Whole-genome sequencing of *Chlamydia trachomatis* isolates from persistently infected patients

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Abstract

Background: Current understanding of the causes of treatment failure in *Chlamydia trachomatis* is poor and antimicrobial susceptibility data are lacking. We used genome sequencing to seek evidence of antimicrobial resistance in isolates sourced from patients who were persistently infected.

Methods: Genomic DNA was extracted from *C. trachomatis* isolates cultured in McCoy cell monolayers. Sequencing libraries were prepared using the SureSelect^{XT} Illumina paired-end

protocol. Paired reads were mapped against a reference genome and single nucleotide variants (SNVs) were identified.

Results: Seven isolates from persistently infected patients and five isolates from successfully treated patients were sequenced. No previously reported SNVs associated with antimicrobial resistance were found. A unique SNV was identified in the *gyrA* gene of one treatment failure isolate, but was located outside of the quinolone resistance determining region; this SNV has been previously reported in other members of the Chlamydiaceae family.

Conclusion: No genomic evidence was found to explain the differences in clinical outcome for our two groups of patients. A mutation unrelated to antimicrobial susceptibility was found in an isolate from a persistently infected patient. The cause of these persistent infections with *C. trachomatis* remain unclear.

Introduction

In 2019, 229,411 diagnoses of *Chlamydia trachomatis* were reported to Public Health England (PHE) [1]. Whilst infection with *C. trachomatis* is readily managed by antimicrobial therapy, anecdotal reports of treatment failure occur [2-4]. Understanding the causes of these treatment failures is poor and standardised antimicrobial susceptibility testing data are lacking. Where susceptibility data are available, there have been reports of multiple drug resistant strains recovered from treatment failures [5]. Repeat infections after clinical diagnosis and treatment occur in up to 20% of *C. trachomatis* infections [6]. Historically, genotyping of the *ompA* gene, which encodes a major outer membrane protein, has been used to differentiate treatment failure from re-infection [7]. However, using *ompA* genovars to type *C. trachomatis* to track persistent or repeat infections is flawed. Recombination within this gene can mask the true

evolutionary history of that strain [8] and so longitudinal identification of a persistent infection becomes difficult without the use of whole-genome sequencing.

Antimicrobial susceptibility testing at PHE showed a significant difference in the doxycycline MICs obtained for *C. trachomatis* isolates sourced from patients with persistent infections to those who cleared infection with 1 g azithromycin (Table 1) [9]. The cause of this difference was unclear. Prior to 2018 the preferred first-line recommended therapy for treatment of uncomplicated *C. trachomatis* infection was a single oral dose of 1 g azithromycin [10]. However, in response to growing concern about the effect of single-dose therapy on antimicrobial resistance in *Mycoplasma genitalium*, an update to the national guideline was issued by the British Association for Sexual Health and HIV (BASHH). The update preferentially recommended the alternate first-line therapy (100 mg doxycycline twice daily for seven days) over an extended 2 g course of azithromycin (given over three days).

To investigate the possibility of persistence of infection due to antibiotic resistance, we sequenced the genomes of *C. trachomatis* isolates referred to PHE from patients who were persistently infected despite treatment with recommended antimicrobials [10] and were at low risk of re-infection. This was carried out using targeted enrichment of *C. trachomatis* DNA to allow sequencing of low level infections from patients exposed to multiple rounds of antimicrobial treatment, with greater sensitivity than other methods [11].

Methods

Isolates

C. trachomatis isolates from patients who were persistently infected, despite a minimum of two rounds of first-line recommended antimicrobials and a low risk of re-infection, were

referred to PHE (between 2010 and 2014). Specific treatment regimens varied (Table 1) but all were in-line with the national treatment guidelines at the time [10]. Risk of re-infection was assigned using self-declared sexual behaviour since initial diagnosis. Low risk of re-infection was assigned when the patient had no sexual contact, protected contact only or unprotected contact with a regular partner who had also tested positive and been treated or had not tested positive. Control isolates (collected in 2013) from patients who were successfully treated with a single dose of 1 g azithromycin were also sequenced.

Clinical and antimicrobial susceptibility data for some of these patients have been reported previously [4, 9].

Tissue culture and DNA extraction

Isolates were cultured in McCoy cell monolayers in shell vials in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% gamma-irradiated foetal bovine serum (Gibco), 200 mM L-Glutamine (Sigma), 100 µg/mL Gentamicin (Gibco), 25 U mL-1 Nystatin (Sigma) and 100 µg/mL Vancomycin (Sigma). Cell lines were screened for the presence of contaminating *Mycoplasma* species using the MycoFluor™ Mycoplasma Detection kit (Invitrogen). After infection monolayers were incubated at 35°C, 5% CO_2 for 48 hours before further passage. The MicroTrak *Chlamydia trachomatis* culture confirmation test (Trinity Biotech) was used to visualise inclusions prior to harvesting. Pooled shell vials were centrifuged at 2300 x g for 10 minutes, supernatant was aspirated, and the pellet re-suspended in 1 mL cold 1:10 PBS (plus glass beads). Samples were vortexed vigorously for 1 minute and were then centrifuged at 284 x g for 5 minutes to remove cell debris. Supernatant containing *C. trachomatis* elementary bodies was aspirated and centrifuged at 13 684 x g for 5 minutes to pellet. Supernatant was

aspirated and genomic DNA (gDNA) was extracted from the pellet using the Wizard Genomic DNA extraction kit (Promega). Sequencing

gDNA was quantified (Qubit [Life technologies]) with carrier human genomic DNA (Promega) added to obtain a final concentration of 200 ng for shearing. Shearing was carried out using a Covaris E210 (6 x 60 seconds, duty cycle 10%, intensity 5 and 200 cycles per bust using frequency sweeping). The SureSelect^{XT} Illumina Paired-End Sequencing Library protocol was used for library preparation and sequencing was performed on a MiSeq (Illumina). RNA baits used to capture and enrich the *C. trachomatis* genome were designed previously [11]. SNV calling (point mutations, insertions [including acquired antimicrobial resistance genes e.g. *tet* genes] and deletions) and phylogenetic tree reconstruction was carried out as previously described [12] except reads were mapped against the *C. trachomatis* D/UW-3/CX reference genome (GenBank accession number: NC_000117.1).

Ethics

Patients with persistent infections were referred to PHE as part of an enhanced surveillance programme; therefore, ethical approval was not required as PHE is able to handle these data under the Health Service (Control of Patient Information) regulation 2002, overseen by the Confidentiality Advisory Group. Control specimens were recruited through a sexual health clinic under ethics application reference 13/WM/0088.

Results

Sequencing

Seven isolates from persistently infected patients and five isolates from successfully treated patients were sequenced (EBI European Nucleotide Archive accession number: PRJEB45721). Phylogenetic reconstruction of the genomes showed that chromosomal and plasmid lineages generally separated on the *ompA* genotype (8 genotype E; 4 genotype D) (Figure 1). However, one genovar D isolate from a successfully treated patient (con28) was found in the genovar E lineage in both trees (Figure 1). Likely due to a recombination event within the *ompA* sequence of that isolate. We found no evidence of previously reported point mutations [13-21], acquired antimicrobial resistance genes or changes to efflux pumps and porin genes associated with antimicrobial resistance in any of our specimens. Nor did we find any major consensus variants across all treatment failure isolates only. A single, non-synonymous SNV was identified in *gyrA* in one treatment failure isolate (cc83) at position G1642T (amino acid change Alanine-548-Serine). This SNV was also confirmed by an independent PCR and sequencing of the product across that position. A BLASTP search against all non-redundant protein sequences on GenBank showed that this was a novel SNV not previously identified in *C. trachomatis.* Whilst this mutation was novel for *C. trachomatis* it was found in other members of the Chlamydiaceae family, namely *Chlamydia suis* and *Chlamydophila felis*.

Discussion

The aim of this study was to sequence the genomes of isolates from patients with persistent infections and patients who resolved infection after treatment with 1 g azithromycin to explore whether there was genetic evidence to explain the differences in clinical outcome for the patients. We did not identify any mutations, in isolates from either patient group, which had been reported previously as associated with *in vitro* antimicrobial resistance and/or

treatment failure in *C. trachomatis* [13-21]. Further to this, no major consensus variants were identified across all treatment failure isolates that were not also identified in the isolates from the successfully treated patients. We did identify a non-synonymous SNV in *gyrA,* a gene known to be involved in fluoroquinolone resistance, of one treatment failure isolate. However, the locus where the mutation was identified was outside the quinolone-resistancedetermining-region of the gene so is unlikely to affect antimicrobial activity.

The DNA sequenced in this study was extracted from isolates which had undergone multiple passages in tissue culture. It is not known how subjecting the isolates to passaging affects the genome and whether culturing in the absence of antimicrobials selects out mutations that have a potential fitness cost to the organism *in vitro*. Direct sequencing from clinical specimens may resolve this. To complicate matters, heterotypic resistance has been described in *C. trachomatis* [22, 23]. This is the result of adaptations by the bacteria to become less susceptible to antimicrobial therapy e.g. induction of slow-growing non-replicative forms in the presence of antimicrobials. These revert to replicative forms once the antibiotic pressure has been removed resulting in a relapse in infection. As stated previously phenotypic antimicrobial susceptibility testing carried out for some of these isolates [9] demonstrated reduced susceptibility of the isolates from persistently infected patients for doxycycline in comparison with the isolates from the successfully treated patients (Table 1). No genetic cause for this difference was detected in this study. Whilst cell lines were screened for the presence of contaminating organisms, generally as part of cell line maintenance and specifically for *Mycoplasma* species; due to the clinical origin of the specimens tested it is not possible to completely rule out co-infection with another organism which may have influenced the susceptibility results obtained. However, it may be that the isolates from the

persistently infected patients are exhibiting heterotypic resistance *in vitro* and this is the cause of the differing susceptibility results a theory also hypothesised recently by Shao *et al* [24]. This 'resistance' is not inherited and the genes involved in its induction are unknown at present.

To conclude, for our cohort there was no evidence that repeat treatment and/or single-dose therapy for *C. trachomatis* infection resulted in antimicrobial resistance. Evidence of genomic recombination was found, but was unrelated to susceptibility. If heterotypic resistance was the cause of the treatment failures, we would not necessarily expect to find genetic evidence of homotypic resistance. The causes of persistent *C. trachomatis* infection remain unclear.

Data Repository: EBI European Nucleotide Archive (PRJEB45721)

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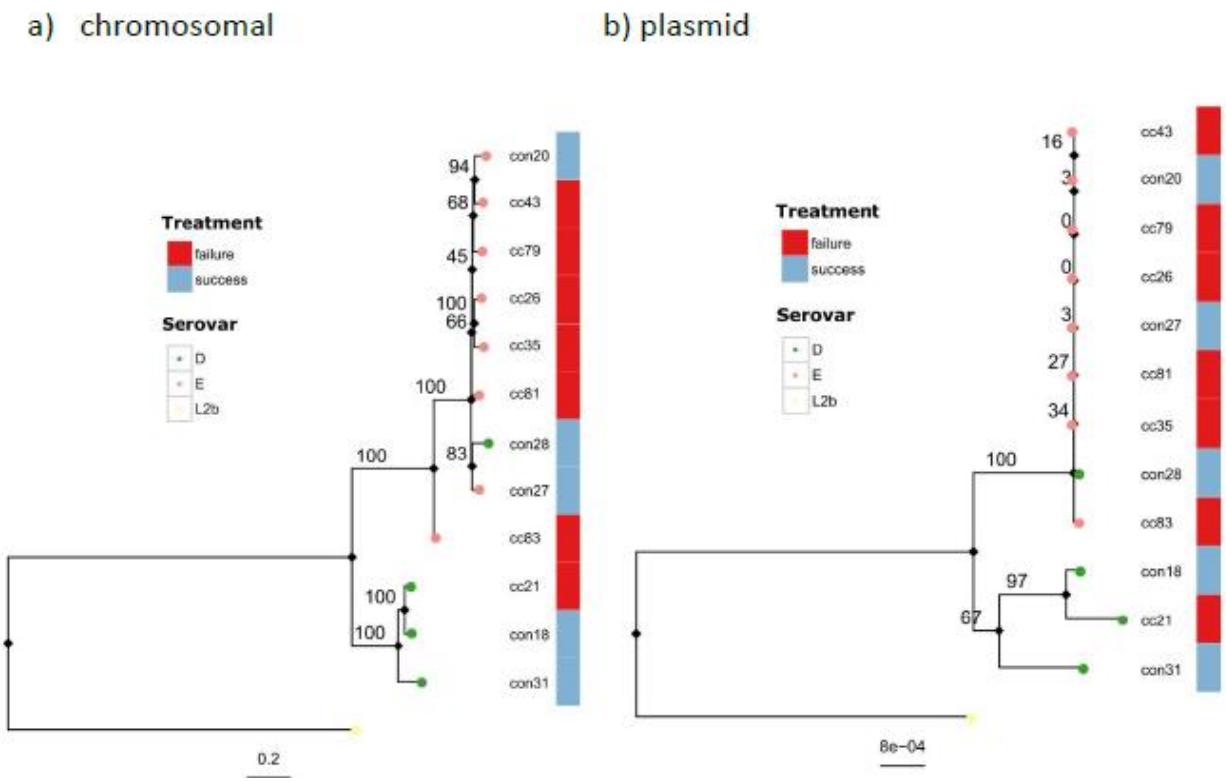

Figure 1 – Maximum likelihood phylogenetic trees constructed from the 12 C. trachomatis chromosome (a) and plasmid (b) sequences in this study, with regions that have possibly undergone recombination removed. The tree is rooted against a chromosome sequence from LGV strain L2b/434/Bu (GenBank accession number: AM884176). “cc” denotes an isolate from a patient who has failed treatment whilst “con” denotes an isolate from a successfully treated patient.

Table 1 – Isolate characteristics

ID ()[9]	Site	*ompA* genotype	Treatment	Azithromycin MIC (mg/L)[9]	Doxycycline MIC (mg/L)[9]
Persistently infected					
CC21 (Pt.6)	Ure	D	2 x 1 g azithromycin	≤0.125	0.125
CC26 (Pt.8)	Ure	E	2 x 1 g azithromycin	≤0.125	0.125
CC35 (Pt.2)	Ure	E	2 x 1 g azithromycin	≤0.125	>1
CC43 (Pt.3)	Cer	E	3 x 1 g azithromyc 14/7 bd	0.125	0.125
CC79 (Pt.10)	Ure	E	2 x 1 g azithromycin, 1 x 100 mg doxycycline 7/7 bd	≤0.125	>1
CC81 (Pt.11)	Cer	E	1 x 1 g azithromycin, 1 x 500 mg erythromycin 7/7 qd	0.5	>1
CC83	Cer	E	1 x 1 g azithromycin, 1 x 100 mg doxycycline 7/7 bd, 2 x 1 g azithromycin + 500 mg azithromycin od 4/4	Not available	Not available

Successfully treated					
Con18 (Ctrl.6)	Cer	D	1 x 1 g azithromycin	≤0.125	≤0.064
Con20 (Ctrl.7)	Cer	E	1 x 1 g azithromycin	≤0.125	1
Con27 (Ctrl.9)	Cer	E	1 x 1 g azithromycin	≤0.125	0.064
Con28 (Ctrl.10)	Cer	D	1 x 1 g azithromycin	≤0.125	0.064
Con31 (Ctrl.11)	Cer	D	1 x 1 g azithromycin	0.25	0.25

Table detailing site of infection (Ure – urethral, Cer – cervical), *ompA* genotype, treatment regimen prescribed and minimum inhibitory concentration (MIC) of azithromycin and doxycycline (where available) for *C. trachomatis* isolates sourced from patients persistently infected and those successfully treated with 1 g azithromycin. MIC data is taken from Pitt *et al.* 2017, () in ID column indicate nomenclature in this reference. MIC data was unavailable