Microbiology (2010), **156**, 1394–1404 **DOI** 10.1099/mic.0.036830-0

The Swedish new variant of *Chlamydia trachomatis*: genome sequence, morphology, cell tropism and phenotypic characterization

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Chlamydia trachomatis is a major cause of bacterial sexually transmitted infections worldwide. In 2006, a new variant of *C. trachomatis* (nvCT), carrying a 377 bp deletion within the plasmid, was reported in Sweden. This deletion included the targets used by the commercial diagnostic systems from Roche and Abbott. The nvCT is clonal (serovar/genovar E) and it spread rapidly in Sweden, undiagnosed by these systems. The degree of spread may also indicate an increased biological fitness of nvCT. The aims of this study were to describe the genome of nvCT, to compare the nvCT genome to all available *C. trachomatis* genome sequences and to investigate the biological properties of nvCT. An early nvCT isolate (Sweden2) was analysed by genome sequencing, growth kinetics, microscopy, cell tropism assay and antimicrobial susceptibility testing. It was compared with relevant *C. trachomatis* isolates, including a similar serovar E *C. trachomatis* wild-type strain that circulated in Sweden prior to the initially undetected expansion of nvCT. The nvCT genome does not contain any major genetic polymorphisms – the genes for central metabolism, development cycle and virulence are conserved – or phenotypic characteristics that indicate any altered biological fitness. This is supported by the observations that the nvCT and wild-type *C. trachomatis* infections are very similar in terms of epidemiological distribution, and that differences in clinical signs are only described, in one study, in women. In conclusion, the nvCT does not appear to have any altered biological fitness. Therefore, the rapid transmission of nvCT in Sweden was due to the strong diagnostic selective advantage and its introduction into a high-frequency transmitting population.

Received 3 December 2009
Revised 17 January 2010
Accepted 19 January 2010

Abbreviations: CDS, coding sequence; EBs, elementary bodies; LGV, lymphogranuloma venereum; MLST, multilocus sequence typing; NAAT, nucleic acid amplification test; nvCT, new variant of *C. trachomatis*; PZ, plasticity zone; TEM, transmission electron microscopy; SNPs, single nucleotide polymorphisms; VNTR, variable number tandem repeats; wtCT, wild-type *C. trachomatis*.

The GenBank/EMBL/DDBJ accession number for the genome sequence of the nvCT isolate reported in this paper is FN652779.

Three supplementary tables and two supplementary figures (including one showing high quality digital time lapse video photomicroscopy of infection of McCoy cells with various *C. trachomatis* strains) are available with the online version of this paper.

INTRODUCTION

Chlamydia trachomatis is the major cause of bacterial sexually transmitted infections worldwide and it can give rise to severe complications such as pelvic inflammatory disease, ectopic pregnancy and infertility. Genital tract infections are caused by a limited number of *C. trachomatis* serovars (genovars) that appear to represent a stable pool of pathogens. However, *C. trachomatis* strains resistant to several antimicrobials have been induced *in vitro* (Wang *et al.*, 2005), raising concerns that *C. trachomatis* strains resistant to antimicrobials used in routine treatment of chlamydial infection or *C. trachomatis* strains with enhanced pathogenic properties may emerge naturally, as observed with *Chlamydia suis* (Lenart *et al.*, 2001). In October 2006, a new variant of *C. trachomatis* (nvCT) was first reported in Sweden (Ripa & Nilsson, 2006); it has spread rapidly in Sweden and has also appeared in other European countries (de Barbeyrac *et al.*, 2007; Health Protection Agency, 2008; Herrmann *et al.*, 2008; Hoffmann & Jensen, 2007; Lynagh *et al.*, 2007; Moghaddam & Reinton, 2007; Reinton *et al.*, 2009; Unemo *et al.*, 2007; Westh & Jensen, 2008).

C. trachomatis has a unique developmental cycle which takes place within a specialized cytoplasmic compartment known as an inclusion (Ward, 1983). The obligate intracellular nature of chlamydial development places severe restrictions on studying the biology of these micro-organisms. Most *C. trachomatis* isolates carry a small plasmid which encodes eight proteins; the role of the plasmid has recently come under close scrutiny since it was demonstrated to be a transcriptional regulator of chromosomal genes and a virulence factor (Carlson *et al.*, 2008). Attempts to cure *C. trachomatis* of its plasmid have been unsuccessful (Pickett *et al.*, 2005). However, live, naturally occurring, plasmid-free isolates of *C. trachomatis* do exist but are exceedingly rare, and they do not spread in the population (Farencena *et al.*, 1997; Peterson *et al.*, 1990; Stothard *et al.*, 1998), which may indicate a reduced biological fitness. Plasmid-free strains are unable to accumulate glycogen in their inclusions, which is a unique characteristic of *C. trachomatis* that can be visualized using iodine staining (Matsumoto *et al.*, 1998). Thus, this unique property is closely linked to the presence of the plasmid, which is now taken as an indicator of virulence (Carlson *et al.*, 2008). Other potential virulence factors have been described, e.g. a type III secretion system that delivers chlamydial effector proteins such as Incs (involved in controlling interactions with the host cell, inclusion development and avoidance of lysosomal targeting), Pmps (autotransporters that interact with the host cell e.g. in attachment) and Tarp (potentially involved in invasion) into the inclusion vacuole and membrane or into the host cytoplasm, which then subvert the host cell for the benefit of the bacterium (Kleba & Stephens, 2008; Rockey *et al.*, 2000).

Preliminary analyses showed that the Swedish nvCT carries a plasmid with a 377 bp deletion within coding sequence (CDS) 1 (Ripa & Nilsson, 2007). Subsequent full sequence characterization of the plasmid showed that it additionally carries a 44 bp duplication immediately upstream of both CDS2 and CDS3 (Seth-Smith *et al.*, 2009). The 377 bp deletion covers a region including the target sequences for the independently produced single-target nucleic acid amplification tests (NAATs) manufactured by Roche Diagnostics and Abbott Laboratories, which were used in two-thirds of the counties in Sweden. Failure to detect nvCT in samples submitted for routine diagnosis resulted in false-negative reports with a consequent huge impact on the overall national detection rates in Sweden, especially in counties where these tests were used (Söderblom *et al.*, 2006; Velicko *et al.*, 2007).

Screening for nvCT showed that its proportion, compared with wild-type *C. trachomatis* (wtCT) strains, varied widely from 7 to 64 % between different counties across Sweden (Herrmann *et al.*, 2008; Unemo *et al.*, 2007). The lowest proportions were found in the one-third of Swedish counties that were using a commercial NAAT (from Becton Dickinson) which targets a plasmid sequence outside the deletion in nvCT. Abbott and Roche designed new dual-target assays that simultaneously target the affected sequence of the nvCT plasmid and another sequence of the plasmid and the chromosomal DNA (*ompA*); these are Abbott RealTime CT/NG and the Roche Cobas *Taq*Man CT v2.0, respectively (Hadad *et al.*, 2008).

Several sequence-based typing systems are now available for application to *C. trachomatis* (Pedersen *et al.*, 2009). Use of variable number tandem repeats (VNTR; Pedersen *et al.*, 2008) and multilocus sequence typing (MLST; Klint *et al.*, 2007) typing systems with the highest resolving power showed that nvCT is clonal; it belongs to serovar E and the *ompA* sequence is identical to the prototype *C. trachomatis* reference strain E/Bour. The nvCT was assigned to MLST sequence type 21 (*hctB*), 19 (CT058), 1 (CT144), 2 (CT172) and 1 (*pbpB*) (Herrmann *et al.*, 2008; Unemo *et al.*, 2007) and VNTR type 8.7.1 (CT1335, CT1299 and CT1291, respectively) (Pedersen *et al.*, 2008).

To determine when nvCT first appeared in the Swedish population, the only two archival collections of *C. trachomatis* available, one from Örebro county in central Sweden (1999–2000) and the other from Malmö (2000–2001) in southern Sweden, were screened but no nvCT was found. This result suggested that the nvCT likely arose after 2000. The subsequent rapid and wide transmission of nvCT across Sweden and the initial observation that the prevalence of nvCT remained high despite the introduction of the Cobas *Taq*Man CT v2.0 in some counties using the Roche NAATs (Hadad *et al.*, 2008) suggested that nvCT, once established and in addition to the diagnostic selective advantage, may have novel biological properties that give it further selective advantage by conferring an increased biological fitness (Hadad *et al.*, 2008; Herrmann *et al.*, 2008; Unemo *et al.*, 2007). A comprehensive case control study involving 1878 patients compared nvCT with wtCT infections; this study showed a significant reduction of

symptomatic urethral infection and abdominal pain amongst nvCT-infected women (Bjartling *et al.*, 2009). This difference in symptoms would confer a selective advantage on nvCT, as patients would be less likely to seek diagnosis allowing greater opportunities for transmission. These data taken together suggest that nvCT is a strain which has recently emerged as a result of a rare genetic event from amongst the established population of highly conserved *C. trachomatis* strains, and hints at potential new biological properties of nvCT.

The aims of our study were to characterize the nvCT strain from Sweden. Here, we present its complete genome sequence (which is the first for a serovar E strain), comparisons with genome sequences of other *C. trachomatis* serovars (A, B, D and L2) and we investigate the biological properties of nvCT in terms of morphology, developmental cycle and cell tropism.

METHODS

***C. trachomatis* isolates.** All *C. trachomatis* isolates examined in this study are described in Table 1. Briefly, the clinical nvCT isolate (Sweden2) was recovered in McCoy cells in October 2006 from a urethral sample from a Swedish man, suffering from symptomatic chlamydial urethritis. The similar clinical 'wild-type' serovar E strain (Sweden3) was isolated in McCoy cells in 2001 in the same area of Sweden, i.e. circulated in Sweden prior to the undetected expansion of nvCT. Compared with nvCT, this strain that we refer to as wtCT has an identical *ompA* sequence and only differs by one nucleotide in VNTR typing (Pedersen *et al.*, 2008). Furthermore, the prototype reference strain E/Bour (also with an identical *ompA* sequence to nvCT), one plasmid-free E strain (C599) and one lymphogranuloma venereum (LGV) strain (L2/434/BU) were included for phenotypic comparisons.

Preparation of DNA for genome sequencing. A large quantity of the nvCT isolate was prepared in mycoplasma-free McCoy cell cultures by expansion of the culture from the original swab; elementary bodies (EBs) were harvested and gradient-purified prior to storage at −80 °C, as described previously (Skipp *et al.*, 2005). Genomic DNA was extracted and purified from the EBs using the wizard genomic purification kit (Promega) according to the manufacturer's instructions. The only modification was that purified EBs were incubated with proteinase K for 1 h at 60 °C prior to addition of the nuclei lysis solution. The quality of the genomic DNA recovered by using this method was assessed by agarose gel electrophoresis and by spectrophotometric analysis. An accurate determination of plasmid and genome copies for each DNA preparation was obtained by performing 5′-exonuclease (*Taq*Man) assays, as described previously (Pickett *et al.*, 2005).

Genome sequencing. The genome of the nvCT isolate was sequenced using paired-end library preparation and shotgun sequencing LR70 on Genome Sequencer FLX (GS FLX; Roche Diagnostics) in two lanes. Using GS FLX Newbler Assembler (Roche), a *de novo* assembly produced three contigs representing the chromosome, including one contig representing assembled rRNA operons and a single contig for the plasmid. These chromosomal contigs comprised 1 037 359 bp and 510 844 reads, representing a coverage of 119×. For confirmation, the nvCT isolate was also sequenced using Illumina Genome Analyser (Illumina). The assembly of these sequences produced six contigs covering the chromosome, totalling 1 054 452 bp, with the largest contig representing 458 705 bp. The coverage for these contigs ranged from 165× to 498×. Again, the *de novo* assembly was confounded by repeats, with reads covering the two rRNA operons assembled as a single contig. Other gaps between the chromosomal contigs occurred at homopolymeric tracts (13Cs, 12As, 12Ts) and repeats associated with the *tarp* gene. The plasmid was present as a separate contig. Integration of the datasets was performed using ICORN (http://icorn.sourceforge.net/), with the ordered GS FLX contigs as a scaffold upon which the Illumina Genome Analyser reads were mapped, and to which corrections were performed. Only one iteration was required to find all of the discrepancies: 90 % of the reads (26 845 892/29 752 262) mapped uniquely, covering 99.9994 % of the genome and representing a genome coverage of greater than 40×, allowing a very high confidence in the final sequence. The contigs were joined using single mapping reads spanning contig boundaries. The nvCT genome was annotated, analysed and compared with all previously sequenced *C. trachomatis* genomes (Table 2) using the Artemis (Berriman & Rutherford, 2003) and ACT (Carver *et al.*, 2005) software packages. The genome sequence and annotation have been deposited in the EMBL database under accession number FN652779.

Growth characteristics. Phase-contrast microscopy of the nvCT isolate, Sweden3 (wtCT), prototype reference strain E/Bour and L2/434/BU strain were used to follow the development cycle. Quantitative real-time PCR was used to compare the growth characteristics of nvCT and Sweden3 (wtCT). To accurately compare the characteristics of these two isolates during early and exponential phases of growth, confluent monolayers of mycoplasma-free McCoy cells were infected at an m.o.i. of 1.0 and cultured at 37 °C in 5 % CO_2 for 54 h, as described previously (Skilton *et al.*, 2009). For each time point, cells were infected in triplicate and the infection was stopped at 6, 18, 30,

Table 1. *C. trachomatis* strains examined in this study

Strain	Origin	Site	Date	Serovar	Plasmid	Reference
nvCT (Sweden2)	Malmö, Sweden	Male urethra	2006	E	pSW2	Seth-Smith *et al.* (2009)
Sweden3 (wtCT)	Malmö, Sweden	Female cervix	2001	E	pSW3	Seth-Smith *et al.* (2009)
Bour (wtCT)	USA	Conjunctiva	1959	E	pBour	Hanna *et al.* (1959)
C599 (plasmid-free)	Indianapolis, USA	Male urethra	1996	E	Plasmid-free	Stothard *et al.* (1998)
L2/434/BU (wtCT)	San Francisco, USA	Bubo	1969	L2	pL2	Schachter & Meyer (1969)

Table 2. General features of the genomes of the nvCT and all other available genome sequences of *C. trachomatis* strains

All genome sequences have a G+C content of 41.3 %, two rRNA operons and 37 tRNA operons.

Strain	Serovar	Biovar	Origin	EMBL accession no.	Chromosome (bp)	No. predicted CDSs	Coding density (%)	Average gene size (bp)	No. pseudo-genes*	Plasmid size (bp)	Reference
nvCT (Sweden2)	E	Trachoma (genitotropic)	Sweden	FN652779	1 042 839	889	89	1056	14	7169	This study
UW-3/CX†	D	Trachoma (genitotropic)	USA	AE001273	1 042 519	894	90	1050	5	7493	Stephens *et al.* (1998)
TZ1A828/OT	B	Trachoma (oculotropic)	Tanzania	FM872307	1 044 282	879	89	1051	14	7502	Seth-Smith *et al.* (2009)
Jali20	B	Trachoma (oculotropic)	The Gambia	FM872307	1 044 352	875	89	1056	18	7506	Seth-Smith *et al.* (2009)
HAR-13†	A	Trachoma (oculotropic)	Saudi Arabia	CP000051	1 044 459	920	90	1032	8	7510	Carlson *et al.* (2005)
434/BU	L2	LGV	USA	AM884176	1 038 842	889	89	1052	15	7499	Thomson *et al.* (2008)
UCH-1	L2b	LGV	England	AM884177	1 038 869	889	89	1052	15	7500	Thomson *et al.* (2008)

*Pseudogene nos do not include cytotoxin gene fragments.

†These genomes have been annotated elsewhere, thus predicted CDSs and pseudogene numbers may not be comparable.

42 and 54 h post-infection. Samples were then stored at −80 °C after snap-freezing. Subsequently, genomic and plasmid DNA was extracted in a microplate format, the residue was resuspended in 100 µl nuclease-free water, and the samples were diluted 1 in 100 prior to quantitative real-time PCR analysis. The quantitative real-time PCR protocol to determine the absolute number of chlamydial plasmids and genomes in samples using 5′-exonuclease (*Taq*Man) assays with unlabelled primers and carboxyfluorescein/carboxytetramethylrhodamine (FAM/TAMRA) dual-labelled probes has been described previously (Pickett *et al.*, 2005).

Iodine staining. The nvCT isolate, Sweden3 (wtCT), prototype reference strain E/Bour and plasmid-free E strain (C599; as a staining control) were cultured on coverslips as described above. Inclusions were examined by phase-contrast microscopy then washed several times with PBS and fixed with methanol. Coverslips were stained with 5 % iodine stain (containing both potassium iodide and iodine in 50 % ethanol) for 10 min. The stain was then changed for 2.5 % iodine stain for 10 min and mounted in 5 % iodine stain in glycerol (1 : 1) for microscopy.

High quality digital time lapse video photomicroscopy. The nvCT isolate and prototype reference strain E/Bour were cultured as described above. Time lapse video microscopy was performed on these strains in McCoy cells as described previously (Skilton *et al.*, 2009).

Cell tropism study. Confluent monolayers of Hep2, McCoy, BGMK, Vero and 293A cell lines were infected at an m.o.i. of 1.0 with the nvCT isolate, Sweden3 (wtCT), prototype reference strain E/Bour and L2/434/BU (the inocula were prepared in McCoy cells) and cultured at 37 °C in 5 % CO_2. At the end of the developmental cycle, the chlamydial growth (inclusion formation) was scored. EBs were harvested and used to infect a fresh culture of the same cells. This was repeated four times.

Transmission electron microscopy (TEM). The nvCT isolate, Sweden3 (wtCT), and prototype reference strain E/Bour were cultured in BGMK cells at 37 °C in 5 % CO_2 for 48 h, as described previously (Skilton *et al.*, 2009).

Antimicrobial susceptibility. The MIC of tetracycline, erythromycin and ciprofloxacin was determined for the nvCT isolate and Sweden3 (wtCT) in McCoy cells. Briefly, the strains were propagated in McCoy cell cultures on coverslips in plastic vials. When the cultures showed at least 10 inclusions in each high power field (magnification of ×200), a 24-well plate was inoculated. After centrifugation, the inoculum was removed and the wells were replenished with 1.5 ml RPMI medium containing 1 % fetal calf serum and 1 µg cycloheximide ml^{-1}. A two-step dilution series of each antibiotic was also added, starting from 0.05 and then 0.1, 0.25, 0.5 and 1.0 µg ml^{-1}. After fixation in methanol, the inclusions were stained using a fluorochrome-labelled *C. trachomatis*-specific monoclonal antibody and were examined by using fluorescence microscopy.

RESULTS

The nvCT (Sweden2) genome sequence and genomic comparisons

The genome of the unique and currently circulating nvCT (Sweden2) is the first genome sequence of a *C. trachomatis* serovar/genovar E strain. Genomic DNA was sequenced on both Genome Sequencer FLX and Illumina Genome Analyser, with the data integrated using ICORN software to produce a single contig. The general features of the nvCT genome are summarized in Table 2, with all the complete *C. trachomatis* genomes included for comparison. The nvCT chromosome consists of 1 042 839 bp (Table 2); it has a high level of sequence identity to, and is syntenic with, all other available genomes from *C. trachomatis* strains. The plasmid (7169 bp) sequence was as described previously (Seth-Smith *et al.*, 2009). A comparative phylogeny of the genomes and their plasmids confirmed the previous observation (Seth-Smith *et al.*, 2009) that the plasmids have co-evolved with their cognate chromosome (Fig. 1). Compared with the most closely related *C. trachomatis* genome (D/UW-3/CX), the nvCT has 5896 single nucleotide polymorphisms (SNPs) (Table 3), and no whole functional gene differences were observed.

The nvCT genome is closest in size to that of D/UW-3/CX, with the genomes of serovars A and B containing an additional 1–2 kb, and the genomes of serovars L2 and L2b strains containing approximately 4 kb less than the nvCT (Table 2). This difference is accounted for by two large indels in the plasticity zone (PZ), including one within the cytotoxin locus, which is 3745 bp.

This trend in genome size is not reflected in the number of CDS, with the nvCT predicted to encode 889 genes, the same as the L2/L2b strains, whereas the serovar B strains TZ1A828/OT and Jali20 are predicted to carry fewer genes, 879 and 875, respectively. However, the numbers of CDSs present in the genomes of A/HAR-13 and D/UW-3/CX may reflect alternative annotation methods rather than genuine whole gene differences (Table 2).

Within the genomes of serovar E (nvCT), B, L2 and L2b strains, 14–18 pseudogenes have been identified (Table 2), with B/Jali20 harbouring the most. Of the 14 pseudogenes found within the nvCT genome, those that are unique to nvCT include ΨSW2_6011, encoding succinate dehydrogenase subunit B; all genomes sequenced to date contain at least one defunct component within this gene cluster. The nvCT also carries other pseudogenes; these include ΨSW2_8191, which would have encoded a putative exported protease, and four conserved hypothetical/membrane/exported proteins of unknown function: ΨSW2_0111, ΨSW2_1021, ΨSW2_4821 and ΨSW2_8891 (Supplementary Table S1, available with the online version of this paper).

Within the PZ, which is the region in which the greatest sequence variation amongst chlamydiae has been identified (Thomson *et al.*, 2008), the sequence of nvCT resembles most closely that of D/UW-3/CX (Fig. 2). Both of these strains have the same disruptions to the phospholipase D operon, with SW2_1551, SW2_1561 and SW2_1571 intact and SW2_1581 carrying the same mutation as the D/UW-3/CX homologue. The nvCT and D/UW-3/CX also encode complete *trp* operons, which are usually intact in genitotropic strains, but non-functional in oculotropic (e.g. B/Jali20) and LGV strains (Caldwell *et al.*, 2003). In addition, nvCT displays a cytotoxin locus nearly identical to that of D/UW-3/CX, which produces an active cytotoxin (Belland *et al.*, 2001), indicating that the nvCT may also

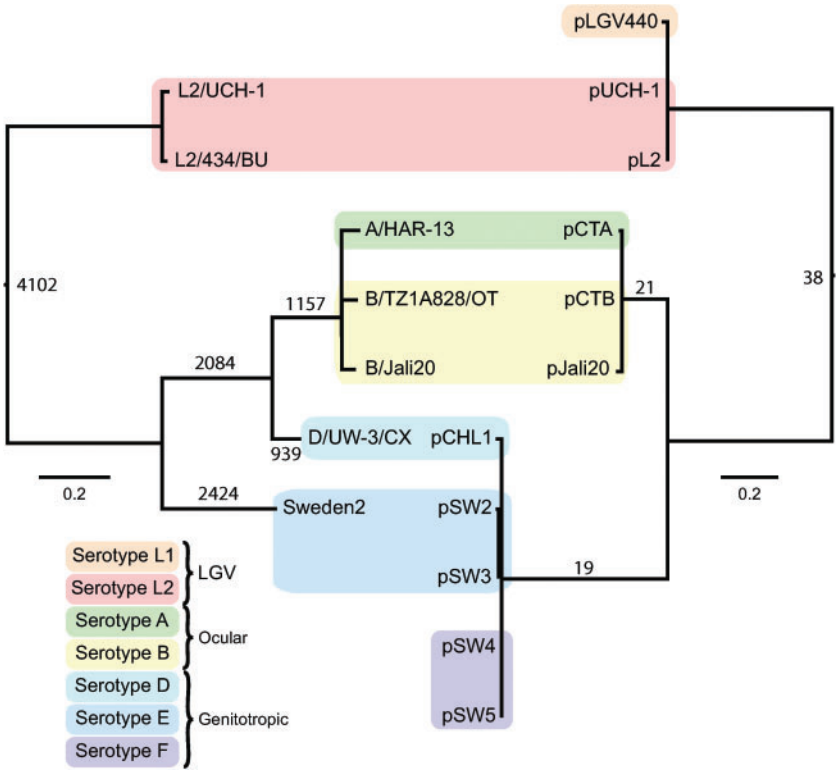

Fig. 1. Phylogenetic relationships within *C. trachomatis*. Comparison of tree topologies produced by maximum-likelihood analyses of plasmid (right) and full genome (left) sequences. The serotypes (serovars/genovars) are indicated. Bars, number of substitutions per SNP site. Numbers on nodes represent numbers of SNPs occurring on that branch.

express this activity. One difference in this operon is the absence of the start codon for CT_165 (D/UW-3/CX notation); thus, for expression of the homologous gene in nvCT, SW2_1641, the protein product would need to be translated from a start codon 35 aa downstream. However, no defined functional domain is affected by this alteration.

Additional sequence variations within CDSs of the nvCT genome were observed (these are summarized in Supplementary Table S2), including in *hctB*, *tarp* and *ompA*; the higher diversity in these genes is consistent with previous observations. There are also examples of sequence variation creating alternate C and N termini in several CDSs. The CDS SW2_1731 contains a large insertion of 307 bp relative to other strains, forming a hypothetical protein of 290 aa. Orthologues of this gene appear to be truncated or otherwise disrupted in other *C. trachomatis* strains. Unlike the situation in strain A/HAR-13, the *secF* gene, SW2_4571, is present as a full-length copy as described for the serovar B strains (Seth-Smith *et al.*, 2009).

Accordingly, the nvCT genome does not contain any major genetic polymorphisms and the genes for central metabolism, development cycle and virulence, etc. are conserved, giving no indication of altered biological fitness. Furthermore, no new genes or genetic polymorphisms were found that would confer the nvCT with antimicrobial resistance. This is also consistent with our phenotypic antimicrobial susceptibility testing, which showed that the nvCT does not have reduced susceptibility to the antimicrobials used routinely to treat *C. trachomatis* infections, i.e. the MICs of tetracycline (0.25 mg l^{-1}), erythromycin (0.25 mg l^{-1}) and ciprofloxacin (0.25 mg l^{-1}) were low.

Table 3. Pairwise comparison of SNP numbers between complete genome sequences of all available genome-sequenced *C. trachomatis* strains

	L2b/UCH-1	L2/434/BU	A/HAR-13	B/TZ1A828/OT	B/Jali20	D/UW-3/CX
L2/434/BU	487					
A/HAR-13	8688	8693				
B/TZ1A828/OT	8603	8588	1335			
B/Jali20	8565	8554	1258	1148		
D/UW-3/CX	8138	8102	3652	3623	3534	
nvCT (Sweden2; E)	7705	7670	7115	7023	7000	5896

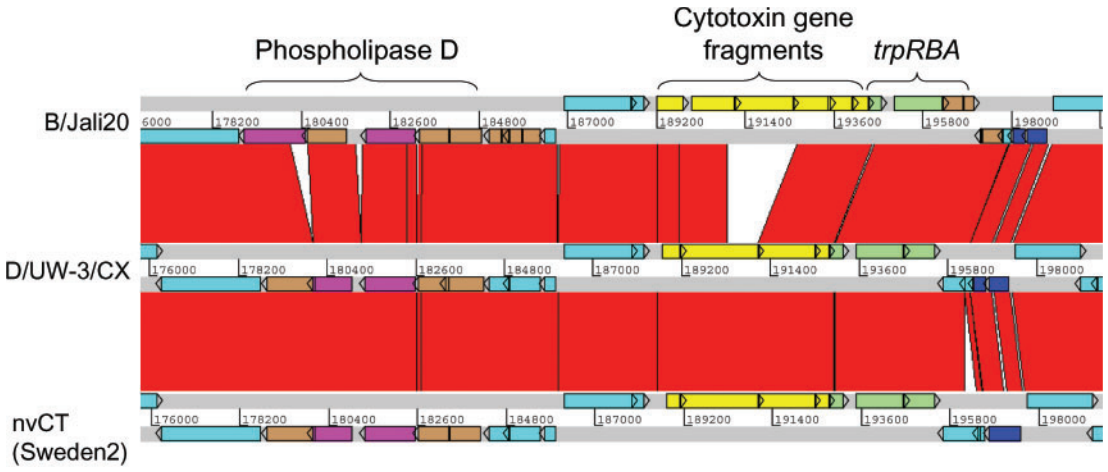

Fig. 2. Comparison of the PZ in *C. trachomatis* nvCT (Sweden2, serovar E), Jali20 (serovar B) and D/UW-3/CX (serovar D). The grey lines indicate forward and reverse reading frames of sequenced genomes, with predicted CDSs superimposed. The red bars indicate regions of 95–100 % nucleotide identity. Brown CDSs denote pseudogenes. The phospholipase D locus (purple) contains pseudogenes in all strains. The cytotoxin locus (yellow) in D/UW-3/CX produces an active cytotoxin (Belland *et al.*, 2001). This locus is identical in the nvCT, except for an SNP causing a putative 35 aa N-terminal truncation in the leftmost CDS. The *trp* operon (green) is complete in strains D/UW-3/CX and the nvCT, but has pseudogene *trpA* in Jali20. The nvCT carries an insertion of 307 bp in a CDS encoding a hypothetical protein downstream of the *trp* operon, causing an N-terminal extension of 127 aa. Additionally, an SNP in the nvCT relative to D/UW-3/CX causes read-through of CDSs encoding two conserved hypothetical proteins to create one CDS of 247 aa (dark blue). Other CDSs are turquoise.

Inclusion formation and growth characteristics of nvCT (Sweden2)

The accumulation of glycogen in mature inclusions was originally used to distinguish *C. trachomatis* from *Chlamydophila psittaci* by staining inclusions with iodine. Attempts to reveal the molecular mechanism by which glycogen synthesis is regulated by the plasmid have been unsuccessful, although it is clear that the plasmid itself does not encode the genes for glycogen synthesis. The lack of a system for manipulating the *C. trachomatis* genome makes natural variants or mutant strains such as nvCT potentially valuable for determining structure–function relationships; whilst the nvCT genome is essentially conserved when compared with other chlamydial isolates, this strain was primarily characterized by a significant deletion within CDS1 of the plasmid. CDS1 encodes a replication protein that is thought to regulate plasmid replication through an iteron binding mechanism and thus is potentially a critical factor in plasmid maintenance (Thomas *et al.*, 1997). Thus, it was of interest to investigate whether the naturally occurring CDS1 deletion within the nvCT plasmid has an effect on the ability of this strain to accumulate glycogen, which might reflect altered biological fitness and virulence. Fig. 3 shows that mature nvCT inclusions appear normal when compared with the reference strain E/Bour and Sweden3 (wtCT).

The growth characteristics of nvCT and Sweden3 (wtCT) were analysed by quantitative real-time PCR and phase-contrast microscopy in McCoy cells; both strains exhibited similar growth kinetics (Supplementary Fig. S1). They also grew at a similar rate to the E/Bour strain, but as expected, grew more slowly than L2, as judged by phase-contrast microscopy. Cell infection by the nvCT and E/Bour was analysed by high-quality digital time lapse video photo-microscopy (Supplementary Fig. S2); the video showed that, for both E/Bour and nvCT, the inclusion development is similar and that nvCT has a typical *C. trachomatis* phenotype.

Co-cultivation of nvCT and Sweden3 (wtCT) showed that these strains can be co-propagated and coincide and, at least *in vitro*, seemed to be equally transmissible, i.e. they did not out-compete each other during many passages. In several Swedish sample collections, rare co-infections of nvCT and wtCT strains have also been identified, which supports the hypothesis that the nvCT does not out-compete the wtCT strains *in vivo* (data not shown).

Cell tropism

The cell tropism experiments, using the commonly available Hep2, McCoy, BGMK, Vero and 293A cell lines, are summarized in Supplementary Table S3. These data suggest that there are only some subtle differences in the cell tropism of the nvCT and Sweden3 (wtCT). Briefly, the reference *C. trachomatis* E/Bour strain (also a serovar E isolate) grew well in all cells except Vero cells. The nvCT and Sweden3 also displayed the same cell tropism towards Vero cells, i.e. they could initially infect Vero cells but only at a very low level and then could not be passaged or

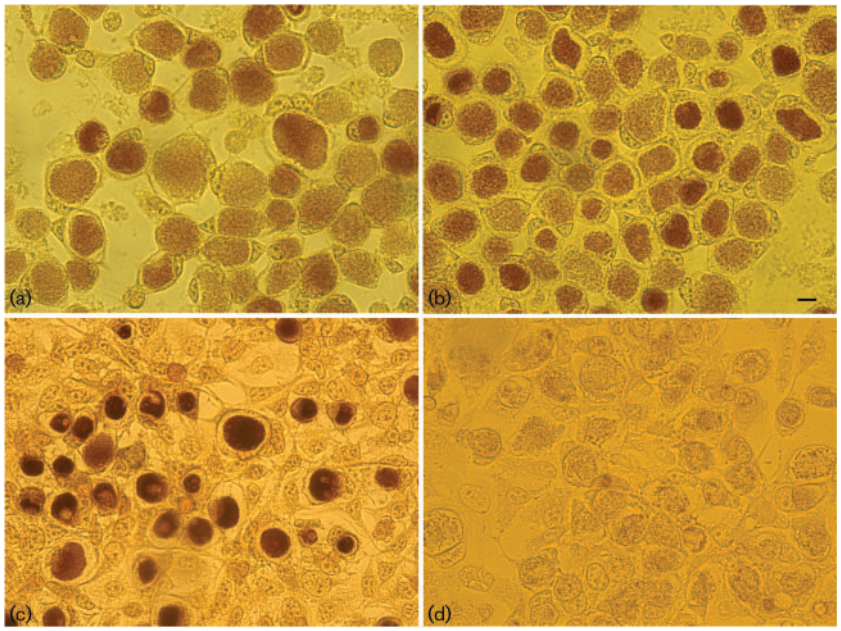

Fig. 3. Iodine-stained inclusions of the *C. trachomatis* strains nvCT [Sweden2 (a)], Sweden3 [wtCT (b)], E/Bour [prototype reference E strain (c)] and C599 [plasmid-free E strain (d)] in McCoy cells. Bar, 4 μm. Stained inclusions containing glycogen are dark brown, as seen in (a)–(c).

expanded further. In contrast, E/Bour grew well and expanded rapidly in BGMK cells (a monkey cell line as Vero cells), whereas both the nvCT and Sweden3 could only initially infect BGMK cells and complete a single developmental cycle; further attempts at passage of Sweden3 were unsuccessful whilst the nvCT rapidly adapted to grow in BGMK cells following the second passage. Therefore, we looked closely at the developmental cycles of nvCT, Sweden3 and E/Bour by TEM to see if there were any major differences. However, these strains looked similar both at the mid stage of the developmental cycle (Fig. 4a and c) and when mature inclusions had formed (Fig. 4b and d). We noticed that some inclusions contained EBs with membrane blebs; this was not an artefact of the fixation process because adjacent inclusions in the same preparation displayed this property and the E/Bour strain also had some inclusions, with EBs carrying blebs which occurred regardless of cell type. Furthermore, TEM preparations performed independently in two different laboratories made the same observations.

DISCUSSION

The results of this study show that the emergence and rapid and nationwide transmission of the nvCT in Sweden was due to the strong diagnostic selective advantage. This is supported by our data which show that the nvCT does not have any obvious biological advantage, or even disadvantage, i.e. the nvCT does not have any altered biological fitness. The nvCT belongs to the most commonly reported genitotropic serovar (serovar E) worldwide; in Sweden, serovar E strains constitute about 40–50 % of the *C. trachomatis* population (Fredlund *et al.*, 2004; Jurstrand *et al.*, 2001; Lysén *et al.*, 2004; Pedersen *et al.*, 2009; Suchland *et al.*, 2003). Comparisons of the nvCT genome, which is also the first genome sequence for serovar E, were made with all the complete genome sequences available (serovars A, B, D and L2). Compared with these genomes and several other relevant wtCT isolates, nvCT does not display any genetic polymorphisms that appear to result in an altered biological fitness; the genes for central metabolism, development cycle and virulence were conserved. Phenotypic characteristics, such as growth kinetics, development cycle, morphology, glycogen accumulation, plasmid copy number (Seth-Smith *et al.*, 2009) and antimicrobial susceptibility were unchanged. In addition, co-cultivation of nvCT and Sweden3 (wtCT serovar E) showed that these strains can coincide *in vitro*; additionally, co-infections of nvCT and wtCT strains have been identified and it has previously been shown that in Örebro county in Sweden, after excluding all the nvCT infections, the serovar distributions before and after the introduction of the nvCT were similar (Jurstrand *et al.*, 2010). These data further support the notion that the nvCT does not out-compete the wtCT serovar E strains. All this is also supported by the observations that the nvCT and wtCT infections are very similar in terms of epidemiological distribution and that differences in clinical signs have been rare (Anagrius & Loré, 2008; Marions *et al.*, 2008; Unemo *et al.*, 2007); these have only been described in women in one large and well-designed study (Bjartling *et al.*, 2009). Nevertheless, the effect of small-scale gene variation such as single amino acid alterations, and alterations in timing and level of gene transcription and translation cannot be completely excluded based on the present genomic and phenotypic data.

The transmission of the nvCT has had a huge impact on the national detection rates of *C. trachomatis* in Sweden

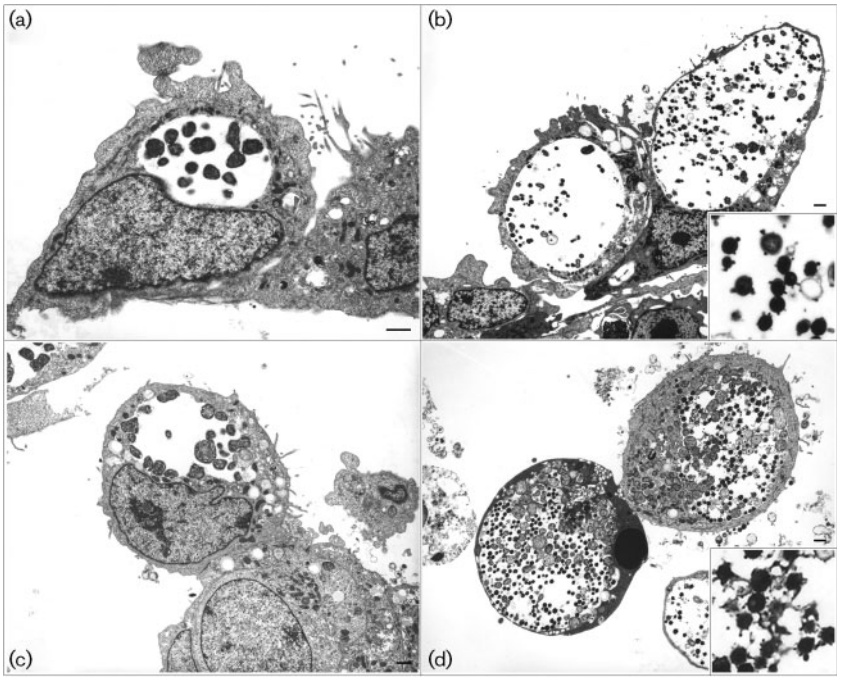

Fig. 4. TEM of the *C. trachomatis* strains nvCT [Sweden2 (a) and (b)] and E/Bour [prototype reference E strain (c) and (d)] in BGMK cells. Electron micrographs were taken at 24 h [mid stage of developmental cycle (a) and (c)] and 48 h [mature inclusions (b) and (d)], and showed no major differences between the strains. The enlarged images in the lower right corner of (b) and (d) show EBs with membrane blebs.

(Söderblom *et al.*, 2006; Velicko *et al.*, 2007), with higher proportions of nvCT in the counties using Roche/Abbott diagnostics systems than those using the BD system, which was able to detect the nvCT throughout the period that it arose. However, the recent national Swedish data show that the increase in the *C. trachomatis* incidence, i.e. after 'catch-up' of the previously missed nvCT-positive cases in early 2007 (Velicko *et al.*, 2007), followed a similar trend to that observed from the mid-1990s (Swedish Institute for Infectious Disease Control, 2009), and the proportion of the nvCT strain compared with wtCT has now started to decline after the selective diagnostic advantage for nvCT was removed (Klint *et al.*, 2009). Single cases of nvCT have been described in a few countries beyond Scandinavia; these include Ireland, France and Scotland (de Barbeyrac *et al.*, 2007; Health Protection Agency, 2008; Herrmann *et al.*, 2008; Lynagh *et al.*, 2007; Unemo *et al.*, 2009). However, current knowledge on the presence and prevalence of nvCT beyond Sweden is limited due to the small number of recent studies and the fact that many laboratories in European countries still cannot detect the nvCT (Reischl *et al.*, 2009; Unemo *et al.*, 2009). Furthermore, the commercial NAATs that can detect nvCT do not allow its discrimination from wtCT, and nvCT-discriminatory in-house NAATs are rarely used. Now that nvCT infections are being routinely diagnosed and treated, and because nvCT has similar biological fitness to the wtCT, presumably the nvCT will not be eradicated but will reach equilibrium with the wtCT strains in the *C. trachomatis* population. The proportions of nvCT infections from different Swedish counties have started to converge towards an equilibrium, following removal of the selective diagnostic advantage for nvCT (Klint *et al.*, 2009). The nvCT is already spreading in other countries, and it could be in early or even late transmission in several. Laboratories using commercial NAATs or in-house NAATs that do not detect the nvCT are encouraged to (i) monitor their *C. trachomatis* incidence rate to identify unexplained significant declines, (ii) frequently participate in effective internal and external quality assurance and control schemes and (iii) consider changing to a testing system that detects the nvCT.

In conclusion, the most plausible explanation for the emergence of the nvCT is that it was due to a recent single genetic event, which appears to be neutral with regard to biological fitness. It is likely to have occurred within a single bacterial cell that clonally expanded and initially existed as a co-infection together with the wild-type parent/progenitor *C. trachomatis*. The nvCT and progenitor were initially transmitted simultaneously; however, at some time, the nvCT was able to separate from the progenitor and cause a single clonal infection, by chance rather than by out-competition of the progenitor due to increased biological fitness. The nvCT was then rapidly and widely transmitted due to the strong diagnostic selective advantage. Accordingly, nvCT escaped detection and thus could spread rapidly, especially in high-frequency transmitting populations. Effective control measures (treatment and contact tracing) that were only applied to the wild-type allowed nvCT to proliferate. This may also be a scenario and a model for expansion and wide transmission of antimicrobial-resistant *C. trachomatis* strains that may possibly emerge in the future, but the only difference is, that if this occurs, the selection will be biological and not diagnostic.

ACKNOWLEDGEMENTS

The authors are grateful to the core sequencing facilities and especially Kicki Holmberg and Joakim Lundeberg, KTH Genome Center, Royal Institute of Technology, Stockholm, Sweden, and Thomas Dan Otto, The Wellcome Trust Sanger Institute, Cambridge, UK, for help with ICORN. The authors are also grateful to Dr B. Van der Pol for the generous gift of the plasmid-free *C. trachomatis* strain C599. This study was supported by grants from the Örebro County Council Research Committee, the Foundation for Medical Research at Örebro University Hospital, Örebro, Sweden, and the Wellcome Trust grant no 080348 to I. N. C.

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Edited by: K. Fields