Chlamydia trachomatis in Andrologic Patients – Direct and Indirect Detection

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Abstract

Background: *Chlamydia trachomatis* is considered to be the most common sexually transmitted disease in Germany. It is currently unclear whether chlamydial infection causes pathological conditions of the male accessory glands with consequences for male infertility.

Patients and Methods: Within the framework of several prospective studies the association between sperm quality, male accessory gland function and infection with *C. trachomatis* was investigated in men of couples with unexplained infertility. Chlamydial infection was determined by serologic methods and by proof of *Chlamydia*-specific DNA. As a marker of infection the direct determination of granulocytes in the ejaculate or the measurement of the polymorphonuclear (PMN) elastase concentration was used. The male accessory gland function was evaluated using the markers fructose, citric acid and α-glucosidase in the seminal plasma.

Results: *Chlamydia*-specific DNA in the ejaculate was present in between 3–5% of the subjects, which corresponds to its prevalence in the normal population. *Chlamydia* IgA antibodies were demonstrated with a frequency of 38% in seminal plasma (n = 834) using a genus-specific test (rELISA). Using other species-specific tests (MIF, SeroCT, IgA pELISA and ImmunoComb), *Chlamydia* IgA antibodies were found at frequencies of between 8 and 22%.

Conclusion: Only in a few individual cases was it possible to show a connection between reduced sperm quality, disturbed male accessory gland function and indication of infection with *Chlamydia*, bacteria or *Ureaplasma*.

Introduction

As in other Western countries, in Germany infection with *Chlamydia trachomatis* is considered to be the most com-mon sexually transmitted disease, although there is little current German epidemiologic data available. Only two studies provide some information on the current prevalence in the normal population, one from Berlin [1], the other from Freiburg [2]. In the Berlin study, in a representative sample of the normal female population (asymptomatic woman drawn from the routine patient population of practicing gynecologists) the prevalence of *Chlamydia* infection was 3.6%, with considerable differences depending on age and marital or partnership status [1]. In the Freiburg study, the prevalence in asymptomatic men and women aged between 15 and 29 years was 5.2% and 4.8%, respectively [2].

While there is agreement on the manifestations and, in particular, the consequences in women with regard to fertility of chronic, ascending *Chlamydia* infection of the genital tract – for example, salpingitis, inflammatory pelvic disease, tubal occlusion and extra-uterine pregnancies – currently it is not clear whether *Chlamydia* infection causes pathologic conditions of the male accessory glands, with consequences for male fertility. It is uncontested that in the male *C. trachomatis* causes urethritis [3]; it is generally accepted that it causes acute epididymitis [4, 5] and probably chronic prostatitis as well [6–8]. All other diseases of the male accessory glands, especially chronic, ascending, clinically asymptomatic infection with consequences for male fertility, have to be considered hypothetical as long as there are only individual reports of questionable rigor and the causal connection between infection of the male accessory glands and/or testicles and disturbed fertility or spermatogenesis cannot be demonstrated or the pathogenic

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processes involved explained. Such insufficiently rigorous findings, which are mainly interpreted as clinically asymptomatic infections, have been the demonstration of *Chlamydia* in culture or by electron microscope in the testicles or the epididymis [9], in culture or by PCR in the ejaculate of semen donors for artificial insemination [10, 11], in the ejaculate of asymptomatic infertile men by means of in situ hybridization [12] as well as the detection of *Chlamydia* antibodies in the serum and/or the seminal plasma [13–16]. In this regard, the employment of methods that can only detect genus-specific antibodies does not permit particularly useful conclusions to be drawn.

Generally, in the absence of clinical signs, leukocytospermia is considered to be the primary symptom of male accessory gland infection, although the absence of leukocytospermia does not exclude an infection and leukocytospermia is often present without a corresponding microbiological finding [7, 17].

Indicators of a dysfunction of the male accessory glands [18], independent of the cause, are the markers of the function of the epididimides (α-glucosidase, carnitine), the prostate gland (citric acid, zinc, acid phosphatase) and the vesicular gland (fructose).

The goal of the present investigation was to determine, on the basis of a series of prospective studies, whether there was a connection in andrologic patients between the direct detection of *C. trachomatis*, its indirect demonstration in serum and seminal plasma by means of various serologic methods and findings that deviated from the norm for the markers of inflammation, the markers of male accessory gland function and the basic spermiogrammatic parameters.

Patients and Methods

The subjects of the study were consecutive patients (age range: 19–58 years; median age: 30 years) of our andrologic outpatient clinic with clinical (palpatory/sonographic) and/or historical findings suggesting a disease of the male accessory glands (quasi-symptomatic patients) as well as male patients who on the basis of gynecological indications had been recommended for assisted reproduction, regardless of whether there was a clinical or historical finding or not (asymptomatic patients). All the groups, therefore, contained both quasi-symptomatic and asymptomatic patients. None of the patients had subjective symptoms. In none of the cases was the clinic referral for a special diagnostic procedure due to a spermiogram parameter that deviated from the norm.

In the first series of investigations the genus-specific rELISA test was used to determine the presence of *Chlamydia* antibodies in the semen (n = 324) and seminal plasma (n = 834); at the same time, as a marker of infection, the number of granulocytes in the ejaculate and the markers for male accessory gland function (n = 273, taken from the 834 seminal plasma samples) were determined. In addition, an expanded microbiological investigation for detection of *Ureaplasma urealyticum* and facultative pathogenic bacteria was carried out.

In the second series of investigations 43 of the seminal plasma samples from the first part of the study which were positive for the genus-specific rELISA test were investigated for comparison using two species-specific tests (ImmunoComb and MIF).

In the third series of investigations the species-specific SeroCT test was used for detection of *Chlamydia* antibodies (IgA) in the seminal plasma of a further 179 patients.

In the fourth series of investigations the ligase chain reaction (LCR) was used to determine the presence of *Chlamydia*-specific DNA in 35 ejaculates that showed positive results in the SeroCT test of the previous part of the study and in 26 ejaculates that showed negative results on testing with SeroCT.

In the fifth series of investigations LCR was used to determine *Chlamydia*-specific DNA in the ejaculate and urine of 77 patients from unexplained childless unions and, for comparative purposes, in 23 ambulant patients with acute urethritis.

In the sixth series of investigations only the seminal plasma of a further patient group (n = 230) was investigated for *C. trachomatis* antibodies using a further species-specific test, the *C. trachomatis* IgA pELISA. A group of semen donors (n = 47) served as control.

In the seventh series of investigations all the seminal plasma samples which were positive for antibodies with the IgA pELISA test in the previous part of the study (n = 43) and 62 samples which were negative for this test were investigated with a new amplification method (BDProbe Tec ET system). In addition, all the semen donors in the sixth series of investigations (n = 47) were investigated as a control group.

The sequence of the individual parts of the study was determined by the diagnostic possibilities available at a given time. The varying numbers of patients included in the different parts of the study were due to the different amounts of time and financial resources available, since all the investigations were carried out within the context of routine diagnosis. The investigations were carried out over a period of 7 years (1993–1999). The ejaculate was obtained by means of masturbation in the outpatient clinic after a period of sexual abstinence of 5 days. The ejaculate samples were investigated according to the WHO standard [18]. In addition, the progressive motility was determined 1 and 3 h after the ejaculate was obtained according to the method described in [19].

The granulocyte concentration was determined using the peroxidase reaction according to the WHO standard [18]. The following biochemical investigations of the seminal plasma samples were carried out: measurement of fructose (UV method, Boehringer Mannheim, Germany), citric acid (UV method, Boehringer Mannheim, Germany), α-glucosidase (colorimetric assay, Boehringer Mannheim, Germany) and PMN elastase (Latex Immuno Assay, Merck, Germany).

All the microbiological investigations were carried out at the Institute for Microbiology and Hygiene of the Medical Faculty (Charité) of the Humboldt University, Berlin, Germany. For determination of facultative pathogenic bacteria 10 µl of ejaculate were inoculated onto Columbia, McConkey and Columbia nalidixic acid agar. A germ count $\geq 10^4$ cfu/ml was considered to be significant. The "Mycoplasma Duo" kit (Sanofi Diagnostics Pasteur, France) was used for semiquantitative determination of *U. urealyticum* and *Mycoplasma hominis*).

The genus-specific antibodies in serum and seminal plasma were determined by rELISA (medac, Hamburg, Germany). Species-specific antibodies were determined using ImmunoComb (Savyon Diagnostics, Israel), SeroCT (Orgenics, Israel) and microimmunofluorescence (MIF), using *C. trachomatis* and *C. pneumoniae* as antigens [20] and by *C. trachomatis* IgA pELISA (medac, Hamburg, Germany). The sera were diluted according to the manufacturer's instructions. The following antibody titers in serum were considered to be positive: rELISA IgG $\geq 1:100$, IgA $\geq 1:100$; ImmunoComb IgG $\geq 1:8$, IgA $\geq 1:8$ (*C. trachomatis*) and IgG $\geq 1:16$,

IgA ≥ 1 : 64 (*C. pneumoniae*); MIF IgG ≥ 1 : 16, IgA ≥ 1 : 20 (*C. trachomatis*) and IgG ≥ 1 : 16, IgA ≥ 1 : 80 (*C. pneumoniae*). The limits for seminal plasma were defined on the basis of experience with other extravasal fluids and following the investigation of semen donors. Seminal plasma was used in a 1 : 4 dilution. The following limit values were defined or IgA: rELISA ≥ 1 : 50; ImmunoComb ≥ 1 : 8 (*C. trachomatis*) and ≥ 1 : 64 (*C. pneumoniae*); Sero CT an index > 1 (*C. trachomatis*) and MIF ≥ 1 : 20 (*C. trachomatis* and *C. pneumoniae*). For the *C. trachomatis* IgA pELISA a titer ≥ 1 : 50 was considered positive (the cutoff was defined as mean value of the optical density of the negative controls multiplied by 2). *C. trachomatis* DNA in the ejaculate and urine were determined by LCR (Abbott, USA). For each LCR test 10 µl of undiluted ejaculate and 10 µl of a 1 : 5 dilution were used. In addition, for every ejaculate sample an inhibition control (approx. 10–100 IFU *C. trachomatis*/10 µl ejaculate) was simultaneously performed. An additional procedure used was the BDProbe Tec ET system "strand displacement amplification" (SDA) and "fluorescent energy transfer" (Becton Dickinson Co., France). This system employs an internal amplification control. For the SDA reaction 100–200 µl of ejaculate were washed with PBS, treated according to the manufacturer's urine protocol and then evaluated.

Statistical evaluation was by means of descriptive statistics, regression analysis and correlation calculations using the standard statistics software application SPSS.

Results

The seven test series yielded the following results:

Series 1

Using a genus-specific rELISA, *Chlamydia* antibodies were demonstrated with a frequency of 68% (IgG) and 19% (IgA) in serum (n = 324) and a frequency of 16% (IgG) and 38% (IgA) in seminal plasma (n = 834). The same test showed *Chlamydia* antibodies in the serum of blood donors (n = 100) with a comparable frequency (50% and 25% for IgG- and IgA antibodies, respectively).

Table 1
Test results for patients with an unfulfilled desire for children (n = 273).

Parameters	Normal values	Results beyond the normal limit (%)	
Granulocytes/ml	< 10^6	22	(8)
Granulocytes, total	3×10^6	26	(10)
Citric acid, total	> 52 µmol	48	(17)
Fructose, total	> 13 µmol	16	(6)
Glucosidase, total	> 20 mE	9	(8) [a]
Aerobic bacteria	< 10^4 cfu/ml	18	(7)
Ureaplasma urealyticum	< 10^4 cfu/ml	37	(14)
Chlamydia IgA[b]	< 1 : 400	22	(8)

[a] n = 110; [b] genus-specific rELISA (medac, Hamburg, Germany)

If one lists all the relevant ejaculate findings (n = 273) for a diagnosis of chronic adnexitis (granulocyte count, biochemical markers of the male accessory gland function, microbiological findings) there is a deviation from the currently accepted normal values for the individual parameters in 6% to 17% of the cases (Table 1). In a correlation matrix (Table 2) of all ejaculate samples with a granulocyte count ≥ 10^6/ml set up to obtain possible signs of florid adnexitis (n = 22), significant correlations were obtained between a) the granulocyte count/ml and the total number of granulocytes in the ejaculate sample, b) the concentrations of fructose and citric acid, c) the total granulocyte count and the presence of *Ureaplasma*, d) a finding of facultative pathogenic aerobic bacteria and *Ureaplasma*, e) the granulocyte count/ml and IgA antibodies to *Chlamydia* in the genus-specific rELISA test, and f) α-glucosidase and IgA antibodies to *Chlamydia* in the genus-specific rELISA test.

Table 2
Correlation matrix for all ejaculate samples with a granulocyte count ≥ 10^6/ml (n = 22).

	Granulo-cytes/ml	Granulo-cytes/total	Fructose	Age	Citric acid	α-glucosi-dase	Carnitine	Bacteria	*Ureaplasma*	*Chlamydia* IgA[a]
Granulocytes /ml	1									
Granulocytes /total	**0.74**	1								
Fructose	– 0.18	0	1							
Age	– 0.11	– 0.12	– 0.22	1						
Citric acid	– 0.18	0	**0.95**	– 0.28	1					
α-glucosidase	0.22	0.22	– 0.07	0.24	– 0.13	1				
Carnitine	– 0.27	– 22	0.39	0.1	0.36	– 0.14	1			
Bacteria	0.28	0.16	– 0.11	0.06	– 0.11	– 0.1	– 0.1	1		
Ureaplasma	0.1	**0.37**	– 0.02	0.03	– 0.02	– 0.26	– 0.06	**0.45**	1	
Chlamydia IgA[a]	**0.38**	0.28	– 0.3	– 0.22	– 0.13–	**0.45**	– 0.01	– 0.09	– 0.2	1

[a] genus-specific rELISA (medac, Hamburg, Germany)

If an attempt is made to establish a meaningful connection between the raised granulocyte count (n = 22) and both the microbiological findings and the markers of the male accessory gland function, a constellation pointing to adnexitis was only found in four (1.5%) cases (patient no. 68, 91, 52 and 14). Only in one of these cases (0.4%) could *C. trachomatis* be considered as the possible pathogenic agent (this was determined by the genus-specific rELISA, which is a poor indicator of *C. trachomatis* infection) (Table 3).

To exclude the possibility of methodological errors that might have influenced the subsequent determination of the presence of granulocytes using the peroxidase reaction, a parallel granulocyte determination using both the peroxidase reaction and a PMN elastase assay was performed (n = 78). Regression analysis (r = 0.8138) and correlation calculations showed a significant agreement between the results obtained with the two methods ($\alpha \leq 1\%$).

Series 2
In a comparative investigation of seminal plasma samples (n = 43) using a genus-specific rELISA, the species-specific ImmunoComb and MIF, positive findings (IgA antibodies) with rELISA were confirmed in 58.1% of the cases by the ImmunoComb test and in 20.9% of the cases by MIF. Using a species-specific test which is considered to be reliable with respect to sensitivity and specificity (MIF) the number of positive findings (IgA antibodies) is reduced to one-fifth of the original value as determined by the genus-specific rELISA, i.e. to 8% of all investigated samples. At the same time, the MIF showed IgA antibodies to *C. pneumoniae* in 15 of the 43 (35%) seminal plasma samples (in six cases exclusively, in nine cases in addition to *C. trachomatis* antibodies). Of particular interest is that six of the nine *C. trachomatis* samples positive by MIF had titers ≥ 400 in the genus-specific rELISA test and that all of the six samples positive for *C. pneumoniae* antibodies by MIF had titers < 400 by rELISA.

Series 3
In a further series of tests on 179 seminal plasma samples, *C. trachomatis*-specific IgA antibodies were found in 24 samples (13.4%) using the species-specific SeroCT.

Series 4
Evidence of *C. trachomatis*-specific DNA in 35 ejaculate samples which were positive using the SeroCT test was obtained in only one case (2.8%); of 26 serologically negative ejaculate samples two cases (8%) had positive DNA results. In this series of tests, a total of three of 61 andrologic patients (5%) had *Chlamydia*-specific DNA, with all three patients having positive results both in urine and ejaculate.

Table 3
Granulocyte count $\geq 10^6$/ml, biochemical markers and microbiological findings in andrologic patients (n = 22).

Patient no.	Granulocytes > 10^6/ml	Granulocytes, total > 3×10^6	Fructose, total > 13 µmol/l	Age, years	Citric acid, total > 52 µmol/l	α-glucosidase, total > 20mE	Carnitine > 250 µmol/l	Bacteria cfu/ml	*Ureaplasma* cfu/ml	*Chlamydia* IgA[a]
147	1	3	66.3	43	45	31.2	243	0	0	0
135	1	1.8	8.57	42	57	n t	354	0	0	0
139	1	2. 5	44.7	28	73	n t	511	0	0	50
58	1	2.5	54.7	31	48	n t	446	0	0	100
85	1	4	50.4	26	40	n t	235	0	0	200
64	1	6	93	37	113	n t	120	0	0	0
7	1.2	3.6	63	29	118	n t	543	0	0	0
68	**1.4**	**8.4**	**61.2**	28	146	n t	**152**	**0**	10^4	**0**
261	1.4	4.2	93.3	44	55	n t	415	0	10^3	50
82	2	6	47.7	28	70	n t	155	0	10^3	0
156	2.4	4.8	17.5	43	58	39.4	275	0	0	0
45	2.4	13.2	52.8	29	128	n t	114	0	0	0
179	2.6	6.5	13.1	30	62	n t	122	0	0	50
111	2.6	6.5	13.1	30	62	n t	122	0	0	50
185	2.6	1.04	0.34	35	11	0.021	33	0	0	0
115	2.6	6.76	57.5	33	23	n t	212	0	0	0
164	3	15	147.5	30	227	27.3	57	0	0	0
274	3.4	17	39.7	35	48	79.5	191	0	0	200
91	**4**	**36**	113.4	35	185	n t	**247**	0	10^4	0
52	**4.4**	**15.4**	26.9	35	**34**	n t	**171**	10^4	10^4	0
14	**5**	**20**	60.4	30	**29**	43. 2	**187**	0	0	**800**
67	6.8	20.4	53.7	31	86	n t	318	0	0	100

[a] genus-specific rELISA (medac, Hamburg, Germany); n t: not tested

Series 5.

In addition, LCR was used to determine *Chlamydia*-specific DNA in the urine and ejaculate of patients with an unfulfilled desire for children (n = 77) and patients with acute urethritis (n = 23). In the patients with an unfulfilleddesire for children, three of 66 urine samples (4.5 %) and three of 77 ejaculate samples (4.0 %) were positive. The proportions of positive findings in the patients with acute urethritis were clearly higher, with two of 17 (12%) showing positive results in urine and two of 23 (9%) positive in the ejaculate. There were three patients in total with positive findings (13%), with one patient showing positive results in both urine and seminal plasma. Evaluation of all findings of the patients with an unfulfilled desire for children with a positive result gave no indication of a past or florid infection of the male accessory glands.

Series 6

Investigation using a new species-specific antibody test, the IgA-pELISA, showed a positive result for 43 of the 230 samples (18.7%) of seminal plasma of the andrologic patients, while only three samples out of 47 (6.4%) of the semen donors were positive.

Series 7

Investigation by SDA of 105 seminal plasma samples with positive results in the previous antibody test (n = 43) and of 62 samples which were negative, revealed positive results in three of the antibody-positive samples (2.9%) These results were confirmed using LCR. All antibody-negative samples and all 47 samples from the semen donors were negative in the amplification test.

Discussion

Despite significant improvements in chlamydial diagnosis in recent years (species-specific antibody tests, nucleic acid amplification procedures) investigators have not been able to confirm the suspected association between *Chlamydia* infection and disturbed male fertility.

The classical methods (direct immunofluorescence and EIA, which have a relatively low sensitivity and specificity) revealed the presence of the pathogenic agent in 1–4% of the andrologic patients investigated (data not shown). The attempt to obtain evidence with regard to the prevalence of *C. trachomatis* infection in andrologic patients by means of local IgA antibody formation was also not successful, in part because at the outset only genus-specific tests were available. Investigation of the seminal plasma samples which had shown positive results with MIF led to a reduction of the proportion of samples with positive antibody results to about 1/5 of the original value (this corresponded to 8% of the patients investigated). Compared to the highly specific MIF test, the gold standard of *Chlamydia* serology, the other species-specific tests resulted in a significantly higher proportion of positive results; ImmunoComb 22%, SeroCT 13% and IgA-pELISA 19%. These differences are probably due to the quality of the test antigens. Investigation of seminal plasma with the *C. trachomatis* IgA-pELISA test showed significant differences between the patient group (19%) and confirmed fertile men (6%). *Munoz* et al. [21] suggest that the reason for the higher antibody detection in a patient group might be a *Chlamydia*-triggered immune response leading to the formation of auto-antibodies against spermatozoa.

More effective are methods of direct detection, especially methods based on nucleic acid amplification. So far the results of only a few individual studies are available, with the proportion of positive findings lying between 1–39% [22–24]. In our own patient populations, BDProbe Tec ET system and LCR showed positive results of 3–5% (nine of a total of 178 patients in three study groups), which corresponds approximately to the prevalence in the general population. It is possible that the large range of positive results in the literature is due to geographic differences, but it is more likely that they are of methodological provenance (different sample preparation, different PCR protocols).

We were able to show that the more or less definite demonstration of a past or florid *Chlamydia* infection does not entail a manifest disease of the male accessory glands or a disturbance of testicular function with consequent effects on fertility. In our patient population there was an indication of a negative influence on sperm motility only as a result of the presence of granulocytes in the ejaculate as determined by statistical correlation (data not shown). It seems highly likely that reactive oxygen species produced by the granulocytes are responsible for this [25].

While the correlation between the total granulocyte count in the ejaculate and the biochemical markers of the male accessory gland function (data not shown) does seem to point to a disturbance of the male accessory gland function in connection with inflammatory processes, with the exception of a few individual cases this is not due to *Chlamydia* (Table 3).

The correlation between *Chlamydia*-IgA antibodies in the seminal plasma and the granulocyte concentration in the ejaculate (Table 3) is of no value as evidence since the test for detection of the antibodies is a genus-specific test. Furthermore, the agreement of the results of the genus-specific test with those of the species-specific tests (as well as with those of the methods of direct detection) is minimal. In addition, it is not possible to distinguish reliably between a recent and a long past infection using serologic methods only. In agreement with *Wolff* et al. [23], we have also been able to show that the IgA antibody titer in the seminal plasma after adequate therapy does not behave uniformly after a year or does not decrease in a predictable manner [26], so that it is not possible to distinguish between a florid and a past infection based on the titer. An indication for treatment can only be based on a direct detection of the pathogen, which should be done by means of an amplification method [27].

Conclusion

The genus-specific rELISA is not suitable for determination of *Chlamydia* infection of the male genital tract. Detection of *Chlamydia* antibodies (IgA) in the seminal plasma of a patient only rarely corresponds to a pathologically significant finding for the markers of inflammation, for the biochemical markers of male accessory gland function or with a relevant clinical finding. In all probability, a direct connection between disturbed male fertility, impaired function of the male accessory glands and *Chlamydia* infection only exists in isolated cases. Furthermore, the low detection rate in andrologic patients of about 4% using the methods of direct detection means that at best only a small proportion of cases of male infertility are due to *Chlamydia* infection. Determination of the local *C. trachomatis* antibody formation would seem to be of use with respect to the triggering of auto-immune processes against spermatozoa. The currently available species-specific tests differ in sensitivity. Since MIF is not suitable for routine diagnosis due to the amount of work it involves, a species-specific ELISA is currently recommended.

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