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ORIGINAL ARTICLE

Impact of antiseptics on *Chlamydia trachomatis* growth

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Significance and Impact of the Study: We measured the antichlamydial effects of various antiseptics. These antiseptics are being used for the treatment of bacterial vaginosis, but their effect on the bacterial vaginosis-related sexually transmitted infections, particularly the most frequent *Chlamydia trachomatis* (*C. trachomatis*) infections has not been investigated. We showed that povidone-iodine (Betadine) inhibited the chlamydial growth in concentrations that was not toxic to the epithelial cells. We concluded that due to its additional antichlamydial effect, povidone-iodine could be a preferable antiseptic in bacterial vaginosis treatment.

Keywords
antiseptic, Betadine, Chlamydia, chlorhexidine, growth, povidone-iodine, quantitative PCR.

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Abstract

Bacterial vaginosis is a frequent dysbiosis, where the normal lactobacillus-dominated flora is replaced by an anaerob/aerob polymicrobial flora. Bacterial vaginosis increases the risk of acquiring sexually transmitted infections (STI) including the most frequent *Chlamydia trachomatis* infections. Intravaginal antiseptics are part of the bacterial vaginosis treatment, and ideally they should also inhibit the bacterial vaginosis-related STI. Therefore, we tested the antichlamydial activity of four antiseptics: iodine aqueous solution, povidone-iodine, chlorhexidine and borax. First, we measured the impact of antiseptics on the viability of the HeLa cervical epithelial cells, and calculated the maximum nontoxic concentrations. Next, we infected the cells with *C. trachomatis* preincubated for 1 h with the particular antiseptic. The chlamydial growth was measured by direct quantitative PCR (qPCR) of the infected cells. The minimal inhibitory concentrations (MIC) of chlorhexidine and povidone-iodine were 3·91 and 97 μg ml^{-1} respectively; however, the MIC of chlorhexidine was close to its maximum nontoxic concentration. The iodine aqueous solution and the borax showed no antichlamydial activity. Our *in vitro* studies showed that chlorhexidine and particularly povidone-iodine are potentially able to limit the bacterial vaginosis-related *C. trachomatis* infection.

Introduction

Chlamydia trachomatis is an obligate intracellular bacterium with a cell tropism to epithelial cells of the conjunctiva and the urogenital tract. The bacterium is particularly capable of establishing persistent infections and chronic inflammation. The local inflammation could lead to tarsus and oviduct fibrosis potentially leading to blindness and infertility. Sexually transmitted infections (STI) caused by the urogenital tract pathogens, *C. trachomatis* serovars D-K and L1-L3 are the most frequent STIs in the world (CDC Sexually Transmitted Disease Surveillance 2013; http://www.cdc.gov/std/stats13/std-trends-508.pdf). While antiseptics are not part of the antichlamydial chemotherapy, they have various intravaginal applications including the prevention of postoperative infections before caesarean section (Memon *et al.* 2011; Haas *et al.* 2013), trans-vaginal ultrasound-guided ovum pick-up (Bhandari *et al.* 2015), surgical treatment of HPV-generated cervical lesions (Gerli *et al.* 2012) and other invasive procedures (Gornall *et al.* 1999; Velasco *et al.* 2009), and prevention of early-onset neonatal group B streptococcal infection during labour (Ohlsson *et al.* 2014).

Besides these applications, antiseptics are being used to prevent/treat prechlamydial states such as bacterial vaginosis. Bacterial vaginosis is a state where the normal lactobacillus flora of the vagina is disappearing and is replaced by a polybacterial flora. Several studies showed that bacterial vaginosis is a significant risk factor of acquiring STIs including chlamydial STIs with a relative risk of 2·0–3·4 (Wiesenfeld *et al.* 2003; Aghaizu *et al.* 2014; Abbai *et al.* 2015). The connection between bacterial vaginosis and chlamydia infection is not clear, but one of the possibilities is the increased indole production of the bacterial vaginosis-related bacteria (Aiyar *et al.* 2014). Urogenital *C. trachomatis* serovars likely propagate in a tryptophan-deprived environment due to the interferon-gamma-induced indole 2,3-dioxygenase, but the bacterium can survive by producing tryptophan from the exogenous indole. The treatment of bacterial vaginosis is complex including the antibiotics metronidazole, clindamycin; the antiseptics benzydamine, chlorhexidine, hydrogen peroxide, povidone-iodine, borax (Reichman *et al.* 2009; Novakov Mikic and Budakov 2010; Verstraelen *et al.* 2012); acidification; prebiotics and recolonizing the cervicovaginal region with lactobacilli (Donders *et al.* 2014). Despite these treatments the long-term cure rate of bacterial vaginosis can be as low as 20–50% (Sobel *et al.* 2006; Mastromarino *et al.* 2013), therefore search for more effective therapies including effective antiseptic therapies is necessary. Importantly, an ideal antiseptic should treat the bacterial vaginosis and inhibit the underlying STIs, including the frequent chlamydial STI.

To test the antichlamydial activity of frequently used antiseptics, we infected HeLa cervical epithelial cells with *C. trachomatis* in the presence of iodine aqueous solution, povidone-iodine, chlorhexidine and borax. For chlamydial growth measurement we used the recently published direct qPCR-based approach (Eszik *et al.* 2016).

Results and discussion

Cell viability of HeLa cells incubated with antiseptics

First, we tested the long-term impact of the antiseptics on the viability of the HeLa human cervical epithelial cells. HeLa cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay (MTT assay) after 48 h of incubation with the particular antiseptic dissolved in cell culture medium (Fig. 1a–d). Maximum cytotoxicity was observed at concentrations of =>1562 μg ml^{-1} for povidone-iodine, while the viability reached its maximum at concentration of =<390 μg ml^{-1}. We considered this value the maximum nontoxic concentration. Maximum cytotoxicity was observed at concentrations of =>8 μg ml^{-1} for chlorhexidine, its maximum nontoxic concentration was 2 μg ml^{-1}. Maximum cytotoxicity was observed at concentrations of 800 μg ml^{-1} for iodine aqueous solution, its maximum nontoxic concentration was 200 μg ml^{-1}. Maximum cytotoxicity was observed at concentrations of 800 μg ml^{-1} for borax, its maximum nontoxic concentration was 25 μg ml^{-1}, however, the viability reached 80% at 400 μg ml^{-1}.

Direct qPCR measurement of the impact of antiseptics on *Chlamydia trachomatis* growth

The direct qPCR method was used to determine the antichlamydial activity of antiseptics (Fig. 2a–d). HeLa cells were infected with *C. trachomatis* multiplicity of infection 8 (MOI 8) after preincubation (37°C, 1 h) with serial 1 : 2 dilutions of povidone-iodine, chlorhexidine, iodine aqueous solution and borax starting with the maximum nontoxic concentrations. The antiseptics' minimal inhibitory concentrations (MIC) were calculated as it was described before (Eszik *et al.* 2016): briefly, the chlamydial DNA concentrations (threshold cycle (C_t) values) measured in the three parallel wells of a given antiseptic concentration were compared with the C_t values measured in the three parallel wells of the highest antiseptic concentration (we considered it as the inoculum) using Student's *t*-test. The lowest antiseptic concentration, where the C_t values did not change significantly compared with the inoculum was considered the minimum inhibitory concentration (MIC) value. The MIC value of povidone-iodine was 97 μg ml^{-1}, the MIC value of chlorhexidine was approximately 4 μg ml^{-1}. The iodine aqueous solution and the borax did not show antichlamydial activity in the tested concentration range. The difference of antichlamydial activity of iodine and povidone-iodine was striking, considering that the microbiologically active compound was the iodine in both substances. Povidone-iodine is an iodophor compound where the free iodine is complexed with the amphipathic polyvinyl pyrrolidone polymer. There are several differences between iodine aqueous solution and povidone-iodine. Povidone-iodine is more stable, releases the iodine more slowly and importantly could bring the iodine close to membranes due to its amphipathic nature. The increased membrane solubility could be one of the reasons of the higher antichlamydial activity. *Chlamydia trachomatis* is a Gram-negative bacterium, with an outer membrane containing a variety of proteins possibly involved in attachment/entry such as MOMP, polymorphic outer membrane proteins B-H, and other proteins involved in the early manipulation of the host cells, such as the type III secretion system ring protein PulD/YscC (Birkelund *et al.* 2009; Liu *et al.* 2010). Interfering with the functions of these proteins

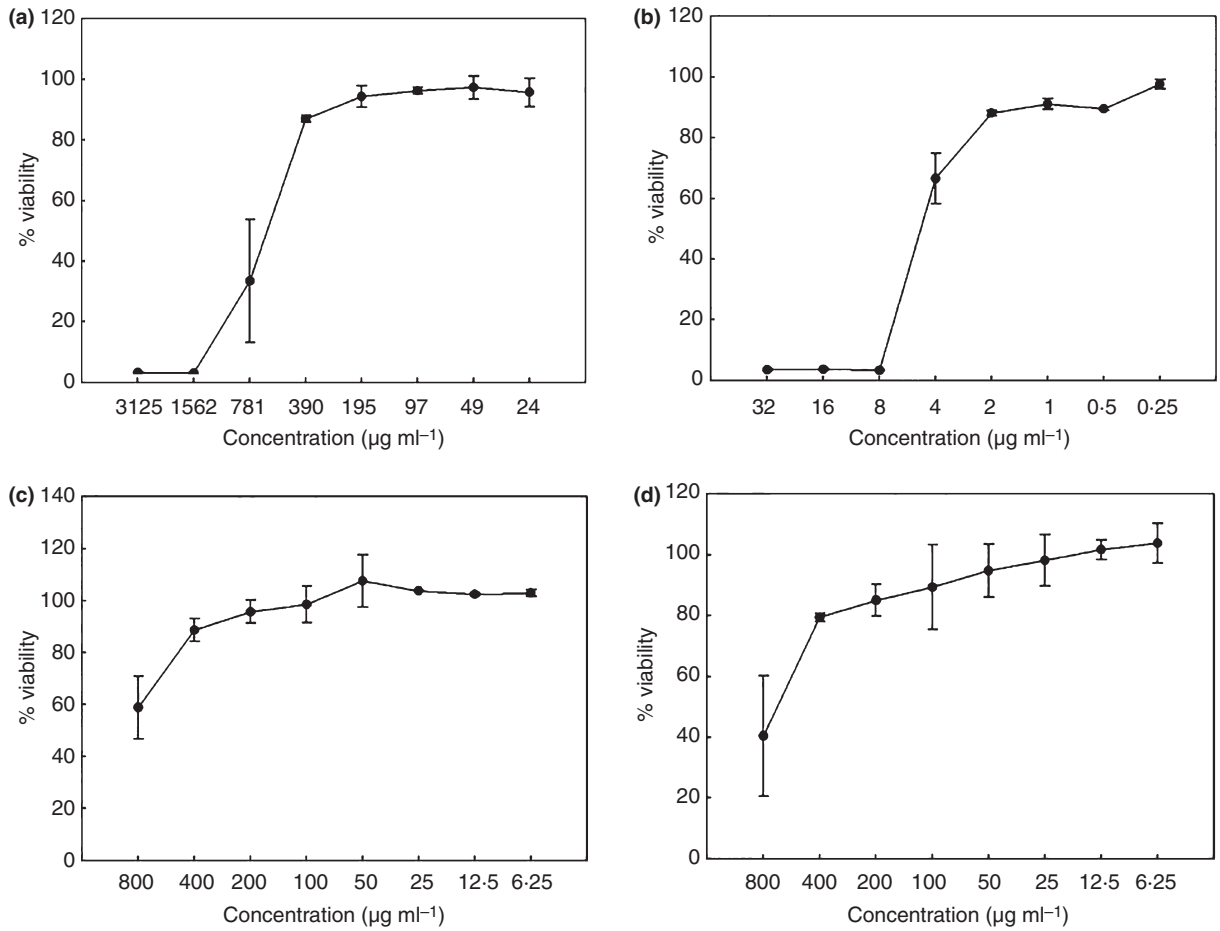

Figure 1 MTT cell viability assay of HeLa cells incubated for 48 h with (a) povidone-iodine, (b) chlorhexidine, (c) iodine aqueous solution and (d) borax dissolved in cell culture medium. Viabilities of the treated cells were compared to the untreated controls. MTT assay was performed as described in the Materials and methods. Three parallel measurements were performed for each antiseptic concentration. Data are means ± standard deviations.

directly or by destabilizing the outer membrane could lead to decreased infectivity. The measured MIC of the chlorhexidine was lower than that of the povidone-iodine; however, its MIC was close to the 4 μg ml^{-1} concentration that decreased the viability of the epithelial cells after 48 h incubation. These data indicate, that the chlorhexidine could be an effective antichlamydial agent, but should be applied as a short-term rinsing, rather than as a long-term vaginal gel. On the other hand, the povidone-iodine had a MIC of 97 μg ml^{-1}, while its maximum nontoxic concentration was 390 μg ml^{-1}, suggesting that this antiseptic can be applied long-term intravaginally. Also, it should be noted that the longer presence of povidone-iodine and its longer interaction with the chlamydial elementary bodies might result in an even lower MIC measured after the 1 h coincubation.

Estimation of the qPCR inhibitory activity of the antiseptics

Since the growth-related chlamydial DNA synthesis was measured by a qPCR method, we tested whether the applied antiseptics had a direct inhibitory impact on the qPCR. This effect could appear as a false-positive antichlamydial activity. We mixed cell lysates of HeLa cells infected with untreated *C. trachomatis* with cell lysates from uninfected HeLa cells treated with the maximum concentration of antiseptic applied for the Chlamydia inhibition. If there was no PCR inhibition then the C_t level of the 1 : 1 mixture (basically a twofold dilution of the chlamydial DNA) of the infected and uninfected but antiseptic containing cell lysates would have been approx. 1 cycle higher (50% less chlamydial DNA concentration) than the *C. trachomatis* infected cells lysate's alone. The

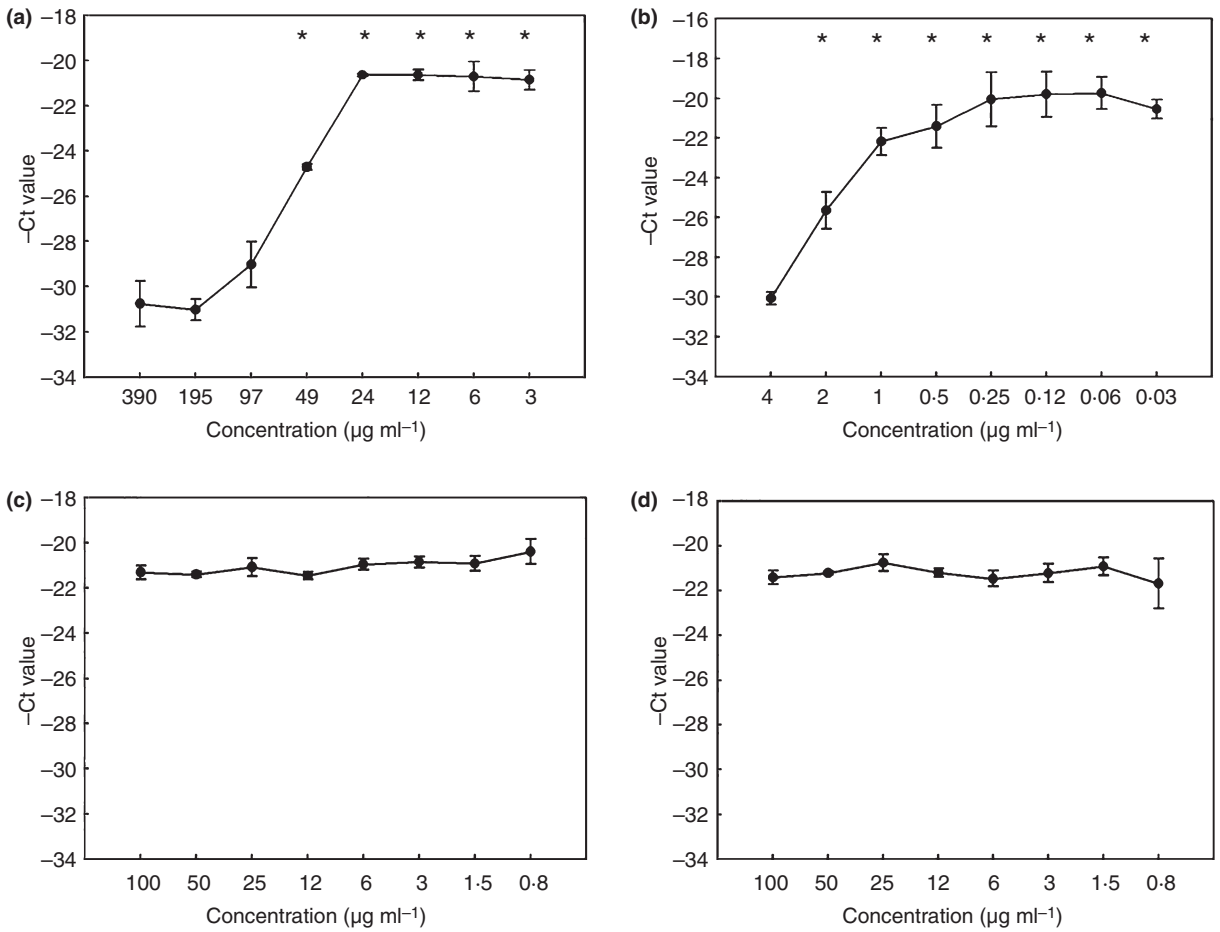

Figure 2 Measurement of antichlamydial activity of the antiseptics by direct qPCR. HeLa cells were infected with *Chlamydia trachomatis* (MOI 8) preincubated with various concentrations of (a) povidone-iodine, (b) chlorhexidine, (c) iodine aqueous solution and (d) borax for 1 h 37°C. Each infection at a particular antiseptic concentration was performed in three parallel wells. At 48 h post infection, the cells were lysed and the chlamydial DNA concentration was measured by direct qPCR. Data are the average $-C_t$ values ± standard deviations. *: the C_t values are significantly different ($P < 0{\cdot}05$) from the values measured in the presence of the highest concentration of antiseptics using Student's *t*-test.

C_t levels of the povidone-iodine, chlorhexidine, iodine aqueous solution and borax mixtures were 1·33, 0·79, 0·85 and 1·28 cycles higher than the untreated *C. trachomatis*-infected cell lysate's (Fig. 3). These results indicate that the antiseptics did not inhibit markedly the qPCR and the observed Chlamydia growth inhibition of povidone-iodine and chlorhexidine could not be due to the inhibition of the qPCR itself.

ChlamyCount immunofluorescent measurement of the impact of antiseptics on *Chlamydia trachomatis* growth

To validate the qPCR results with an independent chamber slide infection system method (Bogdanov *et al.* 2014), we performed *C. trachomatis* infections (MOI 8) in the presence of the antiseptics with the highest concentrations used for qPCR. Infected and control cells were fixed 48 h post infection, and the chlamydial inclusions were labelled with an Alexa-647-labelled anti-chlamydia LPS antibody. The slide was scanned with a DNA-chip scanner, and chlamydial inclusions were enumerated by the ChlamyCount software (Fig. 4). ChlamyCount inclusion number data showed that the povidone-iodine and chlorhexidine treatment decreased the chlamydial inclusion number approximately 94 and 94%, respectively, while the iodine aqueous solution and borax decreased 13 and 43%, respectively the number of chlamydial inclusions. These data validated the qPCR measurements in the case of povidone-iodine and chlorhexidine, although the extent of detected growth reduction was lower than that determined by qPCR. The reason could be that the dynamic range of our immunofluorescent ChlamyCount method

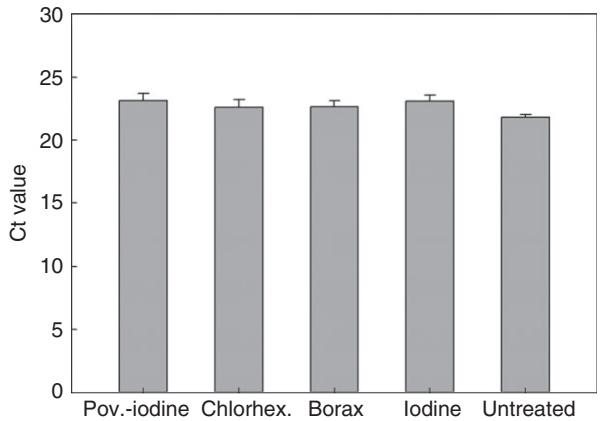

Figure 3 Estimation of the qPCR inhibition by the antiseptics. Cell lysates of HeLa cells infected with untreated *Chlamydiatrachomatis* (MOI 8) mixed with cell lysates from uninfected HeLa cells treated with SPG solution containing 390 μg ml^{-1} povidone-iodine, 4 μg ml^{-1} chlorhexidine, 100 μg ml^{-1} borax and 100 μg ml^{-1} iodine aqueous solution respectively. Untreated *C. trachomatis* (MOI 8)-infected cells are also processed (n = 3). Data are the average C_t values ± standard deviations.

was about 2 $\log_{10}$ (Bogdanov *et al.* 2014), while the qPCR method's dynamic range was approx. 5 $\log_{10}$ (Eszik *et al.* 2016). Different from the qPCR results, the ChlamyCount method also showed a limited chlamydial growth inhibition in the case of iodine aqueous solution and borax. The fact that the chlamydial DNA synthesis remained constant after the application of these latter two compounds, although the inclusion numbers slightly decreased, might indicate that a small portion of the iodine aqueous solution and borax-treated chlamydial EBs become persistent, maintaining the chlamydial DNA synthesis (Gérard *et al.* 2001; Belland *et al.* 2003), but formed smaller/less intense inclusions that was not detected by the ChlamyCount method.

In conclusion, our results showed that povidone-iodine had the widest antichlamydial therapeutic index and could maintain an antichlamydial effect when used intravaginally. Intravaginal povidone-iodine has already been used as a preoperative antiseptic to reduce postsurgery endometritis after caesarean sections (Asghania *et al.* 2011) and histerectomy (Sowapat *et al.* 2006) and as a treatment of bacterial vaginosis. Povidone-iodine also can be attractive in bacterial vaginosis treatment because it has no significant antimicrobial activity against lactobacilli (Sakakura *et al.* 1993; Wewalka *et al.* 2002). Since *C. trachomatis* infection could be linked to bacterial vaginosis, povidone-iodine may treat/limit these two clinical entities at the same time. Also it is worth to note, that povidone-iodine and chlorhexidine has been used intravaginally at a significantly higher concentrations than we used in our *in vitro* tests (van der Meijden *et al.* 1987; Onderdonk *et al.* 1992; Yu and Tak-Yin 1993). Since the maximum *in vivo* tolerable/nontoxic concentrations of these compounds are higher than in our *in vitro* toxicity assay, this could result in an even higher antichlamydial effect *in vivo*.

Materials and methods

Chlamydia strain propagation, HeLa cell culture

Chlamydia trachomatis serovar D strain (UW-3/CX, ATCC) was used, the strain was propagated and partially purified as described previously (Sabet *et al.* 1984). HeLa 229 cells (ATCC) were transferred into 96-well plates (Sarstedt, Nümbrecht, Germany) at a density of 6×10^4 cells per well in 100 μl of minimal essential medium (MEM) with Earle salts supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol l^{-1} L-glutamine, 1× MEM vitamins, 1× nonessential amino acids, 0·005% Na-pyruvate, 25 μg ml^{-1} gentamycin, 1 μg ml^{-1} fungisone. HeLa cells were incubated overnight at 37°C, 5% CO_2 to obtain a 90% confluent cell layer.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was performed to characterize the maximum nontoxic concentration of the antiseptics. HeLa cells were transferred into the wells of the 96-well plate (Sarstedt) at a density of 6×10^4 cells per well in 100 μl of culture medium (see above). The slides were incubated for 1 h at room temperature (RT) and then overnight at 37°C, 5% CO_2 to obtain a 90% confluent cell layer. Next day the medium was supplemented with the serial twofold dilutions of the antiseptics in three parallel wells for each concentration. After 48 h of incubation, 10 μl of the MTT (SIGMA, St. Louis, MO) labelling reagent (final concentration 0·5 mg ml^{-1}) was added to each well. The plate was incubated for 4 h at 37°C, 5% CO_2. After the incubation, 100 μl of the solubilization solution (10% SDS in 1 N HCl) was added into each well. The plate was allowed to stand overnight in the incubator at 37°C, 5% CO_2. Next day the optical density of the wells were measured by a microtitre plate reader (Labsystems Multiskan Ex 355; Thermo Fisher Scientific, Waltham, MA). The absorbance of the formazan product was measured at 540 nm. The average viability (OD 540) of three wells with untreated HeLa cells was considered the 100% viability. Viabilities of the treated cells were compared to the untreated controls as follows: Cell Viability (%) = (OD 540 of treated cells/OD 540 of untreated cells) × 100.

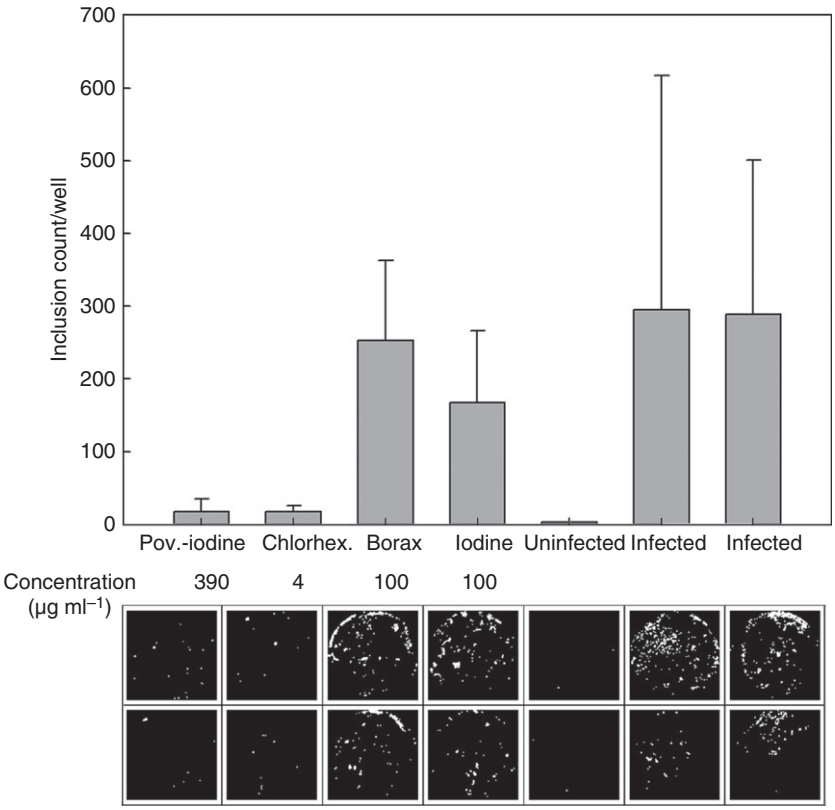

Figure 4 Measurement of antichlamydial activity of the antiseptics by immunofluorescent detection of the chlamydial inclusions. HeLa cells were infected with *Chlamydia trachomatis* (MOI 8) in the presence of the tested antiseptics. Four untreated *C. trachomatis* infected wells and uninfected wells were also included as controls. Each infection with a particular antiseptic was performed using parallel wells. The chlamydial inclusions were enumerated by the ChlamyCount software. The ChlamyCount processed well images and the counted inclusion numbers are shown. Data are means ± standard deviations.

Antiseptics used for *Chlamydia trachomatis* growth inhibition, *Chlamydia trachomatis* infection and DNA extraction

Iodine aqueous solution, povidone-iodine (Betadine, Egis, Budapest, Hungary), chlorhexidine-digluconate (Chlorhexamed; GlaxoSmithKline, Brentford, UK) and borax were diluted in sucrose-phosphate-glutamic acid buffer (SPG). Concentration ranges of 100–0·78 μg ml^{-1} for iodine aqueous solution and borax, 390–3 μg ml^{-1} for povidone-iodine and 4–0·003 μg ml^{-1} for chlorhexidine with two-fold dilutions were tested. Before infection, *C. trachomatis* elementary bodies were incubated in the antiseptics for 1 h, 37°C, 5% CO_2. HeLa cells were washed twice with 200 μl per well of phosphate-buffered saline (PBS), pH 7·4 and were infected at MOI 8 in 100 μl 0·5% (w/v) glucose medium for 60 min, RT. The cells were washed twice with PBS and culture medium containing 1 μg ml^{-1} cycloheximide was added. The plates were incubated at 37°C, 5% CO_2 for 48 h. The cells were washed with PBS twice, resuspended in 100 μl Milli-Q (MQ) water (Millipore, Billerica, MA), and subjected to two freeze–thaw cycles and mixing as it was described before (Eszik *et al.* 2016). The mixed lysates were used as a template in the qPCR. All reagents were purchased from SIGMA, unless otherwise indicated.

Direct quantitative PCR (qPCR)

Direct qPCR was performed as described before (Eszik *et al.* 2016) in a Bio-Rad CFX96 real-time system, using the SsoFast™ EvaGreen® qPCR Supermix (Bio-Rad, Hercules, CA) master mix and the *C. trachomatis* primer pairs *pykF*: 5′-GTTGCCAACGCCATTTACGATGGA-3′, 5′-TGCATGTACAGGATGGGCTCCTAA-3′. The PCR mixture consisted of 5 μl SsoFast™ EvaGreen® supermix, 1–1 μl forward and reverse primers (10 pmol each), 1 μl template and 2 μl MQ water to 10 μl final volume. After the 10 min at 95°C polymerase activation step, 40 PCR cycles of 20 s at 95°C and 1 min at 64°C were performed, with measurement of the fluorescence intensity at the end of the annealing–extension step. The qPCR ended with a melting curve analysis. Student's *t*-test has been used to

compare the statistical differences in C_t values between the two experimental conditions as it was described before (Eszik *et al.* 2016).

Immunofluorescent monitoring of *Chlamydia trachomatis* growth

Chlamydia trachomatis growth was evaluated by immunofluorescent staining as it was described before (Bogdanov *et al.* 2014). Briefly, semiconfluent layers of HeLa cells (6×10^4 cells per well) in chamber slides (Lab-Tek chamber slide system; Thermo Fisher Scientific) were infected with untreated *C. trachomatis* MOI 8 (preincubated in SPG buffer at 37°C for 1 h) or *C. trachomatis* preincubated with iodine aqueous solution (100 μg ml^{-1}), povidone-iodine (390 μg ml^{-1}), chlorhexidine (4 μg ml^{-1}) and borax (100 μg ml^{-1}) in SPG buffer at 37°C for 1 h. Before infection the wells were washed with 200 μl per well of PBS. After removal of the PBS solution, the treated and untreated chlamydiae were added and the cells were incubated at 37°C under 5% CO_2 for 1 h. After infection, the inocula were replaced with a culture medium containing 1 μg ml^{-1} cycloheximide and were incubated at 37°C, 5% CO_2 for 48 h. After removing the culture medium from the slides, the cells were washed twice with PBS (200 μl per well). After detaching the chamber structure from the slides, the cells were fixed with precooled 100% acetone at −20°C for 10 min. Anti-chlamydia LPS antibody (AbD Serotec, Oxford, United Kingdom) labelled with Alexa-647, was used at 1 : 200 dilution for detection of chlamydial inclusions. Following an incubation of 1 h at 37°C, the cells were washed three times with PBS for 7 min and at last with distilled water. Fluorescence signals were analysed with an Axon GenePix Personal 4100A DNA chip scanner and GenePix Pro (ver. 6.1) software (Molecular Devices, Sunnyvale, CA) using the Cy5 channel and a 5-μm resolution. Inclusion counts were determined by the ChlamyCount software as it was described before (Bogdanov *et al.* 2014).

Conflict of Interest

The authors declare no potential conflicts of interest.

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