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RESEARCH ARTICLE

Growth characteristics of *Chlamydia trachomatis* in human intestinal epithelial Caco-2 cells

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10 **One sentence summary:** Our analyses of growth characteristics of *Chlamydia trachomatis* in intestinal epithelial Caco-2 cells support the possibility of the gastrointestinal tract behaving as a source of reinfection of the urogenital tract.

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ABSTRACT

15 *Chlamydia trachomatis* is an obligate intracellular bacterium causing infections of the eyes, urogenital and respiratory tracts. Asymptomatic, repeat and chronic infections with *C. trachomatis* are common in the urogenital tract potentially causing severe reproductive pathology. Animal models of infection and epidemiological studies suggested the gastrointestinal tract as a reservoir of chlamydiae and as a source of repeat urogenital infections. Thus, we investigated the growth characteristics of *C. trachomatis* in human intestinal epithelial Caco-2 cells and the infection-induced defensin production.
20 Immunofluorescence staining and transmission electron microscopy showed the presence of chlamydial inclusions in the cells. Chlamydial DNA and viable *C. trachomatis* were recovered from Caco-2 cells in similar quantity compared to that detected in the usual *in vitro* host cell of this bacterium. The kinetics of expression of selected *C. trachomatis* genes in Caco-2 cells indicated prolonged replication with persisting high expression level of late genes and of heat shock protein gene *groEL*. Replication of *C. trachomatis* induced moderate level of β-defensin-2 production by Caco-2 cells, which might
25 contribute to avoidance of immune recognition in the intestine. According to our results, Caco-2 cells support *C. trachomatis* replication, suggesting that the gastrointestinal tract is a site of residence for these bacteria.

Keywords: Caco-2; *Chlamydia*; defensin; gastrointestinal tract; gene expression

INTRODUCTION

30 Chlamydiae are Gram-negative bacteria, obligate, intracellular pathogens that cause different acute and chronic human diseases. *Chlamydia trachomatis* infects the urogenital and ocular mucosa of humans, and it causes the most common sexually transmitted infection and trachoma as well (Mpiga and
35 Ravaoarinoro 2006).

Chlamydiae have a unique developmental cycle involving two main forms. They exist as infective and metabolically inactive elementary bodies (EBs) or as non-infective but metabolically active reticulate bodies (RB; Bastidas *et al.* 2013).

40 Several publications have described a third, persistent phase of the chlamydial developmental cycle, in which the RB are enlarged and pleomorphic in morphologically abnormal inclusions. In persistence, chlamydiae show altered gene expression patterns (Schoborg 2011). The persistent form of *Chlamydia* was induced by interferon-gamma (IFN-γ; Kokab *et al.* 2010), 45
antibiotics (Kintner *et al.* 2014; Phillips-Campbell, Kintner and Schoborg 2014), iron depletion (Mäurer *et al.* 2007), and it could

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also be induced in different cell types, e.g. in monocytes (Datta *et al.* 2014).

Chlamydia trachomatis persistence has been associated with chronic infections (Wyrick 2010).

Animal pathogenic *Chlamydia* species were isolated from various animals, for example, porcines, ruminants and avians, and they were detected in different organs as well as in feces. In most animals, chlamydiae persist in the gastrointestinal (GI) tract and are transmitted via the fecal–oral route (Rank and Yeruva 2014). Oral infection with *Chlamydia muridarum* has resulted in a long term, persistent infection of the mouse GI tract (Igietseme, Portis and Perry 2001). The GI tract seems to be an ideal site, in which *Chlamydia* can persist because of a down regulated host immune response. In Caco-2 cells, inclusions have indicated a substantial growth of porcine *Chlamydia pecorum* and *Chlamydia suis*; therefore, Caco-2 cells can be regarded as suitable hosts for animal *Chlamydia* (Schiller *et al.* 2004).

Chlamydia trachomatis urogenital strains have been associated with persistent or recurrent infection of the genital tract in women, the nature of which has not been identified. It has been suggested that genital tract infection can be accompanied by infection of the GI tract either via oral infection or by autoinoculation from genital secretions or during sexual activity (van Liere *et al.* 2014). Anorectal samples taken from both men and women have been tested and found positive for *C. trachomatis* (Bax *et al.* 2011). Studies by Yeruva *et al.* have suggested that reinfection of the genital tract may occur via contamination of the genital tract from the infected GI tract, especially in women (Yeruva *et al.* 2013). Furthermore, *C. trachomatis* might have a role in irritable bowel disease (IBD) as suggested by the detection of *C. trachomatis* antigen in the intestine of IBD patients (Dlugosz *et al.* 2010).

Caco-2 cell line could be a suitable model for the investigation of *Chlamydia* infection in the human GI tract. If chlamydiae can persist in the GI tract, it is of relevance to examine the growth characteristics of human *Chlamydia* in Caco-2 cells, and the induced innate immune response in these cells.

In the colonic mucosa, defensins, and among them human β-defensin (hBD)-2 represent the important effectors of the innate host defenses not only by their microbicidal activity but by providing a link to the adaptive immune system as they attract immature dendritic cells and memory T-cells (Kim 2014).

In this study, we applied several morphological and molecular approaches to investigate the growth characteristics of *C. trachomatis* in the intestinal epithelial cell line Caco-2, and we examined whether *Chlamydia* infection induce hBD-2 production in these cells.

MATERIALS AND METHODS

Cell lines

Caco-2, HeLa 229 and McCoy cells were maintained in minimal essential medium (MEM) with Earle's salts completed with 10% FBS, 2 mmol/liter L-glutamine, 1× nonessential amino acids, 25 μg/mL gentamicin and 0.5 μg/mL fungizone. The cell lines were purchased from American Types Culture Collection (ATCC).

Bacterial strains

Chlamydia trachomatis serovar D, UW-3/CX strain from ATCC was used, and it was propagated in McCoy cell line. The infected cells were purified by density gradient centrifugation, as previously described, with some modification (Sabet, Simmons and Caldwell 1984; Burián *et al.* 2003).

The partially purified and concentrated EBs were aliquoted in sucrose–phosphate–glutamic acid buffer (SPG) and stored at –80°C until use. Infective chlamydiae were quantitated by inoculating 10-fold serial dilutions of the EB-containing preparations onto McCoy cells; the inclusions were detected by indirect immunofluorescent method applying anti-Chlamydia lipopolysaccharide (cLPS) monoclonal antibody (AbD Serotec, Oxford, United Kingdom) and FITC-labeled anti-mouse IgG (Sigma-Aldrich, St. Louis, MO). The concentration of infective EBs was expressed as inclusion forming units/mL (IFU/mL).

Bacterial infection

The cells were grown in 6-well (1×10^6 cells/well), 96-well (4×10^4 cells/well), or 24-well culture plates (2.5×10^5 cells/well) with 13 mm glass coverslips. The plates were kept for 1 h at room temperature (RT), and then incubated overnight in 5% CO_2 atmosphere at 37°C to reach 90% confluency. The cells were then infected with *Chlamydia* at a multiplicity of infection (MOI) of 1 or 5 in complete MEM with 0.5% glucose and centrifuged at 800 × *g* for 1 h RT. The medium was replaced in the wells with a cycloheximide-containing one (1 μg/mL). Cycloheximide was not added to the medium of cells in 6-well plates infected for testing defensin secretion or RNA expression analyses. For DNA quantitation, the infected cells in 96-well plates were incubated in cycloheximide-containing medium or cycloheximide-free medium. The culture plates were incubated for different time periods in CO_2 incubator at 37°C.

Assessment of the infectivity of *C. trachomatis* D replicating in Caco-2 or HeLa cells was done by inoculation of the infected cell lysates onto McCoy cells in 24-well plates with glass coverslips. Cell lysates were prepared by scraping the infected cells into the culture medium at each examination time point. After two freeze-thaw cycles and sonication in water bath, the lysates were centrifuged at 800 × *g* for 1 h onto McCoy cells grown in 24-well plates with 13-mm glass coverslips. After 48 h, the cells were fixed with acetone at –20°C for 10 min.

Immunofluorescent staining of infected cells for visualization of inclusions and quantitation of recoverable *Chlamydia*

The staining of *Chlamydia*-infected cells on coverslips was performed via using anti-cLPS and FITC-labeled anti-mouse IgG as secondary antibody. The coverslips were treated with Evan's Blue at RT for 1 min. The chlamydial inclusions were photographed and counted under a fluorescent microscope.

Transmission electron microscopy

The cells were cultured in 6-well plates and infected with *Chlamydia* at an MOI of 1. After 24, 48 and 72 h, the infected cells were washed in plates with 3 mL phosphate buffered saline (PBS) and collected after trypsin treatment. After sedimentation with 400 × *g* for 5 min, the cells were fixed with 2% glutaraldehyde and 1% osmium tetroxide overnight at 4°C. Samples were embedded in Embed 812 (EMS, USA) using a routine transmission electron microscopy (TEM) embedding protocol. Ultrathin sections (70 nm) were cut with an Ultracut S ultra-microtome (Leica, Austria). After staining with uranyl acetate and lead citrate, the sections were examined with a Philips CM10 electron microscope. Images were acquired by using Olympus Soft Imagine Viewer program.

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Table 1. Primer sequences used in qRT-PCR analysis.

C. trachomatis target gene	Sequence	PCR product size (bp)
16S rRNA F	5'-CACAAGCAGTGGAGCATGTGGTTT-3'	191
16S rRNA R	5'-ACTAACGATAAGGGTTGCGCTCGT-3'	
euo F	5'-TCCCCGACGCTCTCCTTTCA-3'	263
euo R	5'-CTCGTCAGGCTATCTATGTTGCT-3'	
ftsK F	5' CGGAAGAAAGCAAGCGTTTC 3'	70
ftsK R	5' GGGCTAGATACACGCATGTTTTAAC 3'	
groEL F	5'-TCACTCTAGGGCCTAAAGGACG-3'	115
groEL R	5'-TCATGTTTGTCGGCAAGCTC-3'	
omcB F	5' TGAAGCAGAGTTCGTACGCAGTG 3'	179
omcB R	5' AACGGATCTCTGGACAAGCGCAT 3'	
ompA F	5'-TCGACGGAATTC TGTGGGAAGGTT-3'	171
ompA R	5'-TATCAGTTGTAGGCTTGGCACCCA-3'	
pyk F	5'-GTTGCCAACGCCATTTACGATGGA-3'	81
pyk R	5'-TGCATGTACAGGATGGGCTCCTAA-3'	

Chlamydial DNA quantitation

For the quantitative assessment of chlamydial replication, we followed a direct DNA quantitation method described previously (Eszik *et al.* 2016). The cells cultured in 96 or 24-well plates were infected with *Chlamydia*. After 0, 24, 48 and 72 h, the infected cells in 3 parallel wells were washed in the plates twice with 200 μL/well PBS. Then, either 100 or 625 μL Milli-Q water was added to the wells, and the plates were stored at –80°C. Two freeze-thaw cycles were applied to free the DNA from the cells. Thoroughly mixed lysates were used as templates directly for quantitative PCR (qPCR) using SsoFast EvaGreen ®Supermix (Bio-Rad). Pyk primers were used for the detection of *C. trachomatis* D genomes (Table 1).

RNA extraction

For the analysis of gene expression, total RNA was extracted from the infected cells in 6-well plates at 2, 24, 48 or 72 h after infection (3 parallel cultures at each time point) with GenElute Mammalian Total RNA Miniprep Kit (Sigma) according to the manufacturer's protocol. Concentration of RNA was determined by spectrophotometry. The extracted RNA was treated with DNase I (Sigma). cDNA was synthetized from DNase-treated RNA with qScript cDNA Supermix synthesis kit (Quanta Biosciences). RNA and cDNA were stored at –80°C until use.

Quantitative RT-PCR

By using cDNA as template, ~~qRT-PCR~~ was performed with PerfeCTa SYBR Green Supermix (Quanta) in CFX96 Real Time C1000 Thermal Cycler (BioRad). 16S rRNA was used as the internal standard for counting the relative expression of *Chlamydia* genes as this gene was previously shown to be an accurate normalizing gene for gene expression analysis in *C. trachomatis* (Borges *et al.* 2010). The relative expression of *euo*, *groEL*, *ftsK*, *omcB*, *ompA* and *pyk* transcripts of *C. trachomatis* was evaluated. The sequences of all primers used for RT-PCR are shown in Table 1. All primers were synthetized by Integrated DNA Technologies Inc. (Montreal, Quebec, Canada). The PCR cycles consisted of a 3-min denaturation at 95°C followed by 55 cycles of 10 s of denaturation at 94°C; 10 s of annealing at 61.5°C for *16S rRNA*, *groEL*, *omcB*, *ompA*, *pyk*, 54°C for *ftsK* and 10 s of extension at 72°C. The samples were tested in triplicates, and no-template controls with distilled water were run in each case. The melt curve analysis was performed to prove the specificity of the amplification.

The relative gene expression levels (RQ) were given by calculating the delta-delta Ct ($\Delta\Delta$Ct) value. The lowest cycle number, at which the various transcripts were detectable, referred to as Ct, was compared with that of the 16S rRNA, and the difference was referred to as ΔCt (Borges *et al.* 2010). The relative expression level was given as $2-(\Delta\Delta Ct)$, where $\Delta\Delta Ct = \Delta Ct$ for the experimental sample minus ΔCt for the control sample at 2 h.

~~ELISA~~ for detection of hBD-2

The supernatant of the infected cells from 3 parallel wells' 24-well plates were harvested at different time points post infection. For the detection of hBD-2 production, the supernatants of the cells were tested by using hBD-2 ELISA kit (Alpha Diagnostic, San Antonio, TX, USA). The level of the hBD-2 (12.5–200 pg/mL detection range) was determined according to the manufacturer's instructions. The supernatant of Caco-2 cells treated with heated (1 h, 56°C) overnight culture of *Escherichia coli* Nissle 1917 strain (MOI of 100), a potent inducer of HBD-2, was used as positive control. Q3

Statistical analysis

Data are expressed as mean ± SD. Independent-samples t-test was used with SigmaPlot for Windows Version 11.0 software. A P value of less than 0.05 was considered to indicate statistically significant difference.

RESULTS

Detection of *Chlamydia* growth by immunofluorescence staining in Caco-2 and conventional host cells

After indirect immunofluorescence staining with anti-cLPS antibody, inclusions of *Chlamydia trachomatis* were seen in Caco-2 and HeLa cells. The detection of the inclusions suggested ongoing replication in both cell types; however, the morphology of the inclusions demonstrated different growth kinetics in the different cell types. *Chlamydia trachomatis* D formed compact inclusions in Caco-2 cells with cLPS appearing in the cell membrane (Fig. 1A), and Hela cells showed inclusions with dense core and expanding cLPS signal at 24 h post-infection (Fig. 1B); at 48 h in Caco-2 cells (Fig. 1C), the inclusions grew larger but in the permissive HeLa cells expanding fluorescing areas had shown the final stage of replication cycle by this time (Fig. 1D). Q4

Transmission electron microscopy of *Chlamydia*-infected Caco-2 and conventional host cells

With TEM, *C. trachomatis* D inclusions were observed both in Caco-2 (Fig. 2A, B) and HeLa (Fig. 2C, D) cells. The developmental stage of the bacteria in the inclusions at 48 h post-infection was rather heterogeneous in Caco-2 cells; however, in HeLa cells fully developed inclusions with numerous EBs were seen.

Chlamydia genome accumulation in Caco-2 and conventional host cells

The quantitative features of *Chlamydia* replication in the intestinal epithelial cells in comparison with that in conventional host cells were investigated with a novel DNA quantitation method (Eszik *et al.* 2016). We followed the accumulation of *Chlamydia*

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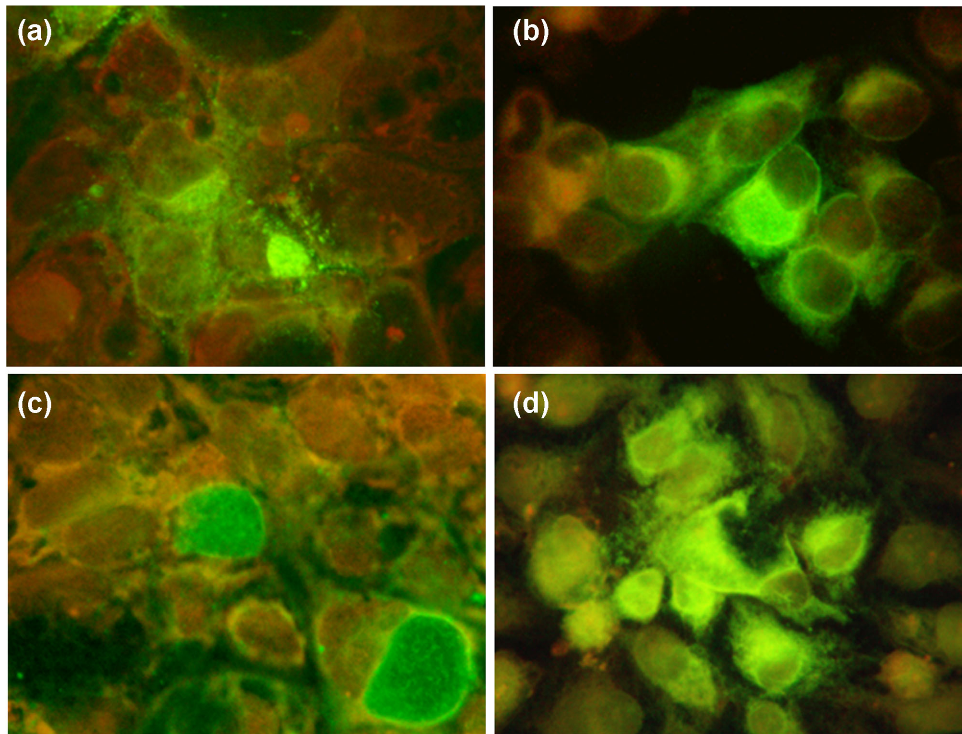

Figure 1. Immunofluorescence-stained inclusions of *C. trachomatis* D in Caco-2 and HeLa cells. The cells were grown on 13-mm coverslips, and the monolayers were infected at an MOI of 1. *Chlamydia trachomatis* D-infected Caco-2 cells were incubated for 24 h (**A**) or for 48 h (**C**), and *C. trachomatis*-infected HeLa cells were incubated for 24 h (**B**) and for 48 h (**D**). After the indicated times, the cells were stained by indirect immunofluorescence using anti-cLPS antibody and FITC-labeled anti-mouse IgG secondary antibody. Pictures were acquired by a digital camera attached to a fluorescence microscope using 625-fold magnification.

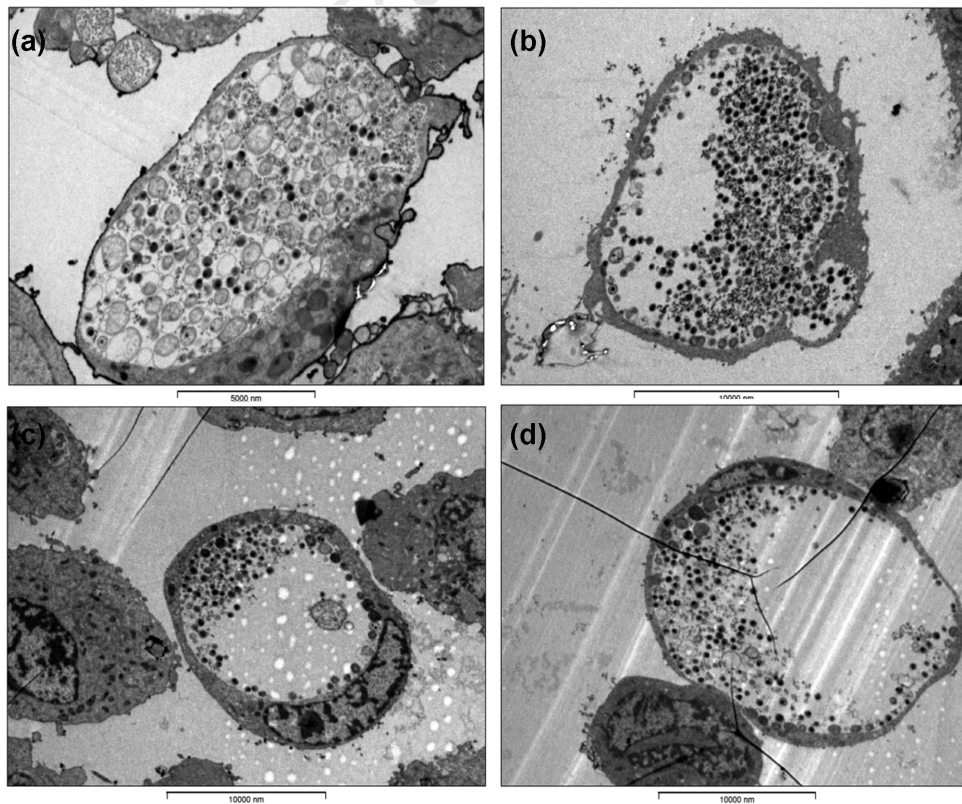

Figure 2. TEM images of *C. trachomatis* D inclusions in infected cells. Cells grown in 6-well plates were infected with chlamydiae at an MOI of 1. At the indicated time points after infection, the cells were fixed and processed for electron microscopy. Chlamydial inclusions in (**A**, **B**) *C. trachomatis* serovar D-infected Caco-2 cells 48 h post-infection; (**C**, **D**) *C. trachomatis* D-infected HeLa cells 48 h post-infection. Magnification is shown by the scale bars.

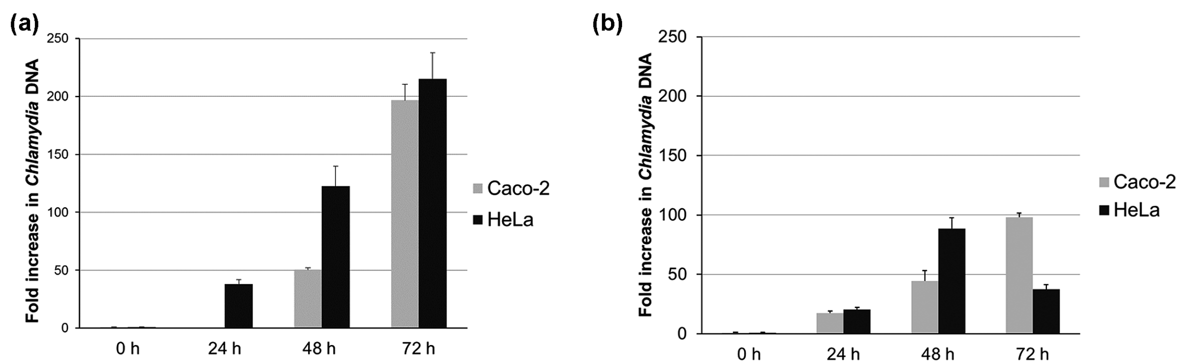

Figure 3. Analysis of *C. trachomatis* D growth based on the quantitation of chlamydial DNA by qPCR in *Chlamydia*-infected cells. Caco-2 and HeLa cells were infected in 96-well plates at an MOI of 5 in a medium with cycloheximide or without cycloheximide; direct detection of *Chlamydia* genes in the lysate of infected cells was done at 0, 24, 48 and 72 h post-infection. Increase in the quantity of chlamydial DNA was compared to the quantities detected at 0 h of infection. (**A**) Increase in the amount of *C. trachomatis* D *pyk* gene in the presence of cycloheximide, and (**B**) in the absence of cycloheximide. The mean of fold change in 3 parallel cultures and SD is shown. The data represent the results of one of three independent experiments.

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genomes during a 3-day culture period in Caco-2 and in HeLa cells. The lysates of infected cells were used as templates, and the quantity of chlamydial genomes was estimated with qPCR at different time points after infection. Fold increase in the amount of *pyk* gene of *C. trachomatis* was calculated in comparison with the amount detected at 0 h of infection. *Chlamydia trachomatis* growth seemed unrestricted in Caco-2 cells, *Chlamydia* genomes propagated to similar amount by 72 h as in HeLa cells (Fig. 3A). As suggested by the microscopic findings, the kinetics of replication followed a slower course in Caco-2 cells. In cycloheximide-free conditions, the yield was lower in Caco-2 cells and in HeLa cells too (Fig. 3B). In HeLa cells the replication peaked at 48 h and declined thereafter; the latter was not seen in Caco-2 cells. In Caco-2 cells, the effect of cycloheximide did not cause any major change in the course of the replication opposite to that in HeLa cells.

Production of infective *Chlamydia* progeny in Caco-2 and conventional host cells

In order to see whether the production of infective chlamydiae paralleled DNA accumulation, the infected cells were collected together with their supernatant, and the recoverable viable *C. trachomatis* bacteria were quantitated by inoculation of the sonicated cell in their media onto McCoy cells. Infective chlamydiae were recoverable showing that a full replication cycle takes place in Caco-2 cell line. The growth of *C. trachomatis* was somewhat delayed in Caco-2 cells, but at 72 h post-infection, similar amount of *C. trachomatis* was cultured from Caco-2 cells to that from HeLa cells (Fig. 4).

Transcript patterns for selected *Chlamydia* genes during infection of Caco-2 and conventional host cells

As the kinetics of *C. trachomatis* replication exhibited difference in Caco-2 cells compared to that in HeLa cells, we investigated the expression of selected *Chlamydia* genes during a 3-day period. Intrinsic characteristics of the intestinal epithelial cells as growth environment could be reflected in a change of gene expression pattern of chlamydiae. The relative gene expression levels normalized to 16S rRNA expression are shown in Fig 5. At 24 h post-infection, the earliest time point evaluated, the relative expression of the early cluster gene *euo* (Fig. 5A) was at much lower level in HeLa cells than in Caco-2 cells, and it increased at

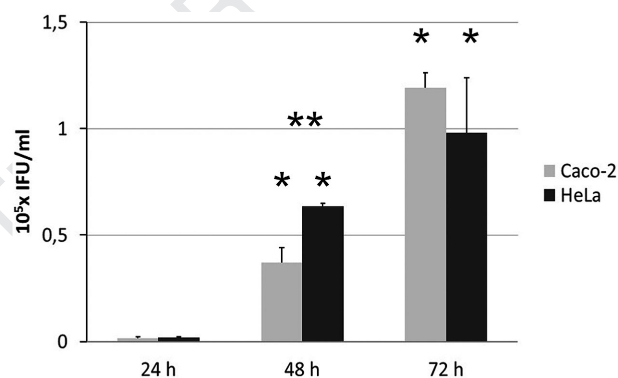
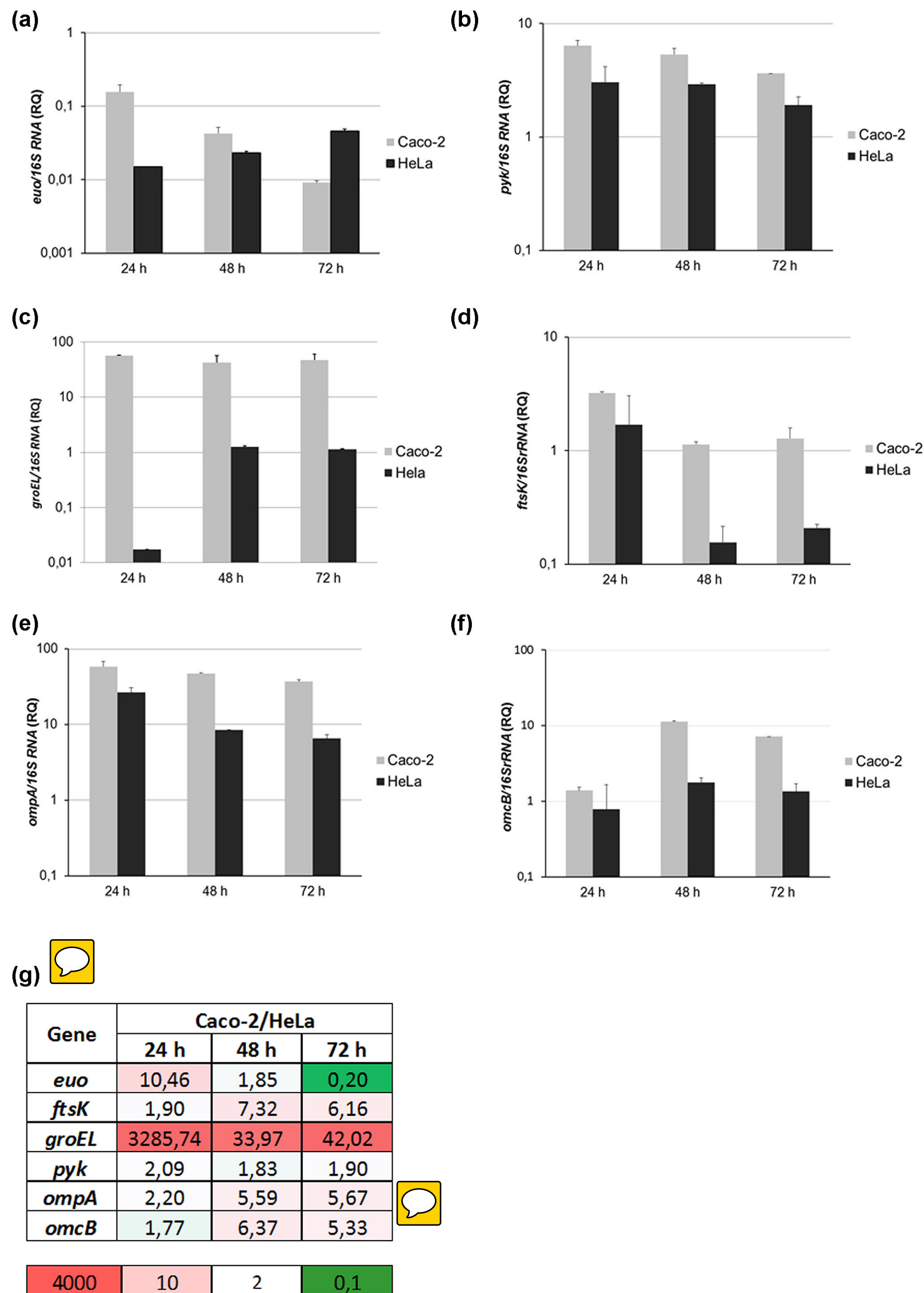


Figure 4. Kinetics of replication of *C. trachomatis* D in Caco-2 cells and in the conventional host cells as assayed by quantitation of recoverable infective bacteria. Lysates of *C. trachomatis*-infected (MOI 5) Caco-2 and HeLa cells in their supernatants were collected at different time points after infection, and they were inoculated onto McCoy cells grown on cover slips. After 48 h incubation, the inclusions were visualized by immunofluorescence staining. The mean of titers expressed as IFU/mL in 3 parallel cultures and SDs are shown. The single asterisk (*) shows statistically significant differences between IFU values in the same cell type at different time points; the double asterisk (**) indicates statistically significant difference between values measured in supernatants of different cell types: $P < 0.05$. The data represent the results of one of three independent experiments.

later time points, when the replication was already at lower rate. After 24 h, *euo* expression level moved to the opposite direction in Caco-2 cells in parallel with the continued replication.

The relative expression of *pyk*, *ompA*, *ftsK* and *omcB* (Fig. 5) genes of *C. trachomatis* D followed similar trends in both examined cell lines, but the relative expression persisted at higher levels in Caco-2 cells than in HeLa cells. After 24 h, the level of *pyk* gene expression decreased gradually over time at similar rates (Fig. 5B) in both examined cell lines. The highest level of *ompA* gene expression was observed at 24 h and persisted at high level for a longer time in Caco-2 than in HeLa cells (Fig. 5E). The highest level of *ftsK* gene expression was observed at 24 h after infection, at the time of frequent cytokinesis, and it remained at high level in Caco-2 cells at later time points too (Fig. 5D). The expression of the late gene *omcB* peaked at 48 h post-infection with again a higher level in Caco-2 cells than in HeLa cells (Fig. 5F).

Constantly, high relative amount of *groEL* transcripts was observed in Caco-2 cells, including the earliest time point tested at



Gene	Caco-2/HeLa		
	24 h	48 h	72 h
euo	10,46	1,85	0,20
ftsK	1,90	7,32	6,16
groEL	3285,74	33,97	42,02
pyk	2,09	1,83	1,90
ompA	2,20	5,59	5,67
omcB	1,77	6,37	5,33

4000	10	2	0,1

Figure 5. The time course of relative expression of different *C. trachomatis* D genes in Caco-2 cells and in conventional permissive HeLa cells. cDNA was prepared from Caco-2 and HeLa cells at different time points (24, 48 and 72 h) after infection with *C. trachomatis* D at an MOI of 5 in 6-well plates in a medium without cycloheximide. Real time qRT-PCR for quantitation of relative expression of (A) *euo*, (B) *pyk*, (C) *groEL*, (D) *ftsK*, (E) *ompA* and (F) *omcB* genes was performed; the expression levels were normalized to *16S rRNA* gene expression. The fold change in relative expression levels is shown (RQ). The data represent the mean values and SD measured in three parallel samples of one of two independent experiments. (G) Calculated ratios of relative gene expression values detected in Caco-2 cells versus in HeLa cells are shown. A bar indicating the color scale for calculated differences is given: boxes with different shades of red through white color denote increased expression; green colored boxes denote decreased expression.

24 h (Fig. 5C). An increased relative high rate of expression of *groEL* gene occurred only from 48 h post-infection in HeLa cells. Further analysis and summary of the above data are shown in Fig. 5G on the ratios of gene expression values seen in Caco-2 cells versus that in HeLa cells. These data demonstrate the delayed and prolonged replication cycle in Caco-2 cells with higher *euo* expression at early time point (24 h) and decreased *euo* expression at late time point (72 h), with higher cytokinesis related (*ftsK*) and membrane protein gene (*ompA*, *omcB*) expressions at later time points in Caco-2 cells. An outstanding *groEL* gene transcription, especially at 24 h post-infection in Caco-2 cells, is detectable.

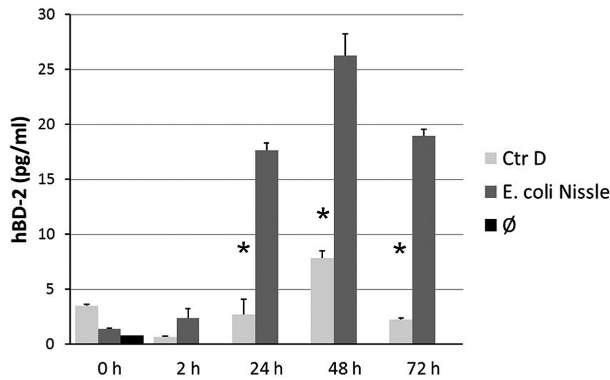


Figure 6. hBD-2 production by Caco-2 cells in response to *Chlamydia* infection. The supernatants of non-infected cells (ø) and the cells infected with *C. trachomatis* D (Ctr D) at an MOI of 5 or treated with heat-treated *E. coli* Nissle strain in 24-well plates were harvested at different time points post-infection. For the detection of hBD-2 production, the supernatants of the cells were tested by hBD-2 specific ELISA. The mean concentration values in three parallel cultures and SDs are shown. The asterisk (*) shows statistically significant differences between values measured in the supernatant of *Chlamydia*-infected cells and values measured in supernatants of *E. coli* Nissle-treated cells; $P < 0.05$.

HBD-2 inducing capability of *C. trachomatis* in Caco-2 cells

Since Caco-2 cells are intestinal mucosal epithelial cells, and this cell type has an important role as a physiological barrier for pathogens in the colon, and hBD-2 is induced by microbial molecules, it was investigated whether *C. trachomatis* was able to induce production of this antimicrobial peptide by Caco-2 cells. By hBD-2 ELISA, hBD-2 protein was detectable in high levels in the supernatant of Nissle-stimulated control Caco-2 cultures with a peak at 48 h post-treatment (Fig. 6). *Chlamydia trachomatis* D-infected cells showed similar time course of hBD-2 release, however at a significantly lower level than in the case of stimulation with *~~Escherichia~~ coli* Nissle.

DISCUSSION

In this study, we investigated the morphological and molecular features of *Chlamydia trachomatis* infection in Caco-2 human intestinal cells. We selected this cell line for our investigation because it shows the characteristics of enterocytes, and in recent publications, the hypothesis of chlamydial persistence in the GI tract and the infected GI tract behaving a source of genital tract reinfection has been proposed (Bavoil *et al.* 2017). Furthermore, *C. trachomatis* has been implicated in GI tract pathologies. As hBD-2 is produced by epithelial cells in the GI tract in response to infection as part of the innate defense (~~Eduardo R.~~ Cobo and ~~Kris~~ Chadee 2013), we tested the defensin inducing capability of *Chlamydia* in Caco-2 cells.

Chlamydia trachomatis D inclusions in Caco-2 cells visualized by immunofluorescence staining and TEM suggested similar but prolonged replication cycle of this *Chlamydia* strain compared to that in HeLa cells. The slower accumulation of chlamydiae was also detectable by finding lower amount of genomes and infective chlamydiae at 48 h post-infection in Caco-2 cells. However, by 72 h post-infection, the values had reflected similar level of chlamydial reproduction in the two cell lines. These results allow us to conclude that for this human genital *Chlamydia* strain, Caco-2 cells provide a growth-conducive environment. In case, the growth medium of the host cells is supplemented with cycloheximide, *Chlamydia* replication cycle occurs in optimal conditions, where the host cell protein synthesis has less influence on the bacterial growth. When cycloheximide was omitted from the medium, chlamydial genome accumulation reached a lower level and suffered early decline in HeLa cells, but this phenomenon was not as pronounced in Caco-2 cells. Gene expression analyses were done in the absence of cycloheximide, when the natural cellular environment would prevail.

It has been described that chlamydial developmental cycle is regulated at transcriptional level (Nicholson *et al.* 2003). The change in the transcription profile has been demonstrated in *in vitro* models, where different stimuli (IFN-γ, penicillin) induced a persistent phase of chlamydiae. Certain cell types proved to be non-permissive for normal *Chlamydia* growth, and the transcription pattern suggested a persistent form of infection in these cells (Gérard *et al.* 1998). Since the growth kinetics in Caco-2 cells was found different from that in the optimal *in vitro* host cell line, we aimed to examine the expression of *C. trachomatis* genes in Caco-2 cells and compared it to that in HeLa cells. The expression level of selected chlamydial genes representing early (*euo*), mid-cycle (*ompA, groEL, pyk*) and late stage (*omcB*) replication cycles, and cell division~~s~~ related *ftsK* gene were analyzed and was normalized to the expression level of *16S rRNA* gene. As the *eou* gene products were described as suppressors of the late genes (Rosario and Tan 2012; Rosario, Hanson and Tan 2014), decreased expression of this gene could explain the higher expression level of *omcB* gene encoding the membrane protein of EBs (~~Eduardo R.~~ Cobo and ~~Kris~~ Chadee 2013) in Caco-2 cells, even at later time points during the infection. As EUO protein binding to *ftsK* promoter has been detected earlier (Rosario and Tan 2012), the persisting high level of the cytokinesis related *ftsK* gene might be associated with this decreased *euo* expression in Caco-2 cells. Transcripts from the *ftsK* gene required for cytokinesis were detectable at a relatively high level during the first day of *C. trachomatis* growth, when bacterial division occurs at the highest rate. It seems that cytokinesis dropped earlier in HeLa cells than in Caco-2 cells. The expression of the ATP synthesis-related *pyk* gene of *C. trachomatis* declined in HeLa cells after 48 h. It occurred synchronously with the decrease in the amount of the chlamydial chromosome detected in this cell line. In concordance, longer high level of *pyk* expression paralleled a more extended genome accumulation in Caco-2 cells. The tendency of the change in *ompA* gene expression level was similar in both cell lines. *pyk* and *ompA* genes are regarded as genes of the mid-cycle (Nicholson *et al.* 2003), when RB accumulate and EBs start to be formed. The *groEL* gene of *C. trachomatis*, a heat shock protein 60 (GroEL-1) encoding gene was highly upregulated throughout the observation period in Caco-2 cells. A stress response related increase in the production of GroEL-1 has been described for *C. trachomatis* (Gérard *et al.* 2004). Our results suggest that replication of *C. trachomatis* in Caco-2 cells evoked a certain stress response by the bacteria.

It has been suggested that in the male and female reproductive tracts, small antibacterial molecules may contribute to a sustained inflammatory response (Redgrove and McLaughlin 2014), or they may control inflammatory and adaptive immune response (Hemshekhar, Anaparti and Mookherjee 2016). According to Niyonsaba *et al.* (Niyonsaba, Ogawa and Nagaoka 2004), hBD-2 can be regarded as a potent chemoattractant of neutrophils. Increased hBD-2 expression could contribute to host defense by recruiting neutrophils. The role of hBDs in the attraction of immature dendritic cells and memory T cells to the site of microbial invasion (Yang *et al.* 1999) has been described. Expression of hBD has been reported in women with *C. trachomatis* positive cervicovaginal samples, albeit at a significantly lower

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level than in non-infected controls (Noda-Nicolau *et al.* 2017). In our experiments with Caco-2 cell line, *C. trachomatis* infection induced the production of hBD-2 at a moderate level compared to the effect of a strong inducer, the *E. coli* Nissle strain. In a murine model of *Chlamydia muridarum* infection, oral application of the bacterium resulted in long carriage and a lack of inflammatory response in the large intestine; however, the infection of the genital tract was cleared after a short period and following inflammation at the infection site (Igietseme, Portis and Perry 2001). The low level of hBD-2 production by the *Chlamydia*-infected Caco-2 cell line might be a sign of suppressed innate immune response, and potentially, a subsequently suppressed adaptive response as well.

In conclusion, Caco-2 cell line representing the epithelial cell lining in the large intestine seems to be a sufficiently permissive host cell for *C. trachomatis*, which primarily infects the genital tract, thus allowing these bacteria to survive at this body site. Together with the low hBD-2 inducing capacity, *C. trachomatis* might be able to replicate there without provoking an inflammatory response. These results seem to support the theory that the GI tract could serve as a site of extra genital survival of chlamydiae, and it could potentially serve as a source of reinfection in the genital tract, especially in women. Our data have relevance to the published data on high co-occurrence of urogenital *Chlamydia* infection with anorectal infection in women (van Liere *et al.* 2014). Our data are also in concordance with the previously published data that more infected men are detected among homosexuals when rectal samples were taken (Bax *et al.* 2011).

Conflicts of interest. None declared.

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GRAPHICAL ABSTRACT

Our analyses of growth characteristics of *Chlamydia trachomatis* in intestinal epithelial Caco-2 cells support the possibility of the gastrointestinal tract behaving as a source of reinfection of the urogenital tract.