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Semen Quality of Men With Asymptomatic Chlamydial Infection

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ABSTRACT: We have shown previously that the in vitro exposure of spermatozoa to elementary bodies (EBs) of *Chlamydia trachomatis* can lead to sperm death over a number of hours of incubation. As such, we have hypothesized that the ejaculates of men with a chlamydial infection could contain increased numbers of nonmotile (dead) spermatozoa if they are exposed to EBs prior to ejaculation. To test this hypothesis, the ejaculates of 642 men undergoing diagnostic semen analysis as part of ongoing infertility investigations with their partner were examined. All men were without symptoms of genitourinary infections and semen analysis was performed according to World Health Organisation (WHO) 1999 methods after a 3–5 day abstinence period. In addition to semen analysis, nested plasmid polymerase chain reaction (PCR) was undertaken on the ejaculate to detect the presence of *C trachomatis* DNA. A total of 31 semen specimens (4.9%) were found to be positive, and in 28 of these, the diagnosis was confirmed using the ligase chain reaction (LCR). Men whose ejaculates were PCR positive for chlamydial DNA had a significantly ($P < .05$) higher mean concentration of leukocytes ($1.71 \pm 2.20 \times 10^6$ per mL) and a higher mean ejaculate volume (3.45 ± 1.52 mL) than in those whose ejaculates were PCR negative (leukocyte concentration: $0.67 \pm 2.59 \times 10^6$ per mL; volume 2.93 ± 1.38 mL). Leukocytospermia was twice as common in men that were PCR positive for chlamydial DNA ($P < .05$) but it was not always associated with the presence of chlamydial DNA in semen. However, there was no difference in the mean percent motility between the 2 groups and the proportion of asthenozoospermia also did not differ. Because these results do not confirm the hypothesis proposed from our in vitro experiments, further work needs to be undertaken to understand whether human spermatozoa are actually exposed to elementary bodies of *C trachomatis* in an infected individual prior to ejaculation.

Key words: *Chlamydia trachomatis,* infertility.

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Although *Chlamydia trachomatis* infections are the most prevalent sexually transmitted bacterial infections throughout the world (World Health Organisation, 1996), it is still relatively unclear whether they have a serious affect on male reproductive health. While some authors are convinced that chlamydial infection in men can lead to serious problems (Purvis and Christiansen, 1995), others are less certain and have argued that its effects on female reproduction are far more important (Keck et al, 1998).

In an attempt to link chlamydial infection in men with male reproductive problems, a number of studies have investigated the relationship between infection and semen quality. However, while some studies have shown that infection is associated with poorer semen quality (Custo et al, 1989; Wolff et al, 1991; Witkin et al, 1995; Cengiz et al, 1997), others have claimed that it is not (Gregoriou et al, 1989; Nagy et al, 1989; Eggert-Kruse et al, 1990, 1996, 1997; Soffer et al, 1990; Dieterle et al, 1995; Weidner et al, 1996; Habermann and Krause, 1999). This has led to confusion in the literature, but in reality, the studies are frequently not comparable because different, and often inadequate, diagnostic methods and test specimens have been used to identify those men with a chlamydial infection.

For example, a number of studies have based their diagnosis on an attempt to culture *C trachomatis* from ejaculates or urethral swabs. However, seminal plasma is known to be inhibitory to the effective culture of *C trachomatis* (Mardh et al, 1980). Other studies have attempted to overcome these problems by attempting to use serological methods although the detection of serum antibodies to *C trachomatis* in asymptomatic men is not particularly helpful (Paavonen and Eggert-Kruse, 1999). Moreover, because the immune response differs between individuals, it is not always clear whether a single elevated antibody titer represents an active or current infection or a previous infection to which a strong immune response was generated. In this situation, all that can be said is that this is suggestive of a diagnosis but it is not unequivocal evidence (Taylor-Robinson, 1996). Further-

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more, the antibody response is not always specific for *C trachomatis* and could in some instances detect cross-reactive antibodies to *Chlamydia pneumonaie*, an organism not found in the genital tract (Moss et al, 1993). Also, the presence of the blood-testes/epididymal barrier may mean that serum antibody levels may not accurately reflect the local situation within the testes or epididymis (Eggert-Kruse et al, 1996). It is now recommended that chlamydial infections be diagnosed using a nucleic amplification method (Black, 1997), as such a test shows good specificity as well as being highly sensitive.

In addition to the problems with chlamydia diagnosis, many studies have not used WHO methodology in their analysis of semen (eg, Custo et al, 1989; Soffer et al, 1990). Moreover, none of the studies have demonstrated that they have performed semen analysis with adequate levels of quality control, yet this is crucial in this type of study given the large measurement errors that can occur with the type of semiquantitative methods used in semen analysis (Dunphy et al, 1989; Clements et al, 1995).

In our recent work, we have examined the effect of *C trachomatis* on human sperm function and have shown that *C trachomatis* serovar E can stimulate the tyrosine phosphorylation of 2 major sperm epitopes (Hosseinzadeh et al, 2000) and can also cause sperm death within a few hours of exposure (Hosseinzadeh et al, 2001). As such, we have hypothesized (Hosseinzadeh et al, 2001) that, if in an infected male, spermatozoa are exposed to *C trachomatis* during spermatogenesis or epididymal transit and storage, then the ejaculate might contain increased numbers of nonmotile spermatozoa. To investigate this possibility, we have undertaken a study of men attending for semen analysis as part of infertility investigations. We have used strict methods of semen analysis, according to WHO (1999) guidelines in combination with stringent internal and external quality control. For the laboratory diagnosis of *C trachomatis* infection, we used 2 amplification-based methods, 1 for detection and another for confirmation.

Materials and Methods

A total of 642 men attending the University Research Clinic (Jessop Hospital for Women, Sheffield, United Kingdom) for diagnostic semen analysis were recruited to the study. All men were undergoing semen analysis as part of a work-up for infertility investigations after failing to conceive with their partner after 1 year of unprotected intercourse. None of the men reported any symptoms of genitourinary infections and were therefore considered asymptomatic of sexually transmitted disease. There were no age restrictions for inclusion in the study, although men were not included if they had a history of treatment by chemotherapy (or radiotherapy to the groin) or if they had undergone a vasovasostomy or orchidectomy.

Techniques of Semen Analysis

Prior to semen analysis, the men were provided with written information and were asked to abstain from sexual intercourse or masturbation for 3–5 days before attending the clinic. All samples for analysis were produced on site and collected into standard containers that had previously been shown not to have any cytotoxic effects on human spermatozoa according to the methods outlined in WHO (1999). Immediately following semen production, the sample was placed in an incubator and allowed to liquefy at 37°C for up to 30 minutes before the analysis commenced. Semen analysis was performed according the WHO (1999) guidelines with all of the measures of semen quality, apart from sperm morphology and the quantification of leukocytes, being completed within 1 hour. The identification and quantification of leukocytes was carried out by immunocytochemistry according to the methods outlined in Kessopoulou et al (1992). Sperm morphology was performed on Papanicolaou-stained smears according to WHO (1999) criteria.

The laboratory employed a comprehensive internal quality control program for semen analysis based on that described by Clements et al (1995). In addition, the laboratory was a member of an external quality-assessment scheme for andrology (UK NEQAS, St. Mary's Hospital, Manchester, United Kingdom) and was shown to be consistently within the target range for all variables.

Detection of C trachomatis in Semen Samples by Polymerase Chain Reaction—At the end of the semen analysis, 300 μL of semen was retained in an Eppendorf tube for the detection of *C trachomatis* DNA. An amplification-based method was used to determine the presence or absence of *C trachomatis* DNA in these samples according to the following protocol. Each 300-μL aliquot was centrifuged at 8000 × *g* for 10 minutes and the pellet was resuspended in 0.3 mL of lysis buffer containing proteinase K (200 μg/mL). Solutions were then placed on a heating block at 55°C for 1 hour, and subsequently at 95°C for 20 minutes for extraction of DNA by a standard phenol/chloroform/iso-amyl alcohol method (Frost et al, 1991). Initially, the extracted DNA was tested for β-globin according to the method of Saiki et al (1985) to check that there were no PCR inhibitors in the samples. When samples were shown to be β-globin positive, they were then tested by nested plasmid PCR according to the method of Claas et al (1990) using initial primers directed against the cryptic plasmid of *C trachomatis* and secondary primers internal to the initial primers as described by Oxley (1997). Products were analyzed by gel electrophoresis in 1.5% agarose with ethidium bromide staining. Each PCR run included *C trachomatis* DNA as a positive control, giving a band size of 320 bp, and distilled water as a negative control. The amplicon was shown to be specific to the pCHL1 plasmid by cloning it into pUC-19 (Yanisch-Perron et al, 1985) and comparing it with GenBank accession number 144462.

DNA extracts from semen specimens that were positive by nested plasmid PCR were further tested by nested major outer membrane protein (MOMP) PCR to determine the serovar status as described by Lan et al (1994). Briefly, 2 sets of primers were used, NLO and NRO, and pCTM3 and SER02A (R&D Systems, Ltd, Abingdon, United Kingdom). The primary PCR products were transferred to a fresh reaction mixture containing primers

Table 1. *Relationship between semen parameters and chlamydial infection in 642 men attending for semen analysis. Data shown is the mean ± standard deviation for the major variables measured at semen analysis (in addition to age) for 611 chlamydia-negative individuals and a further 31 individuals whose ejaculates were shown to contain chlamydial DNA by PCR*

	Chlamydial DNA-negative Individuals (n = 611)	Chlamydial DNA-positive Individuals (n = 31)	Statistical Significance
(a)			
Age (y)	33.58 ± 6.85	34.00 ± 6.77	$P = .7391$
Semen volume (mL)	2.93 ± 1.38	3.45 ± 1.52	$P = .0421$
pH	8.11 ± 0.22	8.13 ± 0.20	$P = .6202$
Sperm concentration ($\times 10^6$/mL)	50.68 ± 50.45	47.88 ± 48.67	$P = .7628$
Percent motile sperm	54.34 ± 26.50	56.42 ± 21.58	$P = .6675$
Percent immotile sperm	41.57 ± 22.83	43.58 ± 21.63	$P = .6318$
Concentration of progressively motile sperm ($\times 10^6$/mL)	82.65 ± 110.80	92.21 ± 100.91	$P = .6381$
Percent of normal morphological forms	17.21 ± 10.42	19.16 ± 10.18	$P = .3093$
Concentration of leukocytes ($\times 10^6$/mL)	0.67 ± 2.59	1.71 ± 2.20	$P = .0285$

pCTM3 and SERO2A and the following nested PCR products were then subjected to Alu I digestion. The products were analyzed on a 7.5% (wt/vol) polyacrylamide gel prior to silver staining and compared with control serovars of *C trachomatis* to genotype the clinical isolates.

Detection of C trachomatis in Semen Samples by Ligase Chain Reaction—All samples found to be positive with plasmid PCR (see above), were further tested using ligase chain reaction (LCR) at the Clinical Pathology Accreditation Ltd, accredited Public Health Laboratory in Liverpool (University Hospital, Aintree, Liverpool, United Kingdom). This was to confirm the diagnosis obtained by PCR but for reasons of cost could not be performed on every sample obtained in the study. The LCR method used was that specified by the manufacturer (Abbott, Berkshire, United Kingdom) for the assay of urine samples except that the starting point was to add 50 μL of test sample (instead of the usual centrifuged urine deposit) to 1 mL of urine resuspension buffer. The test sample was the same DNA extract prepared for nested plasmid PCR (see above). Test specimens were considered chlamydia positive by this method if their fluorescence was greater than a cut-off value calculated automatically by the analyses as 45% of the mean fluorescence of two calibrator positives provided with each kit.

Correlation of Semen Variables With Chlamydial Infection

The mean values for each variable of the semen analysis were compared by an unpaired Student's *t* test between those ejaculates that were shown to contain chlamydial DNA (by plasmid PCR) compared with those that did not. For those data expressed as a percentage (eg, percent motility), the data were first log transformed to $\log_{10}$ before statistical testing. In addition, a Fisher's exact test was used to compare the proportions of samples with asthenozoospermia (<50% motile sperm in their ejaculate) and leukocytospermia ($>1 \times 10^6$ leukocytes/mL) between the chlamydia DNA-positive and -negative samples.

Results

From the nested plasmid PCR analysis of the ejaculates of 642 men, 31 (4.9%) were found to be positive. Of these, 28 were found to be positive by LCR, giving a concordance between the 2 tests of above 90%.

Semen Parameters

A comparison of each semen variable between those men shown by PCR to have DNA for *C trachomatis* in their semen and those that did not revealed 2 interesting differences (Table 1). First, men who were *C trachomatis* positive had an increased semen volume ($P = .0421$), and second, they also had a higher concentration of leukocytes in their ejaculate ($P = .0285$) than did men who were PCR negative for *C trachomatis* DNA. No other statistical differences were apparent between the 2 patient groups. Interestingly, the mean ages of men who were *C trachomatis* DNA positive (33.58 ± 6.85 years) was not statistically different from those that were *C trachomatis* DNA negative (34.00 ± 6.77 years). Nor were there any differences between the percent of motile sperm in the ejaculates of men who were PCR positive (54.34 ± 26.50) or negative (56.42 ± 21.58) for *C trachomatis* DNA.

Incidence of Asthenozoospermia and Leukocytospermia

Eleven out of the 31 *C trachomatis* DNA-positive samples (34.4%) were asthenozoospermic compared with 157 out of 569 *C trachomatis* DNA-negative samples (27.6%) that contained spermatozoa (73 men were azoospermic). Although more frequent in the *C trachomatis* DNA-positive men, the incidence of asthenozoospermia was not statistically different between the 2 groups ($P = .4107$). By comparison, 8 out of the 31 *C trachomatis* DNA-

Table 2. *Relationship between semen parameters and chlamydial serovar in the 31 samples found to be positive for chlamydial DNA. Data shown is the mean ± standard deviation for the major variables measured at semen analysis. No significant differences were observed*

	Serovar				
	D (n = 3)	E (n = 8)	F (n = 3)	Mixed (n = 2)	Unknown (n = 15)
Semen volume (mL)	4.33 ± 1.53	3.84 ± 1.43	2.10 ± 1.01	2.60 ± 0.57	3.45 ± 1.63
pH	8.27 ± 0.29	8.00 ± 1.14	8.07 ± 0.06	8.10 ± 0.00	8.17 ± 0.21
Sperm concentration ($\times 10^6$/mL)	33.40 ± 26.91	48.99 ± 46.86	51.77 ± 49.76	41.80 ± 34.50	50.21 ± 57.36
Percent motile sperm	63.33 ± 23.54	55.12 ± 18.56	62.00 ± 19.52	59.00 ± 35.36	54.27 ± 24.05
Percent immotile sperm	36.33 ± 24.03	45.12 ± 18.44	38.00 ± 19.08	42.00 ± 35.36	45.53 ± 24.19
Concentration of progressively motile sperm ($\times 10^6$/mL)	99.30 ± 94.73	115.60 ± 144.22	49.13 ± 37.74	86.28 ± 114.30	87.73 ± 91.64
Percent of normal morphological forms	22.67 ± 9.87	19.75 ± 12.03	21.67 ± 4.04	29.50 ± 13.44	16.27 ± 9.59
Concentration of leukocytes ($\times 10^6$/mL)	0.70 ± 0.61	1.68 ± 3.10	2.41 ± 3.23	2.30 ± 3.03	0.61 ± 1.53

positive samples (25.8%) were leukocytospermic in comparison with 73 out of the 611 *C trachomatis* DNA-negative samples. This was statistically different ($P = .0449$).

Genotyping

Of the 31 *C trachomatis* DNA-positive men, only 14 could be clearly genotyped by nested MOMP PCR. Of these, 8 specimens were found to be serovar E, 3 were serovar F, and 3 were serovar D. A further 2 specimens were found to contain 2 serovar mixtures of E and D, and E and F, respectively. Table 2 summarizes the semen parameters for each serovar and, although each group was relatively small, there were no obvious trends or statistical differences between them. Furthermore, there was no correlation between the incidence of asthenozoospermia or leukocytospermia in any of the samples that could be genotyped. Nor was there any concordance between asthenozoospermia or leukocytospermia.

Discussion

In our study population of 642 men attending for diagnostic semen analysis as part of infertility investigations, the ejaculates of 4.9% were shown to contain chlamydial DNA. Our interpretation of this is that these men had an active chlamydial infection as indicated by the many studies in which the presence of chlamydial DNA in urine correlated with a chlamydial infection (Chernesky et al, 1994; Lee et al, 1995).

In our diagnosis of chlamydial infection, we chose to use nested plasmid PCR as our primary diagnostic tool. This was in preference to cell culture or the detection of serum antibodies to *C trachomatis*, that have been used previously in studies of this type and are known to be problematic (see Purvis and Christiansen, 1993, 1995; Keck et al, 1998). Most authors now agree that amplification techniques are the preferred test method (Black, 1997). We tested all plasmid PCR-positive samples by LCR and found that 28 out of the 31 were also positive by this method. This gives a correlation of 90% and confirms the reliability of the PCR method.

In addition to nested plasmid PCR, we also chose to perform nested MOMP PCR to determine the chlamydial serovar(s) responsible for the infection. This was because our previous work has shown that serovar E and not Lymphogranuloma venereum (LGV) leads to premature sperm death (Hosseinzadeh et al, 2001). Unfortunately, however, we were only able to obtain definitive data on the genotypes in half of the chlamydia-positive samples. This was because the MOMP PCR method is known to be less sensitive than the plasmid PCR method (Mahony et al, 1993). However, the serovars that we were able to identify were all of types D to F. This is as would be expected because these are the most common causes of infection in the United Kingdom (Eley et al, 1993). No cases of LGV infection were recorded and this is not surprising given that LGV infections are quite rare outside the tropics (Moulder, 1991; Morré et al, 2000).

As our test specimen, we chose to examine the ejaculate itself. This seemed prudent given that the hypothesis that we were trying to test was that, if spermatozoa are exposed to EBs either before or at the time of ejaculation, this might lead to asthenozoospermia as a consequence of premature sperm death by a mechanism similar that observed in our in vitro experiments (Hosseinzadeh et al, 2001). In only analyzing the ejaculate, however, we were unable to determine the site of infection in these men and whether it was urethral or elsewhere in the male reproductive tract (eg, the accessory glands). This is important to ascertain because spermatozoa exposed to EBs only at the time of ejaculation (in the case of an infection in the urethra, seminal vesicles, or prostate) may still be motile at the time of semen analysis because this was completed within 1 hour of ejaculation. However, in our in vitro experiments (Hosseinzadeh et al, 2001), it was shown that

chlamydial-induced sperm death (and hence the corresponding decline in motility) is not instantaneous but takes place over several hours after sperm are first exposed to EBs. As such, it is possible that sperm exposed to EBs only at the time of ejaculation may take several hours to die and this would not normally be observed in a sample provided for diagnostic analysis.

The fact that no difference was found in the motility characteristics (or the incidence of asthenozoospermia) between those individuals found to be positive or negative for chlamydial DNA would tend to lend support to the above explanation. However, it cannot be completely ruled out that, in some individuals at least, spermatozoa may well be exposed to EBs in the time prior to ejaculation (either in the epididymis or in the testicular portion of the vas deferens). If this were the case, then based on the results of our in vitro experiments (Hosseinzadeh et al, 2001), it would be predicted that a significant number of spermatozoa would die unless there are specific mechanisms in place to either protect such spermatozoa from exposure to EBs or to neutralize the active (spermicidal) component. Such possibilities should be considered in any future experiments and diagnostic tests should be conducted to more accurately pinpoint the likely site of chlamydial infection within the male reproductive tract.

Although no differences in the sperm motility characteristics were observed between the positive and negative chlamydial DNA groups, two other differences were observed. The first was a statistically higher mean seminal volume of chlamydia-positive individuals. This has not been reported previously and it is difficult to see how methodological differences in such a simple part of the semen analysis could go unnoticed. However, the only study to consider seminal volume was Wolff et al (1991), who found that the semen volume of men with a chlamydial infection was lower than that of those men who did not have an infection. However, because this study relied on semen culture to make a diagnosis and we know that the culture of chlamydia from semen is notoriously unreliable (Mardh et al, 1980), we cannot be certain that all chlamydia-positive men were accurately diagnosed. Differences in seminal volume between chlamydia DNA-positive and -negative samples could be simply explained by unequal abstinence periods in the 2 groups of men, although it is interesting to speculate whether chlamydial infection might be associated with an increased secretory activity of the accessory glands or the reproductive epithelium. In support of this suggestion, it has been shown how some components of bacteria and neutrophils can lead to elevated secretory activity in gland cells of the airways (Cardell et al, 1999; Dwyer and Farley, 2000). This clearly requires further investigation if it proves to be a consistent feature of the ejaculates of men with a chlamydial infection.

The second statistical difference in the semen characteristics was an increased mean number of leukocytes (and corresponding increased frequency of leukocytospermia) in samples that were PCR positive for chlamydial DNA. To observe an elevation in leukocyte numbers in the ejaculates of men with a bacterial infection is not a surprise. However, not all samples that were PCR positive for chlamydial DNA were leukocytospermic and conversely there were samples that were PCR negative for chlamydial DNA that had elevated numbers of leukocytes in the semen. The latter could be explained by the presence of other genitourinary infections that were not screened for in this study. The lack of concordance between leukocyte numbers and infection has been observed by other authors (Weidner et al, 1996) and there is often a poor link between infection and the number of leukocytes in an ejaculate (Barratt et al, 1990).

In conclusion, this study was unable to support our initial hypothesis that the ejaculates of men that were positive for chlamydial DNA would be more likely to contain dead sperm and therefore would more likely be asthenozoospermic. As such, this study is in agreement with the bulk of previous studies that have concluded that semen quality is unaffected by chlamydial infection. However, we would argue if spermatozoa are only exposed to EBs at the time of ejaculation that there is the possibility that sperm viability may be compromised in the hours after ejaculation, within the female reproductive tract, and therefore any effects of chlamydia on sperm viability may go unnoticed at semen analysis. Therefore, more work is required to dissect the precise sequence of events at ejaculation if we are to fully understand the nature of any sperm damage for the fertility of men infected with chlamydia.

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