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Standardisation is necessary in urogenital and extragenital Chlamydia trachomatis bacterial load determination by quantitative PCR

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ORIGINAL ARTICLE

Standardisation is necessary in urogenital and extragenital *Chlamydia trachomatis* bacterial load determination by quantitative PCR: a review of literature and retrospective study

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ABSTRACT

Objectives Pathogen load has been linked to disease severity in patients infected with HIV, resulting in international standards to adequately and reproducibly quantify load. *Chlamydia trachomatis* (CT) load has been inconsistently linked to disease severity since extensive differences exist in quantification methods (14 methods in 28 articles). Differences include normalisation for human cell load due to CT's intracellular nature, despite the inability to distinguish inflammatory cells from epithelial cells with molecular techniques. We compared the human cell load in CT-positive men and women at the genital and anal site to a CT-negative control group to estimate the impact of inflammatory cells in these samples.

Methods 188 women (tested at genital and anal site) and 519 men (207 tested at the anal site and 312 tested at the urogenital site) were included from our STI-clinic in the Netherlands. Specimens were self-collected vaginal swabs, anal swabs and urine samples. Quantitative-PCR targeting the HLA-gene quantified human cell load. Mann-Whitney-U-test was used for statistical analyses.

Results The genital cell load had a similar range and median (6.5 log10) between CT-negative and CT-positive women . The urogenital cell load was significantly higher than the anal cell load (median 3.6 log10). The anal cell load was significantly higher in men with- than without anal CT infection (median 4.5 versus 3.9 respectively). The anal cell load is significantly higher in CT-positive men than in women. Both Neisseria gonorrhoeae-co-infections and reported anal intercourse significantly increased the human cell load in anal samples.

Conclusion Standardisation in CT load studies is necessary as current studies show 14 different quantification methods in 28 studies . In this study we demonstrate the inappropriateness of normalising the CT load for the human cell load using molecular techniques, as the presence of inflammatory cells cannot be excluded.

INTRODUCTION

Chlamydia trachomatis (CT) is the most common bacterial sexually transmitted infection (STI) in the world, with approximately 100 million people infected at any point in time.[1] The clinical picture of CT is highly variable, and while the majority of patients experience no symptoms, approximately 20% of patients will develop an ascending uterine infection, resulting in pelvic inflammatory disease and possibly infertility.[2 3] It is currently unknown which factors control the natural course of a CT infection.

The bacterial load, defined as the 'measurable quantity of bacteria in an object, organism, or organism compartment' [Medical Subject Headings, Pubmed] has been linked to disease severity and transmission in other STIs like HIV,[4] *Mycoplasma genitalium*[5] and *Neisseria gonorrhoeae* (NG).[6] In CT however, there is conflicting evidence on the influence of the bacterial load on disease, transmission and immunity.[7–11] Hampering decisive conclusions are the many different methods used by studies investigating the CT load. Viral load quantification has become standard care in HIV, hepatitis B, hepatitis C and cytomegalovirus-infections, where commercial tests with international standards for calibration are available. Bacterial CT load studies have no such standards, nor do they adhere to more universal quantitative PCR (qPCR) guidelines like the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE).[12]

A literature search of current human CT load studies (7 July 2017) results in 28 hits. In the 28 included articles, the CT load is reported in 14 different ways (table 1): chlamydia load, chlamydia/mL, chlamydia/5 µl DNA, chlamydia/swab, elementary bodies (EB)/mL, EB/100 µl, inclusion-forming units (IFU)/mL, IFU DNA equivalents/mL, IFU/nL, IFU/swab, genome equivalents/mL, genome copies/reaction mixture, plasmids/mL or plasmids/swab. The CT load also shows large variety in range and mean as illustrated by figure 1. The reported mean or median varies from 22 to 5.6×10^6, while the load ranges from 0.003 to 2.6×10^9. This difference can in part be explained through natural variety, but is likely amplified by the laboratory methods used, such as the sample type and volume, the volume used for DNA isolation and elution and the PCR protocols used.

When the CT load was normalised for the human cell load, the load was reported as chlamydia/100

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Table 1 CT load in published literature

Ref.	Sex	Sample type				Load unit	PCR target	Load			Footnote
		FVU	GS	AS	OS			Low range	High range	Mean/median	
[W1]	♀		16			Chlamydia/mL	Plasmid & CT8785	19	7.6×10^{6}		*,†
[W2]	♀		602				ompA & HLA	15	8.5×10^{7}		
	♂	287					ompA & HLA	15	4.9×10^{4}		
[W3]	♀		181				ompA & HLA	15	2.2×10^{8}	1.7×10^{5}	
	♂	78					ompA & HLA	15	1.3×10^{5}	2.5×10^{2}	
	⚥			37			ompA & HLA	15	1.4×10^{6}	3.9×10^{3}	‡
[W4]	♂		98				OmpA			3.2×10^{3}	§
	♀		126				OmpA			4.0×10^{5}	§
[W5]	♂			227			OmpA & β-globin			6.3×10^{3}	§
[W6]	⚥		30				Conserved region in CT genome			6.9×10^{4}	‡,§
[W7]	♂	185					OmpA			3.7×10^{3}	
[W8]	♂			90			OmpA & HLA	65	2.1×10^{6}	3.2×10^{3}	
	♀			112			OmpA & HLA	55	2.1×10^{7}	2.0×10^{3}	
[W9]	♂	14					Omp			6.3×10^{2}	
	♀	62					OmpA			4.5×10^{2}	
	♀		44				Omp			1.0×10^{4}	
[W10]	♀		19				OmpA	1.0×10§[2]	1.0×10^{7}		
[W11]	♀		43			Chlamydia load	OmpA & β-globin			68	§,†††
	♀		189				OmpA & β-globin			5.3×10^{2}	§,‡‡‡
	♀	87					OmpA & β-globin			22	§
[W12]	⚥	171				Chlamydia/5 ul DNA	OmpA & β-actin	1	2.3×10^{3}		¶
[W13]	♀		39			Chlamydia/swab	Plasmid	1.3×10^{2}	1.4×10^{7}		**
[W7]	♂			240			OmpA			1.5×10^{4}	
[W14]	♀		52				OmpA	4.2×10^{3}	2.6×10^{9}	5.6×10^{6}	
[W15]	♀	143				EBs/mL	Plasmid	32	2.2×10^{5}	2.2×10^{3}	
[W16]	♂	58				EBs/100 ul	Plasmid			1.2×10^{3}	††
	♂		58				Plasmid			8.2×10^{2}	††
	♀	73					Plasmid			47	††
	♀		73				Plasmid			7.7×10^{2}	††,†††
	♀		73				Plasmid			2.2×10^{3}	††,‡‡‡
[W17]	⚥			15		IFU/mL	Plasmid			33	‡‡
	♀		44				Plasmid			2.8×10^{2}	
[W18]	♀		99				Plasmid & HLA	0.003	6.5×10^{3}		
[W19]	♀		75			IFU DNA equivalents/mL	16S & 16S-23S spacer rRNA			2.4×10^{3}	§§
	♀		7				16S & 16S-23S spacer rRNA			7.5×10^{2}	§§
	♂		70				16S & 16S-23S spacer rRNA			6.6×10^{2}	§§
	♂		17				16S & 16S-23S spacer rRNA			3.0×10^{2}	§§
[W20]	♀		96			IFU/nL	Plasmid	2	3.2×10^{4}	5.0×10^{2}	§
	♀			34			Plasmid	4	4.0×10^{3}	3.2×10^{2}	§
[W21]	♀		70			IFU/swab	Plasmid			4.5×10^{5}	
	♀		70				Plasmid			1.9×10^{4}	
[W22]	♂	65				Genome equivalents/mL	16S rRNA	5.0×10^{2}	7.4×10^{8}	5.9×10^{5}	
[W23]	⚥		95			Genome copies/reaction mixture	OmpA	26	4.0×10^{4}	3.2×10^{2}	‡
[W24]	♂	80				Plasmids/mL	Plasmid	7.3×10^{2}	6.9×10^{6}		¶¶
[W25]	♂	38					Plasmid			3.3×10^{4}	¶¶
	♂	47					Plasmid			5.3×10^{3}	
[W26]	♀		33			Plasmids/swab	Plasmid	6.0×10^{2}	1.1×10^{9}		
[W11]	♀		43			Chlamydia/100 cells	OmpA & β-globin			0.03	§,†††
	♀		189				OmpA & β-globin			0.4	§,‡‡‡
	♀	87					OmpA & β-globin			7	§
[W12]	♂	80					OmpA & β-actin	0.4	1.8×10^{3}	21	¶
	♀	91					OmpA & β-actin	0.3	5.0×10^{3}	27	¶
[W12]	⚥	171					OmpA & β-actin	0.3	5.0×10^{3}	24	¶
[W27]	♀		9				OmpA & β-globin	44	1.3×10^{4}	8.2×10^{3}	

Continued

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Table 1 Continued

Ref.	Sex	Sample type FVU	GS	AS	OS	Load unit	PCR target	Load Low range	High range	Mean/median	Footnote
[W18]	♀		96			IFU/HLA	Plasmid & HLA	0.000002	1.1×10^2		
[W28]	⚥				88	IFU/100 million HLA copies	Plasmid & HLA			2.5×10^3	***

For references see online supplementary file 1.
*CT8785 is a chromosomal gene.
†Baczynska *et al*. (2008) assumed 10 plasmids per chromosomal copy to quantify CT copies/mL.
‡Men and women grouped together as unclear how many samples per gender.
§Absolute CT load is converted from log-value.
¶80 male FVU and 91 female FVU.
**Michel *et al*. (2009) assumed 7 plasmids per organism to quantify bacteria/swab.
††Michel *et al*. (2007) assumed 7.72 plasmids per organism to quantify bacteria/swab.
‡‡8 male AS and 7 female AS.
§§Inclusion forming unit DNA equivalents per mL (IFUde) = the amount of Ct DNA obtained from a preparation containing 1 culturable organism (IFU) of strain ATCC VR-346 serovar F.
¶¶Firstburst urine cup.
***88 Oral samples: 37 men, 51 women.
†††Vaginal samples.
‡‡‡Cervical samples.
AS, anal swab; CT, *Chlamydia trachomatis;* EBs, elementary bodies; FVU, first void urine; GS, urogenital swab; HLA, human leucocyte antigen; IFU, inclusion-forming units; OS, oral swab.

cells, IFU/human leucocyte antigen (HLA) or IFU/100 million HLA copies. Several reasons advocate normalisation for the human cell load, primarily CT's intracellular habitat, but also as a test for adequate sampling and interpersonal sampling variability. Furthermore, normalisation may be necessary when comparing CT load in different locations and thus different sample types (eg, urine and genital sample within one woman).[13] Counterarguments put forward are that the used human genes are not specific enough, as they are present in all cells, not only in columnar cells preferred by CT.[14] It has been demonstrated (in urine samples) that the majority of samples will contain squamous cells, which will confound any attempt to control for number of columnar (transitional) epithelial cells by using the total human cell count.[15] In that case, CT load normalisation for high inflammatory cells could produce artificially low CT loads, thereby distorting possible correlations with clinical

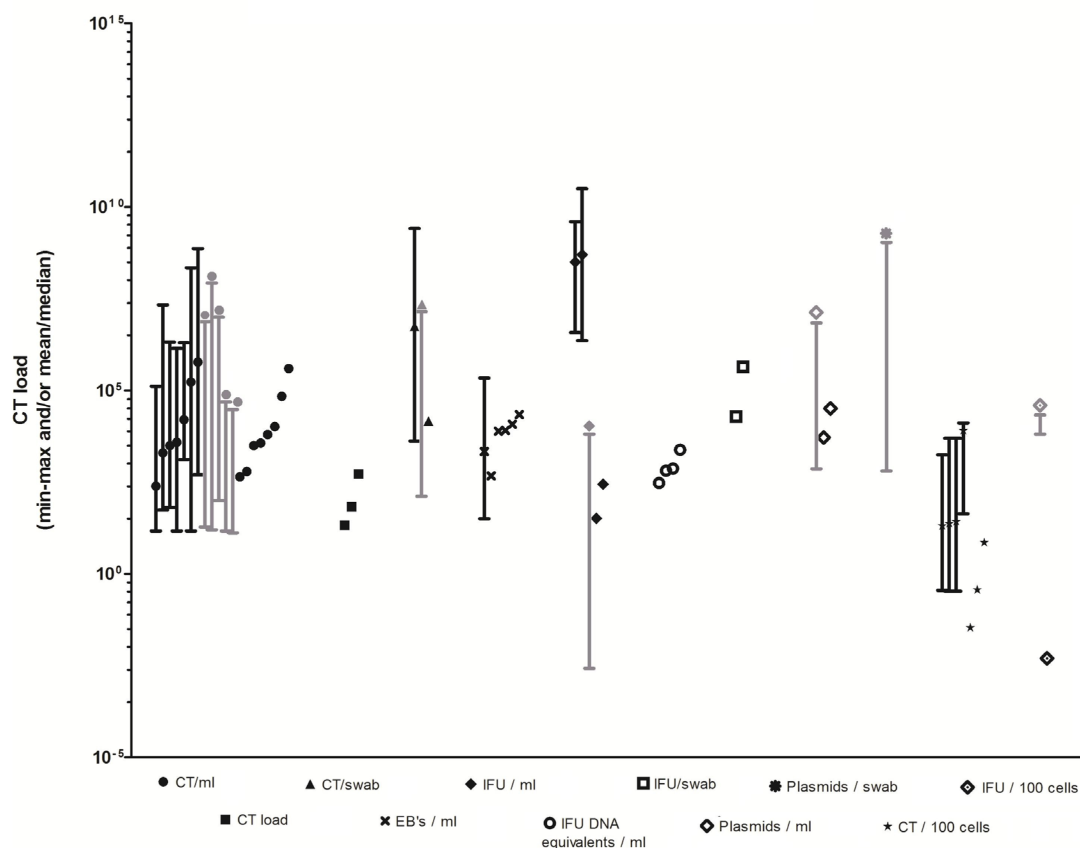

Figure 1 Visual representation of CT load from literature. In this figure, CT load is converted to millilitres or expressed per 100 cells if reported differently in the original study. Black symbols represent the mean/median and black lines indicate load range. Grey lines represent load range when no median/mean was given, and the symbol is used to indicate the method of expressing CT load. CT, *Chlamydia trachomatis*.

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characteristics. Moreover, due to CT's asynchronous life-cycle, intracellular and extracellular CT will be present simultaneously in each sample,[16] making strict CT-per-cell normalisation inadequate and oversimplified.

To test the hypothesis that the presence of inflammatory cells prevents accurate CT normalisation, we compared the human cell load in CT-positive men and women at the genital and anal site to a CT-negative control group.

MATERIALS AND METHODS

A total of 707 patients were included from the STI-clinic in South Limburg from November 2010 to December 2013: 188 women (96 women with a concurrent genital/anal CT infection and 92 women with a negative CT-test at the urogenital and anal site) and 519 men (94CT-positive men and 113 CT-negative men at the anal site, 164 CT-positive men and 148 CT-negative men at the urogenital site). Retrospective review of structured systematic medical history provided information about age, gender and anal intercourse. Controls were matched for age, coinfections with NG and reported sexual behaviour where available. Specimens were self-collected vaginal swabs, anal swabs and urine samples. Samples were tested for CT with either COBAS Amplicor (Roche Diagnostics) or COBAS 4800 (Roche Diagnostics) as per manufacturer's protocol. Swabs are stored in 4.3 mL buffer and approximately 5 mL urine is used. Total nucleic acids from 200 µl sample were isolated using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) and eluted in 120 µl. Eukaryotic cell determination was performed using primers targeting the MHC class II antigen (*HLA-DQA1*)-gene.[7] Sample processing and cell quantification were performed according to a previously described protocol.[17] Two HLA copies are present per cell, thus HLA load was halved to determine the human cell load. Samples were excluded if no cells were present. Human cell load was logarithmically transformed to allow parametric testing using Student's t-test, if data were not normally distributed, Mann-Whitney-U-test for independent samples was used. Associations of human cell load with self-reported anal intercourse and NG-coinfection were assessed.

After reviewing the protocol within the context of the Medical Research Involving Human Subjects Act, the local Medical Ethics Committee of the Maastricht University Medical Centre approved this study and waived the need for informed consent (METC 18-4-161).

RESULTS

Urogenital cell load

In vaginal swabs, the cell load ranged from 2.2 to 7.7 $\log_{10}$ (IQR 6.2–6.7) in CT-negative women and from 3.7 to 7.2 (IQR 5.7–6.7) in CT-positive women (figure 2 (panel A) and table 2). The median was similar (6.5), as was the load distribution in both groups ($p>0.05$). Thus, in women with a genital CT infection, the human cell load was not significantly different from that in women without a genital CT infection. These results suggest that there are few extra inflammatory cells present in a female vagina due to the CT infection.

In urine samples, the load ranged from 1.9 to 6.0 $\log_{10}$ (IQR 2.9–3.8) in CT-negative men and from 1.5 to 6.0 (IQR 3.4–4.7) in CT-positive men (figure 2 (panel C) and table 2). The median and load distribution was significantly different between the two groups ($p<0.001$) with a median of 3.3 in CT-negative urines and 4.2 in CT-positive urines. Urine of men with an NG coinfection (n=2) had a similar human cell load to that of men without an NG coinfection, although numbers were low. Thus, in men with a genital CT infection, the human cell load was significantly higher in men with a CT infection than in men without a CT infection.

Anal cell load

The vaginal cell load (median 6.5 $\log_{10}$) was significantly different from the anal cell load (median 3.6 $\log_{10}$, $p<0.001$) in women. The anal cell load is comparable in women with and without anal CT (median 3.6 in CT+ women and 3.7 in CT-negative women, $p=0.8$).

The urine cell load in men (median 3.7 $\log_{10}$) was significantly different from the anal cell load (median 4.2; $p<0.001$). The distribution of the anal cell load in men and women is demonstrated in table 2 and figure 2 (panel B).

In men, the anal cell load is significantly different between men with and without an anal CT infection (median 4.5 vs 3.9, respectively, $p=0.007$). CT-negatives have a similar anal cell load (median 3.9 in men, 3.7 in women), but the anal cell load is significantly different between CT-positive men and women (median 4.5 and 3.6, respectively).

Further analyses were performed to assess possible confounders, such as coinfections (with NG) or anal intercourse. Men with a CT-only infection had a lower cell load than men with a CT/NG coinfection (4.4 in CT-only infections (n=80), 5.4 in CT/NG coinfections (n=14), 5.1 in NG-only infections (n=12). This suggests that inflammatory cells are present during an NG infection.

To assess the effect of anal sex on the human cell load, further analyses were stratified (missing data for 13 men and 4 women). Of the men, 84% (n=173) reported anal intercourse (not further specified as receptive or insertive) and 43% (n=81) of women reported anal intercourse. When comparing the anal cell load in 81 women and 173 men with anal intercourse, this resulted in a similar cell load (median 3.9 and 4.3, respectively; $p=0.2$). The anal cell load in 103 women and 21 men without anal intercourse was the same (median 3.9 and 3.7, respectively; $p=0.9$). After excluding all NG-positive patients, the results remained the same (only a lower median of 4.2 in men with anal intercourse). Thus, the human cell load is similar in men and women who report anal intercourse, irrespective of a current CT or NG infection. This makes it quite likely that anal intercourse is an independent influence on the human cell load due to friction (with a subsequent influx of inflammatory cells) or it may be due to related causes like anal douching or the presence of semen in the anal canal.

CONCLUSION AND DISCUSSION

The variance in CT loads reported by current studies varies more than million-fold, partly due to methodological differences, such as sample volume, DNA extraction and amplification. More variation is introduced through the ongoing debate whether or not CT load should be normalised for the human cell load. In this study, we demonstrate the inappropriateness of normalising the CT load for the human cell load using molecular techniques, as the presence of inflammatory cells cannot be excluded. Furthermore, we demonstrated significantly different cell counts in genital swabs, anal swabs and urine samples.

With the introduction of PCR, CT load studies have become infinitely easier than culture-based studies. Due to its ease, many research-groups have implemented this technology, but there is no consensus on how to best perform or interpret the data. The accuracy of the quantification relies on the combination

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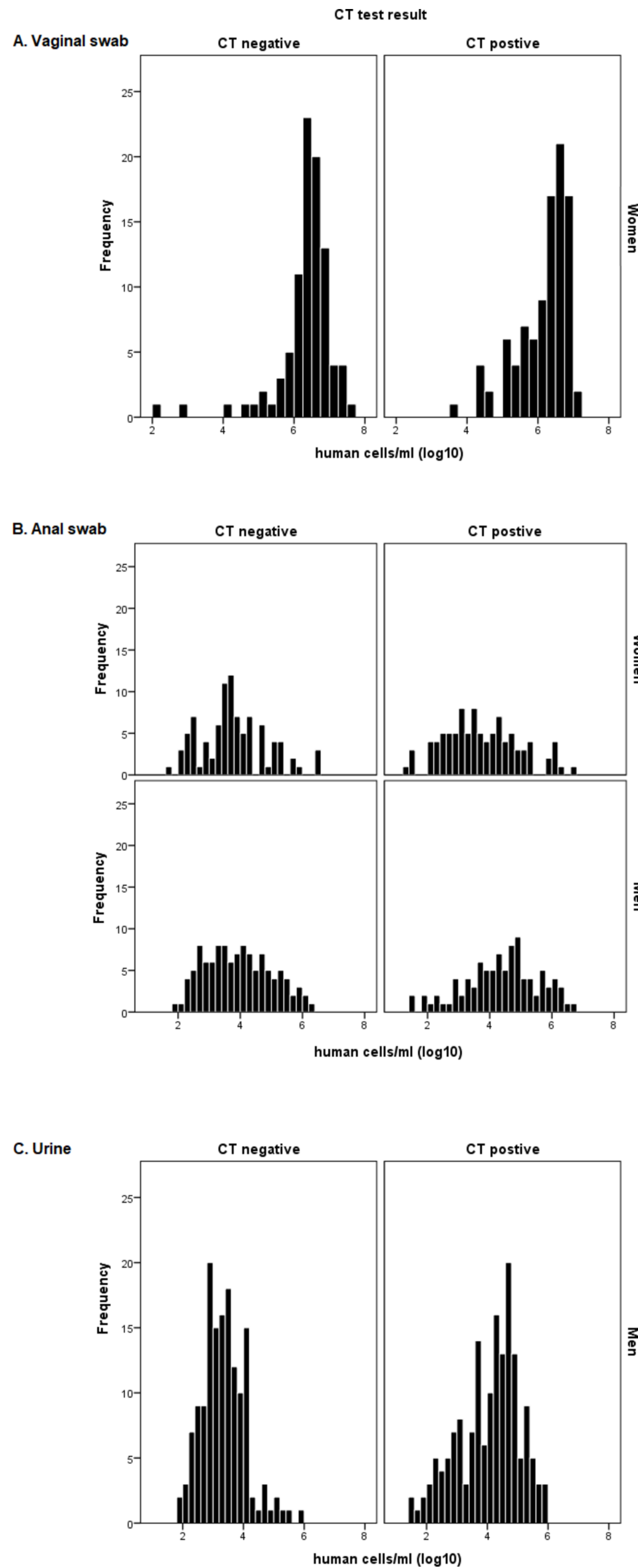

Figure 2 (A) Human cell load distribution in self-collected vaginal swabs in CT-positive (n=96) and CT-negative (n=92) women. (B) Human cell load distribution in anal swabs in CT-positive and CT-negative men and women (113 CT-negative and 94 CT-positive men; 92 CT-negative and 96 CT-positive women). (C) Human cell load distribution in urine samples from CT-positive (n=164) and CT-negative (n=148) men. CT, *Chlamydia trachomatis*.

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Table 2 Human load distribution in genital samples for women and anal samples for men and women

	Women (genital)*		Women (anal)		Men (anal)		Men (anal), NG+excluded		Men (urine)	
	CT– (n=92)	CT+ (n=96)	CT– (n=92)	CT+ (n=96)	CT+ (n=94)	CT– (n=113)	CT+ (n=80)	CT– (n=101)	CT+ (n=164)	CT– (n=148)
Median	6.46	6.45	3.68	3.59†	4.50‡	3.93	4.37	3.81	3.3‡	4.2
Minimum	2.24	3.72	1.65	1.35	1.52	1.94	1.52	1.94	1.5	1.9
Maximum	7.67	7.15	6.57	6.61	6.61	6.32	6.61	6.32	6.0	6.0

*Significant difference in cell load between genital and anal samples in women, p<0.05.
†Significant difference in cell load between CT+ men and women, p<0.05.
‡Significant difference in cell load between CT+ and CT– men, p<0.05.
CT, *Chlamydia trachomatis*.

of preanalytical (specimen collection, transport, processing), analytical (molecular testing, eg, nucleic acid extraction methods, PCR efficiency) and postanalytical (reporting, interpretation of results) characteristics. Studies have demonstrated the importance of variations in these processes on load,[18] but it is difficult to estimate its exact consequence on the CT load. These standardisation problems are widespread, and for some pathogens, such as cytomegalovirus, this has led to international guidelines[19] and interlaboratory comparison of external quality-control panels.[20]

In the current situation, the CT load cannot be compared between studies performed in different laboratories, and consequently, no cut-off for clinical relevance can be established, nor can it be consistently linked to clinical parameters like age, symptoms or transmission.[8 10] We realise that variations will exist between laboratories and studies, but some aspects of CT quantification can easily be streamlined. The first step is a description of the materials and methods to allow replication (collected sample volume, volume used for DNA isolation and elution, and DNA volume used in the PCR). Next, standardisation of the reported CT loads per millilitre (not per swab, per 5 µl DNA or per unspecified parameter) would greatly increase comparability of CT loads. The variable quantification-references result in variable measures of the CT loads, such as bacteria, EB's, IFU or plasmids. Multicopy PCR-targets like the cryptic plasmid and rRNA increase the CT detection-probability[21] but absolute quantification is hampered by variable copy-numbers per bacterium, making single-copy-genes preferable, such as ompA,[22 23] omcB[24] or rpoB.[25] Some studies have deduced the CT load from the plasmid-number, assuming 7–10 plasmids per bacterium[26 27] despite plasmid-copy-variability during the asynchronous developmental cycle.[24] IFU too is highly variable and depends on several factors, including sampling method, transport and laboratory conditions and the methodology used for staining and reading.[28]

Some of the variation in CT load studies comes from normalisation for the human cell load. Normalisation corrects interpersonal sampling variability,[29] ensures adequate sampling,[17 29] allows load-comparison between different sample types[13] and is advocated by CT's intracellular life-cycle.[30] However, with the molecular techniques also used for CT detection, the distinction between epithelial cells (in which CT resides[14 25] and inflammatory cells cannot be made, resulting in a possible underestimate of the CT load during inflammation.

We demonstrated that the genital and anal human cell load is similar in women with and without a CT infection. Several explanations exist for the similar genital cell load in infected and uninfected women, such as the superficial presence of CT bacteria in the vagina without an infection or a sampling error. Furthermore, we did not take other genital infections (eg, candida or bacterial vaginosis) into account. In men however, CT-positive men have a higher anal cell load than CT-negative men. This is partly explained through inflammatory cells, NG-coinfections and possibly the friction/irritation caused by anal intercourse, as demonstrated by a similar cell load in men and women who report anal intercourse. Other, unexplored causes for a raised cell load in patients practising anal intercourse included anal douching, and the presence of semen due to recent unprotected intercourse. Thus, normalisation using commonly used genes, like HLA, will underestimate the anal CT load, preventing an accurate comparison with the genital CT load. Future studies should aim for new methods or genes to be used in CT load normalisation.

To conclude, this article illustrates the wide variety in methodology and consequently CT loads reported in literature. There are almost as many different results as CT load studies published. Future studies should aim for standardisation of the methodology and results, which can be done with current universal qPCR-guidelines like the MIQE.[12] Furthermore, we demonstrated that CT load normalisation is pertinent when comparing different sample types, but currently used genes are not an appropriate method. Further work needs to be carried out to establish methods for more accurate normalisation. With the advice provided here, hopefully, in the future, more definite conclusions can be drawn regarding the CT load and clinical parameters, or to determine a clinical cut-off for disease.

Key messages

- There is currently too much variability in *Chlamydia trachomatis* (CT) load studies (14 ways to report CT load in 28 studies) using PCR.
- The CT load should not be normalised for the human cell load when using molecular techniques as the presence of inflammatory cells cannot be excluded.
- Due to differences in sample types, the CT load cannot directly be compared between genital and anal samples within one person.

Handling editor Catherine A Ison

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