Absence of a *Chlamydia trachomatis* variant, harbouring a deletion in the cryptic plasmid, in clients of a sexually transmissible infection clinic and antenatal patients in Melbourne

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Abstract

A *Chlamydia trachomatis* (CT) variant, harbouring a 377 bp deletion in the cryptic plasmid, recently identified in Europe, has caused false-negative reporting of CT infections by various assays. This report aimed at identifying whether this variant is present among clients of a sexual health clinic, or antenatal screening patients in Melbourne. Two hundred CT-positive specimens (by BDProbeTec™ ET assay) from Melbourne Sexual Health Centre (August 2005–November 2006) were tested by COBAS *Taq*Man 48 PCR assay. Discrepancies were tested by an in-house real-time (Re-Ti) polymerase chain reaction (PCR) assay, amplifying a 274-bp region of the *omp*1 gene. Additionally, 1,071 consecutive specimens from antenatal screening patients at the Royal Women's Hospital (December 2006–April 2007) were tested by COBAS *Taq*Man 48 and *omp*1 Re-Ti PCR. The CT variant was not detected among the 200 CT-positive specimens (95% confidence interval 0–2.3%). Three tested CT-negative by COBAS *Taq*Man 48, *omp*1 Re-Ti PCR and CT mutant-specific PCR, suggesting sample degradation or differential assay sensitivity. Of the 1,071 antenatal screening specimens, 56 tested CT-positive and 1,015 CT-negative by COBAS *Taq*Man 48. All of the CT-negatives tested negative by *omp*1 Re-Ti PCR (95% confidence interval 0–0.5%), with 51 of 56 CT-positives testing positive. These findings show there were no CT variants among attendees of a Melbourne sexual health clinic, nor among antenatal screening patients. It is likely that the variant strain has not yet entered circulation in these populations. However, given the current upsurge in urogenital CT-infections, continued surveillance is necessary to ensure timely detection of this variant, should it be introduced into the population. *Commun Dis Intell* 2008;32:77–81.

Keywords: *Chlamydia trachomatis*, variant, cryptic plasmid, real-time polymerase chain reaction

Introduction

Chlamydia trachomatis (CT) infections are the most frequently reported bacterial sexually transmitted infection in the developed world, with the urogenital tract being the most common site of CT infection.[1,2] However, the majority of CT infections are asymptomatic (approximately 80% in women, and 50% in men) and thereby often remain undiagnosed.[3] Left untreated, chlamydial infections can lead to complications such as epididymitis in men, and pelvic inflammatory disease with long-term concomitant ectopic pregnancy or tubal infertility in women.

The CT bacterium contains approximately 7 to 10 copies of a 7.5-kb cryptic plasmid, which has an unknown function.[4,5] Given the cryptic plasmid is highly conserved in sequence, present in multiple copies and within all serovars, it has been the target for sensitive CT detection with several assays. However, in late 2006, a new CT variant was identified in Sweden, containing a 377-base pair deletion in the cryptic plasmid.[6] This finding came after the observation of an unexpected decrease in CT infections of up to 25% between November 2005 and August 2006 in Halland County, southwest Sweden.[6] To date, the CT variant has been detected throughout Sweden, with single cases reported in Denmark, France and Ireland, and two in Norway.[7–10] None have been detected in other parts of Europe, including the Netherlands and the United Kingdom.[11,12]

This finding has important ramifications in the diagnoses of CT infections, given that several commercially available CT assays target the cryptic plasmid, including the COBAS Amplicor and COBAS *Taq*Man 48 assays (Roche Diagnostics) and Abbott CT/NG test (Abbott Laboratories).[13,14] Assays targeting this deleted region of the cryptic plasmid would result in the false-negative reporting of specimens harbouring the CT variant. According to a recent Microbiology Quality Assurance Program report by the Royal College of Pathologists of Australia, approximately 65% of Australian laboratories are using CT assays potentially susceptible to false-negative CT diagnosis should this CT variant enter circulation.[15] Alternative assays which target areas of the cryptic plasmid outside this deletion region, such as the BDProbeTec™ ET assay (Becton Dickinson); or those targeting the *omp*1 gene, such as the ARTUS CT kit (Qiagen); or rRNA, such as the Aptima

Combo 2 assay (Gen-Probe), can effectively detect this new CT variant. The failure to detect CT infections due to this variant provides a selection advantage over the 'wild-type' CT strains assuming it is at no biological disadvantage. The CT variant would go undetected, the infection left untreated and allowed to persist and potentially spread more widely. Therefore, vigilance to monitor the presence of such variant strains in a given population is of considerable importance. A knowledge of which CT strains are in general circulation, particularly new variants, will improve our understanding of their epidemiology and may improve strategies for diagnosis and disease control.

In this study, we describe a comparative study testing two cohorts from the Melbourne population with the *omp*1 Re-Ti genomic assay and the *Taq*Man 48 and BDProbeTec™ ET assays. We detected no CT variants among the current population, as determined by this alternative target assay for CT detection.

Methods

Specimens

Specimens included in this study consisted of CT-positive specimens from the Melbourne Sexual Health Centre (MSHC) (collected from August 2005 to November 2006). These specimens were collected from both male (88.5%) and female (11.5%) clientele. In addition, consecutive antenatal screening clinical specimens routinely tested at the Royal Women's Hospital (RWH) Department of Microbiology (collected from December 2006 to April 2007) were included. All specimens comprised of first pass urine (FPU). Specimens from the MSHC (n = 200) all previously tested CT-positive by BDProbeTec™ ET assay. Consecutive specimens tested at the RWH (n = 1,071) by COBAS *Taq*Man 48 assay comprised: CT-negative (n = 1,015), or CT-positive (n = 56).

DNA extracts from two CT strains harbouring the deletion mutation were kindly supplied by Dr P Nilsson from the Uppsala University Hospital, Uppsala, Sweden.

The Alfred Hospital ethics committee approved the use of anonymous specimens (from MSHC clients) for research purposes using an 'opt out' consent procedure. Analysis of antenatal screening patient specimens was undertaken with approval covered by public health legislation. All specimens were tested anonymously.

BDProbeTec ET and COBAS TaqMan 48 assay

Two hundred CT-positive FPU specimens, as detected by the BDProbeTec™ ET assay, were tested using the COBAS *Taq*Man 48 PCR Analyzer according to the manufacturer's instructions. Both assays target the CT cryptic plasmid, though at different locations. Given there are up to 10 copies of the cryptic plasmid per bacterium, these assays were designed for their high sensitivity.

CT-consensus real-time PCR assay – Re-Ti PCR assay

All 1,071 consecutive antenatal screening specimens from RWH were tested by CT Re-Ti PCR assay. CT-specific forward and reverse primers and hydrolysis '*Taq*Man' probe were manually designed using alignment of the *omp*1 nucleotide sequences of CT reference strains obtained from GenBank (accession numbers in parentheses): A/Sa1/OT (M58938), B/Alpha-95 (U80075), B-Jali-20 (M33636), Ba/Apache-2 (AF063194), C/TW3/OT (M17343), D/B120 (X62918), D/B185 (X62919), D/IC-Cal8 (X62920), E/Bour-1990 (X52557), F/IC-Cal3 (X52080), G/UW57/Cx (AF063199), H/Wash (X16007), I/UW-12 (AF063200), J/UW36/Cx (AF063202), K/UW31/Cx (AF063204), L1/440-Bu (M36533), L2/434-Bu (M14738), and L3/404-Bu (X55700). The primers (CT-F: 5´-CATGARTGGCAAGCAAGTTTA-3´ and CT-R: 5´-GCAATACCGCAAGATTTTCTAG-3´) were directed to amplify a 274 bp region spanning VD-IV and the hydrolysis probe (5´- HEX-TGTTCACTCCYTACATTGGAGT-BHQ1-3´) targeting a consensus region upstream of VDIV. Underlined nucleotides represent locked nucleic acid bases.

The polymerase chain reaction (PCR) was prepared in a LightCycler® 480 Multiwell Plate 96 containing 1 µM of primer (CT-F and CT-R), 0.2 µM of probe and 1x LightCycler® 480 Probes Master (Roche Diagnostics) per well. The DNA template (7 µl) was added to the appropriate well resulting in a final reaction volume of 20 µl. The PCR was performed under the following parameters: initial enzyme activation at 95°C for 10 minutes; followed by 45 cycles of 95°C for 10 seconds, 60°C for 20 seconds, and 65°C for 30 seconds. Following the amplification phase, a cooling step was performed at 40°C for 10 seconds (ramp rate of 1.5°C per second). Acquisition of the fluorescence signal was performed using the Mono Hydrolysis Probe setting (483–523 nm) following the 65°C extension phase of each cycle.

CT mutant-specific real-time PCR assay

Specimens testing CT-negative by COBAS *Taq*Man 48 and CT-positive by either BDProbeTec™ ET assay or *omp*1 Re-Ti PCR were further tested by a CT mutant-specific real-time PCR assay, previously published.[14] Minor changes to the method included the use of 1.0 µM of each primer and 0.2 µM of each probe. In addition, a melting curve step was incorporated.

Statistical analysis

The 95% confidence intervals (CI) of proportions were calculated using the modified Wald method.[16]

Results

Of the 200 CT-positive specimens identified at the MSHC, as determined by BDProbeTec, three tested CT-negative using the COBAS *Taq*Man 48 assay (Table 1), and were thus potential CT variants. These three specimens subsequently tested CT-negative by both *omp*1 Re-Ti PCR and CT mutant-specific PCR assays. These three discrepancies could be due to sample degradation or differential sensitivity between BDProbeTec and other assays. The fact that none of the three CT-positives (BD ProbeTec)/CT-negative (COBAS *Taq*Man 48) were positive by *omp*1 Re-Ti PCR, confirms that the CT variant was not present among this cohort (95% CI 0–2.3%).

Of the 1,071 clinical specimens derived from consecutive routine antenatal screening at the RWH, 94.8% (1,015/1,071) tested CT-negative by COBAS *Taq*Man 48. Among these 1,015 CT-negative specimens, none tested CT-positive by the Re-Ti PCR assay, confirming the CT variant was not present in this cohort (95% CI 0–0.5%). Conversely, among the 56 CT-positive specimens, five tested CT-negative using the Re-Ti PCR assay (Table 2). The overall concordance between the COBAS *Taq*Man 48 and Re-Ti PCR results was 99.5% (kappa = 0.951), indicating a near perfect agreement.

Two CT strains harbouring the deletion mutation were tested by *omp*1 Re-Ti PCR and CT mutant-specific PCR to confirm that the *omp*1 Re-Ti PCR could detect the CT variant. The *omp*1 Re-Ti PCR was able to detect both variant CT strains, as well as CT-positive specimens, such as from the antenatal screening cohort (Figure). However, the CT

Table 1. Comparative detection of *Chlamydia trachomatis*-positive infections among Melbourne Sexual Health Centre clientele, by BDProbeTec ET assay and COBAS *Taq*Man 48 assay

BDProbeTec ET*	COBAS *Taq*Man 48†		Total
	Positive	Negative	
Positive	197	3	200
Negative	0	0	0
Total	197	3	200

* BD ProbeTec ET assay targets a region of the cryptic plasmid outside of where the mutation occurs.

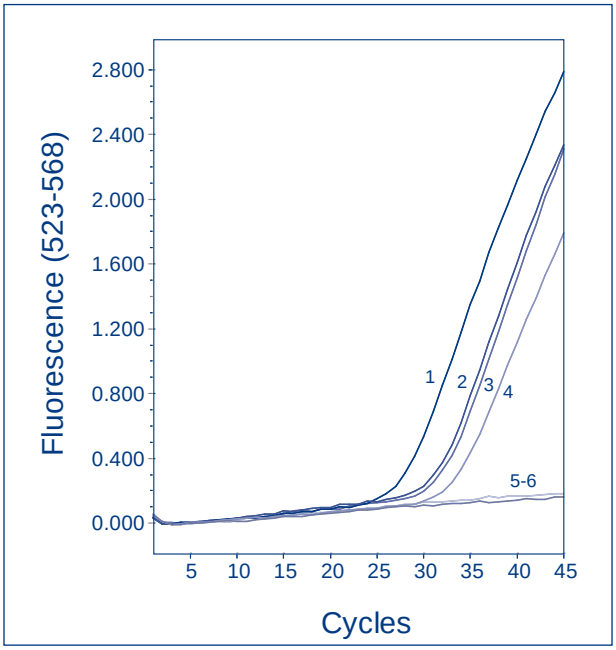
† COBAS TaqMan 48 assay targets the region of the cryptic plasmid where the mutation occurs.

Table 2. Comparative detection of *Chlamydia trachomatis* infections among a Royal Women's Hospital antenatal screening cohort by polymerase chain reaction targeting the cryptic plasmid or *omp*1 gene

Chlamydia trachomatis real-time PCR*	COBAS *Taq*Man 48 assay		Total
	Positive	Negative	
Positive	51	0	51
Negative	5	1,015	1,020
Total	56	1,015	1,071

* *Chlamydia trachomatis* real-time polymerase chain reaction (PCR) assay targets the genomic *omp*1 gene.

Figure. *Omp*1 real-time polymerase chain reaction of *Chlamydia trachomatis* variants and 'wild-type' strains



Confirmation of the *omp*1 real-time PCR assay's capability of detecting the CT variant. Two CT strains, harbouring the deletion mutation, tested positive by *omp*1 Re-Ti PCR (2 and 3), as did 'wild-type' CT-positive specimens from the antenatal screening cohort (1 and 4). Conversely, two CT-negative specimens are shown (5 and 6), in which no amplification curves are present.

Table 3. Comparison of assay sensitivity (using CP values) in the detection of the *Chlamydia trachomatis* variant

Chlamydia trachomatis mutants strain and dilution		CP values		CP difference
		Mutant-specific PCR*	*omp*1 Re-Ti PCR	
1	1/10	26.41	30.56	4.15
	1/100	30.60	33.84	3.24
	1/1000	37.61	39.45	1.84
2	1/10	29.02	31.20	2.18
	1/100	33.39	36.70	3.31
	1/1000	36.91	38.52	1.61

* The *Chlamydia trachomatis* mutant-specific polymerase chain reaction (PCR) targets the region of the cryptic plasmid whereby the mutation occurs.

CP Crossing point.

mutant-specific assay was more sensitive in detecting CT positivity than the *omp*1 Re-Ti PCR, as seen through the later crossing point (average: 2.7 cycles difference) (Table 3).

Discussion

This report describes the absence of CT variants among two study cohorts in the Melbourne population using a novel real-time PCR assay targeting the CT *omp*1 gene encoding the major outer membrane protein. This Re-Ti assay was utilised for the detection of CT infections, including both 'wild-type' and variant strains. The assay was shown to successfully detect CT DNA among 91.1% of antenatal screening specimens previously identified as CT-positive by COBAS *Taq*Man 48. The lower sensitivity of the current assay was not unexpected, given that the COBAS *Taq*Man 48 assay is based on amplification of a region within the multi-copy cryptic plasmid whilst the Re-Ti assay targets the single copy *omp*1 gene. Therefore, we would anticipate a tenfold difference in sensitivity as was indeed demonstrated through comparison of the crossing point values of the Re-Ti *omp*1 assay and Roche mutant-specific assay. In addition, the five discrepancies (negative by Re-Ti *omp*1 positive by *Taq*Man 48) were demonstrated as containing CT DNA in low copy numbers, with *Taq*Man 48 CP values of >40.

Given the lack of detection of the CT variant among the current study cohorts it was imperative to verify that the *omp*1 PCR assay was indeed capable of detecting the CT variant. DNA extracts from two separate CT strains harbouring the deletion mutation, confirmed by DNA sequencing,[6,14] were readily detected by the Re-Ti *omp*1 PCR, providing confidence that we were seeing true negative findings.

It is important to highlight two potential limitations of this study. The first being that collection of the MSHC specimens preceded initial reports of CT variant detection in Sweden. The second is that the antenatal screening cohort could be considered one of lower risk and thus less likely to have the CT variant in circulation.

This was the first description of a CT consensus real-time PCR assay utilising the recently released LC 480 platform. From these results, it is probable that the CT variant strains have not yet entered circulation in Australia. The continued use of CT assays targeting the cryptic plasmid is appropriate in Australia and there is no immediate requirement for performing supplementary assays for CT variants by routine diagnostic laboratories. However, given the current upsurge in urogenital CT infections worldwide, continued surveillance is necessary to ensure timely detection of this variant strain.

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