CHLAMYDIA, PHYSICAL CHARACTERISTICS AND DIAGNOSTIC ASPECTS

CHLAMYDIA, PHYSICAL CHARACTERISTICS AND DIAGNOSTIC ASPECTS

CHLAMYDIAS, FYSISCHE KARAKTERISTIEKEN EN DIAGNOSTISCHE ASPECTEN

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. Dr. P.W.C. Akkermans M. Lit.
en volgens besluit van het College van Dekanen
De openbare verdediging zal plaatsvinden op
woensdag 17 november 1993 om 13.45 uur

door

Johannes Joseph Henricus Theunissen

geboren te Roggel

universiteits
DRUKKERIJ

Promotiecommissie:

Promotores	:	Prof. Dr M.F. Michel
	:	Prof. Dr E Stolz
Co-promotor	:	Dr. J.H.T. Wagenvoort
Overige leden	:	Prof. Dr J. Huisman
	:	Prof. Dr C.P.A. van Boven

The studies presented in this thesis were performed at the institute of Dermato-Venereology and at the institute of Clinical Microbiology and Antimicrobial Therapy, Medical Faculty, Erasmus University Rotterdam, The Netherlands.

Voor Doortje en Ruben die de
grootste offers hebben
moeten brengen

LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
CF	Complement Fixatie
CFU	Colony Forming Units
CPM	Counts Per Minutes
DFA	Direct Fluorescent Assay
DNA	Deoxyribonucleic acid
EBs	Elementary Bodies
ELFA	Enzyme Linked Fluorescent Assay
ELISA	Enzyme Linked Immunosorbent Assay
EMEM	Eagles Modification of Minimal Essential Medium
EMEMS	Eagles Modification of Minimal Essential Medium supplemented with glutamin, vitamins, gentamicin, vancomycin, amphotericin B and fetal calf serum
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
HBBS	Hanks Balanced Salt Solution
IFU	Inclusion Forming Units
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPA	Immunoperoxidase Assay
MIF	Microimmunofluorescentie
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
RBs	Reticulate Bodies
RH	Relative Humidity
RNA	Ribonucleic acid
SEM	Standard Error of the Mean
2-SP	PBS supplemented with glutamin, vitamins, gentamicin, vancomycin, amphotericin B, FCS and glucose
SPG	see 2-SP
STD	Sexually transmitted diseases

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

MICROBIOLOGY

Chlamydia are obligatory intracellular bacteria and together with the *Rickettsia* occupy a unique niche in the microbial world. For a long time, *Chlamydia* were classified as viruses because of their dependence on the host cell. Since *Chlamydia* contain RNA, DNA and ribosomes (1), a double cell wall (2) and are susceptible to antibiotics (1,2), these organisms were later classified as bacteria.

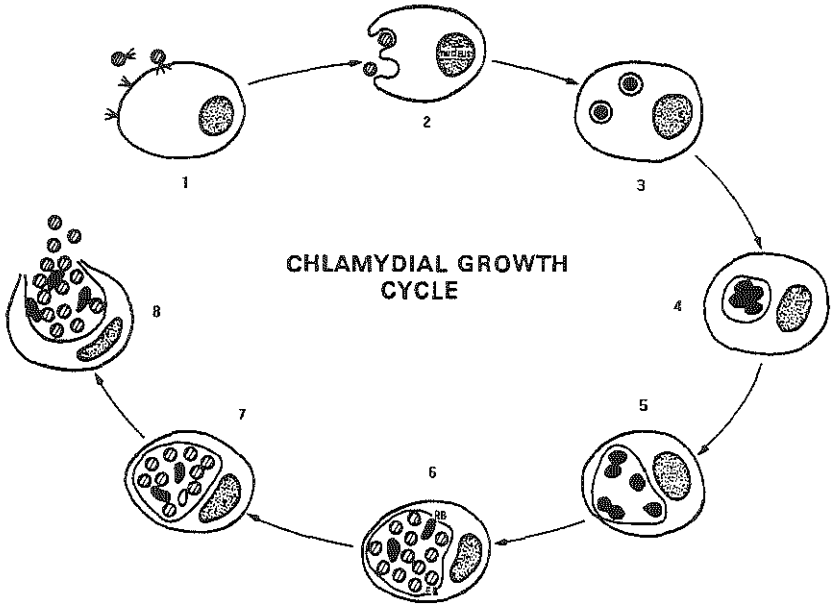

Figure 1. Chlamydial growth cycle*.
1. Elementary body (EB) attachment to host cell (0 hour). 2. Phagocytosis (0 h). 3. Reorganization of EB into reticulate body (RB); start of binary fission (8 h). 4. Continued multiplication; decline of host cell DNA synthesis (12-24 h). 5. Visible Vacuolar appearance of inclusion (24-30 h). 6. Reorganization of RBs to EBs; inclusion contains RBs and EBs (30 h). 7. Infectivity increases; inclusion contains mostly EBs (40 h). 8. Host cell lysis; release of EBs (48-72 h).
*Adapted from Thompson (17)

The growth cycle of *Chlamydia* is shown in Figure 1.The organism has two forms, namely the elementary body (EB) which is 0.3 μm and the reticulate body (RB) which is 0.9 μm. The infectious, non-dividing, metabolically inactive elementary body (EB) can bind to the host cell. According to the presently elucidated mechanism, the EB is then taken up into the host cell via endocytosis (3,4). In the cytoplasm, the EB is surrounded by a membrane derived from the host

cell, but which has been modified considerably by the EB (5). Subsequent fusion with phagosome/lysosome is avoided via a mechanism which is not fully understood at present and the EB then reorganizes into the reticulate body (RB). This RB is strictly intracellular, metabolically active and is able to multiply via binary fission. The fissions occurs in the original phagosome which enlarges during this process and looks like a vacuole in light microscopy. This vacuole is referred to as an inclusion body or in short, an inclusion. During the process of cell division, the RB obtains amino acids, sugars and particularly adenosine triphosphate (ATP) from the host cell. *Chlamydia* are unable to synthesize ATP and therefore depend on the host cell for their energy requirements (6). After 48-72 h, following a large number of fissions, the RBs condense to form EBs and the host cell is lysed. The EBs that are released can then infect new host cells.

CHLAMYDIA SPECIES

At present, the genus chlamydia comprises three species. These are *Chlamydia trachomatis, Chlamydia pneumoniae* and *Chlamydia psittaci*.
To date, 19 different types of *C. trachomatis* are known. Initially, these types were distinguished from each other on the basis of antibodies that were formed in mice after they had been infected with a single type chlamydia (7). The sera of the mice were then investigated for possible cross-reactions with other type of *Chlamydia* using the microimmuunfluorescence assay (MIF). The strength of the (cross)-reactions also influences the identification. Based on this serological distinction, the name serotype or serovar is, therefore, used instead of the type.

C. pneumoniae is a recently discovered species within the genus *Chlamydia* (8). Although, originally the organism was classified as *C. psittaci* on the basis of the form of the inclusion, the differences between *C. trachomatis* and *C. psittaci*, summarized in Table I, clearly indicate that *C. trachomatis* is a separate species. To date, only one serovar of *C. pneumoniae* species is known.

Many different serovars of the species *C. psittaci* are known. Under normal circumstances *C. psittaci* is an animal pathogen. The microbiological differences between the three chlamydia species are summarized in Table I.

CLINICAL SPECTRUM

All *C. trachomatis* serovars are human pathogens, with the exception of mouse pneumonitis serovar. The serovars A, B, Ba and C almost exclusively cause trachoma, the most common preventable form of blindness. In certain cases, a serovar B was isolated from patients with a genital affection (7,10). The serovars

Table I. Some characteristics of *Chlamydia* species.*

Characteristics	Species		
	C. trachomatis	*C. pneumoniae*	*C. psittaci*
Natural host	Human (Mouse)	Human	Birds, Mammals
DNA homology (ref. *C. pneumoniae*)	10	100	10
Mol% G+C of DNA	41/42	40	41
Plasmid	Yes	No	Yes (rare no)
No. of serovars	19	1	Unknown (many)
Inclusion morphology	Oval, vacuolar	Oval, dense	Variable, dense
Glycogen in inclusion	Yes	No	No
Folate biosynthesis	Yes	No	No

*Adapted from Grayston (9).

Table II. Clinical spectrum of human *C. trachomatis* infections*.

Serotype	Host	Infection	Complication
LGV_1, LGV_2, LGV_3	Women Men	Lymphogranuloma venereum	Vulvar/rectal carcinoma Rectal strictures
A, B, Ba, C	Women, Men, Child	Trachoma	Blindness
D-K	Women	Cervicitis Urethritis Conjunctivitis	Salpingitis Perihepatitis
D-K	Men	Urethritis Post-gonococcal urethritis Conjunctivitis	Epididymitis Prostatitis Reiter's syndrome
D-K	Infants	Conjunctivitis Pneumoniae	

*Adapted from Thompson et al (17).

LGV_1, LGV_2 and LGV_3 are responsible for the lymphogranuloma venereum syndrome. Since these 3 serovars grow easily and rapidly in tissue culture, they are frequently used as experimental models. Infections with the serovars D-K are the cause of the most commonly occurring sexually transmitted diseases. In 30% of the cases, such infections follow an asymptomatic course (11-13) which contributes significantly towards the spread of the infections. In the majority of the patients, the serovars D-K cause symptomatic uncomplicated infections such as urethritis or post-gonococcal urethritis in men and urethritis and/or cervicitis in women (14). In certain cases, advanced stages of uncomplicated *C. trachomatis* infection may cause complications such as epididymitis (15) or pelvic inflammatory disease (PID) (16). These complications are important because they can lead to infertility. The most important infections that are caused by *C. trachomatis* are summarized in Table II.

Chlamydia pneumoniae causes respiratory affections varying in severity from bronchitis to atypical pneumonia. The respiratory affections caused by *C. pneumoniae* in relation to the age of the infected patient were reported by Grayston (9). The most important conclusion was that the course of the disease became more severe as the age of the patient increased. Initially, it was assumed that about 6% of all acute pneumonia in the U.S.A. were caused by *C. pneumoniae* (18). Later, it appeared that the period during which the investigations were conducted coincided with an epidemiological out-burst in *C. pneumoniae* infections in the Scandinavian countries (19). In the U.S.A. too, in the early 1980s, there was probably an epidemiological out-burst in *C. pneumoniae* infections (40), so that the figure for pneumonia caused by *C. pneumoniae* after 1985 was probably in the vicinity of 1%. Recently, a relationship between *C. pneumoniae* infection on the one hand and asthma (20), endocarditis (21) and coronary heart disease (22) on the other hand has been established.

C. psittaci can cause numerous infections in animals. Among others, psittacosis (parrot's-disease) and a form of spontaneous abortions in sheep are well known. Transmission of *C. psittaci* from infected animals to humans is possible. Although, in some cases an extremely severe form of pneumonia may result after infection with *C. psittaci*, the organism is not commonly encountered in humane infection. A review of the infections caused by *C. psittaci* in humans was published by Storz et al (23).

DIAGNOSTICS

A number of methods are available for detecting *C. trachomatis* infections. The method most commonly used is the isolation of *C. trachomatis* in the tissue culture. This method is the currently valid 'golden standard' and all the other methods must

be compared with it. The working principle of the method rests on taking a smear from the cervix and the urethra using a chlamydia cotton swab attached to a metal holder. The swab is then transported to the laboratory in 2-SP medium (24). In the laboratory the *Chlamydia* are cultured on HeLa-229 or McCoy cells which are incubated for 3 days at 37°C in an atmosphere containing 5% CO_2 (25). Pretreatment of HeLa-229 and McCoy cells with DEAE-dextran and cycloheximide is essential for optimum harvest (26,27). DEAE-dextran is used to charge the host cells positively. This allows the negatively charged EBs to bind easily to the host cells. Cycloheximide inhibits protein synthesis in eukaryotic cells. In the presence of cycloheximide, the amino acids and sugars present in the host cells are almost exclusively available for the growth of *Chlamydia*.

Two of the oldest non-tissue culture methods for detecting *C. trachomatis* are the direct immunofluorescence assay (DFA) and the Chlamydiazyme test. In the

Table III. Comparison of the DIF and the tissue culture for the detection of cervical or urethral *C. trachomatis* infections*.

Study no.	Man(M)/ Women(W)	No. of patients	Prevalence	Sensitivity (%)	Specificity (%)
1	W	715	5	76	76
2	W	253	27	79	88
3	W	1494	13	73	95
4	W	527	4	70	62
5	W	249	9	100	96
6	W	642	7	60	74
7	W	968	13	76	65
8	W	1004	14	90	93
9	W	301	11	56	56
10	W	303	23	96	91
11	M	536	20	78	95
12	M	236	11	100	82
13	M	576	20	92	86
14	M	623	22	90	89
15	M	200	17	74	98

*Adapted from Barnes (28).

DFA, a direct smear of the swab taken from the patient is prepared on a glass slide. After fixing, the slide is stained with fluorescein isothiocyanate-labeled monoclonal antibody directed against the major outer membrane protein of *C. trachomatis*. The slides are then examined using a fluorescence microscope. In the Chlamydiazyme test, the *Chlamydia* present in the sample taken from the patient are allowed to react with the anti-chlamydia monoclonal antibody coated onto beads. Successive incubations are then carried out with rabbit anti-chlamydia IgG and horseradish peroxidase-labeled goat anti-rabbit IgG. After incubation with a substrate, the developed reaction is read using a spectrophotometer. A comparison of the results obtained using these two tests and those obtained using tissue culture are shown in Tables III and IV. The sensitivity and the specificity of the DFA was generally higher than that of the Chlamydiazyme. The performance of both the tests increased with an increase in the prevalence of *C. trachomatis* in the population that was investigated (28). Other tests for the detection of *C. trachomatis* are the gene-probe and the polymerase chain reaction (PCR). In de gene-probe test, a labeled-DNA sequence is hybridized with *C. trachomatis* specific rRNA. The DNA-RNA complex is then isolated from the rest of the sample and the amount of label is determined. The sensitivity and the specificity of the gene-probe test as

Table IV. Comparison of the Chlamydiazyme and the tissue culture for the detection of cervical or urethral *C. trachomatis* infections*.

Study no.	Man(M)/ Women(W)	No. of patients	Prevalence	Sensitivity (%)	Specificity (%)
1	W	715	5	78	57
2	W	1489	13	83	84
3	W	255	21	96	79
4	W	1059	10	86	39
5	W	299	16	100	72
6	W	484	6	89	45
7	W	514	7	89	72
8	M	336	20	83	76
9	M	1011	20	86	80
10	M	403	19	70	91

*Adapted from Barnes (28).

compared with that of tissue culture were 70.6% & 92.7% respectively in men and

98.2% & 97.7% respectively in women (29). In the PCR, DNA is first isolated from the patient sample after which a *C. trachomatis*-specific DNA sequence is amplified and is made visible in the last step either on a gel or in a blot. Results that were obtained using the PCR to detect uncomplicated *C. trachomatis* infections are summarized in Table V. The sensitivity and the specificity were either comparable with or better than those obtained using the tissue culture (30-32).

Table V. Comparison of the PCR and the tissue culture for the detection of cervical or urethral *C. trachomatis* infections.

Reference no.	Men(M)/ Women(W)	No. of patients	Prevalence (%)	Sensitivity (%)	Specificity (%)
30	M/W	156	16.7	107	98.5
31	M/W	220	21.8	104	98.8
32	M/W	215	11.2	100	93.0

Another approach for establishing acute infections is the detection of antibodies directed against *C. trachomatis*. The presence of an IgM titer and/or a highly increased IgG titer and/or a four-fold seroconversion in IgM, IgG or IgA titer in the patient sera indicate a recent infection. The oldest and at the same time also the reference method for detecting antibodies directed against *C. trachomatis* is the microimmuunofluorescence (MIF) assay developed by Wang and Grayston (8,33). One of the disadvantages of the MIF is that 12 different antigens must be used because in this test system, different *C. trachomatis* serovars show individual characteristic reactions. Another disadvantage of the MIF is the subjective interpretation which is inherent to fluorescence microscopy. During the past years, different attempts based on enzyme linked immunosorbent assay (ELISA) or immunoperoxidase assay (IPA) have been made in order to overcome the disadvantages of the MIF (34-36). In general, in these enzyme reactions based and thus objective tests, *C. trachomatis* LGV_2 was used as the sole antigen. Views on the use of MIF, ELISA or IPA for detecting acute infections vary considerably. Some authors reported that there was a good correlation between serology and the tissue culture (33,34,36,37), whereas others reported that the correlation was poor (35,38).

The detection of an acute infection caused by *C. pneumoniae* using the MIF is based on serology (39). Tissue culture has several limitations for detecting *C. pneumoniae*. The growth of *C. pneumoniae* in cell lines is slower than of *C. trachomatis*. The initial isolation from patient samples is also very laborious due to the low infectivity of cell line unadapted *C. pneumoniae*. In addition, as compared with *C. trachomatis*, taking of samples is difficult because the infected region is not

always easily accessible. In some studies, a good correlation between the two was observed in those cases in which both the results of the serology and the tissue culture were available (40,41), whereas a poor correlation was reported in another study (42). A possible alternative for detecting *C. pneumoniae* in patient samples is the PCR. To date, the data available on the comparison of the sensitivity and the specificity of the PCR with that of the MIF or the tissue culture in patients with respiratory affections are rather limited. Therefore, it is not possible to evaluate the value of the PCR as a diagnostic tool.

Complement fixation (CF) is mostly used for detecting *C. psittaci.* One of its disadvantages is the low sensitivity. An additional objection is that antibodies directed against the lipopolysaccharide (LPS) of *C. psittaci* are detected in the CF. The LPS in all *Chlamydia* species is the same. The prevalence of IgG antibodies directed against *C. trachomatis* is about 3% and that against *C. pneumoniae* is about 50%. Since in humans infections caused by *C. psittaci* are rare, the presence of an antibody titer observed in the CF is mostly due to an infection with *C. pneumoniae* or *C. trachomatis*.

AIM OF THE STUDY

The investigations described in this thesis had several aims. As discussed in the diagnostics section, the detection of antibodies directed against *C. trachomatis* using the MIF is not only laborious, but the interpretation of the results is also subjective. The main objection against using an ELISA or an IPA in which serovar LGV_2 is used as the sole antigen is that antibodies directed against other serovars are either not detected at all or detected unsatisfactorily (34). In the literature too, there is no general consensus on the sensitivity and the specificity of serology as compared with that of the tissue culture for detecting acute *C. trachomatis* infections.

In order to overcome these objections, we developed a sensitive enzyme linked immunofluorescent assay (ELFA). Antibodies directed against 6 different antigens of *C. trachomatis* can be detected using the ELFA. The antigens are chosen in such a way that antibodies against all *C. trachomatis* serovars can be detected. The contribution made by serology in the diagnostics of acute *Chlamydia* infection was investigated in two groups of patients. Initially, a group of men and women with uncomplicated infections who visited the outpatient department of the clinic for sexually transmitted diseases in Rotterdam was investigated in order to determine whether there was any correlation between the results of serology and those of the tissue culture. Later, a group of patients with complicated infections was also investigated. This group consisted of patients suffering from pelvic inflammatory disease who had been admitted to the department of Gynecology of

the Dijkzigt Hospital in Rotterdam. It was also determined whether there was any correlation between the results of serology and those of the tissue culture and the polymerase chain reaction (PCR).

Little is known on the manner in which *C. pneumoniae* is transmitted from one host to another. A possible clue for transmission occurring via aerosols was reported by Hyman et al (42). He deduced this from the *C. pneumoniae* infections acquired by the laboratory personnel after an accident involving a centrifuge. Undoubtedly, an aqueous intermediate also influences the survival of *C. pneumoniae* during transmission. The spread of the organism must occur very efficiently as illustrated by the fact that about 50% of the population in the industrialized world has already acquired *C.pneumoniae* infection between the age of 5 and 15 years (39). Therefore, the aim of the subsequent studies was to investigate the influence of pH, ion concentrations and temperature on the survival of *C. pneumoniae*. The survival of *C. pneumoniae* in aerosols under various conditions was also investigated using a 114.5 L stainless steel aerosol chamber in which both the relative humidity and the temperature could be carefully controlled. The survival of other microorganisms including *C. trachomatis* in aerosols was also investigated in order to determine whether there was any correlation with the survival of *C. pneumoniae* under the same conditions.

C. trachomatis can be stored best for prolonged periods in SPG medium at a minimum temperature of -70°C (43). Optimum survival of *C. pneumoniae* can be achieved by cooling it at 4°C for 4 h prior to freezing (44). However, this method is cumbersome and expensive for transport and culture collections. A large number of pathogenic bacteria can be stored for a long time with a negligible loss in viability by lyophilization in a suitable medium (45,46). Consequently, investigations into the optimum media for lyophilization and different methods of freezing that may be suitable for the transport and storage of *Chlamydia* (chapter 6) were also pursued.

REFERENCES

1. Moulder JW. The relation of the psittacosis group (*Chlamydia*) to bacteria and viruses. *Ann Rev Microbiol* 1966;**20**:107-130.
2. Manire GP, Tamura A. Preparation and chemical composition of the cell walls of mature infectious dense forms of meningopneumonitis organisms. *J Bacteriol* 1967;**94**:1178-1183.
3. Lawn AM, Blyth WA, Taverne J. Interactions of TRIC agents with macrophages and BHK-21 cells observed by electron microscopy. *J Hyg (Lond)* 1973;**71**:515-528.
4. Hodinka RL, Wyrick PB. Ultrastructural mode of entry of *Chlamydia psittaci* into L-929 cells. *Infect Immun* 1987;**54**:855-863.
5. Zeichner SL. Isolation and characterization of macrophage phagosomes containing infectious and heat inactivated *Chlamydia psittaci*: Two phagosomes with different intracellular

behaviors. *Infect Immun* 1983;**40**:956-966.

6. Bavoil P, Ohlin A, Schachter J. Role of disulfide bonding in the outer membrane structure and permeability in *Chlamydia trachomatis*. *Infect Immun* 1984;**44**:479-485.
7. Wang SP, Grayston JT. Immunological relationship between genital TRIC, lymphogranuloma venereum and related organisms in the new microtiter indirect immunofluorescent test. *Am J Ophtalmol* 1970:**70**:367-374.
8. Grayston JT, Kuo CC, Wang SP, Altman J. A new *Chlamydia psittaci* strain called TWAR from acute respiratory tract infections. *N Engl J Med* 1986;**315**:161-168.
9. Grayston JT. *Chlamydia pneumoniae*, strain TWAR. *Chest* 1989;**95**:664-669.
10. Wagenvoort JHT, Suchland RJ, Kluytmans JAJW, Stamm WE. Detection of different *Chlamydia trachomatis* serovars of urogenital origin by enzyme-immunoassay. *Eur J Microbiol Infect Dis* 1989;**6**:319-320.
11. McCormack WM, Alpert S, McComb DE, Nichols RL, Semine DZ, Zinner SH. Fifteen month follow-up of women infected with *Chlamydia trachomatis*. *N Engl J Med* 1979;**300**:123-125.
12. Podgore JK, Holmes KK, Alexander ER. Asymptomatic urethral infections due to *Chlamydia trachomatis* in male U.S. military personnel. *J Infect Dis* 1982;**146**:828.
13. Karam GH, Martin DH, Flotte TR, Bonnarens FO, Joseph JR, Mroczkowsky TF, Johnson WD. Asymptomatic *Chlamydia trachomatis* infections among sexually active men. *J Infect Dis* 1986;**154**:900-903.
14. Schachter J, Grossman M. Chlamydial infections. *Ann Rev Med* 1981;**32**:46-61.
15. Berger RE, Alexander ER, Harnish JP, Paulsen CA, Monda GD, Ansell J, Holmes KK. Etiologies and therapy of acute epididymitis. Prospective study of 50 cases. *J Urol* 1979;**121**:750-754.
16. Mårdh PA, Ripa T, Svensson L, Weström L. *Chlamydia trachomatis* infections in patient with acute salpingitis. *N Engl J Med* 1977;**269**:1377-1379.
17. Thompson SE, Washington AE. Epidemiology of sexually transmitted *Chlamydia trachomatis* infections. *Epidemiol Rev* 1983;**5**:96-123.
18. Marrie TJ, Grayston JT, Wang SP, Kuo CC. Pneumoniae associated with the TWAR strain of Chlamydia. *Ann Intern Med* 1987;**106**:507-511.
19. Persson K. Epidemiological and clinical aspects of infections due to *Chlamydia pneumoniae* (strain TWAR). *Scand J Infect Dis* 1990;**69**:63-67.
20. Hahn DL, Dodge RW, Golubjadnikov R. Association of *Chlamydia pneumoniae* (strain TWAR) infection with wheezing, asthmatic bronchitis and adult-onset asthma. *JAMA* 1991;**266**:225-230.
21. Marrie TJ, Harczy M, Mann OE, Landymore RW, Raza A, Wang SP, Grayston JT. Culture negative endocarditis probably due to *Chlamydia pneumoniae*. J Infect Dis 1990;**161**:127-129.
22. Saikku P, Leinonen M, Tenkanen L, Linnanmaki E, Ekman MR, Manninen V, Mänttäki M, Frick MH, Huttunen JK. Chronic *Chlamydia pneumoniae* infection as a risk factor for coronary heart disease in the Helsinki heart study. *Ann Intern Med* 1992;**116**:273-278.
23. Storz J. *Chlamydia psittaci*: Diversity and emerging immunological classification. *In:* Bowie WR, Caldwell HD, Jones RP, Mårdh PA, Ridgway GL, Schachter J, Stamm WE, Ward ME (eds). *Chlamydial infections*. Cambridge: Cambridge University Press, 1990:363-373.
24. Tjiam KH, van Heijst BYM, de Roo JC, de Beer A, van Joost Th, Michel MF, Stolz E. Survival of *Chlamydia trachomatis* in different transport media and at different temperatures: diagnostic implications. *Br J Ven Dis* 1984;**60**:92-94.

25. Thewessen EPAM, Freundt I, van Rijsoort-Vos JH, Stolz E, Michel MF, Wagenvoort JHT. Comparison of HeLa-229 and McCoy cells for the detection of *Chlamydia trachomatis* in clinical specimens. *J Clin Microbiol* 1989;**27**:1399-1400.
26. Ripa KT, Mårdh PA. Cultivation of *Chlamydia trachomatis* in cycloheximide treated McCoy cells. *J Clin Microbiol* 1977;**6**:328-331.
27. Darougar S, Treharne JD. Cell culture methods for the isolation of *C. trachomatis*-A review. *In:* Mårdh PA, Holmes KK, Oriel JD, Piot S, Schachter J eds. *Chlamydial infections*, **Vol 2.** Amsterdam: Elsevier Biomedical Press, 1982:265-274.
28. Barnes RC. Laboratory diagnosis of human chlamydia infections. *Clin Microbiol Rev* 1989;**2**:119-136.
29. Kluytmans JAJW, Niesters HGM, Mouton JW, Quint WGV, Ijpelaar JAJ, van Rijsoort-Vos JH, Habbema L, Stolz E, Michel MF, Wagenvoort JHT. Performance of a non-isotopic DNA probe for detection of *Chlamydia trachomatis* in urogenital specimens. *J Clin Microbiol* 1991;**29**:2685-2689.
30. Claas HJC, Wagenvoort JHT, Niesters HGM, Tio TT, Van Rijsoort-Vos JH, Quint WGV. Diagnostic value of the polymerase chain reaction for *Chlamydia* as determined in a follow-up study. *J Clin Microbiol* 1991;**29**:42-45.
31. Ossewaarde JM, Rieffe M, Rozenberg-Arska M, Ossenkoppele PM, Nawrocki RP, van Loon AM. Development and clinical evaluation of a polymerse chain reaction for detection of *Chlamydia trachomatis*. *In:* Mårdh PA, La Placa M, Ward ME, eds. *Proceedings of the European Society for Chlamydia Research*, **Vol 2.** Bologna: Societa Editrice Esculapia, 1992:235.
32. Østergaard L, Birkelund S, Christiansen G. Use of polymerase chain reaction for detection of *Chlamydia trachomatis*. *J Clin Microbiol* 1990;**28**:1254-1260.
33. Wang SP, Grayston JT. Microimmunofluorescence antibody responses in *Chlamydia trachomatis* infections. *In:* Mårdh PA, Holmes KK, Oriel JD, Piot S, Schachter J (eds). *Chlamydial infections*, **Vol 2.** Amsterdam: Elsevier Biomedical Press, 1982:301-316.
34. Mahoney JB, Schachter J, Chernesky MA. Detection of antichlamydial immunoglobulin G and M antibodies by enzyme linked immunosorbent assay. *J Clin Microbiol* 1983;**18**:270-275.
35. Schoenwald E, Schmidt BL, Steinmetz G, Hosmann J, Pohla-Gubo G, Luger A, Gasser G. Diagnosis of *Chlamydia trachomatis* infection. Culture versus serology. *Eur J Epidemiol* 1988;**4**:75-82.
36. Hagay ZJ, Sarov B, Sachs J, Shaked O, Sarov I. Detecting *Chlamydia trachomatis* in men with urethritis: serology versus isolation in cell culture. *Genitourin Med* 1989;**65**:166-170.
37. Evans RT, Taylor-Robinson D. Development and evaluation of an enzyme linked immunosorbent assay (ELISA), using chlamydial group antigen to detect antibodies to *Chlamydia trachomatis*. *J Clin Pathol* 1982;**35**:1122-1128.
38. Schachter J, Cles L, Ray R, Hines PA. Failure of serology in diagnosing chlamydial infections in the female genital tract. *J Clin Microbiol* 1979;**10**:647-649.
39. Grayston JT, Wang SP, Kuo CC, Campbell CA. Current knowledge of *Chlamydia pneumoniae*, strain TWAR, an important cause of pneumonia and other acute respiratory diseases. *Eur J Clin Microbiol Infect Dis* 1989;**8**:191-202.
40. Thom DH, Grayston JT, Wang SP, Kuo CC, Altman J. *Chlamydia pneumoniae* strain TWAR, *Mycoplasma pneumoniae* and viral infections in a university student health clinic population. *Am J Epidemiol* 1990;**132**:248-256.
41. Hammerschlag MR, Chirgwin K, Roblin PM, Gelling M, Dumornay W, Mandel L, Smith P,

Schachter J. Persistent infection with *Chlamydia pneumoniae* following acute respiratory illness. *Clin Infect Dis* 1992;**14**:178-182.

42. Hyman CC, Augenbraun MH, Roblin PM, Schachter J, Hammerschlag MR. Asymptomatic respiratory tract infection with *Chlamydia pneumoniae* TWAR. *J Clin Microbiol* 1991;**29**:2082-2083.
43. Mahoney JB, Chernesky MA. Effect of swab type and storage temperature on the isolation of *Chlamydia trachomatis* from clinical specimens. *J Clin Microbiol* 1985;**22**:865-867.
44. Kuo CC, Grayston JT. Factors affecting viability and growth in HeLa-229 cells of *Chlamydia pneumoniae* sp. strain TWAR. *J Clin Microbiol* 1988;**26**:812-815.
45. Lapage SP, Shelton JE, Mitchell TG. Media for the maintenance and preservation of bacteria. *In:* Norris JR, Ribbons DW (eds). *Methods in Microbiology*, **Vol 3.** London: Academic Press London and New York, 1970:1-134.
46. Lapage SP, Shelton JE, Mitchell TG, MacKenzie AR. Culture collections and the preservation of bacteria. *In:* Norris JR, Ribbons DW eds. *Methods in Microbiology*, **Vol 3.** London: Academic Press London and New York, 1970:135-228.

CHAPTER 2

DETECTION OF IgG, IgM AND IgA ANTIBODIES IN PATIENTS WITH UNCOMPLICATED *CHLAMYDIA TRACHOMATIS* INFECTION: A COMPARISON BETWEEN ENZYME LINKED IMMUNOFLUORESCENT ASSAY AND ISOLATION IN CELL CULTURE

J.J.H. Theunissen[1], B.Y.M. van Heijst[2], R.A.M. Chin-A-Lien[1], J.H.T. Wagenvoort[2], E. Stolz[1], M.F. Michel[2].

Departments of Dermato-Venereology[1] and Clinical Microbiology[2], Erasmus University, Rotterdam, the Netherlands.

Published in International Journal of *STD & AIDS* 1993;4:43-48.

SUMMARY

The diagnostic value of serum IgG, IgM and IgA in patients with uncomplicated urogenital *Chlamydia trachomatis* infection was compared with isolation in cell culture. *C. trachomatis* specific antibodies were determined with an Enzyme Linked Immunofluorescent Assay using elementary bodies from *C. trachomatis* serotypes E,F,H,I,J and LGV_2 as antigens. At least two sera from each patient were tested and cultures were also established on the same day. Excluding the IgM titers in men, significantly more IgG,IgA and IgM and combinations of these antibodies were observed in culture positive patients. The sensitivity with which IgG titers in men or IgG and/or IgM titers in men and women could be determined, was significantly lower using *C. trachomatis* LGV_2 as the only antigen than when all six antigens were used. The presence of ten or more leucocytes in the urine sediment of men correlated positively with an IgG or an IgG and/or IgM titer.

INTRODUCTION

At present, *Chlamydia trachomatis* infection is one of the commonest sexually transmitted diseases (STD). *C. trachomatis* infection can cause cervicitis, urethritis, salpingitis and conjunctivitis[1]. Tissue culture is mainly used to detect *C. trachomatis* infections and to isolate the microbe. However, tissue culture is only possible at centers where the specialized facilities and expertize are available.

The microimmunofluorescence method (MIF), developed by Wang and Grayston[2,3] is considered to be sensitive and specific for the detection of serum antibodies against *C. trachomatis*. The necessity to use 12 different antigens in the MIF and the interpretation with the aid of fluorescent microscopy renders the MIF unsuitable for screening large numbers of sera from patients.

In order to circumvent the disadvantages of the MIF, several serological tests for detecting IgG/IgM antibodies in which a single broadly reactive serotype (usually LGV_2) is used in an enzyme linked immunosorbent assay (ELISA) have been described[4,5,6]. An immunoperoxidase assay (IPA) is mainly used for the detection of IgA (mostly in combination with IgG) antibodies in patients with uncomplicated *C. trachomatis* infection[7,8]. In the IPA also, *C. trachomatis* LGV_2 is the only antigen that is used. The use of only a single antigen brings with it the danger that infections caused by other serotypes may not be detected serologically[3,5].

Several years ago, an enzyme linked immunofluorescent assay (ELFA) with a higher sensitivity than of the ELISA has been reported[9]. We have developed an ELFA in which the *C. trachomatis* serotypes E,F,H,I,J and LGV_2 were used as antigens. The six serotypes were chosen to enable the detection of antibodies against all *C. trachomatis* serovars[3]. Using this ELFA, titers of IgG, IgM, and IgA

antibodies against *C. trachomatis* were determined in patients with a high prevalence of *C. trachomatis* infections. In addition, the results obtained using the six antigens were compared with those obtained using LGV_2 as the only antigen.

Since cross-reactivity in ELISA reactions between the antibody response to *C. trachomatis* and *C. pneumoniae* have been described[10], the IgG response of 54 and the IgM response of 42 sera against *C. pneumoniae* antigen was determined and correlated with ELFA titers. Finally, ELFA and microimmunofluorescence (MIF) were compared using 51 sera and *C. trachomatis* E as only antigen.

MATERIALS AND METHODS

Patients

The patient group comprised 140 women and 207 men who attended the clinic for sexually transmitted diseases in Rotterdam. At least two sera were obtained from each patient at approximately ten days interval. On the same day that serum was obtained, the patients were also examined for the presence of other sexually transmitted diseases and *C. trachomatis* cultures were established. Further, the numbers of leucocytes in cervical gram preparations from women and in the urine sediment of men were determined. Ten or more leucocytes at a magnification of x1000 in the cervical gram preparation and ten or more leucocytes at a magnification of x250 in the urine sediment were regarded as positive. Presense of ten or more leucocytes in the urine sediment of men, in the absence of gonococci, was clinically diagnosed as urethritis.

Chlamydia Strains and Antigen Purification

C. trachomatis serotypes E (strain UW-5/Cx), F (strain IC-Cal-3), H (strain UW-4/Cx), I (strain UW-12/Ur), J (strain UW-36/Cx), LGV_2 (strain 343/Bu) were cultured in cycloheximide treated McCoy cells as described by Ripa and Mårdh[11]. After culturing for 48-72 h, the cells were sonicated and the chlamydia elementary bodies (EBs) were partially purified by differential centrifugation at 500 x g for ten min and at 30000 x g for 30 min and centrifuged at 53000 x g for 1 h through a 30% urografine solution (3.9 g/100 ml sodium amidotrizoate, 26 g/100 ml meglumine amidotrizoate, 146 mg/100 ml Iodine). Chlamydia that were more than 95% pure were subsequently washed once with PBS and stored in PBS at -80 °C. Protein concentration of the solution was determined according to the method of Lowry[12].

Chlamydia trachomatis culture

C. trachomatis was isolated from patient material according to the method of Thewessen et al[13]. Briefly, after mixing, a 0.2 ml sample was distributed over

monolayers of HeLa 229 cells in two wells of a microtiter plate. Prior to inoculating the patient material, the monolayers were pretreated with 15 µl/ml DEAE in Hanks balanced salt solution (HBSS) (Flow Lab., Irvine, Scotland). After centrifugation for 1 h at 3000 x g, the medium was replaced with Eagles modification of minimal essential medium containing 10% FCS (Hyclone Laboratories Inc.,Logan, USA), 1% glutamin (Flow), 1% vitamins (Flow), 0.42 M glucose, 0.5 µg/ml cycloheximide (Sigma Chem., St Louis, Miss.), 18 µg/ml gentamicin, 23 µg/ml vancomycin and 2.5 µg/ml amphotericin B. Plates were incubated for 48 h at 37 °C and subsequently fixed and stained with anti-*C. trachomatis* monoclonal antibody (Microtrak; Syva Co., Palo Alto, Calf.) and examined for inclusions.

Microimmunofluorescence

The MIF was performed using a modified procedure as that described by Wang and Grayston[3]. Dots of viable *C. pneumoniae* or *C. trachomatis* strain E EBs were spotted on a 8 slide multitest well (Flow). After drying the slides were fixed in acetone and air dried. Subsequently, the slides were incubated for 30 min at 37 °C with 30 µl serum starting at a 1:8 dilution. The slides were rinsed thrice with PBS and air dried. Hereafter, the slides were incubated for 30 min at 37 °C with 30 µl 1:40 anti-human IgG FITC (Boehringer, Mannheim, Germany) or with 30 µl 1:40 anti-human IgM (Boehringer), rinsed thrice and examined for fluorescent EBs. Positive sera were quantified using two fold dilutions. The MIF IgG titers of 54 sera and the MIF IgM titers of 42 sera against *C. pneumoniae* were determined in the same range as those observed for ELFA IgG or IgM titers. The correlation between *C. trachomatis* and *C. pneumoniae* titers was calculated. The sera for comparing ELFA and MIF were chosen on the basis of known ELFA titers such that al observed ELFA titers were represented. *C. trachomatis* E was chosen as antigen since it occurs most frequently in urogenital infections[14].

ELFA

Wells of PVC microtiter plates (Dynatech Lab. Inc., Alexandria VA) were coated with 50 µl of a 10 µg/ml EB suspension in 0.1 M carbonate/bicarbonate buffer (pH 9.6). After an overnight incubation at 4 °C, the unoccupied binding sites were blocked by incubation for 2 h at 37 °C with PBS containing 2% BSA (Sigma). Subsequently, the plates were rinsed once with PBS containing 1% BSA and 0.05% Tween 20 (PBST) and subsequently incubated with 50 µl diluted serum (1:250 IgM/IgA and 1:1000 IgG) at 22 °C for 45 min. The plates were then rinsed thrice with PBST and incubated at 22 °C for 45 min with either 50 µl of 1:8000 dilution of anti-human IgG biotin (Boehringer) or with 50 µl of 1:1000 dilution of anti-human IgA (Amersham, Bucks., U.K.) or with 50 µl of 1:250 dilution of anti-human IgM which had been coupled to β-galactosidase (Sigma) via N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP, Boehringer). The plates were then rinsed thrice

with PBST and those for IgG and IgA titer determination were incubated for a further 45 min at 22 °C after addition of 50 μl of 1:4000 dilution of streptavidin β-galactosidase (Boehringer) and subsequently rinsed thrice with PBST. The final step comprised a 1 h incubation at 37 °C after addition of 100 μl of 0.25 mg/ml 4-methylumbelliferyl β-D-galactoside (Sigma) in a solution containing 44 mM K_2HPO_4, 55 mM KH_2PO_4, 4 mM $MgCl_2$ (pH 7.6). The reaction was stopped by adding 100 μl of 1 M Na_2CO_3. Fluorescence was measured at 480 nm using FLUOROSKAN 1 (Flow). The reproducibility of the ELFA was tested for every antigen by measuring a positive and an intermediate patient serum with a MIF Ig titer of 1/1024 and 1/64 respectively.

The negative cut-off point was determined using 80 sera from blood donors (35 women and 45 men). The sera were tested individually using the same protocol as above with a 1:250 dilution for IgM/IgA determination and a 1:1000 dilution for IgG determination. The negative cut-off point was set at the value at which 95% (76 of the 80) of the donor sera were negative. Positive sera titers from the patient group were quantified using two-fold dilutions such that the test values were on or just above the cut-off point.

Statistical analysis

Statistical significance was calculated using the Fisher exact test. The correlation between ELFA and MIF, and between *C. pneumoniae* and *C. trachomatis* titers was calculated by linear regression after a ^{2}log transformation.

RESULTS

Reproducibility of the ELFA

Reproducibility of the ELFA was tested for every antigen by measuring the absolute fluorescence of a positive and an intermediate serum ten times over a period of four days. Using *C. trachomatis* LGV_2 as the only antigen in the MIF, the positive serum had an Ig titer of 1:1024, whereas the intermediate serum had an Ig titer of 1:64. The reproducibility of the test for determining IgG antibodies is shown in Table 1. The mean, the standard deviation (SD) and the coefficient of variance SD/mean) were within the criteria set by Hart[15]. The reproducibility of the ELFA for determining IgA and IgM antibodies also satisfied similar criteria (results not shown). The positive and the intermediate sera were subsequently included in every test. The test was repeated if deviations of larger than 10% in the mean value of the positive serum and/or larger than 30% in the mean value of the intermediate serum were observed.

Table 1: Reproducibility of enzyme linked fluorescent assay (ELFA) for determining IgG antibodies.

Antigen	Pos. serum (fluor. units)	CV% *	Interm. serum (fluor. units)	CV% *
E	6530±335	5.1	2958±514	17.4
F	6379±405	6.3	2682±412	15.4
H	6674±252	3.8	2571±416	16.2
I	6018±496	8.2	2519±514	20.4
J	7412±226	3.0	3056±314	10.3
LGV_2	7198±324	4.5	2972±294	9.9

* Coefficient of variance (standard deviation/mean).

Comparison between ELFA and MIF

Using *C. trachomatis* E EBs as antigen there was a high correlation between IgG titers of 51 tested sera in MIF as compared with the titers obtained in ELFA (fig 1). The correlation coefficient was 0.88. There seemed to be no major differences in the sensitivity of ELFA and MIF.

Cell culture

C. trachomatis was isolated from 42 out of the 140 (30.0%) women and from 47 out of the 207 (22.7%) men.

ELFA

1. IgG, IgM or IgA antibodies directed against *C. trachomatis*.

Antibodies of the IgG class directed against the *C. trachomatis* antigens E,F,H,I,J and LGV_2 at least in one of the tested sera of the patients were observed in sera of 23 (48.9%) men who had positive culture and in sera of 32 (20.0%) man who had negative culture ($p = 0.0003$). In women, 31 (73.8%) and 39 (39.8%) respectively were observed to be positive ($p = 0.0004$). When *C. trachomatis* LGV_2 was used as the only antigen, the sensitivity of IgG antibody determination in men was lower than that using all six antigens (E,F,H,I,J and LGV_2), but the specificity remained the same (Table 2). In this respect, no statistically significant differences were observed in women. IgG antibodies against *C. trachomatis* were more frequently observed in the sera of men (43.1%) with an increased number of leucocytes in the urine sediment than in sera of men (20.1%) without any increase

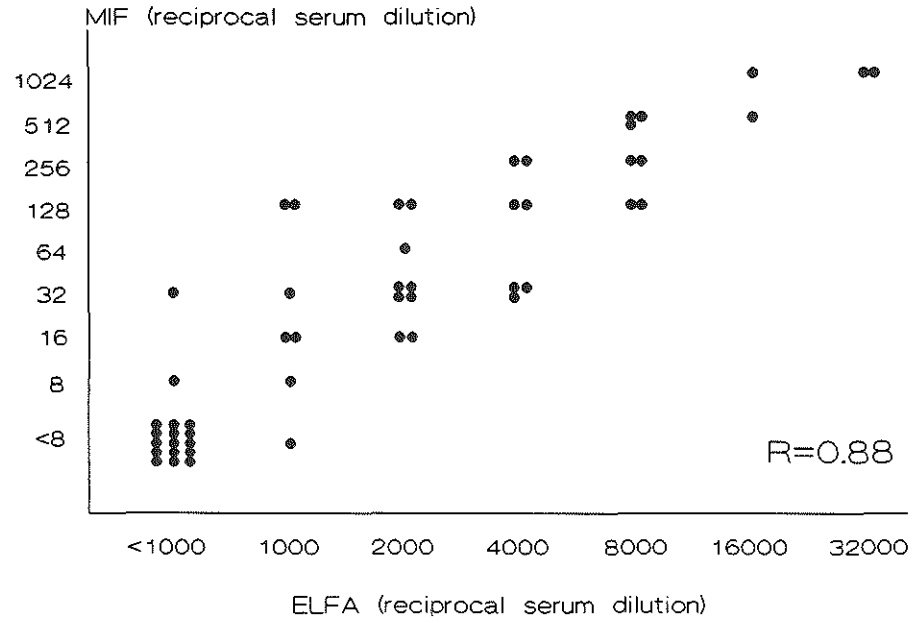

Figure 1. Scatter diagram of IgG titers against *C. trachomatis* strain E EBs obtained by ELFA and Mif.

in the number of leucocytes ($p = 0.025$). In women there was no correlation between the number of leucocytes in the gram preparation of the cervix and an IgG titer in the serum. Serum IgA antibodies directed against *C. trachomatis* antigens E,F,H,I,J and LGV_2 were observed in 23 (48.9%) men with positive culture and in 46 (28.8%) men with negative culture ($p = 0.013$) and in 23 (54.8%) women with positive culture and in 28 (28.6%) women with negative culture ($p = 0.004$). Both in men and women, there was no correlation between the increased number of leucocytes and the presence or the absence of an IgA titer in the serum.

Since only in 3 of 47 (6.4%) culture positive men and in 9 of 160 (5.6%) culture negative men, IgM antibodies were found (see also Table 2), no differences in the frequency between culture positives and culture negatives were observed. In women there was a significant difference ($p = 0.016$).

2. IgG and/or IgM and/or IgA antibodies directed against *C. trachomatis*.

Using all six antigens, IgG and/or IgM antibodies were observed more frequently in the group of patients that had positive cultures than the group that had negative cultures (men 53.2% and 29.0% respectively, $p = 0.0001$, and women 83.3% and 48.0% respectively, $p = 0.00008$). In the group of patients (men and women) that had positive cultures, IgG and/or IgA, IgA and/or IgM, IgG and/or IgA and/or IgM

Table 2: Sensitivity and specificity of ELFA compared with tissue culture.
1. IgG, IgM or IgA antibodies directed against *Chlamydia trachomatis*.

	IgG		IgA		IgM	
	Sens.(%)	Spec.(%)	Sens.(%)	Spec.(%)	Sens.(%)	Spec.(%)
Men						
LGV_2 as the only antigen #	25.5 *	83.8	42.6	73.8	6.4	96.3
All antigens ♦	48.9	80.0	48.9	71.3	6.4	94.4
Women						
LGV_2 as the only antigen #	54.8	69.4	50.0	72.4	31.0	86.7
All antigens ♦	73.8	60.2	54.8	71.4	38.1	82.7

Sensitivity and specificity of the tissue culture is set at 100%.

* P=0.032 as compared with sensitivity 'all antigens'

\# Titer against *C. trachomatis* LGV_2 in at least one serum/patient.

♦ Titer against at least one of the antigens E, F, H, I, I, J, LGV_2 in at least one serum/patient.

The differences in the sensitivity are significant for the difference > = 23.4 % in men and > = 21.4 % in women (see also Table 3).

The differences in the specificity are significant for the difference > = 11.2 % in men and > = 15.3 % in women (see also Table 3).

Table 3: Sensitivity and specificity of ELFA compared with tissue culture.
2. IgG and/or IgM and/or IgA antibodies directed against *Chlamydia trachomatis*.

	IgG and/or IgA		IgG and/or IgM		IgA and/or IgM		IgG and/or IgA and/or IgM	
	Sens.(%)	Spec.(%)	Sens.(%)	Spec.(%)	Sens.(%)	Spec.(%)	Sens.(%)	Spec.(%)
Men								
LGV_2 as the only antigeen #	55.3	64.4	29.8*	81.9	46.8	71.3	57.4	62.5
All antigens ◆	63.8	61.3	53.2	77.5	51.5	67.5	63.8	58.8
Women								
LGV_2 as the only antigen #	66.7	57.1	61.9**	62.2	66.7	66.3	78.6	45.9
All antigens ◆	83.3	51.0	83.3	52.0	69.0	61.2	88.1	44.9

Sensitivity and specificity of the tissue culture is set at 100%.

* P = 0.036 as compared with sensitivity 'all antigens'.

** P = 0.049 as compared with sensitivity 'all antigens'.

\# Titer against *Chlamydia trachomatis* LGV_2 in at least one serum/patient.

◆ Titer against at least one of the antigens E, F, H, I, J, LGV_2 in at least one serum/patient.
The differences in the sensitivity are significant for the difference > = 23.4 % in men and > = 21.4 % in women (see also Table 2).
The differences in the specificity are significant for the difference > = 11.2 % in men and > = 15.3 % in women (see also Table 2).

were more frequently observed than in the group that had negative cultures (results not shown).

The sensitivity with which IgG and/or IgM antibodies were determined in men ($p = 0.036$) and in women ($p = 0.049$) using LGV_2 as the only antigen was lower than that using all six antigens (Table 3). In men (49.0%) with an increased number of leucocytes in the urine sediment, IgG and/or IgM titer was observed more frequently than in men (22.2%) without an increased number of leucocytes ($p = 0.014$).

In women, the determination of IgG antibodies, in contrast to the determination of IgM and IgA antibodies directed against *C. trachomatis*, contributed significantly towards the sensitivity of the test in comparison with cell culture (Tables 2 and 3). However, the specificity was lowest as compared with IgA and IgM.

We found no correlation between IgG or IgM antibodies against *C. trachomatis* determined with ELFA in respectively 54 and 42 sera compared with IgG or IgM antibodies in the same sera against *C. pneumoniae* determined with MIF. The correlation coefficients ranged, depending on the *C. trachomatis* antigen used, from 0.26 to 0.31 for IgG antibodies and from 0.0 to 0.1 for IgM antibodies. In 48.1% of the sera an IgG titer indicating a previous *C. pneumoniae* infection was observed. This percentage is in agreement with that reported in previous studies[10].

DISCUSSION

When the men in the present study were divided into urethritis positive/tissue culture positive and urethritis positive/tissue culture negative groups, a sensitivity of 51.9% and a specificity of 66.7% as compared with tissue culture were observed in the urethritis positive group. Schoenwald et al[6] reported an ELISA test which had a sensitivity of 72.9% and a specificity of 54.0% in men with urethritis, of whom 32.3% had a positive *C. trachomatis* culture. In women with cervicitis (12.7% with positive tissue culture), Schoenwald et al[6] observed a sensitivity of 91.1% and a specificity of 53.9%. In the present study, a sensitivity of 81.3% and a specificity of 66.6% were observed in women with cervicitis (50% with positive tissue culture). In order to determine the negative cut-off point, sera that were known to be negative were used in ELISA by Schoenwald et al[6]. In the general population, the incidence of *C. trachomatis* is 5-13%[16]. In our study, the negative cut-off point that was established using sera of blood donors was probably higher thereby decreasing the sensitivity and increasing the specificity of the test.

In the current study, the values of serum IgA antibodies in *C. trachomatis* culture positive patients were lower than those observed by others. Cevenini et al.[17] and Hagay et al.[7] reported a sensitivity of 94.1% and 83.0% respectivily and a

specificity of 78.0% in tissue culture positive men with urethritis. Although in our group of patients only 53.3% of the tissue culture positive men suffered from urethritis, an IgA titre was observed in only 48% of these urethritis positive men. The difference in the cut-off point, may be a possible explanation for the observed discrepancy in values obtained for IgA.

The results of IgM antibody titer determination in women, that were obtained using the ELFA, corroborated the results reported by others[18,19]. The sensitivity of the test was low, but the specificity was high.

In this study it has been demonstrated that an ELFA in which *C. trachomatis* LGV_2 was used as the only antigen had a significantly lower sensitivity for detecting IgG and/or IgM antibodies as compared with ELFA in which a selected number of *C. trachomatis* antigens were used. Mahoney et al.[5] had already indicated that the use of only *C. trachomatis* LGV_2 antigen in an ELISA was insufficient to detect antibodies against all serotypes. These authors were unable to detect antibodies against C-complex antigens.

Significant sero-conversions of IgG, IgA or IgM antibody titers were scarcely observed. On the one hand, it is possible that the period of time over which the sera were collected was too short, whereas on the other hand, in a population in which the incidence of *C. trachomatis* is high, increased background serum titers of IgG exist and anti-Chlamydia antibodies may persist for prolonged periods[20]. Frequently after reinfection, IgM antibodies in the serum cannot be demonstrated, but the IgG antibody titer increases early in the infection. Since the general practitioner is frequently consulted in the later stage, sero-conversion is no longer demonstrable. The half-life of IgA antibodies in the serum is 6-7 days[21]. After the patient has been treated, a decrease in the IgA titer may be used to monitor the effectiveness of the therapy. We observed only a scarce decrease in IgA titer in the period during which the sera were collected.

In comparison with the tissue culture, the best sensitivity of the ELFA both in men and women was observed for determining IgG and/or IgM and/or IgA antibodies, but the specificity was low. Although there were several improvements in our test as compared with those already reported by other authors[4,5,6,7,8], our results corroborated with those of Schachter et al.[19], leading to the conclusion that determination of IgG and/or IgM and/or IgA antibody titers in a patient population with a high prevalence of *C. trachomatis* were unsuitable for tracing acute infections.

After the discovery of *C. pneumoniae* species and the observed high prevalence of IgG antibodies in the western population, it was reported that antibodies directed against *C. trachomatis* and *C. pneumoniae* could only be distinguished using MIF[10]. In ELISA and ELFA, cross-reactions are mainly caused by antibodies directed against lipopolysaccharide (LPS). In principle, these reactions can be avoided in two ways. Ladany et al.[22] described a method whereby LPS was

extracted out after which only specific anti-MOMP antibodies could attach. It is also possible to use native unfixed EBs as antigen instead of formaline fixed EBs. The LPS is then spontaneously removed from the membrane[23]. Since we could not demonstrate any cross-reactions between the species *C. trachomatis* and *C. pneumoniae*, and since we found good agreement between ELFA and MIF IgG titers, it is possible to use an ELFA or eventually an ELISA technique incorporating viable EBs as antigen to trace antibodies directed against *C. trachomatis*.

REFERENCES

1. Schachter J, Grossman M. Chlamydial infections. Ann Rev of Med 1981;**32**:46-61
2. Wang SP, Grayston JT. Immunological relationship between genital TRIC lymphogranuloma venereum and related organisms in a new microtiter indirect immunofluorescence test. Am J Ophtalmology 1970;**70**:367-374
3. Wang SP, Grayston JT. Microimmunofluorescence antibody responses in *Chlamydia trachomatis* infections. In: Mårdh PA, Holmes KK, Oriel JD, Piot P, Schachter J (ed): Chlamydial infections Vol. 2. Amsterdam: Elsevier Biomedical Press, 1982:301-316
4. Evans RT, Taylor-Robinson D. Development and evaluation of an enzyme linked immunosorbent assay (ELISA), using Chlamydial group antigen to detect antibodies to *Chlamydia trachomatis*. J Clin Pathol 1982;**35**:1122-1128
5. Mahoney JB, Schachter J, Chernesky MA. Detection of antichlamydial immunoglobulin G and M antibodies by enzyme linked immunosorbent assay. J Clin Microbiol 1983;**18**:270-275
6. Schoenwald E, Schmidt BL, Steinmetz G, *et al.* Diagnosis of *Chlamydia trachomatis* infection. Culture versus serology. Eur J Epidemiol 1988;**4**:75-82
7. Hagay ZJ, Sarov B, Sachs J, Shaked O, Sarov I. Detecting *Chlamydia trachomatis* in men with urethritis: serology versus isolation in cell culture. Genitour Med 1989;**65**:166-170
8. Csángó PA, Sarov B, Schiøtz VH, Sarov I. Comparison between cell culture and serology for detecting *Chlamydia trachomatis* in women seeking abortion. J Clin Pathol 1988;**41**:89-92
9. Numasaki K, Chiba S, Moroboshi T, Kudoh T, Yamanaka T, Nakao T. Comparison of enzyme linked immunosorbent assay and enzyme linked fluorescent immunoassay for detection of antibodies against *Chlamydia trachomatis*. J Clin Pathol 1985;**38**:345-350
10. Grayston JT, Wang SP, Kuo CC, Campbell LA. Current knowledge on *Chlamydia pneumoniae* strain TWAR, an important cause of pneumoniae and other acute respiratory diseases. Eur J Clin Microbiol Infect Dis 1989;**8**:191-202
11. Ripa KT, Mårdh PA. Cultivation of *Chlamydia trachomatis* in cycloheximide treated McCoy cells. J Clin Microbiol 1977;**6**:328-331
12. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1954;**193**:265-275
13. Thewessen EAPM, Freundt I, Van Rijsoort-Vos JH, Stolz E, Michel MF, Wagenvoort JHT. Comparison of HeLa 229 and McCoy cell cultures for the detection of *Chlamydia trachomatis* in clinical specimens. J Clin Microbiol 1989;**27**:1399-1404.
14. Wagenvoort JHT, Suchland RJ, Kluytmans JAJW, Stamm WE. Detection of different *Chlamydia trachomatis* serovars of urogenital origin by enzyme-immunoassay. Eur J Clin

Microbiol Infect Dis 1989;6:319-320
15. Hart G. Screening to control infectious diseases: evaluation of control programs for gonorrhea and syphilis. Rev of Infect Dis 1980;2:701-712
16. McCormack WM. Infection with *Chlamydia trachomatis* in women college students. In: Mårdh PA, Holmes KK, Oriel JD, Piot P, Schachter J (ed): Chlamydial infections Vol. 2. Amsterdam: Elsevier Biomedical Press, 1982:151-154
17. Cevenini R, Sarov I, Rumpianesi F, *et al.* Serum specific IgA antibodies to *Chlamydia trachomatis* in patients with chlamydial infections detected by ELISA and immunofluorescense test. J Clin Pathol 1984;37:686-691
18. Jones RB, Bruins SC, Newhall WJ. Comparison of reticulate and elementary body antigens in detection of antibodies against *Chlamydia trachomatis* by an enzyme linked immunosorbent assay. J Clin Microbiol 1983;17:466-471
19. Schachter J, Cles L, Ray R, Hines PA. Failure of serology in diagnosing Chlamydial infections of the female genital tract. J Clin Microbiol 1979;10:647-649
20. Puolakkainen M, Vesterinen E, Purola E, Saikku P, Paavonen P. Persistence of Chlamydial antibodies after pelvic inflammatory disease. J Clin Microbiol 1986;23:924-928
21. Tomasi TB, Grey HM. Structure and function of immunoglobulin A. Progr Allergy 1972;16:81-113
22. Ladany S, Black CM, Farshy CE, Ossewaarde JM, Barnes RC. Enzyme immunoassay to determine exposure to *Chlamydia pneumoniae* (strain TWAR). J Clin Microbiol 1989;27:2778-2783
23. Birkelund S, Lundemose AG, Christiansen G. Immunoelectron microscopy of lipopolysaccharide in *Chlamydia trachomatis*. Infect Immun 1989;57:3250-3253.

CHAPTER 3

CHLAMYDIA TRACHOMATIS-SPECIFIC ANTIBODIES IN PATIENTS WITH PELVIC INFLAMMATORY DISEASE: COMPARISON WITH ISOLATION IN TISSUE CULTURE OR DETECTION WITH POLYMERASE CHAIN REACTION

J.J.H. Theunissen[1], W. Minderhoud-Bassie[2], J.H.T. Wagenvoort[3], E. Stolz[1], M.F. Michel[3], F.J.M. Huikeshoven[2].

Departments of Dermato-Venereology[1], Obstetrics and Gynaecology[2] and Clinical Microbiology[3] Erasmus University, Rotterdam The Netherlands.

Submitted for publication.

SUMMARY

Objective: Detection of acute phase antibodies against *C. trachomatis* and its comparison with tissue culture or polymerase chain reaction (PCR) on samples of cervix and urethra obtained from patients with pelvic inflammatory disease (PID).
Study design: In the academic hospital Dijkzigt, Rotterdam, The Netherlands, prospective investigations were performed on 49 consecutive patients who were admitted with the diagnosis of PID. Infections with *C. trachomatis* were traced using tissue culture, PCR or by determining acute phase IgG and IgM antibodies. Differences between the sensitivities of serology and tissue culture or PCR were calculated using the Fisher exact test.
Results: *C. trachomatis* infection was detected more often in PID patients using serolgy in comparison with PCR ($P=0.028$) or tissue culture ($P=0.005$). All patients who were positive for *C. trachomatis* in the PCR and 3 of the 4 patients who were positive in tissue culture also had acute phase antibodies.
Conclusions: Establishing acute *C. trachomatis* infections in PID patients on the basis of serology was superior to either tissue culture or PCR on samples obtained from cervix and urethra.

INTRODUCTION

The role of *Chlamydia trachomatis* as the cause of pelvic inflammatory disease (PID) is well known (1,2). In a Norwegian study, it was observed that more than 50% of the PID cases were caused by *Chlamydia*, whereas *Neisseria gonorrhoeae* played a more subordinate role (3). A complicated *C. trachomatis* infection often begins as a clinical benign condition which in most cases remains undiagnosed. This can, in a later stage of infection, lead to tubal damage and subsequently to infertility. In some cases, *C. trachomatis* can be cultured from the fallopian tubes (4), from the endometrium (5) or the peritoneal fluid (6). In a recent Swedish study, *C. trachomatis* in samples of tubae and endometrium in a group of infertile women were detected more efficiently using direct immunofluorescence (DFA) than in tissue culture (7).
In the Dutch setting, obtaining samples from tubae and endometrium is often regarded as imposing an unnecessary burden on the patient. Moreover, because culturing of *C. trachomatis* from the samples of tubae and cervix in an acute PID appears to be less sensitive (7), one would attempt to establish the infection on the basis of serology. In this study, enzyme-linked fluorescent assay (ELFA) was used to detect acute phase IgM and IgG antibodies instead of the more laborious microimmunofluorescent (MIF) technique (8). The results were compared with *C. trachomatis* cell culture of cervix/urethra samples and with the results of a *C.*

trachomatis specific polymerase chain reaction on cervix/urethra samples.

MATERIALS AND METHODS

Patients

Forty-nine women who had been admitted consecutively at the Department of Obstetrics and Gynecology of the Academic Hospital Rotterdam-Dijkzigt and who satisfied the clinical criteria for acute PID were enrolled into this study. The clinical diagnosis of PID was based on the presence of at least the first four and one or more of the following criteria (5-9):

1. History of lower abdominal pain. 2. Presence of lower abdominal tenderness. 3. Cervical motion tenderness. 4. Adnexal tenderness. 5. Body temperature >38°C. 6. Leucocytosis >10500/mm^3. 7. Erythrocyte sedimentation rate (ESR) >20 mm/first hour. 8. Abnormal vaginal discharge. 9. Abnormal vaginal bleeding. If the diagnosis, on basis of clinical criteria, was doubtful, PID was established by laparoscopy. In 17 patients (34.7%) the diagnosis of PID was confirmed in this manner.

Samples for *C. trachomatis* cultures and for PCR on *Chlamydia*-specific DNA were obtained using a swab with a metal holder (Medical Wire & Equipment Co. Ltd., Wiltshire, UK) and transported in 2SP medium containing 0.2 M sucrose (Merck, Darmstadt, Germany), 49 mM glutamine (Sigma Chem. Co. St. Louis, MO), 10% foetal calf serum (FCS) (Hyclone Lab. Inc. Logan, Utah), 18 μg/ml gentamicin, 23 μg/ml vancomycin and 2.5 μg/ml amphotericin B in 20 mM phosphate buffer and stored at 4°C if cultured within 24 h or frozen at -70°C, if it was not possible to culture within that period. In each patient at least two sera were collected at about 10 days interval for the estimation of IgG and IgM antibodies against *C. trachomatis*. Prior to sampling for *C. trachomatis* and Chlamydia-specific DNA, samples were collected from cervix, urethra and rectum for culture of *N. gonorrhoeae*.

C. trachomatis culture

C. trachomatis was isolated from samples obtained from the patients according to the method described by Thewessen et al (9). A microtiterplate with a monolayer of HeLa 229 cells was preincubated with 15 μg/ml DEAE in Hank's Balanced Salt Solution (Flow Labs., Irvine, Scotland). After mixing, 0.2 ml/well of each cervix/urethra sample from the patient was setup in duplicate. The plates were then centrifuged at 3000 x g for 1 h and subsequently cultured for 48 h in Eagle's modification of minimal essential medium (Flow) containing 10% FCS, 2 mM glutamine, 1% vitamins, 0.42 M glucose, 0.5 μg/ml cycloheximide (Sigma), 18 μg/ml gentamicin, 32 μg/ml vancomycin and 2.5 μg/ml amphotericin B. The plates

were then fixed and stained with anti-*C. trachomatis* monoclonal antibodies (Microtrak, Syva Co., Palo Alto, Calf.) and examined for inclusions.

C. trachomatis-specific polymerase chain reaction (PCR)

The PCR on *C. trachomatis*-specific DNA was performed according to the method described by Claas et al (10). DNA was extracted from 0.5 ml cervix/urethra sample from each patient. Two sets of oligonucleotide primers were used. The first set (R1 = GTGGATAGTCTCAACCCTAT, R2 = TATCTGTCCTTGCGGAAAAC), was derived from 16S rRNA gene sequences of *C. psittaci* (11) and generated 208-bp amplified products with all three *Chlamydia* species. The second set (T1 = GGACAAATCGTATC TCGG, T2 = GAAACCAACTCTACGCTG was derived from sequences of the common endogenous plasmid of *C. trachomatis* (12) and generated 517-bp amplified products with all known *C. trachomatis* serovars. After 40 cycles, the amplification product was analysed on a 2% agarose gel.

Enzyme-linked fluorescent assay (ELFA)

The ELFA was performed as described previously (13). Wells of PVC microtiter plates were coated with 50 μl of a 10 μg/ml elementary bodies (EB) suspension of the *C. trachomatis* serotypes E (strain UW-5/cx), F (strain IC-Cal3), H (strain UW-4/Cx), I (strain UW-12/Ur), J (strain UW-36/Cx) and LGV2 (strain 343/Bu) in 0.1 M carbonate/bicarbonate buffer (pH 9.6). After an overnight incubation at 4 °C, the unoccupied binding-sites were blocked with PBS containing 2% BSA. The plates were subsequently rinsed with PBS containing 1% BSA and 0.05% Tween-20 (PBST) and incubated with 50 μl diluted test serum (1:250 for determining IgM and 1:1000 for determining IgG) at 22°C for 45 min. The plates were then rinsed with PBST and incubated at 22°C for 45 min with either 50 μl of 1:8000 dilution of anti-human IgG biotin (Boehringer, Mannheim, Germany) or with 50 μl of 1:250 dilution of anti-human IgM which had been coupled to β-galactosidase. The plates were then rinsed with PBST and those for IgG titer determination incubated for a further 45 min at 22°C after addition of 50 μl of 1:4000 dilution of streptavidin β-galactosidase (Boehringer) and subsequently rinsed with PBST. The final step comprised a 1 hr incubation at 37°C after addition of 100 μl of 0.25 mg/ml 4-methylumbelliferyl β-D-galactoside (Sigma) in a buffer containing 44 mM K_2HPO_4, 55 mM KH_2PO_4, 4 mM $MgCl_2$ (pH 7.6). The reaction was stopped by adding 100 μl of 1 M Na_2CO_3. Fluorescence was measured at 480 nm using FLUOROSKAN 1 (Flow).

The criteria for an acute infection were: an IgM titer $\geq$ 1:500 (corresponding with a MIF titer $\geq$ 1:32), and/or an IgG titer $\geq$ 1:16000 (MIF titer $\geq$ 1:512), and/or a four-fold increase/decrease in IgM and/or a four-fold increase in IgG titer. The cut-off points of an acute infection as determined by MIF were described by Grayston et al.(14).

Statistical analysis

Statistical differences between the sensitivity of tissue culture and PCR for *C. trachomatis* on the one hand and *C. trachomatis* serology on the other hand were calculated using the Fisher exact test.

RESULTS

Seven out of the 49 patients were positive in *C. trachomatis* culture and/or PCR on *C.trachomatis*- specific DNA. Four patients were positive in culture. In 3 of these patients, *C. trachomatis*-specific DNA could be detected. Three additional patients were positive in PCR only. *N. gonorrhoeae* was cultured from the samples of cervix and/or urethra in 13 out of the 49 (26.5%) patients. In 3 out of of the 49 (6.1%) patients there were, in culture, indications of both chlamydial and gonococcal infections.

The prevalence of acute phase IgM and/or IgG antibodies against *C. trachomatis* is shown in Table I. Combining high IgG/IgM titers with an increase or decrease in IgM titers and an increase in IgG titers showed an acute antibody titer in 32.7% of the patients. A total of nine patients were only positive when using serology. There was a statistically significant difference between the sensitivity of *C. trachomatis* culture as compared with *C. trachomatis* serology ($p = 0.005$) and between the sensitivity of PCR as compared with serology ($p = 0.028$). In all 6 patients in whom *C. trachomatis*-specific DNA was demonstrated in the PCR and in 3 out of the 4 patients in whom *C. trachomatis* cultures were positive, acute phase antibodies were also observed. In 2 out of the 6 PCR-positive patients, an IgG titer $\geq$ 1:16000 was observed, in 2 patients a four-fold increase in the IgG titers were observed and in 2 patients, both a four-fold increase in the titers of IgM and IgG as well as an IgM titer of 1:1000 and an IgG titer of 1:16000 were observed. Three Chlamydia culture/PCR negative patients were found to be positive in both *N. gonorrhoeae* culture as in *C. trachomatis* serology.

DISCUSSION

In the literature the best methods to trace the cause of PID appears to be tissue culture or direct immunofluorescence on the samples from tubae or endometrium (7,15). In the Netherlands, obtaining samples from tubae for detection of *C. trachomatis* is generally considered as imposing an unnecessary burden on the patient. Moreover, Thejls et al (7) reported that the sensitivity of primary culture for *C. trachomatis* established from the samples from tubae or endometrium was limited as compared with the sensitivity with which chlamydia antigens were demonstrated using direct immunofluorescence. In another study, it was reported

Table I. Distribution of acute phase antibodies against *C. trachomatis* in patients with pelvic inflammatory disease.

Antibody group		Number of patients (%)
1.	IgM ≥ 1/500	6/49 (12.2%)
2.	IgG ≥ 1/16000	5/49 (10.2%)
3.	four-fold increase/decrease in IgM titer	4/49 (8.2%)
4.	four-fold increase in IgG titer	9/49 (18.4%)
5.	1 and 2 combined	9/49 (18.4%)
6.	3 and 4 combined	11/49 (22.4%)
7.	1,2,3 and 4 combined	16/49 (32.7%)
8.	Negative	33/49 (67.3%)

that the samples from tubae and endometrium had to be passaged several times in culture before *C.trachomatis* could be detected (15).

In our study, *C. trachomatis* was cultured from the cervix in 8.2% of the patients with PID, whereas in other studies *C. trachomatis* was isolated from the cervix in 36-49% of patients with the same diagnosis (1,16). As the sensitivity of the *C. trachomatis*-specific PCR for culture confirmed urogenital specimens was shown to be excellent (10), the demonstration of *C. trachomatis*-specific DNA in the PCR might have increased the sensitivity of tissue culture of samples from the cervix. Therefore, together with Chlamydia-culture we performed PCR on all samples. Chlamydia-specific DNA was demonstrated in 6 (12.2%) of the patients. Combination of tissue culture and PCR demonstrated infection with *C. trachomatis* in the cervix as a possible cause of PID in 7 (14.3%) patients but was lower than that reported previously (1,5,15). However, in our opinion, this percentage (14.3%) was the maximum that could be achieved using this form of detection in the patients in this study.

In order to improve the sensitivity for detection of acute *C. trachomatis* infection in PID patients without using invasive techniques, acute IgG/IgM titers against a representative set of chlamydial antigens were determined. From the figures in Table I, it can be seen that a combination of high IgM/IgG titers and four -fold increase/decrease of IgM titer and a four-fold increase in IgG titer had the highest sensitivity for detecting acute phase antibodies against *C. trachomatis* (32.7% of

the patients). Determining the conversions in IgG titers (18.4%) made the largest contribution. This was followed by high IgM titer (12.2%). Determining high IgG titer (10.2%) and conversion in IgM titer (8.2%) made minor contributions. In two patients with an acute antibody titer, only high IgG titers were observed. It was previously reported that IgG antibodies can remain in the circulation for a long period after *C. trachomatis*-caused PID (17). Although these two patients had satisfied the clinical criteria for an acute PID, it could not be totally excluded that both these patients had chronic *C. trachomatis* infection. Serum conversions, often observed in our group of PID patients, were hardly observed in patients with uncomplicated urogenital *C. trachomatis* infections (13). To our knowledge, serum conversions were not determined in the previous investigations on PID patients. However, it was observed, that the average titer of IgM/IgG antibodies in a group of PID-patients in whom *C. trachomatis* cultures were positive was higher than that in a group of PID-patients from whom no *C. trachomatis* could be cultured (17). This was in accordance with our results, as we observed acute phase titers in all but one culture/PCR positive patients.

In 26.5% of the patients, *N. gonorrhoeae* was isolated from the cervix. In our study, the incidence of gonorrhea was similar to that observed by others (1,4,5). Alltogether in 24 (49%) out of 49 PID patients an infection caused by *C. trachomatis* and/or *N. gonorrhoeae* could be detected.

The best method to establish the diagnosis for the suspected causes of PID was DFA on samples from tubae and endometrium (7). In the present study, the number of infections demonstrated using serology was comparable to that demonstrated by Thejls et al. using DFA (7). In our hands, the role of *C. trachomatis* as a possible cause of PID was demonstrated with a higher sensitivity using serology than either culturing of or PCR on samples from the cervix. As compared with DFA on the samples from tubae or endometrium, serology also hardly imposes any unnecessary burden on the patient.

REFERENCES

1. Mårdh P-A, Ripa T, Svensson L, Weström L. Role of *Chlamydia trachomatis* infection in acute salpingitis. N Engl J Med 1977;269:1377-9.
2. Brunham RC, Peeling R, Maclean I, McDowell J, Persson K, Osser S. Postabortal *Chlamydia trachomatis* salpingitis: correlating risk with antigen-specific serological responses and with neutralization. J Infect Dis 1987:155:749-55.
3. Gjønnaess H, Dalaker K, Ånestad G, Mårdh P-A, Kvile G, Bergan T. Pelvic inflammatory disease: etiologic studies with emphasis on chlamydial infection. Obstet Gynecol 1982;59:550-5.
4. Stacey C, Munday P, Thomas B, Gilchrist C, Taylor-Robinson D, Beard R. *Chlamydia trachomatis* in the fallopian tubes of women without laparoscopic evidence of salpingitis.

Lancet 1990;336:960-3.

5. Sweet RL, Schachter J, Robbie MO. Failure of antibiotics to eradicate *Chlamydia trachomatis* in the endometrium despite apparent clinical cure of acute salpingitis. JAMA 1983;250:2641-5.
6. Henry-Suchet J, Catalan F, Loffredo V, *et al. Chlamydia trachomatis* associated with chronic inflammation in abdominal specimens from women selected for tuboplasty. Fertil Steril 1981;36:599-605.
7. Thejls H, Gnarpe J, Lundkvist Ö, Heimer G, Larsson G, Victor A. Diagnosis and prevalence of persistent chlamydia infection in infertile women: tissue culture, direct antigen detection, and serology. Fertil Steril 1991;55:304-10.
8. Wang SP, Grayston JT. Microimmunofluorescence antibody responses in *Chlamydia trachomatis* infections. In: Mårdh PA, Holmes KK, Oriel JD, Piot P, Schachter J eds. Chlamydial infections. Amsterdam: Elsevier Biomedical Press, 1982 Vol. 2:301-16.
9. Thewessen EAPM, Freundt I, Van Rijsoort-Vos JH, Stolz E, Michel MF, Wagenvoort JHT. Comparison of HeLa 229 and McCoy cell cultures for the detection of *Chlamydia trachomatis* in clinical specimens. J Clin Microbiol 1989;27:1399-1400.
10. Claas HJC, Wagenvoort JHT, Niesters HGM, Tio TT, Van Rijsoort-Vos JH, Quint WGV. Diagnostic value of the polymerase chain reaction for *Chlamydia* as determined in a follow-up study. J Clin Microbiol 1991;29:42-5.
11. Wiesburg WG, Hatch TP, Woese CR. Eubacterial origin of chlamydiae. J Bacteriol 1986;167:570-4.
12. Sriprakash KS, Macavoy ES. Characterisation and sequence of a plasmid from the trachoma biovar of *Chlamydia trachomatis*. Plasmid 1987;18:205-14.
13. Theunissen JJH, Van Heijst BYM, Chin-A-Lien RAM, Wagenvoort JHT, Stolz E, Michel MF. Detection of antibodies in patients with uncomplicated *Chlamydia trachomatis* infection: a comparison between enzyme linked fluorescent assay and isolation in cell culture. Int J STD & AIDS 1993;4:43-48.
14. Grayston JT, Wang SP, Foy HM, Kuo CC. Seroepidemiology of *Chlamydia trachomatis* infection. In: Mårdh PA, Holmes KK, Oriel JD, Piot P, Schachter J eds. Chlamydial infections. Amsterdam: Elsevier Biomedical Press, 1982 Vol. 2:405-19.
15. Shepard MK, Jones RB. Recovery of *Chlamydia trachomatis* from endometrial and fallopian tube biopsies in women with infertility of tubal origin. Fertil Steril 1989;52:232-8.
16. Miettinen A, Heinonen PK, Teisala K, Punnonen R, Paavonen J. Antigen specific serum antibody response to *Chlamydia trachomatis* in patients with acute pelvic inflammatory disease. J Clin Pathol 1990;43:758-61.
17. Puolakkainen M, Vesterinen E, Purola E, Saikku P, Paavonen J. Persistence of Chlamydial antibodies after pelvic inflammatory disease. J Clin Microbiol 1986;23:924-8.

CHAPTER 4

FACTORS INFLUENCING THE INFECTIVITY OF *CLAMYDIA PNEUMONIAE* ELEMENTARY BODIES ON HL CELLS

J.J.H. Theunissen[1], B.Y.M. van Heijst[2], J.H.T. Wagenvoort[2], E. Stolz[1], M.F. Michel[2].

Departments of Dermato-Venereology[1] and Clinical Microbiology[2], Erasmus University, Rotterdam, The Netherlands.

Published in The Journal of Clinical Microbiology 1992;30:1388-1391.

SUMMARY

The influence of variations in the pH, NaCl concentration, temperature, concentration of calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions on the survival of *Chlamydia pneumoniae* elementary bodies (EB) outside the host cells was investigated. The survival was determined after various incubation periods by counting inclusion forming unit (IFU) after culturing for 72 hr on monolayers of HL cells. The normal physiological conditions were restored prior to infecting the HL cells with *C. pneumoniae*. A decline in the infectivity of *C. pneumoniae* EB were observed at pH values of lower than 5 and higher than 8 or at NaCl concentrations of lower than 80 mM. The viability of *C. pneumoniae* EB in SPG medium decreased as the temperature and/or incubation period increased. Incubation temperatures of up to 20°C and incubation periods of up to 48 hr did not affect the viability of *C. pneumoniae*. One hundred percent of the *C. pneumoniae* EB were infective after a 1 hr incubation at 35°C, whereas 90%, 50% and 40% respectively survived after incubations of 8, 24 and 48 hr. The viability of *C. pneumoniae* was uneffected within the investigated range of Ca^{2+} and Mg^{2+} ion concentration in the medium. The presence of 10% foetal calf serum (FCS) in the incubation medium had a stabilizing effect on the viability of *C. pneumoniae*. This effect became more pronounced as the incubation period increased.

INTRODUCTION

Chlamydia pneumoniae, previously known as *Chlamydia psittaci* TWAR [5], is an important cause of respiratory complaints in adults [11]. *C. pneumoniae* epidemics have been observed both in local and in nation-wide communities [8,13]. Since the initial isolation of *C. pneumoniae* from patient material is tedious, the diagnosis is generally based on the presence of acute phase antibodies [6]. In contrast to *C. trachomatis* which requires mucous membrane to mucous membrane contact for its transmission, *C. pneumoniae* is most probably transmitted via aerosols. *C. pneumoniae* infections are not season dependent [15]. The microbe has to be able to withstand physical changes in its environment in order to survive outside its host.

Changes in pH, salt concentration, temperature, concentrations of Ca^{2+} and Mg^{2+} ions were investigated in order to establish the limit(s) to which these parameters affected the infectivity of *C. pneumoniae* EB on HL cells.

MATERIALS AND METHODS

Chlamydia strain and antigen purification

C. pneumoniae (strain TW-183) was propagated in HL cells as described by Cles & Stamm [3]. Briefly, HL cells were seeded in 25 cm^2 tissue culture flasks (Costar, Cambridge, Ma.). After the cells had grown to a confluent monolayer, they were preincubated for 15 min in Hanks Balanced Salt Solution (HBSS, Flow, Irvine, Scotland) containing 15 μg/ml DEAE dextran (Sigma, USA). The monolayer was subsequently inoculated with *C. pneumoniae*. After centrifugation for 1 hr at 1200 x g, the medium was replaced by Eagles modification of Minimal Essential Medium (EMEM, Flow) containing 10% FCS, 2 mM glutamine (Flow), 1% Vitamin (Flow), 18 μg/ml gentamicin, 23 μg/ml vancomycin, 2.5 μg/ml amphotericin B and 1 μg/ml cycloheximide (Sigma) (EMEMS). Cells containing *C. pneumoniae* were sonicated following a 72 hr incubation at 37°C in an atmosphere containing 5% CO_2. *C. pneumoniae* EB were partially purified by differential centrifugation at 500 x g for 10 min and at 30000 x g for 30 min. After centrifugation, EB were suspended in SPG comprising 20 mM phosphate buffer containing 0.2 M sucrose (Merck, Germany), 49 mM glutamine (Sigma, USA), 10% FCS (Hyclone Lab. Utah), 18 μg/ml gentamicin, 23 μg/ml vancomycin and 2.5 μg/ml amphotericin B at a concentration of $2x10^8$ inclusion forming units (IFU)/ml and stored at -80°C.

Culturing and staining

Tissue culture plates with 24 wells (Greiner, Solingen, Germany) containing confluent monolayer of HL cells for *C. pneumoniae* culture were pretreated with HBBS containing DEAE dextran as described above. The monolayer was then inoculated with *C. pneumoniae* and centrifuged for 1 hr at 1200 x g and incubated in EMEMS at 37°C in an atmosphere containing 5% CO_2 for 72 hr after which the infected monolayer was fixed with 96% ethanol. *C. pneumoniae* inclusions were stained with anti-*C. pneumoniae* monoclonal antibody (Washington Research Foundation, Seattle) and fluorescein isothiocyanate-labeled goat anti-mouse Immunoglobulins (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), in 0.003% Evans Blue (Sigma). Inclusions were counted at a magnification of x320 using a Leitz Ortholux fluorescent microscope.

Incubation with variations in pH

C. pneumoniae was diluted to a concentration of 2×10^5 IFU/ml with SPG at pH from 4-10 increasing in steps of 0.5 units. After incubation for 1,4,8,24 and 48 hr at 22°C, a 0.2 ml sample was withdrawn from each tube into 1.8 ml EMEMS. Prior to infecting HL cells, any deviation from pH of 7.5 was corrected by bubbling air or CO_2. Tissue culture plates with 24 wells containing monolayers of HL cells were

inoculated in triplicate with 0.4 ml samples. As positive controls, 4 wells were inoculated with a 0.4 ml suspension of *C. pneumoniae* in EMEMS. The concentration of *C. pneumoniae* EB in the control tubes was identical to the end-dilution in the tubes which were incubated at varying pH. The number of IFU/well in the positive control was set at 100%.

Incubation with variations in NaCl concentration

C. pneumoniae was diluted to a concentration of 2×10^4 IFU/ml with distilled water containing 10% FCS at NaCl concentrations ranging from 15.4 mM to 400 mM increasing in steps of 40 mM. After different incubation periods (as described above for variations in pH), 1.6 ml distilled water containing 10% FCS and NaCl were added to 1 ml of the sample such that the final concentration of NaCl in each tube was physiological (154 mM). The subsequent steps were the same as those described above for variations in pH.

Incubation with variations in temperature

C. pneumoniae was diluted with SPG to a concentration of 2×10^5 IFU/ml. Incubations were carried out at temperatures ranging from 0°C to 45°C in steps of 5°C. After different incubation periods (as described above variations in pH), 0.2 ml sample from each tube was withdrawn into 1.8 ml EMEMS. The subsequent steps were the same as those described for variations in pH.

Effect(s) of SPG and FCS

i)**SPG.** *C. pneumoniae* was diluted with PBS to a concentration of 2×10^5 IFU/ml at pH 4.5, 7.0 and 9.0 instead of SPG at similar pH values. Incubations were carried out at 5°C, 25°C and 45°C. The subsequent steps were the same as those described above for variations in pH.

ii)**FCS.** *C. pneumoniae* was diluted with distilled water to a concentration of 2×10^4 IFU/ml and at NaCl concentrations of 80 mM, 154 mM, 240 mM, 320 mM and 400 mM instead of 10% FCS in distilled water containing similar NaCl concentrations. Incubation was carried out at 22 °C. The subsequent steps were the same as those described above for variations in NaCl concentration.

Incubation with variations in the concentration of Ca^{2+} and Mg^{2+} ions

C. pneumoniae was diluted to a concentration of 2×10^4 IFU/ml with distilled water containing 10% FCS, 154 mM NaCl at $CaCl_2$ concentrations from 0.346 mM to 4 mM at increasing steps of 0.5 mM or $MgCl_2$ at concentrations from 0 mM to 2.5 mM at increasing steps of 0.5 mM. After different incubation periods (as described above for variations in pH), a 1 ml sample was adjusted to physiological Ca^{2+} and Mg^{2+} concentrations of 2.5 mM and 1 mM repectively, with 0.46 ml of EMEMS containing Ca^{2+} and Mg^{2+}, after which HL cells were infected. The subsequent

steps were the same as those described above for variations in pH.

Statistical methods

Variations within a single experiment were determined by calculating the Standard Error of the Mean (SEM).

RESULTS

Variations in pH

One hundred percent of the *C. pneumoniae* EB survived after incubation for 24 hr at 22°C in SPG medium at pH range 5 to 8 (Fig. 1). After 48 hr incubation at pH range 5 to 8, the viability of *C. pneumoniae* decreased to about 65%. At pH lower than 5 or higher than 8, there was a strong decline in the number of infectious organisms (Fig. 1). The survival of *C. pneumoniae* incubated for 4 hr at 22°C in PBS at different pH was similar to the survival in SPG medium. Incubations for longer periods showed that SPG had a stabilizing effect on the viability of *C. pneumoniae*. After incubations for 8, 24 and 48 hr in PBS at pH 7.0, survivals of respectively 78.2%, 29.1% and 0% were observed.

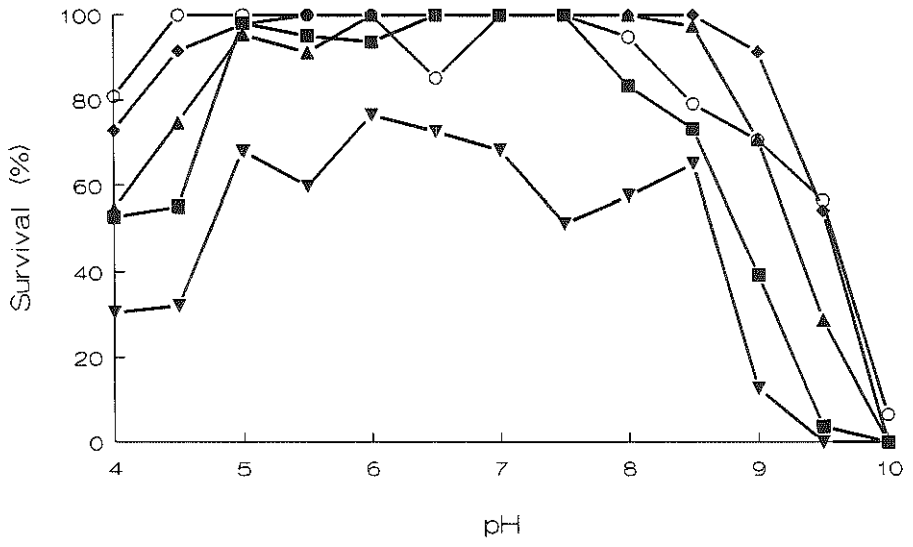

Figure 1

Survival of *C. pneumoniae* in SPG with changing pH and incubation periods at 22°C. Percentage survival after incubations of 1 hr (-○-); 4 hr (-◆-); 8 hr (-▲-); 24 hr (-■-) and 48 hr (-▼-) at pH from 4.0 to 10.0 (increasing in steps of 0.5 units). n = 9; SEM ≤ 15% (results not shown).

Variations in NaCl concentration

The viability of *C. pneumoniae* in medium containing 10% FCS was affected only at NaCl concentrations of lower than 80 mM (Fig. 2). After longer incubation periods (24 and 48 hr), a gradual decline in the viability was observed over the whole range of NaCl concentrations that were tested. The infectivity of *C. pneumoniae* was stabilized by FCS. The effect of FCS on the survival of *C. pneumoniae* EB increased as the incubation periods increased. After incubations of 8, 24 and 48 hr in distilled water at physiological NaCl concentration without FCS, survivals of respectively 54.7%, 16.9% and 0% were observed.

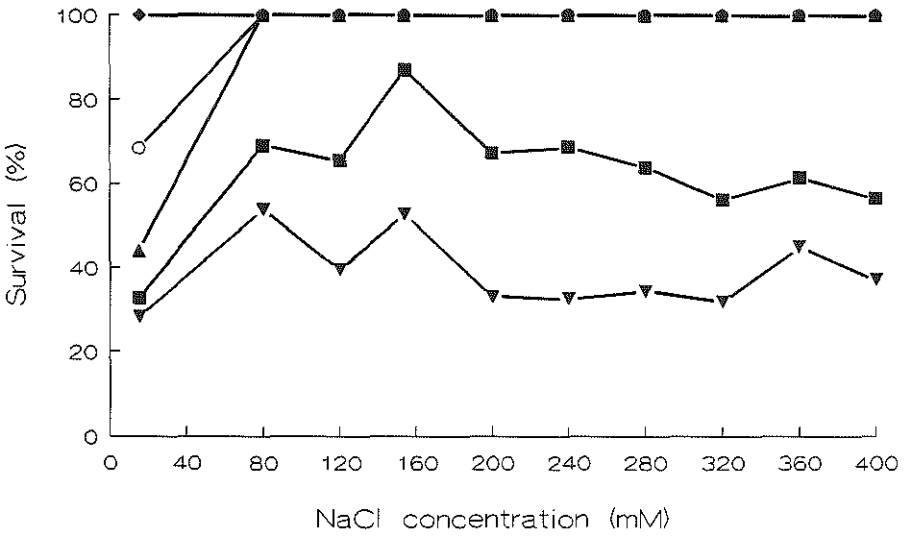

Figure 2

Survival of *C. pneumoniae* in distilled water containing 10% FCS with changing NaCl concentration and incubation periods at 22°C. Percentage survival after incubations of 1 hr (-○-); 4 hr (-◆-); 8 hr (-▲-); 24 hr (-■-) and 48 hr (-▼-) at NaCl concentrations from 15.4 mM to 400 mM (increasing in steps of 40 mM). n = 9; SEM ≤ 20% (results not shown).

Variations in temperature

No adverse effect on the viability of *C. pneumoniae* in SPG medium was observed after incubation at temperatures between 0°C and 20°C (Fig. 3). The viability of *C. pneumoniae* decreased at temperatures above 20°C and this adverse effect of increasing temperature on the survival increased as the incubation periods increased. The stabilizing effect of SPG on the survival of *C. pneumoniae* increased as the temperature and/or the incubation period increased. Incubation in PBS at 25°C for 8, 24 and 48 hr resulted in survivals of respectively 78.2, 29.1 and 0%.

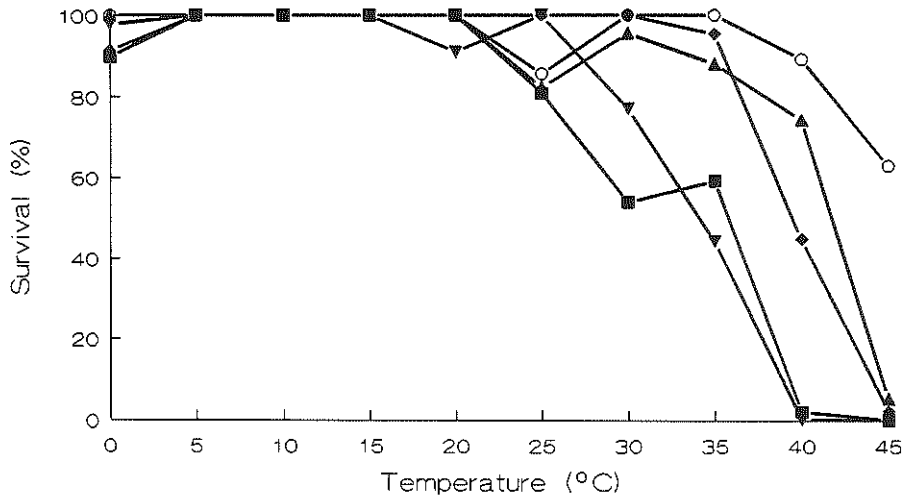

Figure 3 Survival of *C. pneumoniae* in SPG with changing temperature and incubation periods. Percentage survival after incubations of 1 hr (-○-); 4 hr (-◆-); 8 hr (-▲-); 24 hr (-■-) and 48 hr (-▼-) at temperatures from 0°C to 45°C (increasing in steps of 5°C). n = 9;
SEM ≤ 25% (results not shown).

Variations in the concentration of Ca^{2+} and Mg^{2+} ions

Variations of Ca^{2+} ion concentration from 0.346 mM to 4 mM or Mg^{2+} ion concentration from 0 mM to 2.5 mM at pH 7.5 and physiological NaCl concentration had no effect on the survival of *C. pneumoniae* (data not shown).

DISCUSSION

The results of this study showed that the survival of *C. pneumoniae* in SPG medium declined rapidly at pH lower than 5 or at pH higher than 8. Narita et al [12] observed a decrease in the turbidity when *C. psittaci* EB were exposed to an alkaline pH. These authors also demonstrated that the loss in the turbidity was coupled with the loss of EB contents. It is likely that the loss of cell contents results in the death of the organism. However, these authors did not check the viability. It was demonstrated that the major outer membrane protein (MOMP) is acidic [1]. Therefore, it is possible that exposure to an alkaline pH causes irreversible changes in the structure of MOMP. A possible explanation for the loss of viability at pH lower than 5 may be that the reduction of disulphide bonds between cysteine-rich proteins in the outer membrane of *Chlamydiae*, results in the loss of rigidity of EB.

C. pneumoniae EB were well able to withstand the changes in the osmolarity of the medium. In medium containing FCS, the viability was observed to be affected only at NaCl concentrations of lower than 80 mM in the range 15.4 mM to 400 mM that was tested. *C. pneumoniae* EB are probably resistant to hypertonic media since they are metabolically inactive. Loss of water to the hypertonic environment probably has no effect on the membrane proteins which are responsible for the adhesion and invagination of EB to the host cells. In that case, the infectivity of EB remains unimpaired. Rigidity of EB may be lost due to water-uptake in hypotonic media.

The survival of *C. pneumoniae* in SPG medium was observed to be 100% after incubation for 48 hr at temperatures of between 5°C and 20°C. The survival was observed to be 80% after incubation for 48 hr at 25°C. Kuo et al [9] observed a significant decline in the viability of *C. pneumoniae* strain AR-39 in SPG medium after incubation at 22°C. No living organisms were observed after 48 hr. As compared with the results of this study, in their study, the survival declined rapidly also after incubation at 4°C. There are several possible explanations for these discrepancies. Kuo et al cultured on HeLa 229 cells, whereas, later it was reported [3,10] that HL cells were more suitable than HeLa 229 cells for culturing *C. pneumoniae*. Kuo et al used *C. pneumoniae* strain AR-39, whereas in this study we used strain TW-183. Although it is true that both these strains are identical with respect to DNA-DNA hybridization and restriction endonuclease analyses [6], it is possible that both strains may have background anomalies with respect to their adaption in vitro and growth on cell lines. It is well known that the initial in vitro isolation of *C. pneumoniae* from patient material is tedious. However, subsequent propagation after the first few passages is relatively easier [13]. A higher efficiency in the culturing of the TW-183 strain used in the present study could have been due to adaptation to laboratory conditions.

The orientiation of the dipole moments of phospholipids in the membrane is influenced by the presence of divalent cations in the medium. Alterations in the dipole moments may indirectly cause conformational changes in the membrane lipids. The activity of the membrane-bound proteins may be regulated in this way [14]. The infectivity of *C. pneumoniae* EB was not affected by changes in the concentrations of either the Ca^{2+} or the Mg^{2+} ions. Up to 3 successive freeze-thawings of *C. pneumoniae* did not result in any decline in the number of IFU. Other authors [9] reported a decline of 11% in the number of IFU after freeze-thawing.

The survival of *C. pneumoniae* was considerably affected not only by SPG but also by the FCS contained in SPG. The stabilizing effect on the integrity of EB became greater as the incubation period increased. The SPG medium was originally used since it had shown a benificial effect on the survival of *Rickettsiae* outside its host [2]. Since its introduction in 1969 for isolating *C. trachomatis* [4], SPG

medium has been generally accepted as *Chlamydia* 'storage' medium. The role played by various constituents of SPG medium on the stability of *Chlamydia* were never investigated further.

Survival of *C. pneumoniae* outside host cells is limited. Transmission of *C. pneumoniae* between various members of the same family [16] supports a direct host to host transmission, although transmission via fomites cannot be totally excluded. A recent report also supports the possibility of spread by droplet aerosolization [7].

ACKNOWLEDEGMENTS

The authors thank Dr. Kuo for kindly providing *C. pneumoniae* strain TW-183.

REFERENCES

1. **Bavoil, P., A. Ohlin, J. Schachter.** 1984. Role of disulfide bonding in outer membrene structure and permeability in *Chlamydia trachomatis*. Infect Immun. 44:479-485.
2. **Bovarnick, M.R., J.C. Miller, J.C. Snijder.** 1950. Stability of *Rickettsiae*. J. Bacteriol. 59:509-523.
3. **Cles, L.D., W.E. Stamm.** 1990. Use of HL cells for improved isolation and passage of *Chlamydia pneumoniae*. J. Clin. Microbiol. 28:938-940.
4. **Gordon, F.B., I.A. Harper, A.L. Quan, J.D. Traharne, R.St.C. Dwyer, J.A. Garland.** 1969. Detection of *Chlamydia* (Bedsonia) in certain infections of man. 1. Laboratory procedures: Comparision of yolk sac and cell culture for detection and isolation. J. Infect. Dis. 120:451-462.
5. **Grayston J.T., C.C. Kuo, S.P. Wang, J. Altman.** 1986. A new *C. psittaci* strain, TWAR, isolated in acute respiratory tract infections. N. Engl. J. Med. 315:161-168.
6. **Grayston J.T., S.P. Wang, C.C. Kuo, L.A. Campbell.** 1989. Current knowledge of *Chlamydia pneumoniae*, strain TWAR, an important cause of pneumonia and other acute respiratory diseases. Eur. J. Clin. Microbiol. Inf. Dis. 8:191-202.
7. **Hyman C. L., M.H. Augenbraun, P.M. Roblin, J. Schachter, M.R. Hammerschlag.** 1991. Asymptomatic respiratory tract infection with *Chlamydia pneumoniae* TWAR. J. Clin. Microbiol. 29:2082-2083.
8. **Kleemola M., P. Saikku, R. Visakorpi, S.P. Wang, J.T. Grayston.** 1988. Epidemics of pneumonia caused by TWAR, a new *Chlamydia* organism, in military trainees in Finland. J. Infect. Dis. 157:230-236.
9. **Kuo C.C., J.T. Grayston.** 1988. Factors affecting viability and growth in HeLa 229 cells of *Chlamydia pneumoniae sp.* strain TWAR. J. Clin. Microbiol. 26:812-815.
10. **Kuo C.C., J.T. Grayston.** 1990. A sensitive cell line, HL cells for isolation and propagation of *Chlamydia pneumoniae* strain TWAR. J. Infect. Dis. 162:755-758.
11. **Marrie T.J., J.T. Grayston, S.P. Wang, C.C. Kuo.** 1987. Pneumonia associated with the TWAR strain of *Chlamydia*. Ann. Intern. Med. 106:507-511.

12. **Narita T., P.B. Wyrick, G.P. Manire.** 1976. Effect of alkali on the structure of cell envelopes of *Chlamydia psittaci* elementary bodies. J. Bacteriol. **125**:300-307.
13. **Persson K.** 1990. Epidemiological and clinical aspects on infections due to *Chlamydia pneumoniae* (strain TWAR). Scand. J. Infect. Dis. **69**:63-67.
14. **Seelig J.** 1990. Interaction of phospholipids with Ca^{2+} ions. On the role of the phospholipid head groups. Cell Biol. Int. Rep. **14**:353-360.
15. **Thom D.H., J.T. Grayston, S.P. Wang, C.C. Kuo, J. Altmann.** 1990. *Chlamydia pneumoniae* strain TWAR, *Mycoplasma pneumoniae*, and viral infections in acute respiratory disease in a university student health clinic population. Am. J. Epidemiol. **132**:248-256.
16. **Yamasaki T., H. Nakada, N. Sakurai, C.C. Kuo, S.P. Wang, J.T. Grayston.** 1990. Transmission of *Chlamydia pneumoniae* in young children in a Japanese family. J. Infect. Dis. **162**:1390-1392.

CHAPTER 5

INFLUENCE OF TEMPERATURE AND RELATIVE HUMIDITY ON THE SURVIVAL OF *CHLAMYDIA PNEUMONIAE* IN AEROSOLS

J.J.H. Theunissen[1*], N.A. Lemmens-den Toom[2], A. Burggraaf[3], E. Stolz[1], M. F. Michel[2].

Departments of Dermato-Venereology[1] and Clinical Microbiology[2] and Central Research Laboratories[3], Erasmus University, Rotterdam, The Netherlands.

Published in Applied and Environmental Microbiology 1993;59:2589-2593.

SUMMARY

The survival of *Chlamydia pneumoniae* in aerosols was investigated using a chamber with a capacity of 114.5 L. We injected $5x10^7$ inclusion forming units (IFU) of *C. pneumoniae* in aerosols with a droplet-size of 3-5 μm. Samples were taken after 0.5 min and every min thereafter. The survival of *C. pneumoniae* was measured at four temperatures (8.5, 15, 25 and 35°C) and at three different relative humidities (RH) of 5, 50 and 95% for each temperature. The survival of *Streptococcus pneumoniae, Streptococcus faecalis, Klebsiella pneumoniae, Chlamydia trachomatis* LGV2 and cytomegalovirus were also determined at 25°C and 95% RH and compared with that of *C. pneumoniae*. At the mentioned temperatures and RH, a rapid decrease of *C. pneumoniae* IFU was observed in the first 30 s. After this the decrease in the number of IFU was more gradual. The survival of *C. pneumoniae* in aerosols was optimum at 15-25°C and 95% RH; it was good compared with that of other microorganisms. A lower death rate was observed only in *S. faecalis*. In *C. trachomatis*, death rate during the first 30 s was higher than that in *C. pneumoniae* (85% and 53.3% respectively). After the first 30 s, the death rates in the two organosms were identical. It was concluded that transmission of *C. pneumoniae* via aerosols was possible. There is probably a direct transmission from person to person taking into account the relatively short survival period of *C. pneumoniae* in aerosols.

INTRODUCTION

Transmission of microorganisms which cause respiratory infections probably occurs via an airborne route. In contrast to transport over long distances, host-to-host transmission is a relatively rapid process which occurs via sneezing and coughing and which is accompanied by transmission of microorganisms in small droplets of 3-10 μm, the so-called aerosols.

During the aerial passage, the survival of microorganisms in aerosols depends on five different factors: relative humidity (RH), temperature, level of oxygen, presence of UV radiation and constituents of the aerosol and air (12,16). The degree to which these factors influence the survival of microorganisms in aerosols depends strongly on the type of microorganism and the time it has to spend in the aerosol. In general, the following rules apply. Gram-negative bacteria survive the best at low temperatures and RH (4,5). Gram-positive bacteria survive better at low temperatures and high RH (5,6). In almost all cases, gram-positive bacteria survive better and longer than gram-negative bacteria in aerosols. The sensitivity of gram-

negative bacteria to oxygen depends on the species. Viruses with membrane lipids are more stable in aerosols than are viruses without membrane lipids (5). Viruses which possess lipids survive the best at RH below 50%, whereas viruses which do not possess lipids are most stable at RH above 50% (6).

C. pneumoniae is a recently reported microorganism which can cause respiratory infections varying in seriousness from bronchitis to atypical pneumonia (10). Although, no proof has yet been provided, it is assumed that *C. pneumoniae* is transmitted via aerosols. A possible indication for such a mode of transmission was provided by the accidental infection of laboratory personnel with *C. pneumoniae* (14). Transmission must progress particularly efficient because 50-60% of the European and North American population experience an infection with *C. pneumoniae* at the age of 5 to 15 years (10).

In the present study the survival of *C. pneumoniae* elementary bodies (EBs) in air was determined at different temperatures and relative humidity in an aerosol chamber to obtain further insight into its mode of transmission. A possible relationship between climate/seasons and the efficiency of transmission could thus be investigated. In order to make the acceptable assumption that infection with *C. pneumoniae* occurs via the airborne route, survival of other respiratory microorganisms in the same aerosol chamber was also determined.

Transmission of the urogenital pathogen *Chlamydia trachomatis* LGV_2 requires contact between mucous membranes. If transmission of *C. pneumoniae* occurs via an airborne route, an adaptation of *C. pneumoniae* to this mode of transmission is likely and differences in the survival rate in aerosols between *C. trachomatis* LGV_2 and *C. pneumoniae* can be expected. Differences in the survival of *C. trachomatis* LGV_2 and *C. pneumoniae* were determined by comparing the infectivity decrease of both microorganisms in aerosols.

MATERIALS AND METHODS

Microorganisms and antigen purification. *Chlamydia pneumoniae* (strain TW-183) was propagated in HL cells as described by Cles and Stamm (3). HL cells were seeded in 75 cm^2 tissue culture flasks (Costar, Cambridge, Mass.). After the cells had grown to a confluent monolayer, they were pre-incubated for 15 min in Hank's balanced salt solution (HBSS) supplemented with 15 μg/ml DEAE dextran (Sigma, Chem. Comp., St Louis, Miss.). The monolayer was subsequently inoculated with *C. pneumoniae*. After centrifugation at 1200 x g for 1 h, the medium was replaced by Eagle's modification of minimal essential medium (Flow Laboratories, Irvine, Scotland) containing 10% fetal calf serum (FCS; Hyclone Laboratories, Inc., Logan, Utah), 2 mM glutamine (Flow), 1% vitamins (Flow), 18 μg/ml gentamicin, 23 μg/ml

vancomycin, 2.5 μg/ml amphotericin B and 1 μg of cycloheximide (Sigma) per ml (EMEMS). Following incubation at 37°C for 72 h in an atmosphere containing 5% CO_2, *C. pneumoniae* containing cells were sonicated and partially purified by differential centrifugation at 500 x g for 10 min and at 30000 x g for 30 min. The 30000 x g pellet was homogenized in SPG comprising 20 mM phosphate buffer containing 0.2 M sucrose (Merck, Darmstadt, Germany), 49 mM glutamine (Sigma), 10% FCS, 18 μg/ml gentamicin, 23 μg/ml vancomycin and 2.5 μg/ml amphotericin B and centrifuged at 53000 x g for 1 h through a 30% urografin solution (3.9 g/100 ml sodium amidotrizoate, 26 g/100 ml meglumine amidotrizoate, 146 mg/100 ml iodine). Chlamydiae in the resulting pellet were washed once with phosphate-buffered saline (PBS, pH 7,4) and stored in SPG at -80 °C until use.

Chlamydia trachomatis LGV_2 (strain 343/Bu) was propagated in McCoy cells. Culture and purification of *C. trachomatis* were identical to those described for *C. pneumoniae*. We chose *C. trachomatis* so that we could compare two organisms that belong to the same genus but cause different infections.

Streptococcus faecalis, Klebsiella pneumoniae and *Streptococcus pneumoniae* were isolated from throats of patients with respiratory disorders. Pure cultures of the bacteria were established in Todd-Hewitt bouillon (Oxoid, Basingstoke, United Kingdom) by culturing for 16 h and were then stored in Todd-Hewitt bouillon supplemented with 10% glycerol (Merck) at -80°C till use. *S. faecalis* was included because its survival in aerosols is known to be good (13). *K. pneumoniae* and *S. pneumoniae* were included as examples of a gram-negative and a gram-positive microorganism which may cause infections in the respiratory tract.

Cytomegalovirus (CMV, Strain Kerr) was cultured in MRC-5 cells (Flow). The cells were seeded in 75 cm^2 tissue culture flasks (Costar) and were grown to a confluent monolayer. The medium was then removed and the cells were inoculated with 10^5-10^6 plaque forming units (PFU) of CMV in Dulbecco's modification of minimal essential medium supplemented with 2 mM glutamine, 2.5% FCS, 1% amino acids and 18 μg/ml gentamicin (DMEMS) and incubated at 37°C for 2 h under an atmosphere containing 5% CO_2. After incubation, the free virus was rinsed away and the cells were incubated in DMEMS at 37°C for 5 days under an atmosphere containing 5% CO_2. Virus containing cells were subsequently sonicated and the virus was partially purified by centrifugation at 1500 x g for 10 min. The CMV containing supernatant was stored at -80 °C until use. Cytomegalovirus was included as an example of a virus which may cause infections in the respiratory tract.

Culture and staining. Tissue culture plates with 24 wells (Greiner, Solingen, Germany) containing HL cells for *C. pneumoniae* culture or McCoy cells for *C. trachomatis* LGV_2 culture were treated with Hank's balanced salt solution

containing DEAE as described above. Monolayers were inoculated with *Chlamydiae,* centrifuged at 1200 x g for 1 h and incubated in EMEMS for 72 h at 37°C in an atmosphere containing 5% CO_2. After incubation, the infected monolayer was fixed with 96% ethanol (Merck). *C. trachomatis* LGV_2 inclusions were stained with anti-*C. trachomatis* monoclonal antibody (Microtrak, Syva CO., Palo Alto, Calf.). *C. pneumoniae* was stained using anti-*C. pneumoniae* monoclonal antibody (Washington Research Foundation, Seattle, Wash.) and fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulins (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) in 0.003% Evans blue (Sigma). Inclusions were counted with a Leitz Ortholux fluorescent microscope.

S. faecalis and *K. pneumoniae* were cultured in Todd-Hewitt agar (Oxoid) at 37 °C for 16 h. *S. pneumoniae* was cultured on blood agar (Oxoid) at 37 °C for 16 h in an atmosphere containing 5% CO_2.

Tissue culture plates with 24 wells (Greiner) containing MRC-5 cells were inoculated with CMV and incubated at 37°C in an atmosphere containing 5% CO_2. After 1 h the medium was substituted with 1 ml of two-fold strenght Basal Medium (Eagle)-without phenol red (Difco Laboratories, Detroit, Mich), diluted twofold with 1.2% Seakem-ME agarose (FMC Bioproducts, Rockland, Maine) in distilled water, yielding full strenght medium containing 0.6% agarose. After incubation at 37°C for 10 to 14 days under an atmosphere containing 5% CO_2, the cells were fixed by incubation with 10% formalin in PBS for 24 h. After fixing, the agar layer was removed and the cells were stained for 1 min with 1% methylene blue in distilled water. After destaining with distilled water, the plaques were counted at a magnification of x100.

Aerosol Chamber. The aerosol chamber comprised a stainless steel cylindrical container with a capacity of 114.5 Liters (Fig. 1). The temperature in the chamber could be varied from 8.5°C to 70°C by altering the temperature of the surrounding water jacket, which was connected to a water bath with a temperature regulator. The RH could be varied either by using water vapor (increase in RH) or by pumping air which had been passed through silica column (decrease in RH) into the chamber. Eventual over- or underpressure was compensated for via a hydrophobic, autoclavable 0.45 μm or 0.22 μm pore-size filter (Millipore Intertech, Bedford, MA). The RH in the chamber was measured with a MC-2 humidity probe (Panametrics, Waltham, MA.), and the temperature was measured with a PT-100 electrode. Both probes were autoclavable. The temperature and RH could be read on the home made digital display. A FK-8 aerosol gun was used to generate aerosols (7). The pressure (5 kg/cm^2) that was required for injection was obtained from a nitrogen (N_2) gas cylinder. At this pressure of injection, a 1 ml sample was dispersed in 4

s into aerosols with a droplet-size of 3-5 μm. Samples were obtained via a stainless steel capillary impinger with a diameter of 1 mm. The rate of sampling was 10 L/min, and the size of the sample was 5 liters. The content of the aerosol chamber was kept homogeneous using a ventilator with four fins with a diameter of 5.5 cm and a speed of 3000 rpm. The infected compartment of the aerosol chamber was a closed system which could be autoclaved.

In each experiment, 1 ml PBS supplemented with 10% FCS containing either $5x10^7$ inclusion forming units (IFU) of *Chlamydiae* EBs or $1x10^8$ to $5x10^8$ CFU of *S. faecalis, S pneumoniae* or *K. pneumoniae* obtained from a 16-h culture was injected. For CMV, $1,4x10^7$ PFU contained in 1 ml DMEMS was injected. The sampling medium was the same as that used for injection and consisted of PBS supplemented with 10% FCS. The protein concentration of this medium (0.35 g/100 ml) is similar to the protein concentration found in saliva.

In separate experiments, the physical fallout was measured over 5.5 min for every temperature and RH by spraying $5x10^7$ cpm ^{67}Ga labeled gallium citrate in 1 ml PBS containing 10% FCS. A 5-liter sample was taken after 0.5, 1.5, 2.5, 3.5, 4.5 and 5.5 min. The total number of counts per minute in the samples obtained at different times was subsequently set at 100%. In performing the control experiments, with ^{67}Ga, it was observed that the maximum recovery at all RH tested, was 2% of the total that was converted to aerosol. The physical fallout measured over the 5.5-min period appeared to be negligible. Theoretically, the physical fallout is the highest at high RH because droplets can form on the wall of the aerosol chamber. Several experiments for determining this fallout were repeated with ^{125}I-labeled *C. pneumoniae* EBs (9). ^{125}I-labeled *C. pneumoniae* EBs ($5x10^7$ IFU) were injected at 8.5, 15, 25 and 35°C and 95% RH. Subsequently, the physical fallout (decrease in the number of counts per minute) and the survival of *C. pneumoniae* under the tested conditions were determined. The physical fallout measured during 5.5 min was negligible, whereas the decrease in the number of infectious EBs was comparable to that of unlabeled *C. pneumoniae* EBs under the same conditions. On the basis of this information, we decided to express the decrease in the number of infectious organisms in aerosols as a ratio viable microorganisms (mo)/^{67}Ga.

Prior to spraying the microorganisms in the aerosol chamber, we tested whether they could withstand the injection pressure of 5 kg/cm^2 and the high air speeds that are reached during sampling. For this purpose, prior to spraying, the microorganisms were cultured as described above.

The microorganisms were then injected directly into the sampling medium using the aerosol gun and cultured again. To check their survival during sampling, we suspended the microorganisms in sample medium and cultured as them as described

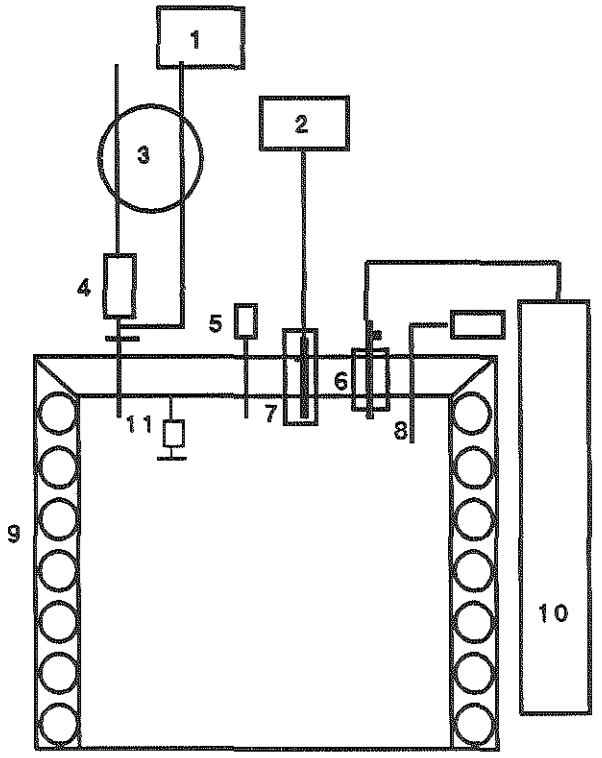

Figure 1. Schematic diagram of the aerosol chamber. **1.** Water vapor generator. **2.** Temperature and RH meter. **3.** Pump. **4.** Silica column. **5.** Filter (0.22 μm or 0.45 μm). **6.** Aerosol gun. **7.** Temperature and RH probes. **8.** Capillary impinger. **9.** Water-jacket. **10.** Nitrogen cylinder. **11.** Ventilator.

above. Subsequently, 5 L of air was sucked via the capillary impinger through the sampling medium containing the microorganisms and the microorganisms were cultured again. All the microorganisms that were used in this study appeared to withstand the injection-pressure as well as the air speeds during sampling.

All experiments and checks were performed at least in duplicate.

Statistic analysis. Differences between survival of *C. pneumoniae* at different temperatures and RH and those of *C. pneumoniae* and other microorganisms tested were analysed by using multiple linear regression analysis after log transformation. The starting point of analysis is from 0.5 minutes after spraying the organisms.

RESULTS

The survival of *C. pneumoniae* during 5.5 min at different temperatures and RH are depicted in Fig. 2.

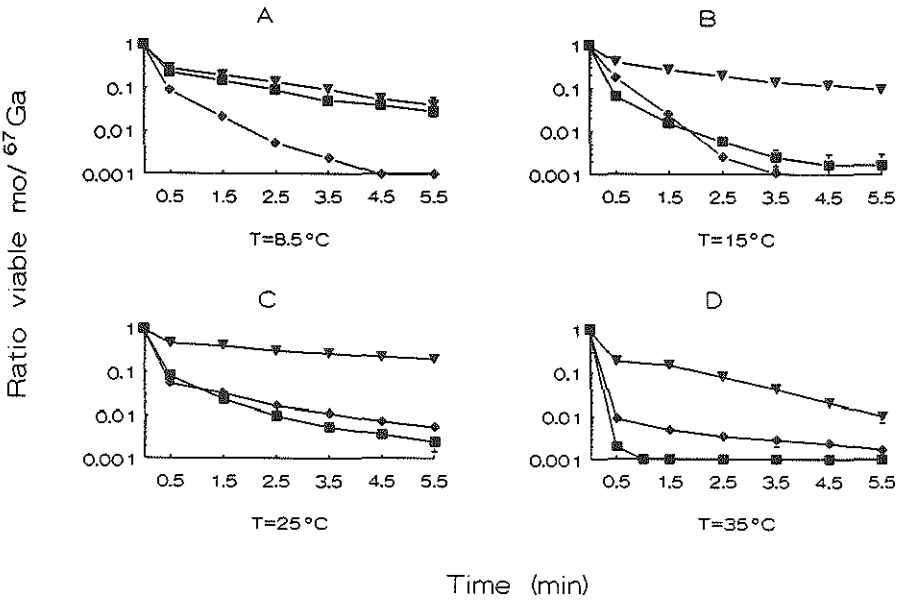

Figure 2. The survival of *Chlamydia pneumoniae* at different temperatures and relative humidity. The survival at 8.5°C (A), 15°C (B), 25°C (C) and 35°C (D) is expressed as a ratio of viable microorganisms/^{67}Ga. Survival of *C. pneumoniae* was measured during 5.5 min at 5% RH (-◆-), 50% RH (-■-) and 95% RH (-▼-). Error bars indicate the variation in the measurements. In the cases where the error bars are not visible, the variation was so small that they are beyond the range of scale that was used.

An initial rapid inactivation of infectious *C. pneumoniae* EBs during the first 0.5 min which was followed by a more gradual decrease in death rate during the next 5 min. It was also observed that the loss of infectious EBs was the lowest at high RH. The survival at 95% RH was significant higher than that observed at 5% or 50% RH ($P < 0.0001$). The best temperature for the survival of *C. pneumoniae* EBs in aerosols was between 15°C and 25°C (Figs. 2B and 2C). However, the survival at 5°C did not differ significantly from that at 15°C or 25°C. At 35 °C only, the survival was lower than the survival at other temperatures ($P < 0.001$). The rate of survival at 50% RH was observed to be dependent on the temperature. The

survival of *C. pneumoniae* EBs at 8.5°C and 50% RH (Fig. 2A), was almost identical to that at 95% RH. The survival at 15°C and 25°C and 50% RH was identical to that at 5% RH, but lower than the survival at 95% RH (Figs. 2B and 2C). Hardly any infectious EBs were observed at 35°C after 0.5 min when measured at 50% RH. The percentage survival was then lower than that observed at 5% and 95% RH (Fig. 2D).

The survival of *C. pneumoniae* in aerosols was compared with that of other microorganisms at 25 °C and 95% RH. A comparison of the results is depicted in Fig. 3. It can be seen that under the tested conditions, only *S. faecalis* in aerosols survived better than *C. pneumoniae*, but the difference was not statistically significant. The survival of the respiratory tract pathogen *K. pneumoniae* was slightly lower ($P = 0.062$), whereas the survival of the pathogens *S. pneumoniae* ($P < 0.0001$) and CMV ($P < 0.0001$) was clearly lower than that of *C. pneumoniae*.

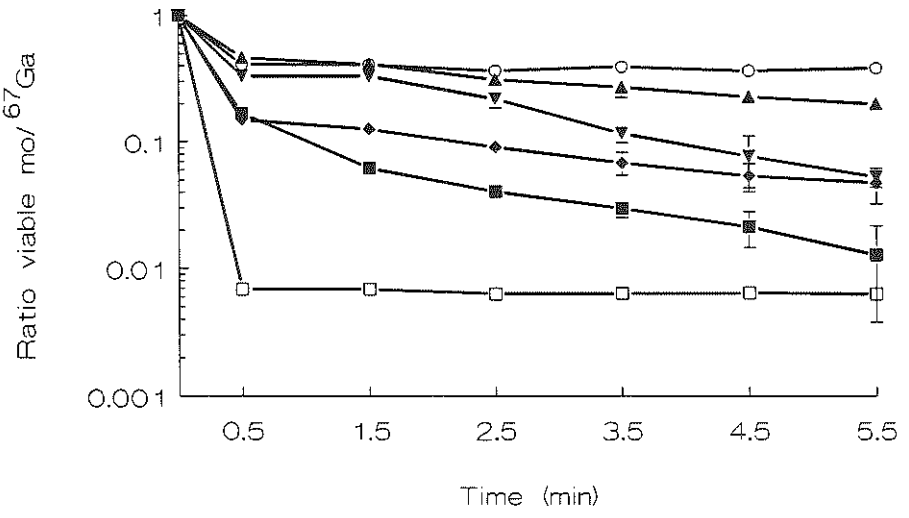

Figure 3. The survival of different microorganisms at 25°C and 95% RH. The survival is expressed as a ratio of viable mo:^{67}Ga. The survival during 5.5 min was measured for *S. feacalis* (-○-), *C. pneumoniae* (-▲-), *K. pneumoniae* (-▼-), *C. trachomatis* LGV_2 (-◆-), Cytomegalovirus(-■-) and *S. pneumoniae* (-□-). Error bars indicate the variation in the measurements.

The high inactivation of *S. pneumoniae* during the first 0.5 min was noteworthy. Thereafter, during the period of measurement, there was no further decrease in the number of *S. pneumoniae* CFU.

The decrease in the number of infectious *C. trachomatis* LGV_2 IFU in aerosols

during the first 0.5 min was much higher than that of *C. pneumoniae* IFU (85.0% and 53.4% respectively). After the first 0.5 min, death rates in both microorganisms were identical (12% per min). Overall there was a significant difference in survival between the organisms
($P < 0.001$).

DISCUSSION

The percent survival of *C. pneumoniae* in aerosols was highest at high RH and at temperatures between 15°C and 25°C. Dehydration appeared to be an important factor in the inactivation of *C. pneumoniae*, whereas extreme temperatures had a more limited influence on the survival. Inactivation was highest immediately after injection of the aerosols. This phenomenon was also observed after spraying of other microorganisms (17,20). The vapor pressure of the aerosol was in equilibrium with that of the surrounding. At low RH, the water in the aerosol immediately evaporated into the surroundings. Increases or decreases in temperature caused higher and lower evaporation rates, respectively. Several membrane reactions may occur depending on the composition of the membrane and presence or absence of a cell wall. Transition of lipids from the liquid/crystalline state to the gel state is a possible inactivation mechanism. The probability of such a phase transition increases with decreasing temperature and RH (8). Since a considerable proportion of the membrane lipids of *Chlamydiae* consist of unsaturated fatty acids (15), it is plausible that the temperature at which the transition to gel state occurs lies between 0 and 10°C. This may possibly explain the lower survival of *C. pneumoniae* in aerosols at 8.5°C and 95% RH as compared with the survival at 15-25°C and 95% RH.

Another inactivation mechanism is the occurrence of Maillard reactions at low to moderate RH (0-50%). The probability of these reactions, which require low activation energy, increases with increasing temperature (5). Such reactions occur either between lipids and proteins or between proteins and proteins and result in the removal of water. Inactivation of the proteins involved in anchoring and invagination of *C. pneumoniae* to the host cell may then occur, resulting in a decreased infectivity of *C. pneumoniae* in aerosols. Inactivation of proteins in the Maillard reaction may be an explanation for the low survival of *C. pneumoniae* EBs at 35 °C, especially at 5% and 50 %RH.

Damage to the outer membrane of gram-negative bacteria is another mechanism by which microorganisms can be inactivated during spraying in aerosols. As such, after spraying in 75% RH, 80% of *Escherichia coli* MRE 160 were susceptible to lysozyme action, indicating membrane damage (11). It was also

reported that in bacteria and viruses inactivation at high RH was the result of surface inactivation due to the (partial) removal of proteins from the outer membrane (2,19). In contrast with bacteria, *C. pneumoniae* EBs, because they are metabolically inactive, possess no repair mechanisms so that any eventual damage to the outer membrane cannot be restored. Because the survival of *C. pneumoniae* was the highest at high RH and because the membrane integrity of *C. pneumoniae* EBs depends on disulfide bridges between proteins in the outer membrane (1), in contrast with that of other gram-negative bacteria, it seems likely that the integrity of outer membrane proteins of *C. pneumoniae* is higher than that of other gram-negative bacteria. This limits the loss of outer membrane proteins during spraying which has a beneficial effect on the survival at high RH.

At low RH, two other mechanisms, namely the toxic effect of salts or amino acids in the aerosol from which the water has evaporated (19) and inactivation due to withdrawal of structural water molecules play a role. Since *C. pneumoniae* EBs are resistant to high salt concentrations in liquid media (18), it is possible that, at low RH, inactivation occurs via the withdrawal of structural water molecules.

The results in Fig. 3 show that the survival of *C. pneumoniae* in aerosols, under the conditions tested, is better than that of most other pathogens. Only *S faecalis*, a bacterium known to survive well in aerosols at 95 %RH (13), had a higher percent survival. Compared with pathogens that are likely to be transmitted by aerosols, i.e., *K. pneumoniae*, *S. pneumoniae* and CMV, *C. pneumoniae* showed better survival. Although it can not be excluded that another order of survival percentages between the organisms would be observed under different test conditions, transmission of infection with *C. pneumoniae* via the airborne route seems probable. The difference between the uropathogenic serovar *C. trachomatis* and the respiratory pathogen *C. pneumoniae* was noteworthy. *C. pneumoniae* was more resistant to the stress which occurred in the aerosol during the first 0.5 min after spraying. It is possible that the tertiary structure of one or more proteins in the outer membrane of *C. pneumoniae* is more resistant to dehydration and/or mechanical damage during the first 0.5 min.

When only the survival of *C. pneumoniae* in aerosols is taken into account, transmission of this pathogen occurs most efficiently when the temperature is between 15°C and 25°C and the RH is high. However, a number of other variables such as UV radiation, host resistance and minimal infectious dose should also be taken into consideration before the results are extrapolated to the real-world situation.

ACKNOWLEDGEMENTS

We thank C.C. Kuo for kindly providing *C. pneumoniae* TW-183, C.A. Bruggeman for kindly providing cytomegalovirus strain Kerr and P.G.H. Mulder for providing the statistical model and performing the statistical analysis.

REFERENCES

1. **Bavoil, P., A. Ohlin, and J. Schachter.** 1984. Role of disulfide bonding in outer membrane structure and permeability in *Chlamydia trachomatis*. Infect. Immun. 44:479-485.
2. **Benbough, J. E., and P. Hambleton.** 1973. Structural, organizational and functional changes associated with envelopes of bacteria sampled from aerosols, p. 135-137. *In* J. F. Ph Hers, K. C. Winkler (ed.), Airborne transmission and airborne infection, Oosthoek Publishing Company, Utrecht, The Netherlands.
3. **Cles, L. D., and W. E. Stamm.** 1990. Use of HL cells for improved isolation and passage of *Chlamydia pneumoniae*. J. Clin. Microbiol. 28:938-940.
4. **Cox, C. S.** 1966. The survival of *Escherichia coli* sprayed into air and into nitrogen from distilled water and from solutions of protecting agents as a function of relative humidity. J. Gen. Microbiol. 43:383-399.
5. **Cox, C. S.** 1989. Airborne bacteria and viruses. Sci. Prog. Oxf. 73:469-500.
6. **Jong, de J. C., T. Trouwborst, and K. C. Winkler.** 1973. Mechanisms of inactivation of viruses and macromolecules in air, p. 124-130. *In* J. F. Ph Hers, K. C. Winkler (ed.), Airborne transmission and airborne infection, Oosthoek Publishing Company, Utrecht, The Netherlands.
7. **Jong de, J. C., and K. C. Winkler.** 1968. The inactivation of poliovirus in aerosols. J. Hyg. Comb. 66:557-565.
8. **Eze, M. O., and R. N. McElhaney.** 1981. The effect of alterations in the fluidity and phase state of the membrane lipids on the passive permeation and facilitated diffusion of glycerol in *Escherichia coli*. J. Gen. Microbiol. 124:299-307.
9. **Fraker, P. J., J. C. Speck.** 1978. Protein and cell membrane iodinations with a sparingly soluble chloramide 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril. Biochem. Biophys. Res. Commun. 80:849-857.
10. **Grayston, J. T., S. P. Wang, C. C. Kuo, and L. A. Campbell.** 1989. Current knowledge of *Chlamydia pneumoniae*, strain TWAR, an important cause of pneumoniae and other acute respiratory diseases. Eur. J. Clin. Microbiol. Infect. Dis. 8:191-202.
11. **Hambleton, P., and J. E. Benbough.** 1973. Damage to the envelopes of gram-negative bacteria recovered from aerosols, p. 131-134. *In* J. F. Ph Hers, K. C. Winkler (ed.), Airborne transmission and airborne infection, Oosthoek Publishing Company, Utrecht, The Netherlands.
12. **Hatch, M. T., and R. L. Dimmick.** 1966. Physiological responses of airborne bacteria to shifts in relative humidity. Bacteriol. Rev. 30:597-603.
13. **Hemmes, J. H.** 1959. The survival of microorganisms in air. PhD Thesis. State University

Utrecht, The Netherlands

14. **Hyman, C.L., M. H. Augenbraun, P. M. Roblin, J. Schachter, and M. R. Hammerschlag.** 1991. Asymptomatic respiratory tract infection with *Chlamydia pneumoniae* strain TWAR. J. Clin. Microbiol. **29**:2082-2083.
15. **Jenkin, H. M.** 1967. Comparative lipid composition of psittacosis and trachoma agents. Amer. J. Ophthalmol. Part II. **63**:1087-1098.
16. **Larson, E. W.** 1973. Environmental variables and microbial survival, p. 81-96. *In* J. F. Ph Hers, K. C. Winkler (ed.), Airborne transmission and airborne infection, Oosthoek Publishing Company, Utrecht, The Netherlands.
17. **Songer, J. R.** 1967. Influence of relative humidity on the survival of some airborne viruses. Appl. Microbiol. **15**:35-42.
18. **Theunissen, J. J. H., B. Y. M. van Heijst, J. H. T. Wagenvoort, E. Stolz, and M. F. Michel.** 1992. Factors influencing the infectivity of *Chlamydia pneumoniae* elementary bodies on HL cells. J. Clin. Microbiol. **30**:1388-1391.
19. **Trouwborst, T., and J. C. de Jong.** 1973. Surface inactivation, an important mechanism of aerosol inactivation for viruses, inactivated at high relative humidity, p. 137-140. *In* J. F. Ph Hers, K. C. Winkler (ed.), Airborne transmission and airborne infection, Oosthoek Publishing Company, Utrecht, The Netherlands.
20. **Webb, S. J.** 1960. Factors effecting the viability of airborne bacteria. II The effect of chemical additives on the behavior of airborne cells. Can. J. Microbiol. **6**:71-87.

Chapter 6

THE EFFECTS OF MEDIUM AND RATE OF FREEZING ON THE SURVIVAL OF CHLAMYDIAS AFTER LYOPHILIZATION

J.J.H. Theunissen[1], E. Stolz[1], M.F. Michel[2].

Departments of Dermato-Venereology[1] and Clinical Microbiology[2], Erasmus University, Rotterdam, The Netherlands.

In Press (Journal of Applied Bacteriology)

SUMMARY

The effects of suspension media and rate of freezing on the survival of *Chlamydia trachomatis* LGV_2 and *Chlamydia pneumoniae* after lyophilization were assessed. The highest loss in infectious elementary bodies (EBs) occurred during lyophilization. The survival was higher after freezing at a rate of 1°C min^{-1} and lyophilization than that after rapid freezing at -70°C or -196 °C. The recovery (± 5%) was higher when fetal calf serum (FCS) containing glucose, saccharose or lactose was used as lyophilization media than that (0.5% - 3%) when yolk-sac, skimmed milk or phosphate buffer containing sucrose, glutamin and 10% FCS (SPG) were used. After lyophilization, the survival was not affected in the tested range from 10^4 to $5x10^6$ inclusion forming units (IFU) ml^{-1} prior to freezing. After storage for 4 months at 4°C, the numbers of IFU of both *Chlamydia* serovars that were recovered were identical to the numbers of IFU immediately after lyophilization. It was concluded that chlamydias can be stored and transported in lyophilized form. However, a loss of 95% in infectious EBs should be taken into account.

INTRODUCTION

Lyophilization is the most suitable method for preserving bacteria, viruses, yeasts and fungi for culture collections, provided that the micro-organisms are able to withstand this procedure (Lapage *et al.* 1970a). In principle, the method is based on a process in which cell suspensions are first frozen and then *ca* 95% of the water in the suspension is sublimated. After this, in certain cases, all but the last 1-2% of the residual water is gradually removed under vacuum. This method is very acceptable because most of the clinically interesting micro-organisms may be lyophilised without any significant loss in their viability. Moreover, because lyophilized micro-organisms may be stored for prolonged periods, it costs little to store them and no special precautions are required for transportation. Possible disadvantages of lyophilization may be damage to DNA (Ashwood-Smith and Grant 1976], to the cell wall (Ohtomo *et al.* 1988) or to lipopolysaccharides (Ray *et al.* 1976). Alterations in the membrane permeability are also possible during lyophilization (Sinskey and Silverman 1970). If lyophilization is not possible because the survival rates are lower than 0.1%, or in cases where survival is not an essential requirement after storage for prolonged periods, storage in liquid nitrogen or at -70 °C may be other suitable alternatives.

Chlamydia pneumoniae is a recently-described respiratory pathogen (Grayston *et al.* 1986). It can cause a variety of respiratory infections varying in seriousness

from bronchitis to atypical pneumonia (Grayston *et al.* 1989). *Chlamydia trachomatis* is a human urogenital pathogen. At present, 18 different serotypes of this pathogen are known. Infections with *C. trachomatis* are among the most widely occurring sexually- transmitted diseases (Martin 1990). It is known that both *Chlamydia* species can be preserved for prolonged periods provided that they are frozen at a minimum of -70°C. For the optimum survival of *C. pneumoniae* it is necessary to cool the microbe at 4°C for 4 h prior to freezing at -70°C (Kuo and Grayston 1988). This method of preservation is unsuitable for the purposes of culture collection, is expensive and is very clumsy for transport. The autors have therefore attempted to find a suitable medium for optimum survival of Chlamydias after lyophilization. Initially *C. pneumoniae* and *C. trachomatis* LGV_2 were frozen in at -70 °C or -196 °C in four different media prior to lyophilization. As fetal calf serum (FCS) supplemented with glucose offered the highest survival rates, additional changes were restricted to other saccharides dissolved in FCS. The rate of freezing was also varied during these experiments.

MATERIALS AND METHODS

Chlamydia strains and antigen purification

Chlamydia trachomatis LGV_2 (strain 434/Bu) was propagated in cycloheximide-treated McCoy cells as described by Ripa and Mårdh (1977). The McCoy cells were seeded in 75-cm^2 tissue culture flasks (Costar, Cambridge, Mass.). After the cells had grown to a confluent monolayer they were pre-incubated for 15 min in Hank's balanced salt solution (HBSS) supplemented with 15 μg/ml DEAE dextran (Sigma). The monolayer was subsequently inoculated with *C. trachomatis* LGV_2. After centrifugation at 1200g for 1 h the medium was replaced by Eagle's modification of minimal essential medium (Flow) containing 10% fetal calf serum (FCS)(Hyclone Laboratories, INC., Logan, Utah), 2 mmol l^{-1} glutamine (Flow), 1% vitamins (Flow), 18 μg ml^{-1} gentamicin, 23 μg ml^{-1} vancomycin, 2.5 μg ml^{-1} amphotericin B and 1 μg ml^{-1} cycloheximide (Sigma)(EMEMS). After incubation at 37°C in an atmosphere containing 5% CO_2 for 72 h, cells containing *C. trachomatis* LGV_2 were sonicated and partially purified by differential centrifugation at 500g for 10 min and at 30000g for 30 min. The pellet was homogenized in SPG comprising 20 mmol l^{-1} phosphate buffer containing 0.2 mol l^{-1} sucrose (Merck) 49 mmol l^{-1} glutamine (Sigma), 10% FCS, 18 μg ml^{-1} gentamicin, 23 μg ml^{-1} vancomycin and 2.5 μg ml^{-1} amphotericin B and centrifuged at 53000g for 1 h through a 30% urografin solution (3.9 g 100 ml^{-1} sodium amidotrizoate, 26 g 100 ml^{-1} meglumine amidotrizoate, 146 mg 100 ml^{-1} iodine). *Chlamydia trachomatis* LGV_2 elementary bodies were washed once with phosphate-buffered saline (PBS) and stored in SPG at -80 °C until use.

Chlamydia pneumoniae (strain TW-183) was propagated in HL cells. Culture and purification of *C. pneumoniae* was the same as that described for *C. trachomatis* LGV_2 (EBs). *Chlamydia pneumoniae* EBs were cooled at 4°C for 4 h before storage at -80°C.

Culture and staining

Tissue culture plates with 24 wells (Greiner, Solingen, Germany) containing HL cells for *C. pneumoniae* culture or McCoy cells for *C. trachomatis* LGV_2 culture were treated with Hank's balanced salt solution containing DEAE as described above. Monolayers were inoculated with chlamydias, centrifuged at 1200g for 1 h and incubated in EMEMS for 72 h at 37°C in an atmosphere containing 5% CO_2. After incubation, the infected monolayer was fixed with 96% ethanol (Merck). *Chlamydia trachomatis* LGV_2 inclusions were stained with anti-*C. trachomatis* monoclonal antibody (Microtrak, Syva CO). *Chlamydia pneumoniae* was stained with anti-*C. pneumoniae* monoclonal antibody (Washington Research Foundation) and fluorescein isothiocyanate-labelled goat anti-mouse immunoglobulins (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service) in 0.003% Evans blue (Sigma). Inclusions were counted with a Leitz Ortholux fluorescent microscope.

Lyophilization

Chlamydias were suspended in different lyophilization media at a concentration of 10^6 inclusion forming units (IFU) ml^{-1} and 0.2 ml of Chlamydias containing lyophilization medium was diluted with 1.8 ml SPG. Three wells of a 24 wells tissue culture plate were inoculated with 0.4 ml of this dilution and cultured as described above. The number of IFU per well observed in this control was set at 100%. Three millilitres of lyophilization medium containing chlamydias at a concentration van 10^6 IFU ml^{-1} was equally divided into 12 glass ampoules and frozen. One ampoule was then thawed, diluted 1:10 with SPG and cultured. The decrease in the total number of IFU after freezing was determined. The other ampoules were lyophilized under 6×10^{-2} mbar in an Edwards Super Modulyo lyophilizer. The numbers of IFU were determined either after 24 h, or in certain cases, 4 months after lyophilization by rehydrating the pellet with distilled water. The chlamydias were then diluted 1:10 with SPG and 0.4 ml of this dilution was cultured.

Controls for the freezing procedure were *Escherichia coli* and *Neisseria gonorrhoeae* (both were patient strains) which were lyophilized in FCS containing 7.5% glucose and the above protocol. The number of colony forming units was determined before and after freezing and after lyophilization of *E. coli* and *N. gonorrhoeae* on Tryptone Soy Agar (Oxoid) and plates containing G.C. hect Agar

(Becton Dickinson) supplemented with antibiotics (Difco) respectively. The results were compared with those already reported (Lapage *et al.* 1970b). Each experiment was performed at least in duplicate. Mean percentages survival are given in the results section.

Variations in the media for lyophilization and the rate of freezing

Initially, four different media were used for freezing. Skimmed milk and FCS containing 7.5% glucose were used because the viability of several bacteria after lyophilization was reported to be optimum in these media (Lapage *et al.* 1970a). Yolk-sac and SPG were used because these media have been traditionally used to store chlamydias after freezing and because it is known that their viability remains satisfactory after thawing (Kuo and Grayston 1988). In addition, during the variations in freezing parameters, FCS containing 7.5% saccharose or lactose and trehalose were also tested for their suitability as media for lyophilization.

The ampoules containing chlamydias for lyophilization were frozen in three different manners. These were instantaneous freezing in dry ice/methanol (-70°C), snap freezing in liquid nitrogen (-196°C) and strictly controlled freezing at a rate of 1°C/min down to -150°C via a Cryoson TRA-5 (Cryoson HiTech, Middenbeemster, The Netherlands).

RESULTS

The recoveries of *C. pneumoniae* and *C. trachomatis* LGV_2 after lyophilization in yolk-sac, SPG, skimmed milk and FCS containing 7.5% glucose are shown in Table 1. The final temperature after rapid freezing till -70°C or -196 °C had no effect on the recovery before and after lyophilization. From the media that were tested, the highest recovery (± 5.5% recovery) after lyophilization was observed when FCS containing 7.5% glucose was used as the preservation medium. Since the survival of chlamydias in skimmed milk was already lower than that in the other media after freezing, its recovery after lyophilization was thus adversely affected. The difference between the survival of *C. pneumoniae* and *C. trachomatis* LGV_2 when yolk-sac was used as the medium for lyophilization was remarkable. The survival of *C. pneumoniae* after freezing in yolk-sac was twice as high.

The survival of chlamydias after lyophilization was similar when 7.5% glucose, lactose and saccharose in FCS were tested for their suitability as a protective agents (Tables 1 and 2). In comparison, the recovery was lower when trehalose was used as a protective agent. In most cases, controlled freezing at a rate of 1°C min^{-1} resulted in a slightly higher recovery than that observed via other methods of rapid freezing.

The size of the chlamydia inoculum (10^4 to $5x10^6$ IFU ml^{-1}), using FCS supplemented with 7.5% glucose as lyophilization medium, had no affect on the

Table I. Influence of preservation media on viability counts before and after lyophilization.

Preservation medium	Bacterial strain	Freezing end-temperature (°C)	Viability (%)	
			Before-lyo	After-lyo
Yolk-sac				
1	*C. trachomatis*	-70	37.0	1.1
2		-196	36.2	1.0
3	*C. pneumoniae*	-70	80.3	0.8
4		-196	75.3	0.6
SPG				
1	*C. trachomatis*	-70	81.2	1.8
2		-196	76.5	2.1
3	*C. pneumoniae*	-70	91.0	2.7
4		-196	88.3	2.6
Skimmed milk				
1	*C. trachomatis*	-70	55.4	2.6
2		-196	58.7	2.9
3	*C. pneumoniae*	-70	45.6	0.9
4		-196	45.1	1.0
FCS glucose				
1	*C. trachomatis*	-70	94.2	5.4
2		-196	90.3	5.9
3	*C. pneumoniae*	-70	73.0	4.4
4		-196	73.5	2.9
5	*E. coli*	-70	92	75
6		-196	88	81
7	*N. gonorrhoeae*	-70	45	10
8		-196	55	9

Table II. Viability of chlamydias at different rates of freezing and subsequent lyophilization after preservation in FCS containing different saccharides.

FCS containing	Chlamydia strain	Rate of freezing	Viability (%)	
			Before-lyo	After-lyo
7.5% glucose				
1	*trachomatis*	slow	74.4	5.6
2	*pneumoniae*		71.2	6.2
7.5% saccharose				
1	*trachomatis*	fast	65.1	5.4
2		slow	77.4	4.9
3	*pneumoniae*	fast	69.1	3.0
4		slow	70.8	5.2
7.5% lactose				
1	*trachomatis*	fast	74.9	5.7
2		slow	76.3	3.8
3	*pneumoniae*	fast	65.9	1.4
4		slow	70.7	5.8
7.5% trehalose				
1	*trachomatis*	fast	70.1	0.8
2		slow	86.3	1.9
3	*pneumoniae*	fast	78.8	1.6
4		slow	88.4	3.4

survival after lyophilization (results not shown). In samples that were first frozen in FCS supplemented with 7.5% glucose and lyophilized no decrease in the numbers of IFU in *C. pneumoniae* and *C. trachomatis* LGV_2 was observed after storage at 4°C for 4 months (results not shown).

DISCUSSION

The factors which have an influence on the survival of micro-organisms during lyophilization are very complex and poorly understood. The process of lyophilization

may be divided into three steps all of which have an influence on the viability of micro-organisms. In the first place the manner of freezing influences the survival, followed by the actual lyophilization and finally the manner of rehydration of the micro-organisms during the recovery after lyophilization.

For an optimum recovery, cryoprotective agents are added to the medium before microorganisms are frozen (Rightsel and Greiff 1967). The protection that these additives offer depends on the exclusion of them from the solution in the immediate vicinity of membrane proteins. In the liquid phase and during freezing the thermodynamic conditions are thus unfavourable for the denaturation of membrane proteins (Carpenter *et al.* 1991). A wide variety of chemical compounds such as proteins, saccharides and inorganic salts can be used as cryoprotective agents. Tables 1 and 2 show that both SPG and FCS containing 7.5% sugar are optimal media for the survival of chlamydias after freezing. Skimmed milk appeared to be less suitable for freezing, whereas the viability of *C. pneumoniae* in yolk-sac was satisfactory. Previous results showed that the protective effect of SPG in an aqueous solution could be ascribed to the serum component (Theunissen *et al.* 1992). Table 2 shows that in general, controlled freezing led to a slightly improved survival as compared with that after rapid freezing.

The inactivation of micro-organisms during lyophilization depends on the denaturation of membrane proteins by withdrawal of water and is, therefore, fundamentally different from inactivation during freezing. The hydrogen bonds between membrane proteins and water are split during lyophilization. Thus, for a good recovery of microorganisms after lyophilization, the cryoprotective agents in the medium must adopt the role of water and re-establish the hydrogen bonds with the membrane proteins (Carpenter *et al.* 1991). Thus, for any given micro-organism, there are only one or two suitable cryoprotective agents. A medium containing lactose and gelatine was optimum for mycobacteria (Šlosárek *et al.* 1976). A medium containing 12% sucrose was optimum for anaerobes (Staab and Ely 1987) and a medium containing 0.2 mol l^{-1} sucrose and 1% bovine serum albumin was optimum for respiratory syncytial virus (Tannock *et al.* 1987). During lyophilization of chlamydias, the particular saccharide that was used hardly affected the survival, although trehalose offered less protection than the other saccharides.

During rehydration of lyophilized bacteria, the viability was affected by the medium that was used (Ray *et al.* 1976; Valdes *et al.* 1985). In the appropriate rehydration medium, the repair mechanisms of bacteria were able to restore non-lethally injured bacteria. Since these repair mechanisms are absent in *Chlamydia* EBs, there was no justification for varying the rehydration medium.

In comparison with that of the other micro-organisms, the survival of chlamydias after lyophilization, was somewhere in the middle. After lyophilization, higher survival was observed in among others, *E. coli* (80%) (MacKenzie 1977;

Table 1) and in Varicella Zoster Virus (100%) (Grose *et al.* 1981). Lower survival was observed in among others in *S. aureus* (0.1%) (Ohmoto *et al.* 1988), several anaerobes (0.01-0.1%) (Staab and Ely 1987), *Campylobacter* (0.001%) (Owen *et al.* 1989) and in a large number of viruses (Rightsel and Greiff 1967).

The recovery of chlamydias was higher after freezing in SPG at -80°C compared with lyophilization as a preservation technique. However, because there was no decrease of infectious chlamydias on day one and four months after lyophilization and because the percentage survival after lyophilization was independent of the initial inoculum, even a 5% survival of chlamydias after lyophilization may be considered as successful. Nevertheless, the results of this study do not exclude the possibility for further improvement in this survival rate. Although, long-term survival after lyophilization was not investigated in this study, lyophilization appears to be a cheap alternative for storage and transport of chlamydias.

REFERENCES

Ashwood-Smith, M.J. and Grant, E. (1976) Mutation induction in bacteria by freeze-drying. *Cryobiology* **13**, 206-213.

Carpenter, J.F., Arakawa, T and Crowe, J.H. (1991) Interactions of stabilizing additives with proteins during freeze-thawing and freeze-drying. In *Developments in Biological Standardization* **74**, ed. May, J.C., Brown, F. pp. 225-239. Basel: Karger.

Grayston, J.T., Kuo, C.C. Wang, S.P. and Altman, J. (1986) A new *Chlamydia psittaci* strain, TWAR, isolated in acute respiratory tract infections. *New England Journal of Medicine* **315**, 161-168.

Grayston, J.T., Wang, S.P, Kuo, C.C. and Campbell, L.A. (1989) Current knowledge on *Chlamydia pneumoniae* Strain TWAR, an important cause of pneumonia and other acute respiratory diseases. *European Journal of Clinical Microbiology and Infectious Diseases* **8**, 191-202.

Grose, C., Friedrichs, W.E. and Smith, K.O. (1981) Cryopreservation of Varicella-Zoster virions without loss of structural integrity or infectivity. *Intervirology* **15**, 154-160.

Kuo, C.C. and Grayston, J.T. (1988) Factors affecting viability and growth in HeLa 229 cells of *Chlamydia* sp. strain TWAR. *Journal of Clinical Microbiology* **26**, 812-815.

Lapage, S.P., Shelton, J.E. and Mitchell, T.G. (1970a) Media for the maintenance and preservation of bacteria. In *Methods in Microbiology* **3a**, ed. Norris, J.R. and Ribbons, D.J. pp. 1-134. Academic press: London and New York.

Lapage, S.P., Shelton, J.E. Mitchell, T.G., and Mackenzie, A.R. (1970b) Culture collections and the preservation of bacteria. In *Methods in Microbiology* **3a**, ed. Norris, J.R. and Ribbons, D.J. pp. 135-228. Academic press: London and New York.

MacKenzie, A.P. (1977) Comparative studies on the freeze-drying survival of various bacteria: gram type, suspending medium, and freezing rate. In *Developments in Biological Standardization* **36**, ed. Cabasso, V.J., Regamey, R.H. pp. 163-277. Basel: Karger.

Martin, D.H. (1990) Chlamydial infections. *Medical Clinics of North America* **74**, 1367-1387.

Ohtomo, T., Yamada, T. and Yoshida, K. (1988) Outermost-cell-surface changes in an

encapsulated strain of *Staphylococcus aureus* after preservation by freeze-drying. *Applied and Environmental Microbiology* **54**, 2486-2491.

Owen, R.J., On, S.L.W. and Costas, M. (1989) The effect of cooling rate, freeze-drying suspending fluid and culture age on the preservation of *Campylobacter pylori*. *Journal of Applied Bacteriology* **66**, 331-337.

Ray, B., Speck, M.L. and Dobrogosz, W.J. (1976) Cell wall lipopolysaccharide damage in *Escherichia coli* due to freezing. *Cryobiology* **13**, 153-160.

Rightsel, W.A. and Greiff, D. (1967) Freezing and drying of viruses. *Cryobiology* **3**, 423-431.

Ripa, K.T. and Mårdh, P.A. (1977) Cultivation of *Chlamydia trachomatis* in cycloheximide treated McCoy cells. *Journal of Clinical Microbiology* **6**, 328-331.

Sinskey, T.J. and Silverman, G.J. (1970) Characterisation of injury incurred by *Escherichia coli* upon freeze drying. *Journal of Bacteriology* **101**, 429-437.

Šlosárek, M., Šourek, J. and Miková, Z. (1976) Results of long-therm preservation of Mycobacteria by means of freeze-drying. *Cryobiology* **13**, 218-224.

Staab, J.A. and Ely, J.K. (1987) Viability of lyophilised anaerobes in two media. *Cryobiology* **24**, 174-178.

Tannock, G.A., Hierholzer, J.C., Bryce, D.A., Chee, C.F. and Paul, J.A. (1987) Freeze drying of respiratory syncytial virus for transportation and storage. *Journal of Clinical Microbiology* **25**, 1769-1771.

Theunissen, J.J.H., van Heijst, B.Y.M., Wagenvoort, J.H.T., Stolz, E. and Michel, M.F. (1992) Factors influencing the infectivity of *Chlamydia pneumoniae* elementary bodies on HL cells. *Journal of Clinical Microbiology* **30**, 1388-1391.

Valdez, G.F., Giori, G.S., Ruiz Holdago, A.P. and Oliver, G. (1985) Effect of the rehydration medium on the recovery of freeze-dried lactic acid bacteria. *Applied and Environmental Microbiology* **50**, 1339-1341.

CHAPTER 7

SUMMARY AND DISCUSSION

SUMMARY AND DISCUSSION

Serological detection of acute *Chlamydia trachomatis* infections.

The development of an enzyme linked immunofluorescent assay (ELFA) for the detection of IgG and/or IgM and/or IgA antibodies directed against *Chlamydia trachomatis* is described in chapter 2. With a correlation coefficient of 0.88, the sensitivity of the ELFA was almost identical to that of the microimmunofluorescence (MIF) which was used as the reference method. The disadvantages of the MIF such as the subjective assessment of the titer and the requirement for 12 different antigens in the test system (1) were overcome in the ELFA. In the ELFA, the results are obtained via an objective optical measurement, whereas the number of antigens which are offered for reaction can be limited to 6. Antibodies directed against every *C. trachomatis* serovar can be detected using these 6 antigens (2).

The possibility of cross-reactions with *Chlamydia pneumoniae* should be taken into account when antibodies directed against *C. trachomatis* are determined. These reactions occur in an enzyme linked immunosorbent assay (ELISA) and are attributed to antibodies which are directed against the lipopolysaccharide (LPS) of *C. trachomatis, Chlamydia psittaci* or *Chlamydia pneumoniae*. The cross-reactions may be avoided by extracting the LPS with sodium desoxycholate after fixing the chlamydias (3). Since unfixed elementary bodies (EBs) shed LPS spontaneously upon binding with antibodies (4), the use of unfixed EBs is also another possibility to avoid cross-reactions. We used unfixed EBs as an antigen in the ELFA and observed that there were no correlations between antibodies directed against *C. trachomatis* and those directed against *C. pneumoniae* (correlation coefficient between 0.0 and 0.1 for IgM and between 0.26 and 0.31 for IgG).

A sensitivity of 63.8% and a specificity of 58.8% as compared with that of cell culture were observed in men with uncomplicated infections using the ELFA when the results of IgG, IgM and IgA were combined. IgM antibodies made a minor contribution towards the sensitivity of the ELFA in this group of patients. As compared with IgG antibodies, IgA antibodies contributed slightly towards the sensitivity of the ELFA. This contribution was associated with a decrease in specificity. The sensitivity was observed to be 48.9% when only IgG antibodies were determined, whereas the sensitivity was observed to be 68.3% when IgG and/or IgA antibodies were determined. The specificity decreased from 80.0% to 61.3%. The sensitivity (48.9%) and the specificity (71.3%) for determining IgA antibodies only were observed to be lower than those reported previously by others in men with uncomplicated infections (5,6). In these studies sensitivities of 83.0% and 94.1% were reported, whereas the specificity in both cases was observed to be 78.0%.

The sensitivity of the ELFA as compared with that of the cell culture in

women with uncomplicated *C. trachomatis* infections was observed to be 88.1% when the results of IgG, IgM and IgA were combined. This was higher than that observed in men. However, the specificity of the reaction was lower than that in men and was observed to be 44.9%. The sensitivity and specificity of the ELFA when determining IgG antibodies as compared with that of the cell culture in women was found to be 73.8% and 60.2 % respectively. In comparison with the results reported by others (7), who found a sensitivity of 91.1% and a specificity of 53.0%, the specificity using the ELFA was lower whereas the sensitivity was higher. This difference could be attributed to the difference in the negative cut-off point. The results of IgM antibody determinations corroborated those already reported using other methods, namely that the sensitivity was observed to be low, whereas the specificity was observed to be high (8,9). In the group of women also, in comparison with the IgG antibodies, IgA antibodies contributed less towards the sensitivity. The sensitivity increased from 73.8% to 83.3% for determining IgG and/or IgA as compared with only IgG antibodies. The specificity decreased from 60.2% to 51.0% for IgG and/or IgA as compared with IgG only.

The sensitivity for determining IgM and/or IgG antibodies directed against *C. trachomatis* using the cell culture as the reference method was significantly lower in the groups of men and women using LGV_2 as the only antigen as compared with the sensitivity using the group of selected antigens. In spite of this difference and the high sensitivity of the ELFA, it was concluded that determining antibodies in patients with an uncomplicated *C. trachomatis* infections was of no value in tracing acute infections. However, the ELFA was suitable for investigating the prevalence of *C. trachomatis* infections in groups with high incidence, namely those consulting the outpatient department of the clinic for sexually transmitted diseases (STD).

Tracing of acute *C. trachomatis* infections in a group of patients suffering from complicated urogenital infections using the ELFA is described in chapter 3. A group of patients comprising 49 women with acute pelvic inflammatory disease (PID) was investigated. The results of serology were compared with those of the cell culture and the polymerase chain reaction (PCR) using samples obtained from the cervix and the urethra. The titers that were observed in the ELFA were correlated with those in the MIF. The cut-off point in the MIF for an acute infection was described previously (10). A fourfold increase or decrease in the IgM titer and/or a fourfold increase in the IgG titer and/or an IgM titer $\geq$ 1:500 (in agreement with a MIF titer $\geq$ 1:32) and/or an IgG titer $\geq$ 1:16000 (in agreement with a MIF titer $\geq$ 1:512) was therefore regarded as an indication for an acute *C. trachomatis* infection. An acute *C. trachomatis* infection was traced in 16 of the 49 patients using serology. An infection was detected in 4 of the 49 patients by cell culture and in 6 of the 49 patients using the PCR. These numbers were significantly lower than that observed in serology. All PCR-positive patients and 3 of the 4 patients with positive cell cultures had an antibody titer or a progression in antibody titer

which indicated an acute *C. trachomatis* infection. An acute infection was demonstrated in 9 of the 49 patients by serology only. The percentage (32.7%) of *C. trachomatis* infections observed in the serology in the group of patients with PID was higher than the percentage (20.0%) that was observed by others in the direct immunofluorescence assay (DFA) using samples obtained from tubae or endometrium (11). At present, the detection of *C. trachomatis* antigen in samples from tubae or endometrium using the DFA is regarded as the most sensitive method for tracing acute *C. trachomatis* infections in patients with PID. However, obtaining samples from tubae or endometrium is only possible via invasive techniques and is often considered as imposing an unnecessary burden on the patients in the Netherlands. Serology is a possible non-invasive alternative for tracing acute *C. trachomatis* infections in patients with PID because its sensitivity is at least comparable with that of the DFA.

Transmission of *Chlamydia pneumoniae*.

Little is known on the mode of transmission of *C. pneumoniae*, an organism causing respiratory tract infections. Without any doubt, an aqueous intermediate plays a role in the survival of *C. pneumoniae* in the host and in its transmission. Therefore, *C. pneumoniae* must be able to survive the physical changes in the environment. The variable physical parameters which may have an influence on the survival of *C. pneumoniae* EBs in aqueous environment were investigated in studies described in chapter 4. The infectivity of *C. pneumoniae* EBs appeared not to be affected at pH of between 5 and 8. Infectivity was also not affected by NaCl concentrations of higher than 80 mM, temperatures between 0°C and 25°C, variations in the Ca^{2+} concentrations of 0.3 mM to 4 mM or in the Mg^{2+} concentrations of 0 mM to 2.5 mM. Rapid inactivation of *C. pneumoniae* was observed to occur at pH of lower than 5 or higher than 8 similar to that reported for *Chlamydia psittaci* (12), at temperatures of higher than 30°C and in the absence of proteins in the suspension medium. The protein component had a predominantly stabilizing effect on the infectivity of *C. pneumoniae* EBs. In a suspension medium containing 35 mg/ml protein, about 60% infectious EBs were observed after incubation for 48 hours, whereas no infectious EBs were observed in suspension medium without any protein. It was concluded that the infectivity of *C. pneumoniae* EBs in an aqueous environment was limited and it is likely that its transmission from one host to another must occur without delay.

Since there were indications in the literature that the transmission of *C. pneumoniae* occurred via aerosols (13,14), it was decided to investigate the decrease in the survival of *C. pneumoniae* EBs in aerosols in time at different temperatures and at different percentages of relative humidity (RH). The decrease in the infectivity or survival in time under these conditions were compared with the decrease in the infectivity or the survival of other respiratory tract microorganisms

such as *Streptococcus pneumoniae*, cytomegalovirus and *Klebsiella pneumoniae*. *S. faecalis* was tested because it could be considered as a positive control, whereas *C. trachomatis* was tested because of an eventual difference between the survivals of chlamydias pathogenic for the urogenital tract and the respiratory tract. The results showed that the survival of *C. pneumoniae* in aerosols was optimum at temperatures between 15°C and 25°C and 95% RH. Two phases were distinguished in the survival curves. First, independent of the temperature or the RH, the infectivity of *C. pneumoniae* EBs was rapidly inactivated during the first 30 seconds after aerosolization. Thereafter, the inactivation progressed more gradually.

K. pneumoniae (a gram-negative respiratory tract pathogen), *S. pneumoniae* (a gram-positive respiratory tract pathogen) and cytomegalovirus (a respiratory tract virus) were inactivated faster than *C. pneumoniae* in aerosols at 25°C and 95% RH. The difference between the respiratory tract pathogen *C. pneumoniae* and the urogenital pathogen *C. trachomatis* was remarkable. The infectivity of *C. trachomatis* in aerosols decreased faster during the first 30 seconds after aerosolization than that of *C. pneumoniae*. This clearly indicated that *C. pneumoniae* is adapted to the host environment and to its mode of transmission to another host.

It was concluded that transmission of *C. pneumoniae* from host to host via aerosols was possible and progressed most efficiently at high relative humidity.

Long-term storage of *Chlamydia*.

Long-term storage of microorganisms is frequently essential for culture collections and for preparing vaccines. Cryopreservation is the only method used for long-term storage of chlamydias. Since this method is both expensive and laborious, survival of chlamydias after lyophilization was investigated in studies described in chapter 6. The viability of chlamydias was approximately 75% after freezing at -70°C (15,16). In comparison, the survival was much lower after lyophilization. A suspension of chlamydias in fetal calf serum (FCS) containing 7.5% glucose, lactose or saccharose and frozen at a controlled rate resulted in a survival of 5% after lyophilization. This survival rate of chlamydias after lyophilization was average as compared with that of other microorganisms. After lyophilization, 80% survival was observed in *Escherichia coli* (17) and 100% survival was observed in varicella zoster virus (18), whereas the survival of *Staphylococcus aureus* (19) and *Campylobacter pylori* (20) after lyophilization was 0.1% and 0.001% respectively. The survival after 4 months was identical to that observed immediately after lyophilization. In addition, it was observed that the survival was not dependent on the initial inoculum. Therefore, in spite of the low survival, it is still possible to use lyophilization for the long-term storage of chlamydias.

SAMENVATTING EN DISCUSSIE

Serologische detectie van acute *Chlamydia trachomatis* infecties.
In hoofdstuk twee werd de ontwikkeling van een enzyme linked immunofluorescent assay (ELFA) beschreven voor het detecteren van IgG en/of IgM en/of IgA antilichamen gericht tegen *Chlamydia trachomatis*. De gevoeligheid van deze ELFA was met een correlatie coëfficiënt van 0.88 nagenoeg gelijk aan die van de microimmuunfluorescentie (MIF) die als referentie methode werd gebruikt. De nadelen van de MIF namelijk de subjectieve beoordeling van de titer en de noodzaak om 12 verschillende antigenen in het testsysteem te gebruiken (1), werden in de ELFA ondervangen. De uitkomst van de ELFA wordt immers bepaald door een objectieve optische meting, terwijl het aantal antigenen dat in de reactie wordt aangeboden beperkt kan blijven tot zes. Met deze antigenen kunnen antilichamen gericht tegen iedere *C. trachomatis* serovar bepaald worden (2).

Bij het bepalen van IgG antilichamen gericht tegen *C. trachomatis* dient rekening te worden gehouden met de mogelijkheid van kruisreacties met *Chlamydia pneumoniae*. Deze reacties treden op bij gebruik van een enzyme linked immunosorbent assay (ELISA) en zijn toe te schrijven aan antilichamen die gericht zijn tegen het lipopolysaccharide (LPS) van *C. trachomatis, Chlamydia psittaci* en *Chlamydia pneumoniae*. De kruisreacties kunnen worden vermeden door het LPS na fixatie van de *Chlamydia* te extraheren met natrium-desoxycholaat (3). Omdat niet gefixeerde Ebs spontaan LPS verliezen bij binding met antilichamen (4), biedt het gebruik van niet gefixeerde Ebs ook een mogelijkheid om kruisreacties te voorkomen. Het bij de ELFA gebruikte antigeen bestond uit niet gefixeerde EBs. Er werden door ons dan ook geen correlaties gevonden tussen antilichamen gericht tegen *C. trachomatis* en antilichamen gericht tegen *C. pneumoniae*.

Bij mannen met ongecompliceerde infecties werd met de ELFA een sensitiviteit en een specificiteit ten opzichte van de celkweek van respectievelijk 63.8% en 58.8% bereikt, als de resultaten van IgG, IgM en IgA werden gecombineerd. Het aantonen van IgM antilichamen leverde in deze patiëntengroep een zeer geringe bijdrage aan de sensitiviteit van de ELFA. IgA antilichamen in de mannenpopulatie leverde, vergeleken met IgG antilichamen, een kleinere bijdrage tot de sensitiviteit van de ELFA . Deze bijdrage was bovendien geassocieerd met een daling van de specificiteit. De sensitiviteit bij bepalen van alleen IgG antilichamen was 48.9% terwijl de sensitiviteit bij bepalen van IgG en/of IgA antilichamen 68.3% bedroeg. De specificiteit nam dan af van 80.0% tot 61.3%. Bij het bepalen van IgA antilichamen alleen was zowel de sensitiviteit (48.9%) als specificiteit (71.3%) lager dan wat door anderen eerder beschreven werd voor patiënten met ongecompliceerde infecties (5,6). In deze studies werden sensitiviteiten van 83.0% en 94.1% gerapporteerd terwijl de specificiteit in beide gevallen 78.0% bedroeg.

De sensitiviteit van de ELFA ten opzichte van de celkweek was bij vrouwen met ongecompliceerde *C. trachomatis* infecties 88.1% indien de resultaten van IgG, IgM en IgA werden gecombineerd. Deze sensitiviteit was hoger dan die bij mannen. De specificiteit van de reactie was echter lager dan bij de mannen namelijk 44.9%. De sensitiviteit en specificiteit van de ELFA bij bepalen van IgG antilichamen was in vrouwen respectievelijk 73.8% en 60.2%. Vergeleken met reeds gepubliceerde gegevens van andere auteurs (7) die een sensitiviteit van 91.1% en een specificiteit van 53.0% vonden, was de sensitiviteit van de ELFA ten opzichte van de celkweek lager terwijl de specificiteit hoger was. Dit verschil kon worden toegeschreven aan een verschil in negatief afsnijpunt. De resultaten van IgM antilichaambepaling kwamen overeen met wat reeds voor andere detectie methoden beschreven is, namelijk dat de sensitiviteit laag is terwijl de specificiteit hoog is (8,9). Ook in de vrouwengroep leverden IgA antilichamen een kleinere bijdrage tot de sensitiviteit vergeleken met IgG antilichamen. De sensitiviteit steeg bij het bepalen van IgG en/of IgA van 73.8% tot 83.3% vergeleken met alleen IgG antilichamen. De specificiteit daalde bij IgG en/of IgA vergeleken met IgG alleen van 60.2% naar 51.0%.

Zowel bij de mannen als bij de vrouwengroep was de sensitiviteit bij het bepalen van IgM en/of IgG antilichamen gericht tegen *C. trachomatis*, met de celkweek als referentiemethode, significant lager bij gebruik van LGV_2 als enig antigeen vergeleken met de groep van geselecteerde antigenen. Ondanks dit verschil en de hoge gevoeligheid van de ELFA kon geconcludeerd worden dat het bepalen van antilichamen in patiënten met een ongecompliceerde *C. trachomatis* infectie ongeschikt is voor het traceren van acute infecties. Wel is de ELFA geschikt om de prevalentie van *C. trachomatis* infectie in een bevolkingsgroep met hoge incidentie, zoals de populatie die een polikliniek voor SOA bezoekt, te onderzoeken.

In hoofdstuk drie werden vervolgens met behulp van de ELFA acute infecties veroorzaakt door *C. trachomatis* opgespoord in een patiëntengroep met gecompliceerde urogenitale infecties. Als patiëntengroep werden 49 vrouwen met acute pelvic inflammatory disease (PID) gekozen. De serologische resultaten werden vergeleken met celkweek en polymerase ketting reactie (PCR) verricht op samples afkomstig van cervix en urethra. De met de ELFA gevonden titers werden gecorreleerd aan die van de MIF. Van de MIF werden de afsnijpunten voor een acute infectie reeds eerder beschreven (10). Een viervoudige stijging of daling van de IgM titer en/of een viervoudige stijging van de IgG titer en/of een IgM titer $\geq$ 1/500 (overeenkomend met een MIF titer $\geq$ 1/32) en/of een IgG titer $\geq$ 1/16000 (overeenkomend met een MIF titer $\geq$1/512) werden daarom als representatief beschouwd voor een acute *C. trachomatis* infectie. Een acute *C. trachomatis* infectie kon bij gebruik van serologie in 16 van de 49 patiënten worden opgespoord. Met de celkweek werd in vier en met de PCR in zes van de 49 patiënten een infectie gedetecteerd wat significant lager was vergeleken met de

serologische resultaten. Alle PCR positieve patiënten en drie van de vier celkweek positieve patiënten vertoonden een antilichaamtiter of een antilichaamtiterverloop dat duidde op een acute infectie met *C. trachomatis*. Bij negen van de 49 patiënten kon alleen met serologie een acute infectie worden aangetoond. Het percentage *C. trachomatis* infecties dat met serologie in de PID patiëntengroep kon worden gevonden (32.7%), was hoger dan het percentage dat door anderen werd gevonden (11) bij gebruik van directe immuunfluorescentie (DFA) op materiaal afkomstig van tubae of endometrium (20.0%). De detectie van *C. trachomatis* antigeen door DFA in tubae of endometrium, werd tot nu toe als de meest sensitieve methode beschouwd voor het opsporen van acute *C. trachomatis* infecties bij PID patiënten. Het verkrijgen van dit antigeen is echter alleen mogelijk door invasieve technieken en wordt in de Nederlandse situatie vaak als te belastend voor de patiënt gezien. Daar met behulp van serologie minimaal een vergelijkbare sensitiviteit behaald kan worden als met DFA, is serologie een mogelijk niet invasief alternatief voor het opsporen van acute *C. trachomatis* infecties in PID patiënten.

Overdracht van *Chlamydia pneumoniae*.

Van *C. pneumoniae*, een verwekker van luchtweginfecties, is weinig bekend over de wijze van overdracht van de ene gastheer op de andere. Een waterige intermediair speelt ongetwijfeld een rol bij de overleving van *C. pneumoniae* in de gastheer en bij de overdracht van het organisme. *C. pneumoniae* moet daarom in staat zijn om te overleven bij fysische veranderingen in de omgeving. In hoofdstuk vier werd onderzocht wat de grenzen waren van de overleving van *C. pneumoniae* EBs voor enkele fysische variabelen in een waterig milieu. De infectivitelt van *C. pneumoniae* EBs bleek niet beïnvloed te worden door een pH tussen de 5 en de 8. Ook een NaCl concentratie >80 mM, een temperatuur van 0 °C- 25 °C, variatie van de Ca^{2+} concentratie van 0.3mM- 4 mM of van de Mg^{2+} concentratie van 0 mM- 2.5 mM had geen invloed op de infectiviteit. Snelle inactivatie van *C. pneumoniae* vond plaats bij een pH lager dan 5 of hoger dan 8 overeenkomend met gegevens voor *Chlamydia psittaci* (12), bij temperaturen >30 °C en indien het suspensiemedium geen eiwit bevatte. Vooral de eiwitcomponent had een stabiliserende invloed op de infectiviteit van *C. pneumoniae* EBs. In een suspensiemedium dat 35 mg/ml eiwit bevatte kon na 48 uur incubatie nog ongeveer 60% infectieuze EBs worden teruggevonden terwijl in een suspensiemedium dat geen eiwit bevatte geen infectieuze Ebs meer werden gevonden. Geconcludeerd werd dat de infectiviteit van *C. pneumoniae* Ebs in een waterig milieu gelimiteerd is en dat voor overleving een snelle overdracht van het organisme van de ene op de andere gastheer dus waarschijnlijk is.

Omdat in de literatuur aanwijzingen bestonden die duidden op een overdracht van *C. pneumoniae* door middel van aërosolen (13,14), werd besloten de afname in de tijd van de overleving van *C. pneumoniae* EBs in aërosolen te onderzoeken bij

een verschillende temperaturen en verschillende graden van luchtvochtigheid. De afname van de infectiviteit of van de overleving in de tijd werd onder dezelfde omstandigheden vergeleken met de afname van de infectiviteit of de overleving van andere respiratoire microörganismen zoals *Streptococcus pneumoniae*, cytomegalovirus en *Klebsiella pneumoniae*, van *Streptococcus faecalis* en van *C. trachomatis*. *S. faecalis* gold als positieve controle. De vergelijking met *C. trachomatis* had als doel een eventueel verschil in overleving tussen een uropathogene en een respiratoire chlamydia aan te tonen. Uit de experimenten bleek dat *C. pneumoniae* in aërosolen het beste overleeft bij een temperatuur tussen de 15 °C en de 25 °C en bij een luchtvochtigheid (RH) van 95%. In de afstervingscurves waren twee fases te onderscheiden. Eerst vindt er, onafhankelijk van temperatuur of RH, een zeer snelle inactivatie van de infectiviteit van *C. pneumoniae* EBs plaats gedurende de eerste 30 seconden na aërosolisatie. Hierna verloopt de inactivatie meer geleidelijk.

K. pneumoniae (een gram-negatieve respiratoire pathogeen), *S. pneumoniae* (een gram-positieve respiratoire pathogeen) en cytomegalovirus (een respiratoir virus) vertoonden allen in aërosolen bij 25 °C en 95 %RH snellere inactivatie curves dan *C. pneumoniae*. Opmerkelijk was het verschil tussen de repiratoire pathogeen *C. pneumoniae* en de urogenitale pathogeen *C. trachomatis*. De infectiviteit in aërosolen van *C. trachomatis* daalde gedurende de eerste 30 seconden na aërosolisatie veel sneller dan de infectiviteit van *C. pneumoniae*. Dit duidt op een duidelijke adaptatie van *C. pneumoniae* aan het gastheermilieu en aan de bijbehorende manier van overdracht naar een andere gastheer.

Geconcludeerd werd dat een overdracht van *C. pneumoniae* van gastheer op gastheer via aërosolen waarschijnlijk is en dat deze overdracht het meest effectief verloopt bij hoge luchtvochtigheid van de omgeving.

Langdurige opslag van *Chlamydia*.

Voor onder andere vaccin bereiding en culture collections is langdurige opslag van microöganismen vaak een vereiste. Voor *Chlamydia* is cryopreservatie de enige in gebruik zijnde methode voor langdurige opslag van organismen. Aangezien deze methode op termijn duur en arbeidsintensief is werd in hoofdstuk zes onderzocht wat de overleving van *Chlamydia* is na lyophilisatie. Na invriezen bij -70 °C bleek ongeveer 75% van de *Chlamydia* nog levensvatbaar (15,16). Lyophilisatie leverde vergeleken hiermee, aanzienlijk lagere overlevingspercentages. Een suspensie van *Chlamydia* in een 7.5% oplossing van glucose, lactose of saccharose in FCS gecombineerd met een gecontroleerde manier van invriezen, leidde na lyophilisatie tot een overleving van 5%. Vergeleken met andere microörganismen is de overleving van *Chlamydia* na lyophilisatie gemiddeld te noemen. Zo vertoonden *Escherichia coli* (17) en varicella zoster virus (18) overlevingspercentages van respectievelijk 80% en 100% na lyophilisatie, terwijl voor *Staphylococcus aureus*

(19) en *Campylobacter pylori* (20) overlevingspercentages van resp. 0.1% en 0.001% werden gevonden. Het percentage overleving na vier maanden was gelijk aan het percentage dat gevonden werd onmiddellijk na lyophilisatie. Bovendien bleek het overlevingspercentage onafhankelijk te zijn van het oorspronkelijk inoculum. Hierdoor is het, ondanks het lage percentage overlevende *Chlamydia*, toch mogelijk om lyophilisatie te gebruiken als methode voor langdurige opslag van deze organismen.

REFERENCES

1. Wang SP, Grayston JT. Microimmunofluorescence antibody responses in *Chlamydia trachomatis* infections. In: Mårdh PA, Holmes KK, Oriel JD, Piot P, Schachter J (ed): Chlamydial infections Vol. 2. Amsterdam: Elsevier Biomedical Press, 1982:301-316.
2. Wang SP, Grayston JT. Immunological relationship between genital TRIC lymphogranuloma venereum and related organisms in a new microtiter indirect immunofluorescence test. Am J Ophtalmology 1970;**70**:367-374
3. Ladany S, Black CM, Farshy CE, Ossewaarde JM, Barnes RC. Enzyme immunoassay to determine exposure to *Chlamydia pneumoniae* (strain TWAR). J Clin Microbiol 1989;**27**:2778-2783.
4. Birkelund S, Lundemose AG, Christiansen G. Immunoelectron microscopy of lipopolysaccharide in *Chlamydia trachomatis*. Infect Immun 1989;**57**:3250-3253.
5. Hagay ZJ, Sarov B, Sachs J, Shaked O, Sarov I. Detecting *Chlamydia trachomatis* in men with urethritis: serology versus isolation in cell culture. Genitourin Med 1989;**65**:166-170.
6. Cevenini R, Sarov I, Rumpianesi F, *et al.* Serum specific IgA antibodies to *Chlamydia trachomatis* in patients with chlamydial infections detected by ELISA and immunofluorescence test. J Clin Pathol 1984;**37**:686-691.
7. Schoenwald E, Schmidt BL, Steinmetz G, *et al.* Diagnosis of *Chlamydia trachomatis* infection. Culture versus serology. Eur J Epidemiol 1988;4:75-82.
8. Jones RB, Bruins SC, Newhall WJ. Comparison of reticulate and elementary body antigens in detection of antibodies against *Chlamydia trachomatis* by an enzyme linked immunosorbent assay. J Clin Microbiol 1983;**17**:466-471.
9. Schachter J, Cles L, Ray R, Hines PA. Failure of serology in diagnosing Chlamydial infections of the female genital tract. J Clin Microbiol 1979;**10**:647-649.
10. Grayston JT, Wang SP, Foy HM, Kuo CC. Seroepidemiology of *Chlamydia trachomatis* infection. In: Mårdh PA, Holmes KK, Oriel JD, Piot P, Schachter J (eds). Chlamydial infections. Amsterdam: Elsevier Biomedical Press, 1982 Vol. 2:405-19.
11. Thejls H, Gnarpe J, Lundkvist O, Heimer G, Larsson G, Victor A. Diagnosis and prevalence of persistent chlamydia infection in infertile women: tissue culture, direct antigen detection, and serology. Fertil Steril 1991;**55**:304-310.
12. Narita T, Wyrick PB, Manire GP. Effect of alkali on the structure of cell envelopes of *Chlamydia psittaci* elementary bodies. J Bacteriol 1976;**125**:300-307.
13. Hyman CL, Augenbraun MH, Roblin PM, Schachter J, Hammerschlag MR. Asymptomatic respiratory tract infection with *Chlamydia pneumoniae* TWAR. J Clin Microbiol 1991;**29**:2082-2083.
14. Yamasaki T, Nakada H, Sakurai N, Kuo CC, Wang SP, Grayston JT. Transmission of

Chlamydia pneumoniae in young children in a Japanese family. J Infect Dis 1991;**162**:1390-1392.

15. Kuo CC, Grayston JT. Factors affecting the viability and growth in HeLa 229 cells of *Chlamydia* sp. strain TWAR. J Clin Microbiol 1988;**26**:812-815.
16. Tjiam KH, van Heijst BYM, de Roo JC, de Beer A, van Joost Th, Michel MF, Stolz E. Survival of *Chlamydia trachomatis* in different transport media and at different temperatures: diagnostic implications. Br J Ven Dis 1984;**60**:92
17. MacKenzie AP. Comparative studies on the freeze-drying survival of various bacteria: gram type, suspending medium, and freezing rate. In: Developments in Biological Standardization **36**, ed. Cabasso, V.J., Regamey, R.H. 1977. pp. 163-277. Basel: Karger.
18. Grose C, Friedrichs WE, Smith KO. Cryopreservation of Varicella-Zoster virions without loss of structural integrity or infectivity. Intervir. 1981;**15**, 154-160.
19. Ohtomo T, Yamada T, Yoshida K. Outermost-cell-surface changes in an encapsulated strain of *Staphylococcus aureus* after preservation by freeze-drying. Appl Environ Microbiol 1988;**54**, 2486-2491.
20. Owen RJ, On SLW, Costas M. The effect of cooling rate, freeze-drying suspension fluid and culture age on the preservation of *Campylobacter pylori*. J Appl Bacteriol 1989;**66**, 331-337.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 19 augustus 1960 te Roggel. Van 1972 tot 1979 werd het Atheneum B doorlopen aan het Bisschoppelijk College te Roermond. In 1979 werd begonnen aan de studie Biologie aan de Rijks Universiteit te Utrecht. In 1982 werd het kandidaatsexamen Chemische Biologie behaald. Hierna werden de hoofdvakken Biochemie (Prof. Dr de Kruijff) en Moleculaire Microbiologie (Prof. Dr Hoekstra) en het bijvak Klinische Immunologie (prof. Dr Ballieux) gevolgd. In november 1986 werd de studie Biologie afgesloten met het doctoraalexamen. Hierna volgde een aanstelling als wetenschappelijk medewerker bij het instituut Dermato-Venereologie, van de Faculteit der Geneeskunde en Gezondheidswetenschappen, Erasmus Universiteit Rotterdam, waar dit proefschrift werd bewerkt onder begeleiding van Prof. Dr Michel en Prof. Dr Stolz. Van februari 1993 tot mei 1993 was schrijver dezes aangesteld als bioloog bij de vakgroep Medische Microbiologie, Academisch Ziekenhuis Leiden. Vanaf juni 1993 tot heden is de auteur werkzaam als projectmedewerker bij het Laboratorium voor Water- en Levensmiddelenmicrobiologie, Rijksinstituut voor Volksgezondheid en Milieuhygiëne, Bilthoven.

DANKWOORD

Last but not least wil ik iedereen die een bijdrage heeft geleverd aan het tot stand komen van dit proefschrift, bedanken. Uit het feit dat op deze pagina slechts enkelen met name genoemd worden, mag geconcludeerd worden dat vele anderen ten onrechte ongenoemd blijven.

Om te beginnen zou al het voorgaande onmogelijk zijn geweest zonder de stimulerende begeleiding van prof Michel. Na zijn emeritaat nam de snelheid waarmee de verschillende delen van het manuscript werden beoordeeld, zomogelijk nog toe.

Prof Stolz heeft, ook in tijden dat het minder ging, altijd in de goede afloop van bovenstaande exercitie geloofd. Om deze reden, vanwege zijn onmisbare hulp bij de klinische onderwerpen en omdat hij altijd wel een "geldpot" vond waaruit mijn salaris betaald kon worden ben ik hem zeer erkentelijk.

Ik heb de commissieleden, Prof. dr C.P.A. van Boven en Prof. dr J. Huisman in een bepaald stadium opgezadeld met een grote hoeveelheid leeswerk. Hen ben ik erkentelijk voor de enthousiaste en accurate manier waarmee zij zich van deze taak gekweten hebben.

Yvonne van Heijst, en Nicole den Toom hebben vele werkzaamheden verricht, zonder welke het manuscript aanzienlijk dunner zou zijn.

De mensen van de Centrale Research Werkplaatsen, met name Arie Burggraaf, Joop Aartsen, Wim Holland, Harvey Wong Long Sing, Gerard Heuvelsland en Hans de Haan wil ik bedanken voor de snelle manier waarop technische problemen voor mij werden opgelost.

De hulp van Willy Minderhoud-Bassie, Roël Chin-A-Lien, Frans Huikeshoven en Hans Wagenvoort was onmisbaar bij het interpreteren van patiëntengegevens.

Zonder Tannies hulp waren er geen steriele media en materialen geweest.

Bob Tank, voor het echte engels.

Irma Bakker ben ik dank verschuldigd voor de ruimte op de afdeling en voor de discussies, die getuigen van de belangstelling waarmee ze het onderzoek gevolgd heeft.

De mensen van het baclab en de aanname ben ik erkentelijk voor de vaak onaangename werkjes die ze voor mij hebben opgeknapt.

Het was gezellig met de collega's van Klinische Microbiologie.

PUBLICATIONS

1. Theunissen JJH, Jackson RL, Kempen HJM, Demel RA. Membrane properties of oxysterols. Interfacial orientation, influence on membrane permeability and redistribution between membranes. Biochim Biophys Acta 1986;**860**:66-74.

2. Theunissen JJH, van Heijst BYM, Chin-A-Lien RAM, Wagenvoort JHT, Stolz E, Michel MF. Detection of IgG, IgM, and IgA antibodies in patients with uncomplicated *Chlamydia trachomatis* infection: A comparison between Enzyme Linked Immunofluorescent Assay and isolation in cell culture.
Int J *STD & AIDS* 1993;**4**:43-48.

3. Theunissen JJH, Minderhoud-Bassie W, Wagenvoort JHT, Stolz E, Michel MF, Huikeshoven M. Detection of antibodies directed against *Chlamydia trachomatis* in patients with pelvic inflammatory disease: Comparison with cervix culture and polymerase chain reaction. Submitted.

4. Theunissen JJH, van Heijst BYM, Wagenvoort JHT, Stolz E, Michel MF. Factors influencing the infectivity of *Chlamydia pneumoniae* elementary bodies on HL cells. J Clin Microbiol 1992;**30**:1388-1391.

5. Theunissen JJH, Lemmens-den Toom NA, Burggraaf A, Stolz E, Michel MF. Influence of temperature and relative humidity on the survival of *Chlamydia pneumoniae* in aerosols. Appl Environ Microbiol 1993;**59**:2589-2593.

6. Theunissen JJH, Stolz E, Michel MF. The effect of cooling rate and suspension medium on freeze-drying survival of *Chlamydiae*. In Press in J Appl Bacteriol.

7. Theunissen JJH, van der Meyden WI, Klufio CA, Stolz E, Michel MF. Prevalence of *Chlamydia trachomatis* among family planning attendees in Papoea New Guinea. Submitted.

8. Theunissen JJH, Lemmens-den Toom NA Burggraaf A, Stolz E, Michel MF. De overleving van *Chlamydia pneumoniae* in aërosolen bij veschillende temperaturen en luchtvochtigheden. Samenvatting van een voordracht op de najaarsvergadering van de Nederlandse Vereniging van (Medische) Microbiologie. Ned Tijdschr Geneeskd 1993;**137**:685-686.

9. Tjhie JHT, Roosendaal R, Walboomers JMM, Theunissen JJH, Soendjojo N, Meijer CJLM, MacLaren DM, van den Brule AJC. A general polymerase chain reaction enables detection of possible substrains of *Chlamydia pneumoniae*. Submitted.