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**NEW VARIANT**

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

Jose Paolo V Magbanua, Beng Tin Goh, Claude-Edouard Michel, Aura Aguirre-Andreasen, Sarah Alexander, Ines Ushiro-Lumb, Catherine Ison, Helen Lee

**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.

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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. Untreated *C trachomatis* infections can lead to complications such as pelvic inflammatory disease and infertility in women and epididymitis in men. Up to 80% of infected women and 50% of infected men are asymptomatic, with most of these individuals remaining undiagnosed. Targeted screening of high risk populations has been recommended to control *C trachomatis* infections, and several countries including the United Kingdom have embarked on screening programmes based on nucleic acid amplification tests (NAATs).

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. 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. 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 polymerase chain reaction; PMNL, polymorphonuclear leucocytes; STI, sexually transmitted infections.

**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
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.

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) that target the cryptic plasmid or outer membrane complex B protein gene (ompC) 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 Ca²⁺ and Mg²⁺ (BioWhittaker, Walkersville, MD, USA). The mixture was centrifuged for 15 minutes at 17 000 g and 25°C (MegaFuge 1.0R). The resulting pellet was washed once with DPBS and then resuspended in 100 µl of 2 M NH₄OH (Sigma). The tube was sealed, incubated at room temperature for 10 minutes, and then uncapped and incubated at 95°C (SD 1°C) 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 using amplification conditions described previously. Another real time PCR assay for the C. trachomatis plasmid was performed with the FVU specimen of the proband as previously described.

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°C (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 MgCl₂, 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°C for 3 minutes; 40 cycles of denaturation (94°C, 40 seconds), annealing (50°C, 35 seconds), and extension (68°C, 2 minutes); and a final extension step at 68°C 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 (Accelrys, Wigan). Another in-house PCR method was also used to determine the presence of omp1 in the FVU specimen.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Nucleotide positions* and direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOMP-108</td>
<td>GCCATTAAATGTCTACAGGACATCTTGTCC</td>
<td>780140-780168, forward</td>
</tr>
<tr>
<td>MOMP87</td>
<td>TGAAACAAAGCCTATGATCAGCGG</td>
<td>779951-779974, forward</td>
</tr>
<tr>
<td>RVS1163</td>
<td>GGAAATTGCGTTAACCTGAG</td>
<td>778886-779007, reverse</td>
</tr>
<tr>
<td>RVS1059</td>
<td>GCCATACCGCAAGAGTCTATGTTTTCAC</td>
<td>778982-779011, reverse</td>
</tr>
<tr>
<td>CT419F</td>
<td>TGGGATCGCTTGTAGTATT</td>
<td>779654-779673, forward</td>
</tr>
<tr>
<td>CTP20F</td>
<td>TACATTGGAATTTAATGTC</td>
<td>779183-779202, forward</td>
</tr>
</tbody>
</table>

*Nucleotide positions correspond to the regions of the genome (for omp1) or of the plasmid pLGV440 (GenBank accession number X06707) targeted by each primer.
**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* and *Chlamydia trachomatis* was also established the presence of *Chlamydia* plasmid free variants have been detected in the proband: (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 results indicated 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 *M. genitalium* remained negative. The NAAT results indicate that the *C. trachomatis* strain detected in the proband is a plasmid free strain.

**DISCUSSION**

NAATs are considered the most sensitive tests for the diagnosis of *C. trachomatis* infection. The targets for nucleic acid amplification in these tests include the cryptic plasmid, major outer membrane protein complex genes (*omp1, omcB, 16S* ribosomal RNA). The plasmid is a preferred target for many NAATs because its presence in multiple copies makes it an ideal target for diagnostic testing. 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, plasmid free variants have been described. 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 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. Investigations conducted in

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

<table>
<thead>
<tr>
<th>Test manufacturer or developer</th>
<th>Test name</th>
<th>Test target</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial tests</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>BD Biosciences</td>
<td>ProbeTec ET</td>
<td>Plasmid</td>
<td>–</td>
</tr>
<tr>
<td>Roche</td>
<td>Amplicor CT/NG PCR</td>
<td>Plasmid</td>
<td>–</td>
</tr>
<tr>
<td>Artus</td>
<td>RealArt CT PCR</td>
<td><em>omp1</em></td>
<td>–</td>
</tr>
<tr>
<td>GenProbe</td>
<td>Aptima Combo 2</td>
<td>16S ribosomal RNA</td>
<td>+</td>
</tr>
<tr>
<td>University of Cambridge</td>
<td>Chlamydia Rapid Test</td>
<td>Lipopolysaccharide</td>
<td>–</td>
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<tr>
<td>Research tests</td>
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<td></td>
</tr>
<tr>
<td>CDC and HPA</td>
<td>Real time PCR</td>
<td>Plasmid</td>
<td>–</td>
</tr>
<tr>
<td>University of Southampton</td>
<td>Taqman PCR</td>
<td>Plasmid</td>
<td>–</td>
</tr>
<tr>
<td>University of Southampton</td>
<td>Taqman PCR</td>
<td><em>ompB</em></td>
<td>+</td>
</tr>
<tr>
<td>LSHTM</td>
<td><em>omp1</em> PCR</td>
<td><em>omp1</em></td>
<td>+</td>
</tr>
</tbody>
</table>

CDC, Centers for Disease Control and Prevention; HPA, Health Protection Agency; LSHTM, London School of Hygiene and Tropical Medicine.
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submit the article for publication. The corresponding author has full access to the data of the study and had final responsibility for the decision to submit for publication.

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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