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

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**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.

**ABSTRACT**

*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. 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 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**

*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 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). 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), antibiotics (Kintner et al. 2014; Phillips-Campbell, Kintner and Schoborg 2014), iron depletion (Mäurer et al. 2007), and it could...
also be induced in different cell types, e.g. in monocytes (Datta et al. 2014).

Chlamydia 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, infections 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 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 persistence 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 x nonessential amino acids, 25 μg/mL gentamicin and 0.5 μg/mL fungizone. The cell lines were purchased from American Type 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; Burían 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 x 10^6 cells/well), 96-well (4 x 10^4 cells/well), or 24-well culture plates (2.5 x 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% CO2 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 x 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 CO2 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 x 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 x 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.
Table 1. Primer sequences used in qRT-PCR analysis.

<table>
<thead>
<tr>
<th>C. trachomatis target gene</th>
<th>Sequence</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA F</td>
<td>5’-CACAGGATAGCTGACGGCCTTGGTTTT-3’</td>
<td>191</td>
</tr>
<tr>
<td>16S rRNA R</td>
<td>5’-ACTAAAGAAAGGTTTGGTGGCGAAGCCTGTG-3’</td>
<td>263</td>
</tr>
<tr>
<td>evo F</td>
<td>5’-TCCCTGCTGCTCTCTCTGC-3’</td>
<td>70</td>
</tr>
<tr>
<td>evo R</td>
<td>5’-TCGGAAAGAAGCAAGGCTTTCC-3’</td>
<td>92</td>
</tr>
<tr>
<td>ftsK R</td>
<td>5’-GGGCTGAGTACCCGGATTTTAAC-3’</td>
<td>115</td>
</tr>
<tr>
<td>groEL F</td>
<td>5’-ACTAAGCTGACGGCCTTGGTTTT-3’</td>
<td>171</td>
</tr>
<tr>
<td>groEL R</td>
<td>5’-TCGGAAAGAAGCAAGGCTTTCC-3’</td>
<td>179</td>
</tr>
<tr>
<td>omcB R</td>
<td>5’-ACCGGATCTTGGACAGGGCAT-3’</td>
<td>81</td>
</tr>
<tr>
<td>ompA R</td>
<td>5’-CGGATCTCAGATGATGCAAAGGCG-3’</td>
<td>191</td>
</tr>
<tr>
<td>pyk F</td>
<td>5’-GGTGCACAGATGCGCTTGGGAAAGGTT-3’</td>
<td>170</td>
</tr>
<tr>
<td>pyk R</td>
<td>5’-CTGGAGGATCTCAGATGCGCTTGGGAAAGGTT-3’</td>
<td>191</td>
</tr>
</tbody>
</table>

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 after infection (3 parallel cultures at each time point) with GenE-180 strain (MOI of 100), a potent inducer of hBD-2, was used as positive control. 

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).

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.
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.
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 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. 3).

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 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
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.
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 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-related fsK gene were analyzed and was normalized to the expression level of 16S rRNA gene. As the euo 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 fsK promoter has been detected earlier (Rosario and Tan 2012), the persisting high level of the cytokinesis related fsK gene might be associated with this decreased euo expression in Caco-2 cells. Transcripts from the fsK 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 chemotactant 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
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 chlamydial, 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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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.