NEW VARIANT

Chlamydia trachomatis variant not detected by plasmid based nucleic acid amplification tests: molecular characterisation and failure of single dose azithromycin

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Objective: To characterise a *Chlamydia trachomatis* variant strain from a patient with non-gonococcal urethritis (NGU) whose first void urine (FVU) displayed discrepant *C trachomatis* test results and describe the clinical response to treatment.
Methods: The FVU specimen was assayed with an immune based *Chlamydia* Rapid Test (CRT) and various nucleic acid amplification tests (NAATs) to establish *C trachomatis* infection. Sequencing of the major outer membrane protein gene (*omp1* also known as *ompA*) was undertaken to identify the serovar of the variant strain. Polymerase chain reaction (PCR) analysis was also conducted to determine whether the strain harboured deletions in the cryptic plasmid or was plasmid free.
Results: The FVU specimen was strongly reactive in CRT but negative with the plasmid based Amplicor PCR (Roche) and ProbeTec ET (Becton-Dickinson) assays. However, NAATs for 16S RNA (Aptima Combo 2, GenProbe), *omp1* (RealArt CT PCR, Artus and in-house NAATs) or the outer membrane complex B protein gene (*omcB*) established *C trachomatis* infection. Sequencing of *omp1* showed that the variant belonged to serovar I. PCR analysis indicated that the variant was plasmid free. The patient did not respond to single dose azithromycin treatment but subsequently responded to a course of doxycycline.
Conclusions: A pathogenic plasmid free *C trachomatis* variant was identified. Clinicians should be alerted to the possibility of undetected *C trachomatis* infection caused by such variants and the potential of azithromycin failure in patients with recurrent chlamydial NGU. The occurrence of this variant is rare and should not form the basis for judgment of the performance or usefulness of plasmid based NAATs for *C trachomatis* detection.

The organism *Chlamydia trachomatis* is the cause of the most commonly reported bacterial sexually transmitted infections (STI) in developed countries such as the United States and United Kingdom. The prevalence of *C trachomatis* infection in these countries ranges from 3% to 20% among sexually active young adults between 16 and 24 years of age.[1 2] Untreated *C trachomatis* infections can lead to complications such as pelvic inflammatory disease and infertility in women and epididymitis in men.[3] Up to 80% of infected women and 50% of infected men are asymptomatic,[4] with most of these individuals remaining undiagnosed. Targeted screening of high risk populations has been recommended to control *C trachomatis* infections,[1] and several countries including the United Kingdom have embarked on screening programmes based on nucleic acid amplification tests (NAATs).[2]

Most commercial NAATs for the detection of *C trachomatis* rely either on conserved regions of 16S ribosomal RNA or on the 7.5 kb cryptic plasmid of the organism as the target for amplification.[5 6] A *C trachomatis* variant with a deletion in the plasmid was recently identified in Sweden, after an apparent 25% decrease in *C trachomatis* infections was noted.[7] This variant has a 377 bp deletion in the region of the plasmid targeted by two NAATs, the M2000 (Abbott Laboratories, Abbott Park, IL, USA) and Amplicor CT/NG PCR (Roche Diagnostic Systems, Branchburg, NJ, USA) tests, and it therefore yielded false negative results with both of these tests. However, this strain remains detectable by another plasmid based NAAT, ProbeTec ET (BD Biosciences, Sparks, MD, USA), because the deletion does not affect the target region of this test. We now describe the clinical presentation and characterisation of a *C trachomatis* variant identified in the United Kingdom that generated false negative results with all plasmid based NAATs but remained detectable with assays based on 16S ribosomal RNA or outer membrane protein genes.

METHODS

Patient and specimen collection

A 28-year-old heterosexual African man attended the Ambrose King Centre (AKC) at the Royal London Hospital in December 2006 complaining of dysuria over a 3-week period. He was one of the 904 male patients recruited for a study at the AKC between March and December 2006 to evaluate the *Chlamydia* Rapid Test (CRT) being developed by the Diagnostics Development Unit at the University of Cambridge. This study was approved by the Moorfields and Whittington research ethics committee. Written informed consent was obtained from the patient, and clinical research guidelines for the relevant institutions were followed in the conduct of this research.

For the study, the patient was requested to provide 30–40 ml of first void urine (FVU) after not having urinated for at least 2 hours. Before urine collection, the patient had a routine urethral smear collected for Gram staining and culture for *Neisseria gonorrhoeae*. Blood tests for treponemal and HIV antibodies were performed. An aliquot of the FVU specimen was tested for *Mycoplasma genitalium* using a real time

Abbreviations: CRT, *Chlamydia* Rapid Test; DPBS, Dulbecco's phosphate buffered saline; FVU, first void urine; hpf, high power field; GNID, Gram negative intracellular diplococci; NAATs, nucleic acid amplification tests; NGU, non-gonococcal urethritis; *omp*, outer membrane protein; PCR, polymerase chain reaction; PMNL, polymorphonuclear leucocytes; STI, sexually transmitted infections polymerase chain reaction (PCR) assay[8] at the Sexually Transmitted Bacteria Reference Laboratory of the Health Protection Agency (HPA).

Chlamydia rapid test

The CRT was performed with 3 ml of the FVU specimen. The urine was diluted with 6 ml water (Sigma, St Louis, MO, USA) and then centrifuged at 3000 *g* for 20 minutes at room temperature (Megafuge 1.0R; Hereaus, Osterode, Germany). The resulting pellet was extracted with 400 µl of lysis agent, 300 µl of analyte stabiliser, and 100 µl of signal enhancer reagent, with thorough mixing after the sequential addition of each reagent. A portion (100 µl) of the resulting extract was tested with a dipstick as previously described.[9]

Commercial NAATs

The following commercial NAATs were used to detect the presence of *C trachomatis* in the FVU of the patient infected with the newly identified variant: Amplicor CT/NG PCR, ProbeTec ET, RealArt CT PCR (Artus, Hamburg, Germany), and Aptima Combo 2 (GenProbe, San Diego, CA, USA). These tests were performed according to manufacturers' instructions.

In-house NAATs

The FVU specimen of the proband was also assayed by Taqman based quantitative PCR (QPCR) tests that target the cryptic plasmid or outer membrane complex B protein gene (*omcB*) of *C trachomatis* . For these tests, 0.5 ml of urine was mixed with 0.5 ml of Dulbecco's phosphate buffered saline (DPBS) without Ca^{2+} and Mg^{2+} (BioWhittaker, Walkersville, MD, USA). The mixture was centrifuged for 15 minutes at 17 000 *g* and 25℃ (Megafuge 1.0R). The resulting pellet was washed once with DPBS and then resuspended in 100 µl of 2 M NH_4OH (Sigma). The tube was sealed, incubated at room temperature for 10 minutes, and then uncapped and incubated at 95° (SD 1℃) for 60–70 minutes. The precipitate was resuspended in 0.5 ml of nuclease free water (Sigma), and incubated for ⩾30 minutes at room temperature before amplification. QPCR was performed by the method of Pickett *et al*[10] using amplification conditions described previously.[11] Another real time PCR assay for the *C trachomatis* plasmid was performed with the FVU specimen of the proband as previously described.[12]

Extraction of DNA for PCR analysis of the cryptic plasmid and *omp1*

A portion (0.5 ml) of the FVU specimen was centrifuged for 15 minutes at 17 000 *g* and 25℃ (Megafuge 1.0R), and the resulting pellet was subjected to extraction with the use of a QIAprep Miniprep Kit (Qiagen, Valencia, CA, USA). The same method was also used for extraction of DNA from the culture supernatant of the *C trachomatis* serovar I strain UW-12/Ur (American Type Culture Collection), which served as control for PCR assays.

PCR and sequence analysis of *omp1*

The entire major outer membrane protein gene (*omp1* or *ompA*), including the four variable sequence regions (VS1 to VS4), was amplified by PCR with the forward primer MOMP-108 (corresponding to position 108 bp upstream of *omp1*) and the reverse primer RVSEND (corresponding to a position 80 bp downstream of *omp1*). This primer set yields a 1327 bp fragment including *omp1*. The primers MOMP87 (corresponding to a site located 87 bp downstream of *omp1*) and either RVS1163 or RVS1059 (corresponding to nucleotide positions 1163 and 1059 of the gene, respectively) were used to amplify fragments of *omp1*. PCR was performed with a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) and an Expand High Fidelity kit (Roche). The 50 µl reaction mixture consisted of 5 µl of DNA template, 15 pmol of each primer, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, and 1.5 µl of Expand polymerase in 1X reaction buffer. The amplification protocol comprised an initial denaturation step at 95℃ for 3 minutes; 40 cycles of denaturation (94℃, 40 seconds), annealing (50℃, 35 seconds), and extension (68℃, 2 minutes); and a final extension step at 68℃ for 10 minutes. The amplification products were separated by electrophoresis on a 1.0% agarose gel, purified with the use of a QIAquick gel extraction kit (Qiagen), and sequenced with an Applied Biosystems 3730xl DNA Analyzer at the Department of Biochemistry, University of Cambridge. The primers used for *omp1* amplification and sequence analysis are shown in table 1. The determined nucleotide sequence was aligned with published *omp1* sequences from various serovars with the use of MacVector V7.2.3 software (Accelerys, Wigan). Another in-house PCR method[13] was also used to determine the presence of *omp1* in the FVU specimen.

Table 1 Polymerase chain reaction and sequencing primers for *omp1* and the cryptic plasmid of *C trachomatis*

Primer	Sequence (5′ to 3′)	Nucleotide positions* and direction
PCR and sequencing primers for *omp1*		
MOMP-108[14]	GGCCATTAATTGCTACAGGACATCTTGTC	780140–780168, forward
MOMP87[14]	TGAACCAAGCCTTATGATCGACGG	779951–779974, forward
RVSEND[15]	AAGYCGAGCCCAGAAAYACGGAT	778798–778020, reverse
RVS1163[14]	CGGAATTGTGCATTTACGTGAG	778886–778907, reverse
RVS1059[14]	GCAATACCGCAAGATTTTCTAGATTTCATC	778982–779011, reverse
CT419F[16]	TGGGATCGTTTTGATGTATT	779654–779673, forward
CT902F[16]	TACATTGGAGTTAAATGGTC	779183–779202, forward
PCR primers for the cryptic plasmid		
Seq30F	TGGTACGAGAAGGTGATTCTAAGCC	5244–5268, forward
LCR20R[17]	CAACAAAAGTCCATTATGACC	91–111, reverse
Seq31F	AGTCTTCTGCTTACAATGCTCTTGC	7046–7070, forward
Seq4F	CCAAGCTTAAGACTTCAGAGGAGCG	1538–1562, forward
Seq6R	GCCAGCACTCCAATTTCTGACTGTG	2742–2766, reverse
Seq21F	TCCCTCGTGATATAACCTATCCG	2131–2153, forward
Seq7F	GTTGGGAAAAATAGACATGGATCGG	3488–3152, forward
CtpFPR[18]	TGGGTGTGACTGTGAATTTTCC	5147–5168, reverse
Seq10F	GTGTCTCACTAATTCTAGGACTGCT	5709–5733, forward
Seq8F	CGTTGTAGAGCCATGTCCTATCTTG	4164–4188, forward
LCR25R[17]	AGAATTCAAAGTTGCTGAGAATA	6785–6807, reverse

*Nucleotide positions correspond to the regions of the genome (for *omp1*) or of the plasmid pLGV440 (GenBank accession number X06707) targeted by each primer.

Table 2 *C trachomatis* tests performed on the urine sample of the proband

Test manufacturer or developer	Test name	Test target	Result
Commercial tests			
BD Biosciences	ProbeTec ET	Plasmid	–
Roche	Amplicor CT/NG PCR	Plasmid	–
Artus	RealArt CT PCR	*omp1*	+
GenProbe	Aptima Combo 2	16S ribosomal RNA	+
University of Cambridge	*Chlamydia* Rapid Test	Lipopolysaccharide	+
Research tests			
CDC and HPA	Real time PCR[12]	Plasmid	–
University of Southampton	Taqman PCR[10]	Plasmid	–
University of Southampton	Taqman PCR[10]	*omcB*	+
LSHTM	*omp1* PCR[13]	*omp1*	+

CDC, Centers for Disease Control and Prevention; HPA, Health Protection Agency; LSHTM, London School of Hygiene and Tropical Medicine.

PCR analysis of the cryptic plasmid

Various regions of the *C trachomatis* cryptic plasmid were amplified by PCR with combinations of primers that together encompass the entire plasmid (table 1). PCR was performed as described above for *omp1*, with the exception that annealing was performed at 54°C and extension time for the 40 cycles was increased to 3 minutes. Seven primer pairs were used for analysis of the plasmid: (1) Seq30F and LCR20R, (2) Seq31F and Seq6R, (3) Seq4F and Seq6R, (4) Seq21F and CtpFPR, (5) Seq7F and CtpFPR, (6) Seq10F and LCR25R, and (7) Seq8F and LCR25R. PCR products were separated by electrophoresis on a 1% agarose gel and were visualised by staining with ethidium bromide.

RESULTS

The patient complained of pain during urination and revealed that he had engaged in unprotected vaginal sexual intercourse with four partners in the United Kingdom and mainland Europe during the preceding 3 months. Genital examination revealed a clear urethral discharge, microscopic examination of which showed 30 polymorphonuclear leucocytes per high power field (PMNL/hpf) ×1000 but no Gram negative intracellular diplococci (GNID), indicating non-gonococcal urethritis (NGU). Culture for *Neisseria gonorrhoeae*, treponemal enzyme immunoassay test, HIV antibody test, and an in-house real time PCR assay for *Mycoplasma genitalium* were all negative. The FVU specimen was also negative for *C trachomatis* by the ProbeTec ET assay, the routine NAAT used to detect *C trachomatis* at the hospital.

The FVU was tested with both the CRT and the Amplicor CT/NG PCR assay; it was found to be strongly reactive in the former but negative in the latter. To examine whether the CRT result was a false positive, we performed two TaqMan PCR assays[10] to detect *omcB* or the cryptic plasmid. The sample was clearly positive for *omcB* (mean 47 770 (SD 7103) copies per millilitre of urine; n = 3) but negative for the plasmid. The FVU was also negative with another in-house plasmid based PCR assay[12] but yielded positive results when tested in two different laboratories by alternative NAATs that target *omp1* (RealArt CT PCR and an in-house PCR assay[13]). The Aptima Combo 2 assay, which targets 16S ribosomal RNA, also established the presence of *C trachomatis* infection in the patient. Sequencing analysis revealed that *omp1* of the *C trachomatis* strain detected in the proband is identical to that of the serovar Ia strain Ia/CL-9 (CS-190/96).[19] The results of the various *C trachomatis* tests performed on the patient's urine are summarised in table 2.

The patient was treated for NGU with azithromycin (1 g immediately). Six weeks later, he reported initial symptomatic improvement, with a recurrence of dysuria and urethral discharge during the previous week. Examination revealed a mucoid urethral discharge, microscopic analysis of which revealed 50 PMNL/hpf (×1000) but no GNID. He was again treated for NGU but with 100 mg of doxycycline twice daily for 1 week. Repeat testing on a FVU sample with the use of plasmid based NAATs (ProbeTec ET, Amplicor CT/NG PCR, and TaqMan PCR) was negative, whereas the Taqman PCR test for *omcB* remained positive, although the *C trachomatis* load (3496 (SD 671) copies/ml) was much lower than that of the initial specimen. Real time PCR for *M genitalium* remained negative. The NAAT results indicate that the *C trachomatis* strain detected during the second clinic visit was the same strain observed earlier.

To investigate further why the variant *C trachomatis* strain was not detected with plasmid based NAATs, PCR was performed with primers designed to yield overlapping products covering the entire 7.5 kb cryptic plasmid (table 1). DNA extracted from a culture supernatant of the serovar I strain UW-12/Ur was used as a positive control. Whereas the seven primer combinations yielded amplification products of the expected sizes with the control strain, no amplification products were detected with DNA isolated from the variant strain (fig 1A). The plasmid map indicating the regions covered by the plasmid amplification products is shown in figure 1B. Twenty-six additional primer combinations that target different regions of the plasmid also yielded amplification products with DNA from the serovar I strain but not with the variant strain (data not shown). The DNA extracts used for amplification of the plasmid for both the serovar I and the variant strains, however, yielded products of the expected size when used to amplify *omp1* (fig 1A). These results suggest that this *C trachomatis* variant is a plasmid free strain.

DISCUSSION

NAATs are considered the most sensitive tests for the diagnosis of *C trachomatis* infection.[5,6] The targets for nucleic acid amplification in these tests include the cryptic plasmid, major outer membrane protein complex genes (*omp1*, *omcB*), and 16S ribosomal RNA.[20] The plasmid is a preferred target for many NAATs because its presence in multiple copies renders plasmid based tests more sensitive than chromosome based ones.[21] Indeed, three of four major commercial platforms for *C trachomatis* detection used in North America and Europe are plasmid based NAATs. Although the plasmid is well conserved among *C trachomatis* strains,[22] plasmid free variants have been described.[23,24] The use of plasmid based NAATs for systematic screening over a long period may result in diagnostic selection pressure and the consequent emergence of plasmid free strains[20] and false negative test results.

The present case reveals a novel *C trachomatis* variant that was not detected with any of the plasmid based NAATs applied and is therefore a plasmid free strain which differs from the variant strain reported in Sweden.[7] Investigations conducted in

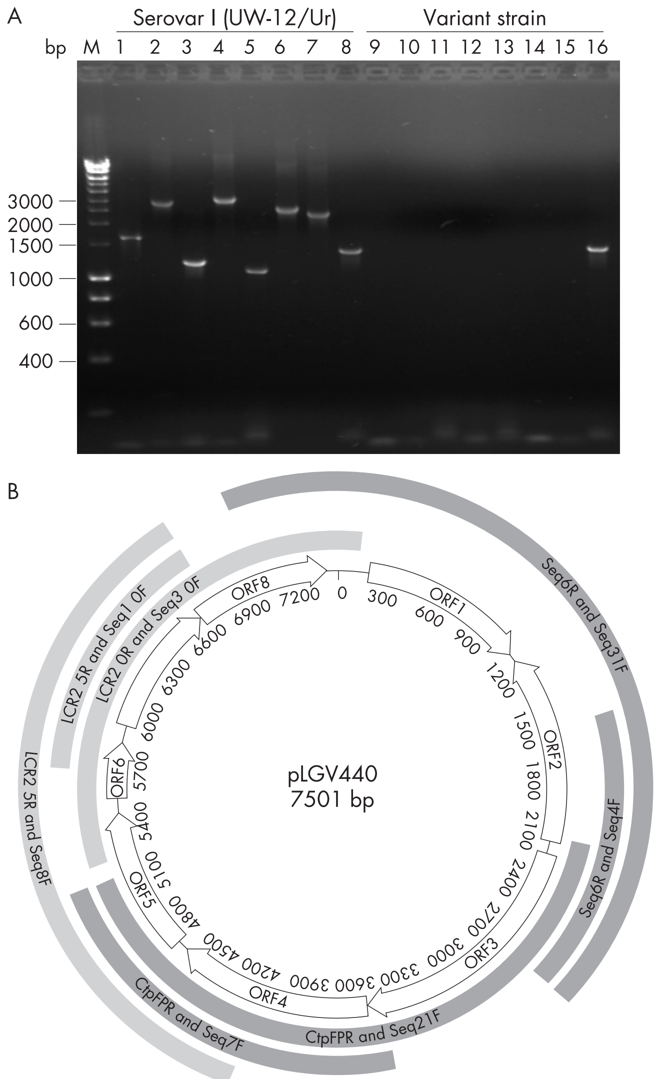

Figure 1 PCR analysis of the *C trachomatis* variant for *omp1* and cryptic plasmid sequences. (A) The serovar I strain UW-12/Ur (lanes 1–8) and the variant strain of the proband (lanes 9–16) were subjected to PCR analysis with seven primer pairs specific for various regions of the 7.5 kb cryptic plasmid (lanes 1–7 and 9–15) or with primers that amplify the entire *omp1* sequence (lanes 8 and 16). The primers and expected sizes of the corresponding amplification products are as follows: lanes 1 and 9, Seq7F and CtpFPR (1680 bp); lanes 2 and 10, Seq21F and CtpFPR (3037 bp); lanes 3 and 11, Seq4F and Seq6R (1228 bp); lanes 4 and 12, Seq31F and Seq6R (3221 bp); lanes 5 and 13, Seq10F and LCR25R (1098 bp); lanes 6 and 14, Seq8F and LCR25R (2643 bp); lanes 7 and 15, Seq30F and LCR20R (2368 bp); and lanes 8 and 16, MOMP-108 and RVS-END (1327 bp). The PCR products were separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide. Lane M, molecular size markers. (B) Plasmid map based on pLGV440 (GenBank accession number X06707) showing the open reading frames (ORFs) and regions covered by the various PCR products obtained from the serovar I strain UW-12/Ur with the primer pairs used in (A).

Ireland,[25] the United Kingdom,[26] and the Netherlands[27] did not identify the latter strain in these countries. However, recent studies have reported detection of the Swedish variant in patients attending STI clinics in Norway[28] and Denmark.[29]

It was not possible to establish a definite source of the patient's infection, given that he had partners both in the United Kingdom and mainland Europe before his first clinic visit. We succeeded in tracing two of his four partners, and both were negative for *C trachomatis* in tests performed elsewhere, although one of them was previously diagnosed and treated for non-gonococcal "cervicitis". Follow-up *C trachomatis* tests on cervical swab and FVU specimens from these two partners were negative for both the plasmid and *omcB* using Taqman PCR.

The persistent urethritis in our patient may have been due either to a poor response to azithromycin treatment or to re-infection from a new partner; the latter possibility is highly unlikely given that he disclosed only protected intercourse between his two clinic visits. More importantly, the NAAT results on the second FVU specimen indicate that the *C trachomatis* strain detected was identical to that observed during his first clinic visit and thus support the possibility of the failure of azithromycin treatment. Azithromycin failure in the absence of re-infection has been reported in women[30] and with multidrug resistant strains.[31] In the present patient, an initial response to azithromycin was followed by symptom relapse and clinical and laboratory evidence of urethritis, albeit with a lower chlamydial load. These observations are suggestive of reactivation of persistent infection resulting from heterotypic resistance associated with a high chlamydial load.[32]

The patient's high risk activities, the inability of plasmid based NAATs to detect the *C trachomatis* strain responsible for his infection, the failure of single dose azithromycin treatment, and the fact that a large proportion of *C trachomatis* infections are asymptomatic suggest that other cases of infection with this variant strain may have gone undetected in settings that rely solely on plasmid based NAATs for *C trachomatis* detection. However, retrospective testing using CRT and Amplicor PCR of almost 300 *C trachomatis* positive urine samples (112 female, 183 male) obtained from screening 3739 individuals attending STI and young people's sexual health clinics in 2006 failed to detect the presence of variant strains, indicating that it is not highly prevalent. A prospective study to determine the frequency of plasmid variants will be initiated by the HPA. Until additional data become available, this report remains an isolated case and should not form the basis for judgment either of the performance of the various NAAT assay systems or of the efficacy of treatment regimens. Nevertheless, it is important to recognise the possible existence of undetected *C trachomatis* infections caused by variant strains in STI clinics or *C trachomatis* screening programmes, especially in settings where plasmid based NAATs are the method of choice for diagnosis of such infection.

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Competing interests: The authors from the University of Cambridge are equity holders of a spin-off company, Diagnostics for the Real World (DRW) Ltd, that was founded to take advantage of rapid test technologies developed at the University of Cambridge. Both the University of Cambridge and the Wellcome Trust are also equity holders of DRW. Other authors declare no competing interests.

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