from low-risk groups. Despite the not very aggressive features of *Chlamydia* indicated by such findings, this organism is associated with such serious conditions in women as salpingitis and subsequent tubal infertility. The role of *Chlamydia* in the pathogenesis of postinflammatory tubal infertility is supported by serological findings obtained in infertile women (chapter 7).
Chapter 8 presents and discusses MIC and MBC values of tetracycline and other agents for 22 chlamydial isolates. These findings show that no in vitro resistance to this antibiotic is demonstrable at this time.

# SAMENVATTING

De kweek van patientenmateriaal op continue cellijnen wordt beschouwd als de meest gevoelige diagnostiek voor Chlamydia-infekies. Afnametechniek, het transport van materiaal van kliniek naar laboratorium en de kweektechniek zelf bepalen in hoge mate de uiteindelijke kwaliteit. Uit hoofdstuk 3 wordt duidelijk, dat het transport van patientenmateriaal onder suboptimale condities een verlies van 25% aan positieve score kan teweegbrengen. In hoofdstuk 4 en 5 staat een verbeterde kweektechniek beschreven op microtiterplaatjes, die veel hoger scoort dan de traditionele methode met vials en kleuring volgens Giemsa. In die hoofdstukken worden twee antigeendetekterende methoden voor Chlamydia geëvalueerd. Levend materiaal is niet langer nodig, zodat het transportprobleem van patientenmonsters naar het laboratorium wordt omzeild. Deze diagnostica, gebaseerd op of de immunofluorescentiemethode of de enzyme immunoassay scoren zeer slecht bij asymptomatici. Juist bij hen bestaat behoefte aan een betrouwbaar diagnosticum om epidemiologische verspreiding van de infektie tegen te gaan.
Zowel het percentage mannen zonder urethritis onder chlamydiadragers als het percentage chlamydia-infekties onder mannen zonder urethritis is hoog: 27%, resp. 12%. Dit blijkt ook uit een onderzoek, beschreven in hoofdstuk 6, waarbij in 6% van de voor KID bestemde spermamonsters Chlamydia werd aangetroffen. De donoren behoorden, in tegenstelling tot de onderzochte populatie van hoofdstuk 4 en 5, juist niet tot de risicogroepen. Ondanks het weinig agressieve beeld, dat zulke bevindingen van Chlamydia oproept, wordt dit organisme ook geassocieerd met ernstige aandoeningen bij de vrouw als salpingitis en de daaropvolgende kans op tubaire infertiliteit. De rol van Chlamydia bij het ontstaan van postinflammatoire infertiliteit wordt gesteund door de resultaten van het serologisch onderzoek van infertiele vrouwen (hoofdstuk 7).
In hoofdstuk 8 worden voor 22 chlamydia-isolaten MIC and MBC waarden van o.a. tetracycline bepaald. Hieruit blijkt, voorlopig, nog geen in-vitro resistentie voor dit antibioticum aantoonbaar.

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## CURRICULUM VITAE

De schrijver van dit proefschrift werd in 1948 geboren te Djakarta, Indonesië. Na het doorlopen van de HBS-B opleiding te Bussum werd in 1965 begonnen met de studie chemische technologie aan de Technische Hogeschool te Delft. In 1972 werd deze afgesloten met het ingenieursexamen in de richting biochemie (Prof. drs. W. Berends). Aan de Medische Faculteit te Rotterdam werd aansluitend de studie in de geneeskunde gevolgd, waar in 1978 het artsexamen werd afgelegd. Gedurende de periode 1972-1979 was hij werkzaam als leraar schei- en natuurkunde aan HAVO en VWO, vervolgens aan de HBO-B opleiding biochemische richting van het Van Leeuwenhoek Instituut te Delft. De opleiding tot dermato-venereoloog werd in 1979 gestart in het Academisch Ziekenhuis te Rotterdam (Prof. dr. E. Stolz, Prof. dr. Th. van Joost). Binnen een samenwerkingsverband tussen de afdelingen Dermatovenereologie en Klinische Microbiologie en Antimicrobiële Therapie (Prof. dr. M.F. Michel) van de Erasmus Universiteit Rotterdam is het chlamydia-onderzoek ter hand genomen, hetgeen heeft geresulteerd in het onderhavige proefschrift. Sinds 1 april 1986 is hij werkzaam als dermatovenereoloog in het Reinier de Graaf Gasthuis te Delft.