Genital chlamydial infection: association between clinical features, organism genotype and load

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The association between the clinical features of genital chlamydial infection and organism genotype and load was evaluated. Chlamydial DNA was detected and quantified in genital swabs from 233 (7%) of 3384 consecutive patients attending a genitourinary medicine clinic. The chlamydia-positive subcohort comprised 132 (57%) females and 101 (43%) males. Clinical features were present in 33% women and 72% men. The chlamydial load was found to be higher in women (median load: 5.6 log) than men (median load: 3.5 log). Single variable analysis failed to show a significant association between chlamydial load and clinical features (P value=0.3). Owing to the limited amount of clinical material, information on chlamydial genotypes was available for 70% ($n=162$) of chlamydia-positive patients. However, multivariable analysis of these samples did show a significant association between chlamydial load and clinical features (P value=0.02). This discrepancy is most probably due to the difference in the amount of data analysed by single variable (data from 233 patients) and multivariable (data from 162 patients) analysis. The distribution of chlamydia genotypes was as follows: type E (46%), F (22%), D (8%), K (8%), G (7%), J (4%), I (1%) and H (0.6%). No statistically significant association was observed between chlamydial genotype and clinical features in either single variable (P value=0.6) or multivariable (P value=0.4) analysis. These findings suggest that chlamydial load and diversity in the *ompA* gene plays little, if any, role in the pathogenesis of genital chlamydial infection.

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INTRODUCTION

The role of organism load in determining the clinical presentation of genital *Chlamydia trachomatis* (CT) infection has been a focus of interest over the past several years. Studies using CT culture and utilizing the technique of quantifying the number of c.f.u. have found higher loads in younger patients, in women compared to men and in those with evidence of genital tract inflammation (Eckert *et al.*, 2000; Geisler *et al.*, 2001). In contrast, molecular studies have reported variable results. Chlamydial load has been positively associated with symptoms and clinical signs in men but only the presence of multiple symptoms or clinical signs has been associated with organism load in

women (Michel *et al.*, 2007). Two studies have reported no association between clinical features and chlamydial load in first void urine specimens from men or women (Gomes *et al.*, 2006; Wiggins *et al.*, 2009). Chlamydial load in vulvovaginal swabs was reported to be higher among women with vaginal discharge as compared to women without discharge (Wiggins *et al.*, 2009).

Considering the role of chlamydial genotypes or serovars in determining clinical presentation, serovar Ga has been found to be associated with dysuria in men, serovar F with abdominal pain and/or dyspareunia in women and serovar K with vaginal discharge (Geisler *et al.*, 2003; Morré *et al.*, 2000). Serovar Ia has been detected more frequently in asymptomatic patients. There appears to be no association between serovars D and E and either symptomatic or asymptomatic infection. CT serovars D, E, F, I and J were the most frequently detected types in these two studies. Young white women infected with B class serovars (B, D,

Abbreviations: BHQ, black-hole quencher; CI, confidence interval; CMO EAG, Chief Medical Officer's Expert Advisory Group; CT, *Chlamydia trachomatis*; FAM, 6-carboxyfluorescein; GU, genitourinary; MGB, minor groove binder; NFQ, non-fluorescent quencher; OR, odds ratio; ROX, carboxy-X-rhodamine.

D⁻, E) have also been found to yield the highest organism load (Eckert *et al.*, 2000). Other studies using either serological or molecular techniques to type CT have reported either no (Batteiger *et al.*, 1989; Gomes *et al.*, 2006; Millman *et al.*, 2006; Van de Laar *et al.*, 1996) or weak (Boisvert *et al.*, 1999; Dean *et al.*, 1995; Van Duynhoven *et al.*, 1998; Workowski *et al.*, 1994) association between CT type and the presence or absence of clinical features.

This study was designed to overcome the two limitations of previous molecular studies (Gomes *et al.*, 2006; Michel *et al.*, 2007; Wiggins *et al.*, 2009), i.e. non-consecutive patients and the adverse effect of sample storage on chlamydial load. We investigated the association between genotype identification by using real-time PCR on fresh genital swabs from consecutive patients attending a genitourinary (GU) medicine clinic.

METHODS

Study population. The study population comprised 3384 consecutive patients attending the Department of Genitourinary Medicine at Addenbrooke's Hospital, Cambridge, UK, from August 2005 to January 2006. There were 1640 males [median age: 26 years (range: 13–73)] and 1744 females [median age: 23 years (range: 12–66)] in this cohort. Two swabs (endocervical and urethral) were obtained from female patients, placed in a single tube and treated as a single sample. The urethral sample was taken from just within the urethra. The cervix was cleaned to remove excess mucus prior to taking the cervical sample. The sample was collected by inserting a cotton swab into the cervical os, rotating firmly, withdrawing and then placing the swab in chlamydia transport medium (CTM). A single urethral swab was analysed from each male patient. This was obtained by inserting a cotton swab 2–4 cm into the urethra, rotating firmly, withdrawing and placing the swab into CTM. All swabs for chlamydia testing were collected after taking swabs for microscopy and *Neisseria gonorrhoeae* culture. All samples were collected by experienced practitioners trained in sample collection. The IDEIA chlamydia specimen collection kit (S600730; Dako) was used for the collection of genital swabs as described previously (Jalal *et al.*, 2006a). Urine specimens were not used for chlamydia detection. Clinical data were recorded for each patient and linked to chlamydia quantification and genotyping results by a code. To ensure anonymity, patient identifiers were removed after coding. Men were classified as symptomatic if they presented with dysuria, urethral discharge or intra-scrotal pain and women if they had symptoms of vaginal discharge, dysuria, pelvic discomfort/pain, or post-coital or intermenstrual bleeding. A record was also made of the presence of cervicitis, defined as mucopurulent secretions with or without contact bleeding. Quantification of polymorphs on microscopy of a Gram-stained smear of cervical secretions was not used to diagnose cervicitis. The presence of urethritis in men, defined as ≥ 5 polymorphs on microscopy ($\times 1000$ magnification) of a Gram-stained urethral smear, was also recorded. The Cambridge Local Research Ethics Committee approved this study.

Chlamydial load and genotyping. The Clinical Microbiology and Public Health Laboratory uses an in-house quantitative real-time PCR for the detection and quantification of CT DNA in clinical samples. This is a triplex assay targeting cryptic plasmid (qualitative detection), *ompA* gene (quantitative detection and confirmation) and an internal

control (synthetic DNA molecule to quality control DNA extraction and amplification) (Jalal *et al.*, 2006b). Three changes were made to the published method: (i) DNA from swabs was extracted using CAS-1820 X-Tractor Gene (Corbett Robotics) instead of MagNa Pure LC Robot (Roche Diagnostics); (ii) the number of amplification cycles was increased from 40 to 45; and (iii) HJ plasmid probe was modified (5'-FAM-CGAACTCATCGGCG-MGB-NFQ-3'; this probe was synthesized by Applied Biosystems). The performance of the assay after these changes was validated on three consecutive runs, each containing 94 samples and controls as described previously (Jalal *et al.*, 2006b). These modifications did not affect the performance of the assay. Agreed turn-around time for CT quantitative results was ≤ 5 days. After the completion of routine diagnostic work, 162 (70%) of 233 samples contained sufficient material to determine CT genotype. These leftover samples were stored at -20°C and CT genotyping was performed by using nested real-time PCR as described previously (Jalal *et al.*, 2007a).

***rpoB* PCR.** The routine diagnostic assay targets the *ompA* gene for the quantification of chlamydia genomic DNA. The primers and probe for this part of the triplex assay are from highly conserved regions of the *ompA* gene. However, there are a few nucleotide variations in the amplicons from different genotypes. We wanted to investigate the effect of these variations on the quantitative ability of the current assay. Hence, a new assay was designed targeting the *rpoB* gene with no nucleotide variation in amplicons from different genotypes of chlamydia. *rpoB* PCR was used to investigate the effect of sample storage at -20°C . After genotyping, 148 samples contained sufficient material for this analysis. This uniplex assay targeted the *rpoB* gene of CT. The new assay was optimized to produce comparable sensitivity with the quantitative part (*ompA*) of the routine diagnostic assay. The concentration of all PCR ingredients and the amplification parameters of the new assay were the same as those for the routine diagnostic assay except that different primers and probe were used (Jalal *et al.*, 2006b): forward primer, 5'-ACGACCAGGAGAGCCTGCAA-3' (location: 1421); reverse primer, 5'-ATAACGTCCAACGCGGCCTA-3' (location: 1511); and probe, 5'-ROX-TGCTCGATCCACAATTATGCG-BHQ-2-3' (location: 1442). The locations of primers and probe were according to GenBank accession no. AY826975. These primers and the probe were synthesized by metabion International. The lower limit of detection for *rpoB* PCR was determined by testing a dilution series of CT type E ATCC VR-348B at the following concentrations: 12, 6, 3, 1.5 and 0.75 genome copies per reaction. Each concentration was tested 10 times and Probit analysis of the results was carried out using StatsDirect version 2.4.1. A total of 14 isolates representing all the genovars of CT and 26 bacterial isolates representing the pathogenic bacteria and commensals of the oropharynx, genital tract and rectum were used to investigate the specificity of the primers and probe. The identity of these organisms is shown in a previous publication (Jalal *et al.*, 2006b – in Tables 1 and 2).

Statistical analysis. The chlamydial load values were converted to natural logarithms and logged values were used in statistical analysis of the data. Two main sets of analyses were performed: one where chlamydial load was the outcome (continuous) and the other where the presence or absence of clinical features was the outcome (binary). The Kruskal–Wallis test was employed to investigate the association between age and CT genotypes. Censored normal linear regression was used when the outcome was continuous, and logistic regression was used for the binary outcome; *P* values were determined by the likelihood ratio test. Each explanatory variable was examined consecutively for an association with the outcome in single variable analysis. This was followed by multivariable analysis in a backwards stepwise procedure wherein the least significant variable was removed at each step, provided it was not a significant confounder, i.e. its removal resulted in a change [in the coefficient for the continuous outcome and odds ratio (OR) when the outcome was binary] of less

than 10 %. This continued until all variables were either significant at the 5 % level or were substantial confounders, at which point interactions between the significant variables were added to the model and removed one at a time if not significant. When using clinical features as the outcome, the logged chlamydial loads were grouped into the following bands: less than 3, 3 to less than 4, 4 to less than 5, 5 to less than 6 and 6 or more. All analyses were performed in STATA 11.

RESULTS

Effect of sample storage at -20°C on chlamydial load

According to Probit analysis, *rpoB* PCR had a 95 % probability of detection at 2 [confidence interval (CI) of 1 to 4] genome copies of CT per reaction. The primers and probe used in *rpoB* PCR did not cross-react with DNA from any of the pathogenic bacteria or commensals from the oropharynx, genital tract and rectum that were tested. *rpoB* PCR amplified DNA from all strains of CT. Although CT DNA was detected in all of the 148 samples before and after storage, there was a significant degradation of DNA in almost all samples after storage at -20°C . There were only three samples with a load of $<1 \log_{10}$ genome copies (ml CTM) $^{-1}$ before storage. This number increased to 27 after storage. Similarly, there were 40 samples with a load of $>6 \log$ before storage. Only one sample had a load of $>6 \log$ after storage. A comparison of chlamydial load before and after storage is shown in Fig. 1. We were unable

to use *rpoB* PCR for its intended purpose due to the limited quantity of DNA in stored samples. The chlamydial load data from stored samples were not used to investigate the association between clinical features and chlamydial load.

Association between covariates (sex, age and genotype) and chlamydial load

CT DNA was detected in genital swabs from 233 (7 %) of 3384 patients. This CT-positive subcohort comprised 132 (57 %) women [median age: 20 years (range: 15–44)] and 101 (43 %) men [median age: 23 years (range: 17–62)]. The accurate quantification of chlamydial load was not possible for six women and three men due to the low quantity of CT DNA in the samples from these patients. Samples from 54 women and 2 men had chlamydial loads of $>6 \log$. Due to limited resources and a fixed turn-around time of ≤ 5 days, these samples were not further titrated to determine the precise value of the chlamydial load.

Genital swabs from women (median load: 5.6 log) had a much higher chlamydial load than those from men (median load: 3.5 log). The distribution of chlamydial load among women and men is shown in Fig. 2. To investigate the effect of age on chlamydial load, the age of participants was categorized into the following bands in line with a published study (Gomes *et al.*, 2006): ≤ 20 years, 21–30, 31–40 and > 41 years. The majority of CT-positive patients were under the age of 30 years [95.4 %

Fig. 1. Histograms showing comparative load for 148 samples before (a; *ompA* PCR) and after (b; *rpoB* PCR) storage at -20°C .

Fig. 2. Histograms of the distribution of clinical samples based on the quantification of CT *ompA* copy number (ml CTM)⁻¹. Chlamydial loads in (a) 132 women and (b) 101 men are shown.

($n=126$) women and 78 % ($n=79$) men]. While there was a weak association between age groups and chlamydial load in single variable analysis ($P=0.09$), with the 21–30 year age group having lower load than those aged under 20, this association was not apparent on multivariable analysis ($P=0.5$) (Table 1).

CT genotyping results were available for 162 (70 %) of the 233 chlamydia-positive patients. This group comprised 90 women and 72 men. The distribution of CT genotypes was as follows: type E (46 %), F (22 %), D (8 %), K (8 %), G (7 %), J (4 %), I (1 %) and H (0.6 %). Two different genotypes were detected in swabs from four patients (2 %). There was no significant difference in the distribution of CT genotype between women and men. The Kruskal–Wallis test revealed no association between age and genotype ($P=0.4$). Table 2 shows the distribution of genotypes between different age groups (a Fisher's exact test yielded a P value of 0.2). Similarly, there was no association between genotype and chlamydial load (P value

of 0.6). The results of single and multivariable censored regression analysis are summarized in Table 1. These results demonstrated that only gender was associated with chlamydial load, with males having a significantly lower load than females.

Association between covariates (sex, age, chlamydial load and genotype) and clinical features

Clinical features of genital infection were found in 44 (33 %) of 132 women and 73 (72 %) of 101 men. Signs of cervicitis and urethritis as defined in Methods were identified in 10 (23 %) of the 44 women and 49 (67 %) of the 73 men, respectively. All women with cervicitis had a chlamydial load of >4.3 log and seven of them had a load of >6 log. A total of 42 of the 49 men with urethritis had a chlamydial load of ≥ 3 log. There was a strong association between sex and clinical features, with the OR (<0.001) for males to have clinical features being much greater than for

Table 1. The association of covariates (sex, age and genotype) with chlamydial load

Covariate	Single variable analysis		Multivariable analysis*	
	Unadjusted coefficient (95 % CI)	Unadjusted <i>P</i> value	Adjusted coefficient (95 % CI)	Adjusted <i>P</i> value
Sex				
Male	−1.85 (−2.27, −1.42)	<0.001	−1.96 (−2.44, −1.47)	<0.001
Female	Reference		Reference	
Age group (years)				
≤20	Reference	0.09	Reference	0.5
21–30	−0.55 (−1.06, −0.04)		−0.24 (−0.73, 0.26)	
31–40	−0.54 (−1.47, 0.39)		0.18 (−0.70, 1.07)	
≥41	NE		NE	
Genotype				
D	Reference	0.6	Reference	0.6
E	0.68 (−0.34, 1.70)		0.83 (0.01, 1.65)	
F	0.74 (−0.37, 1.85)		0.95 (0.04, 1.86)	
G	1.53 (0.14, 2.93)		1.35 (0.21, 2.49)	
H	0.40 (−3.07, 3.88)		−0.24 (−3.05, 2.57)	
I	1.09 (−1.62, 3.79)		0.06 (−2.12, 2.25)	
J	0.21 (−1.37, 1.80)		0.56 (−0.74, 1.85)	
K	1.37 (0.00, 2.74)		1.10 (−0.01, 2.22)	
E + F	1.79 (−0.97, 4.55)		0.65 (−1.61, 2.90)	
E + G	0.70 (−2.77, 4.18)		1.78 (−1.06, 4.62)	
D + E	−0.10 (−3.57, 3.38)		1.22 (−1.60, 4.04)	

NE, Not estimable.

*Adjusted results based on 162 observations.

females. No significant association was found between age and CT genotype and clinical features. Single variable analysis suggested no association between CT load and clinical features, whereas multivariable analysis indicated otherwise. This difference may be due to the smaller number of observations available in the multivariable analysis. The results of single and multivariable analysis for the association between covariates (sex, age, load and genotype) and clinical features are summarized in Table 3.

Table 2. The distribution of age groups and genotypes

Genotype	Age group (years)		
	≤20	21–30	31–40
D	5	7	1
E	24	42	9
F	20	14	1
G	5	5	2
H	0	1	0
I	2	0	0
J	4	3	0
K	8	5	0
E + F	1	0	1
E + G	1	0	0
D + E	0	1	0
Total	70	78	14

DISCUSSION

This study investigated the association between chlamydial load in genital swabs with *ompA* genotypes and clinical features. The design of this study is similar to that performed by Gomes *et al.* (2006) with a few exceptions. Apart from different study populations, Gomes *et al.* (2006) performed their analysis on stored DNA from first void urine. This approach was based on the evidence for chlamydial co-infection of the urethra and cervix in women. However, the chlamydial load in urine may not be a true reflection of the cervical chlamydial load. Their study reported a wide distribution in the number of epithelial cells (ml urine)^{−1} suggesting a variation in the time between urine collection and last previous urination. Using a variable denominator (epithelial cells) to normalize the chlamydial load may affect the accuracy of quantitative data. For this reason, genital swabs were used for quantitative analysis in this study. We did not normalize the chlamydial load data by the quantification of cellular DNA because genital swabs contain a mixture of different types of cells, i.e. columnar, squamous and inflammatory cells. Normalizing chlamydial load by total genomic DNA load does not provide the relevant information, i.e. chlamydial load per columnar cell. The potential bias in our quantitative data, due to differences in sample collection by nurses and doctors, was minimized by a standardized method of sample collection.

Quantification of chlamydial DNA was performed on fresh samples as part of the routine diagnostic service. The

Table 3. The association of covariates (sex, age, chlamydial load and genotype) with clinical features

Covariate	Clinical features		Single variable analysis		Multivariable analysis*	
	Presence	Absence	Unadjusted OR (95 % CI)	Unadjusted <i>P</i> value	Adjusted OR (95 % CI)	Adjusted <i>P</i> value
Sex						
Male	73	28	5.21 (2.96, 9.19)	<0.001	13.87 (4.46, 43.20)	<0.001
Female	44	88	Reference		Reference	
Age group (years)						
≤20	45	65	Reference	0.03	Reference	0.17
21–30	62	43	2.08 (1.21, 3.59)		1.59 (0.73, 3.45)	
31–40	10	8	1.81 (0.66, 4.93)		0.48 (0.11, 2.09)	
≥41	0	0	NE		NE	
Chlamydial load (logged)						
<3	17	20	Reference	0.3	Reference	0.02
3 to <4	28	24	1.37 (0.59, 3.20)		1.05 (0.30, 3.64)	
4 to <5	28	17	1.94 (0.80, 4.69)		3.08 (0.76, 12.50)	
5 to <6	20	23	1.02 (0.42, 2.47)		4.44 (1.02, 19.41)	
≥6	24	32	0.88 (0.38, 2.03)		7.92 (1.73, 36.28)	
Genotype						
D	6	7	Reference	0.6	Reference	0.4
E	43	32	1.57 (0.48, 5.12)		1.24 (0.31, 4.92)	
F	16	19	0.98 (0.27, 3.52)		0.64 (0.14, 2.91)	
G	6	6	1.17 (0.24, 5.62)		1.09 (0.18, 6.58)	
H	1	0	NE		NE	
I	1	1	1.17 (0.06, 22.94)		3.43 (0.11, 111)	
J	3	4	0.87 (0.14, 5.58)		0.72 (0.09, 6.06)	
K	6	7	1.00 (0.21, 4.67)		0.88 (0.14, 5.71)	
E + F	2	0	NE		NE	
E + G	1	0	NE		NE	
D + E	1	0	NE		NE	

NE, Not estimable.

*Adjusted results based on 162 observations.

accuracy of quantification data on stored samples or DNA is undetermined. Two studies investigating chlamydial load have reported considerable decline in the positivity rate of samples after the storage of extracted DNA (Gomes *et al.*, 2006) or unextracted clinical material (Wiggins *et al.*, 2009) at -20°C . A total of 54 (22%) of the 244 Amplicor-positive urine samples did not amplify using *ompA* nested PCR (Gomes *et al.*, 2006). Similarly, RealArt PCR (Qiagen) did not detect CT DNA in 102 (21.4%) of 476 Amplicor-positive samples (Wiggins *et al.*, 2009). The authors concluded that it was not possible to rule out degradation of DNA, in spite of the difference in sensitivity between the qualitative (target: cryptic plasmid, higher sensitivity due to multiple copies) and the quantitative (target: *ompA* gene, lower sensitivity due to a single copy) PCRs. In this study, a significant degradation in CT DNA in genital swabs was observed after storage at -20°C , even when quantitative PCRs of comparable sensitivity were performed on these samples (Fig. 1). A further strength of our study was the unbiased recruitment of patients and complete load dataset for the chlamydia-positive subcohort. The weaknesses of our

study included a single centre for patient recruitment and an incomplete genotyping dataset due to limited clinical material. To avoid the recruitment bias, it was decided not to collect extra samples for the purpose of this study. Hence, the investigations for the association between chlamydial load with genotypes and clinical features were possible for 162 (70%) patients, but the data for chlamydial load, clinical features and other covariates were available for all of the 233 chlamydia-positive patients. The distribution of genotypes in our cohort was similar to that given in a number of published studies from the USA and different European countries (Jalal *et al.*, 2007b; Lysén *et al.*, 2004; Oehme *et al.*, 2003; Sayada *et al.*, 1992; Suchland *et al.*, 2003).

Gomes *et al.* (2006) reported that gender was not associated with a difference in chlamydial load. We report a higher median load (~ 2 logs) for women than for men. Gomes *et al.* (2006) used urine from both men and women to represent loads at one anatomical site, i.e. the urethra. In our study, the load for women represented a cumulative load at two different anatomical sites, i.e. the cervix and

urethra, and that for men represented the load at a single anatomical site, i.e. the urethra. A higher quantity of material in samples from women may have resulted in this finding. A culture-based study that used genital swabs for the quantification of chlamydial DNA reported a sixfold higher load for women (Eckert *et al.*, 2000). Although the discomfort associated with urethral swab collection from men may contribute to the lower load, there are important biological differences between men and women that may have a significant effect on the load. These biological differences include the presence of a greater amount of infection-susceptible epithelium in women and the chlamydial-killing effects of prostatic secretions and the urethral flushing effect of urination in men (Eckert *et al.*, 2000). Culture-based studies have reported a higher chlamydial load at a younger age (Eckert *et al.*, 2000; Geisler *et al.*, 2001). We did not observe any effect of age on load. The immunological principle behind the decrease in load among older patients is partial immunity due to repeated infections. Since 2005, genital chlamydial infection has been diagnosed by nucleic acid amplification techniques for 2239 (7%) of 31 689 patients attending the GU Medicine Clinic at Addenbrooke's Hospital. The median prevalence of genital chlamydial infection among patients attending GU clinics in the UK was reported to be 16.4% (range: 7–29) (CMO EAG, 1998). The prevalence of infection is relatively low in the population attending our GU clinic. This local epidemiology may suggest a lower prevalence of repeated infections among our patients rather than a lower load with increasing age. Gomes *et al.* (2006) reported no effect of age on chlamydial load and no association between clinical features and chlamydial load and genotype.

In summary, we failed to find an association between different chlamydial genotypes and the presence or absence of clinical features. Similarly, single variable analysis did not show a significant association between chlamydial load and clinical features. However, multivariable analysis showed a significant association between chlamydial load and clinical features. This discrepancy was most probably due to the difference in the amount of data analysed by single variable (233 datasets) and multivariable (162 datasets) methods. These results differ from those of culture-based studies (Eckert *et al.*, 2000; Geisler *et al.*, 2001), but are consistent with a published molecular study (Gomes *et al.*, 2006). The lack of association between CT genotypes and clinical features suggests that the polymorphism in *ompA* does not play a significant role in pathogenicity. Similarly, the lack of association between chlamydial load and clinical features indicates the importance of host factors in the pathogenesis of infection. Based on these observations, we have designed future genomic and proteomic studies to investigate the pathogenesis of genital chlamydial infection.

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