AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

In Vitro Activity of Quaternary Ammonium Surfactants against Streptococcal, Chlamydial, and Gonococcal Infective Agents

Ângela S. Inácio,[a] Alexandra Nunes,[b] Catarina Milho,[c]* Luís Jaime Mota,[c]* Maria J. Borrego,[b] João P. Gomes,[b] Winchil L. C. Vaz,[d] Otília V. Vieira[d]

CNC—Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal[a]; Department of Infectious Diseases, National Institute of Health, Lisbon, Portugal[b]; Instituto de Tecnologia Química e Biológica António Xavier, Universidade NOVA de Lisboa, Oeiras, Portugal[c]; CEDOC, NOVA Medical School, Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, Lisbon, Portugal[d]

Quaternary ammonium compounds (QAC) are widely used, cheap, and chemically stable disinfectants and topical antiseptics with wide-spectrum antimicrobial activities. Within this group of compounds, we recently showed that there are significant differences between the pharmacodynamics of *n*-alkyl quaternary ammonium surfactants (QAS) with a short (C_{12}) alkyl chain when *in vitro* toxicities toward bacterial and mammalian epithelial cells are compared. These differences result in an attractive therapeutic window that justifies studying short-chain QAS as prophylactics for sexually transmitted infections (STI) and perinatal vertically transmitted urogenital infections (UGI). We have evaluated the antimicrobial activities of short-chain (C_{12}) *n*-alkyl QAS against several STI and UGI pathogens as well as against commensal *Lactobacillus* species. Inhibition of infection of HeLa cells by *Neisseria gonorrhoeae* and *Chlamydia trachomatis* was studied at concentrations that were not toxic to the HeLa cells. We show that the pathogenic bacteria are much more susceptible to QAS toxic effects than the commensal vaginal flora and that QAS significantly attenuate the infectivity of *N. gonorrhoeae* and *C. trachomatis* without affecting the viability of epithelial cells of the vaginal mucosa. *N*-Dodecylpyridinium bromide (C_{12}PB) was found to be the most effective QAS. Our results strongly suggest that short-chain (C_{12}) *n*-alkyl pyridinium bromides and structurally similar compounds are promising microbicide candidates for topical application in the prophylaxis of STI and perinatal vertical transmission of UGI.

Despite advances in modern medical care, sexually transmitted infections (STI) and perinatal urogenital infections (UGI) are major global causes of illness, infertility, and death (1). If left untreated, curable STI are known to facilitate HIV infection and transmission (2) and are also intimately associated with congenital and perinatal infections in neonates (3). In recent years, the significant increase in multidrug-resistant pathogens, together with the lack of effective vaccines, makes the prevention of STI a challenge. Currently available options for prevention of STI are essentially restricted to condoms (4), but limited economic resources and gender inequality may narrow women's ability to negotiate consistent condom use with their sexual partners. Safe and effective topical microbicides, such as vaginal gels, represent one of the most promising prevention strategies. Perinatal vertically transmitted UGI are also canonically prevented by timely screening and antibiotic treatments of pregnant women, but the enforceability of these procedures is very dependent upon the socioeconomic context. A one-time use of topical microbicides may offer a prophylactic solution for intrapartum transmission of UGI from the mother to the neonate (5), particularly in those cases in which canonical antibiotic treatments are either not available or not adequately carried out. The microbicidal activity of quaternary ammonium compounds (QAC) makes these compounds attractive and cheap prophylactic alternatives (6).

All surfactants are toxic to all cells at concentrations that cause membrane dissolution, but selective toxicity at lower concentrations to bacterial cells, as opposed to mammalian epithelial cells, is observed only for quaternary ammonium surfactants (QAS), a subfamily of the QAC (7, 8). We have recently reported detailed studies on the mechanisms of QAS toxicity to mammalian (9) and bacterial (8) cells at concentrations at which cell membrane dissolution does not occur. Our results indicated that while QAS toxicity to mammalian cells was primarily due to surfactant-induced mitochondrial dysfunction (9), the toxic action on bacteria was considerably more complex (8), involving impairment of bacterial energetics and cell division at low concentrations and membrane permeabilization and electron transport inhibition at slightly higher doses. From the perspective of concentration dependence of QAS toxicity, the differences between mammalian and bacterial cells is more pronounced for the short-chain QAS, contrary to what is frequently stated in the literature (10). QAS having *n*-alkyl groups with 10 to 12 carbons discriminate better between the host cells and the pathogens than do their respective homologues with 14 to 16 carbons (7, 8) and offer a sufficiently large therapeutic window to merit research on their use as topical vaginal antiseptics.

Here, we have expanded our studies on (C_{12}) *n*-alkyl QAS an-

Received 20 January 2016 **Returned for modification** 20 February 2016
Accepted 9 March 2016

Accepted manuscript posted online 14 March 2016

Citation Inácio AS, Nunes A, Milho C, Mota LJ, Borrego MJ, Gomes JP, Vaz WLC, Vieira OV. 2016. *In vitro* activity of quaternary ammonium surfactants against streptococcal, chlamydial, and gonococcal infective agents. Antimicrob Agents Chemother 60:3323–3332. doi:10.1128/AAC.00166-16.

Address correspondence to Otília V. Vieira, otilia.vieira@nms.unl.pt.

* Present address: Catarina Milho, Centre for Biological Engineering, University of Minho, Campus de Gualtar, Braga, Portugal; Luís Jaime Mota, UCIBIO-REQUIMTE, Faculdade de Ciências e Tecnologia, Universidade NOVA de Lisboa, Caparica, Portugal.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.00166-16.

TABLE 1 Bacterial and protozoan strains used in this study

Species and strain ID	Type	Source of isolation	Clinical information	Age (yr)	Gender	Country of origin or ATCC ID	Antibiotic(s) to which organism is resistant
Lactobacillus acidophilus							
Scav						ATCC 4356	
Lactobacillus jensenii							
62G						ATCC 25258	
Streptococcus agalactiae							
681	II-2 ST-109	Cerebrospinal fluid	Meningitis	<12	F	Angola	Erythromycin
5234	III-2 ST-174	Cerebrospinal fluid	Meningitis	<12	F	Angola	
277/10	V	Vaginal fluid		46	F	Portugal	Erythromycin, clindamycin
354/10	II	Vaginal fluid		39	F	Portugal	Erythromycin, clindamycin
1078/09	IV ST-28 CC19	Vaginal fluid		46	F	Portugal	Erythromycin, clindamycin
1109/08	IV ST-291 CC17	Vaginal-rectal exudate		27	F	Portugal	
NEM316	III-3 ST-23	Cerebrospinal fluid	Meningitis	<1		ATCC 12403	
2603V/R	V ST-110 CC19					ATCC BAA-611	
Neisseria gonorrhoeae							
PT07-15	ST-878	Synovial fluid	Arthritis	40	F	Portugal	
PT07-54	ST-3618	Pelvic biopsy	Pelvic mass	33	F	Portugal	Ciprofloxacin
PT09-15	ST-2	Conjunctival exudate	Conjunctivitis	<1	M	Portugal	
PT09-78	ST-1034	Pharynx exudate	Pharyngitis	58	M	Portugal	
PT10-12	ST-2992	Rectal exudate	Proctitis	20	M	Portugal	
PT12-100	ST-8345	Urethral exudate	Urethritis	41	M	Portugal	Ciprofloxacin
PT13-15	ST-2400	Conjunctival exudate	Conjunctivitis	<1	F	Portugal	Ciprofloxacin
PT13-105	ST-10063	Urethral exudate	Urethritis	24	M	Portugal	Ciprofloxacin
Chlamydia trachomatis							
LGV II, strain 434		LGV Bubo				ATCC VR-902B	
Trichomonas vaginalis							
162212		Vaginal fluid		32	F	Portugal	
166391		Vaginal fluid		45	F	Portugal	

timicrobial properties and examined their activity against several STI and UGI pathogens as well as against commensal *Lactobacillus* species. Three analogous *n*-alkyl-QAS families, pyridinium bromide (PB), benzyl-dimethylammonium bromide (BZK), and trimethylammonium bromide (TAB), with the same (C_{12}) *n*-alkyl chain were compared. The reasoning that led to the choice of these analogues has been discussed in previous work (7–9, 11). We show that the studied pathogenic bacteria are much more susceptible to QAS toxic effects than the commensal vaginal bacterial flora and vaginal mucosa cells. C_{12}PB was found to be the most effective among the QAS tested. More importantly, besides reducing the capacity for primary infection (first-round infectivity), QAS also significantly attenuated the second-generation infectivity of *Chlamydia trachomatis* and reduced the viability of the gonococci that were capable of infecting HeLa cells.

MATERIALS AND METHODS

Reagents. QAS of the highest commercially available purity, *N*-dodecylpyridinium bromide (C_{12}PB), *N*-dodecyltrimethylammonium bromide (C_{12}TAB), and *N*-dodecyl-*N*-benzyl-*N*,*N*-dimethylammonium bromide (C_{12}BZK), were purchased from Sigma-Aldrich and used as received. All mammalian cell culture reagents and Hanks' balanced salt solution (HBSS) were purchased from Gibco Life Technologies S.A, agar-agar was obtained from Merck, Todd-Hewitt broth was from Difco, and the *Trichomonas* medium no. 2, Lactobacilli MRS agar plates, and Lactobacilli MRS broth were from Oxoid. Chocolate agar PolyViteX and Columbia agar plus 5% sheep blood agar were purchased from bioMérieux. Anti-*Neisseria gonorrhoeae* conjugated with fluorescein isothiocyanate (FITC) (ab20815) and anti-MOMP (major outer membrane protein of *C. trachomatis* strain L2) antibodies were obtained from Abcam, and antitubulin (clone B-5-1-2) was from Sigma-Aldrich. Secondary fluorescent antibodies were from Molecular Probes Invitrogen Corporation or Jackson ImmunoResearch Laboratories. All other chemicals used were from Sigma-Aldrich.

Microorganism strains, growth conditions, and antimicrobial susceptibility. A total of 21 microorganisms (listed in Table 1) were examined. All isolates belong to the stock culture collection of the STI reference laboratory from the Portuguese National Institute of Health, unless stated otherwise. Before each experiment, frozen stocks of all strains were subcultured at least once to check strain viability, and inocula were prepared accordingly. Briefly, for *Trichomonas vaginalis*, each frozen stock was first centrifuged at 3,000 rpm for 5 min at 24°C before being cultured in fresh sterile medium at 35°C, and inocula of 1.275×10^6 cells/ml were prepared directly from each 48-h subculture. For the fastidious bacteria *Neisseria gonorrhoeae*, *Streptococcus agalactiae*, and *Lactobacillus* spp., each inocu-

TABLE 2 MICs and relative antimicrobial efficacy (MIC/CMC)[a]

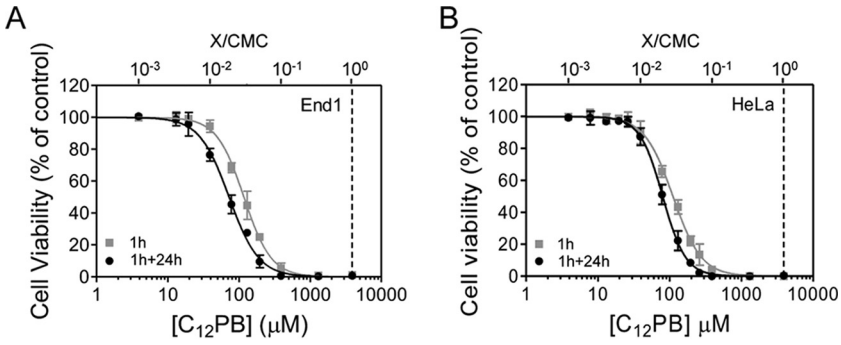
Organism and strain ID	C_{12}TAB		C_{12}BZK		C_{12}PB	
	MIC (μM)	MIC/CMC	MIC (μM)	MIC/CMC	MIC (μM)	MIC/CMC
Lactobacillus acidophilus						
Scav (ATCC 4356)	50	0.014	25	0.015	50	0.013
Lactobacillus jensenii						
62G (ATCC 25258)	100	0.028	100	0.059	100	0.026
Streptococcus agalactiae						
2603V/R (V, ST-110, CC19) (ATCC BAA-611)	25	0.007	6.25	0.004	12.5	0.003
NEM316 (III-3, ST-23) (ATCC 12403)	50	0.014	6.25	0.004	25	0.006
277/10 (V)	12.5	0.003	3.13	0.002	6.25	0.002
354/10 (II)	25	0.007	6.25	0.004	12.5	0.003
1078/09 (IV, ST-28, CC19)	25	0.007	6.25	0.004	12.5	0.003
1109/08 (IV, ST-291, CC17)	25	0.007	3.13	0.002	6.25	0.002
681/04 (II-2, ST-109) (Angola)	25	0.007	3.13	0.002	12.5	0.003
5234/05 (III-2, ST-174) (Angola)	25	0.007	3.13	0.002	6.25	0.002
Neisseria gonorrhoeae						
PT07-15	25	0.007	6.25	0.004	12.5	0.003
PT07-54	25	0.007	12.5	0.007	12.5	0.003
PT09-15	25	0.007	6.25	0.004	12.5	0.003
PT09-78	25	0.007	6.25	0.004	12.5	0.003
PT10-12	50	0.014	25	0.015	25	0.006
PT12-100	25	0.007	6.25	0.004	6.25	0.002
PT13-15	25	0.007	12.5	0.007	12.5	0.003
PT13-105	25	0.007	12.5	0.007	12.5	0.003
Trichomonas vaginalis						
162212	400	0.114	200	0.118	200	0.051
166391	400	0.114	200	0.118	200	0.051

[a] MICs were determined according to the established procedures recommended for each strain. The critical micelle concentration (CMC) values for the QAS used have been previously reported by us (11) and are as follows: C_{12}TAB, 3.5×10^{-3} M; C_{12}BK, 1.7×10^{-3} M; C_{12}PB, 3.9×10^{-3} M.

lum was prepared by making 0.5 McFarland saline–phosphate-buffered saline (PBS) suspensions (~1.5×10^8 CFU/ml) of isolated colonies selected from 24- to 48-h plates of chocolate agar, 5% sheep-blood agar, or MRS agar, respectively, incubated at 35 to 36°C in 5% CO_2. The MIC for each QAS assayed was determined by the broth microdilution method (12, 13), using a 1:20 dilution of the prepared inoculum for all microorganisms, except for *T. vaginalis*, for which a 1:10 dilution was used. Twofold serial dilutions of concentrated stock QAS solutions (0.4 to 1.6 mM) were prepared in the required medium into 96-well plates. A control without QAS was also prepared. All cultures were incubated for 18 to 48 h in an orbital shaker (200 rpm) at 35 to 37°C with 5% CO_2. Purity check and colony or viable cell counts of the inoculum suspensions were also evaluated in order to ensure that the final inoculum density closely approximates the intended number. The MIC was determined as the lowest QAS concentration at which no visible growth was observed. Optical densities were measured at 600 nm, except for *T. vaginalis*, for which microscopic observation was performed. These assays were done in triplicate.

Cell culture and MTT assay. HeLa 229 cells (ATCC CCL-2.1) were seeded in 96-well plates and grown in Dulbecco's modified Eagle medium (DMEM) with GlutaMAX, supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml streptomycin, at 37°C in 5% CO_2 for 24 h, when they reached confluence. The End1/E6E7 immortalized human endocervical epithelial cell line (14) was kindly provided by Raina Fichorova of the Brigham and Women's Hospital, Harvard Medical School (Boston, MA, USA). Cells were maintained in keratinocyte serum-free medium supplemented with 50 μg/ml bovine pituitary extract, 0.1 ng/ml human transferrin, and $CaCl_2$ to a final concentration of 0.4 mM. Cells were seeded in 96-well plates and grown until confluence (~24 h) at 37°C in 5% CO_2. The cells were then incubated with increasing QAS concentrations for 1 h. Stock solutions of surfactants were prepared in Opti-MEM cell culture medium, without serum and antibiotics, as multiples of the respective critical micelle concentration (CMC) (11). At the end of the incubation, QAS-containing medium was replaced by fresh complete culture medium without phenol red. Cell viability was assessed immediately or 24 h after exposure to QAS by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (15). The samples were quantified colorimetrically at 570 nm (background wavelength correction at 620 nm) on a SpectraMax Plus384 microplate spectrophotometer (Molecular Devices Inc.). The background absorbance (culture medium plus MTT without cells) was subtracted from the absorbance of each sample, and data are shown as percentages of control. Dose-response toxicity curves were fitted using the Hill equation (16), through computer-assisted curve fitting (SigmaPlot 11.0). From these analyses, it was possible to calculate the 10% inhibitory concentration (IC_{10}; i.e., the concentration at which cell viability was 10% of that of the control), IC_{50}, and IC_{90} for each data set.

Infection of epithelial cells with *N. gonorrhoeae*. Gonococcal infection assays were performed by a standard method (17, 18), with slight modifications. HeLa cells were grown in glass coverslips or in 48-well plates until reaching confluence. After that, cells were washed and cell culture medium was replaced by Opti-MEM serum- and antibiotic-free culture medium. *N. gonorrhoeae* PT07-15 cells from a 24-h plate were resuspended in Opti-MEM to achieve a final multiplicity of infection (MOI) of 100. Bacteria were allowed to adhere for 1 h at 37°C in a CO_2 incubator, in the presence or absence of each of the QAS. Cells were then washed with PBS, followed by 3 h of incubation with DMEM supple-



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Inhibitory concentrations (IC) of QAS for epithelial cells

	IC (μM) after exposure time of			
	60 min		60 min + 24 h recovery	
QAS	End1	HeLa	End1	HeLa
C_{12}TAB				
IC_{10}	158.3	118.8	94.3	110.3
IC_{50}	304.7	256.9	202.3	197.5
IC_{90}	586.4	555.2	433.9	353.5
C_{12}BZK				
IC_{10}	29.1	17.5	14.3	15.8
IC_{50}	61.7	44.6	25.9	32.7
IC_{90}	130.8	113.8	46.9	67.6
C_{12}PB				
IC_{10}	44.5	40.7	25.3	35.6
IC_{50}	117.1	111.2	72.5	79.9
IC_{90}	308.3	304.2	207.9	179.5

FIG 1 Dose-dependent toxicity of QAS to nonpolarized epithelial cells. The effect of C_{12}PB on the viability of endocervical epithelial cells (End1) (A) and of cervical adenocarcinoma cells (HeLa) (B) was assessed by the MTT assay immediately or 24 h after the cells had been incubated with increasing C_{12}PB concentrations for 1 h. The C_{12}PB concentration is plotted on a logarithmic scale. Cell viability is expressed as a percentage of the viability of control cells. Data are presented as means ± standard deviations (SD) from at least 3 independent experiments, each done in triplicate. The Hill equation was fitted (lines) to the data. The CMC of C_{12}PB surfactant is represented by the black dashed line. (C) Ten percent inhibitory concentration (IC_{10}), 50% inhibitory concentration (IC_{50}), and 90% inhibitory concentration (IC_{90}), i.e., surfactant concentrations at which cell viability was, respectively, 10%, 50%, and 90% of that of the control for each time point, were calculated from the theoretical curves adjusted to the dose-response toxicity plots.

mented with 10% FBS at 37°C and 5% CO_2, to allow the internalization of the attached bacteria. For fluorescence cell imaging assays, cells were fixed with ice-cold methanol for 10 min and immunolabeled with an anti-*N. gonorrhoeae* FITC antibody. Images were taken in a Carl Zeiss laser scanning confocal microscope LSM 510. The percentage of infected cells and the number of infecting particles per infected cell were determined and compared with the values for untreated controls. To assay for bacterial invasion and evaluate the viability of the gonococci that were capable of infecting host cells, cell monolayers were washed with PBS, followed by the addition of 100 μl of PBS supplemented with 0.5 mM EDTA to each well. Cells were scraped off and collected, vortexed vigorously for 1 min, serially diluted, spread on chocolate agar plates, and incubated for 48 h at 37°C in a CO_2 incubator. CFU were counted to determine the number of viable bacteria. Data are presented as CFU/10^5 epithelial cells.

Infection of epithelial cells by *C. trachomatis*. *C. trachomatis* L2/434/Bu (ATCC VR-902B) was propagated in HeLa cells using standard techniques (19). To test the effect of the QAS on *C. trachomatis in vitro* infection, HeLa cells seeded the day before on 24-well plates were equilibrated in HBSS and incubated at 37°C with *C. trachomatis* for 1 h at an MOI of 5, in the presence or absence of each of the QAS. The cells were then washed with culture medium containing 10 μg/ml gentamicin, but without penicillin or streptomycin, and incubated in this medium at 37°C. At 24 h postinfection, cells that had been seeded on glass coverslips were fixed with methanol for 5 min at −20°C and processed for immunofluorescence microscopy using monoclonal antibodies against the major outer membrane protein (MOMP), to enable the estimation of the number of infected cells and the volume of the vacuoles enclosing *C. trachomatis*. At 44 h postinfection, the number of infectious *C. trachomatis* progeny present was estimated after osmotic lysis of the infected cells with H_2O, followed by serial dilutions of the lysates in sucrose-phosphate-glutamate buffer that were used to infect HeLa cells seeded the day before on glass coverslips in 24-well plates. After 24 h of infection, the cells were fixed in methanol for 5 min at −20°C and immunolabeled as described above. Inclusions in 20 to 25 fields of view were counted in the appropriate dilutions (10 to 50 inclusions per field of view) using a Leica DMRA2 microscope with a 40× objective (numerical aperture [NA], 0.75). Inclusions per field of view were transformed into inclusion-forming units (IFU) per milliliter (19).

Morphometric analysis of chlamydial inclusions was carried out in confocal optical sections of cells labeled with an anti-MOMP *C. trachomatis* antibody. Image acquisitions were made in a confocal microscope with an EC-PlanNeofluor 40× oil immersion objective (NA, 1.30) using the Carl Zeiss laser scanning system LSM 510 software. Surface rendering was carried out in 5- to 12-μm z-stack images composed of 0.7-μm optical slices using Imaris (Bitplane AG). The volume of *C. trachomatis* inclusions was calculated from this volumetric reconstruction.

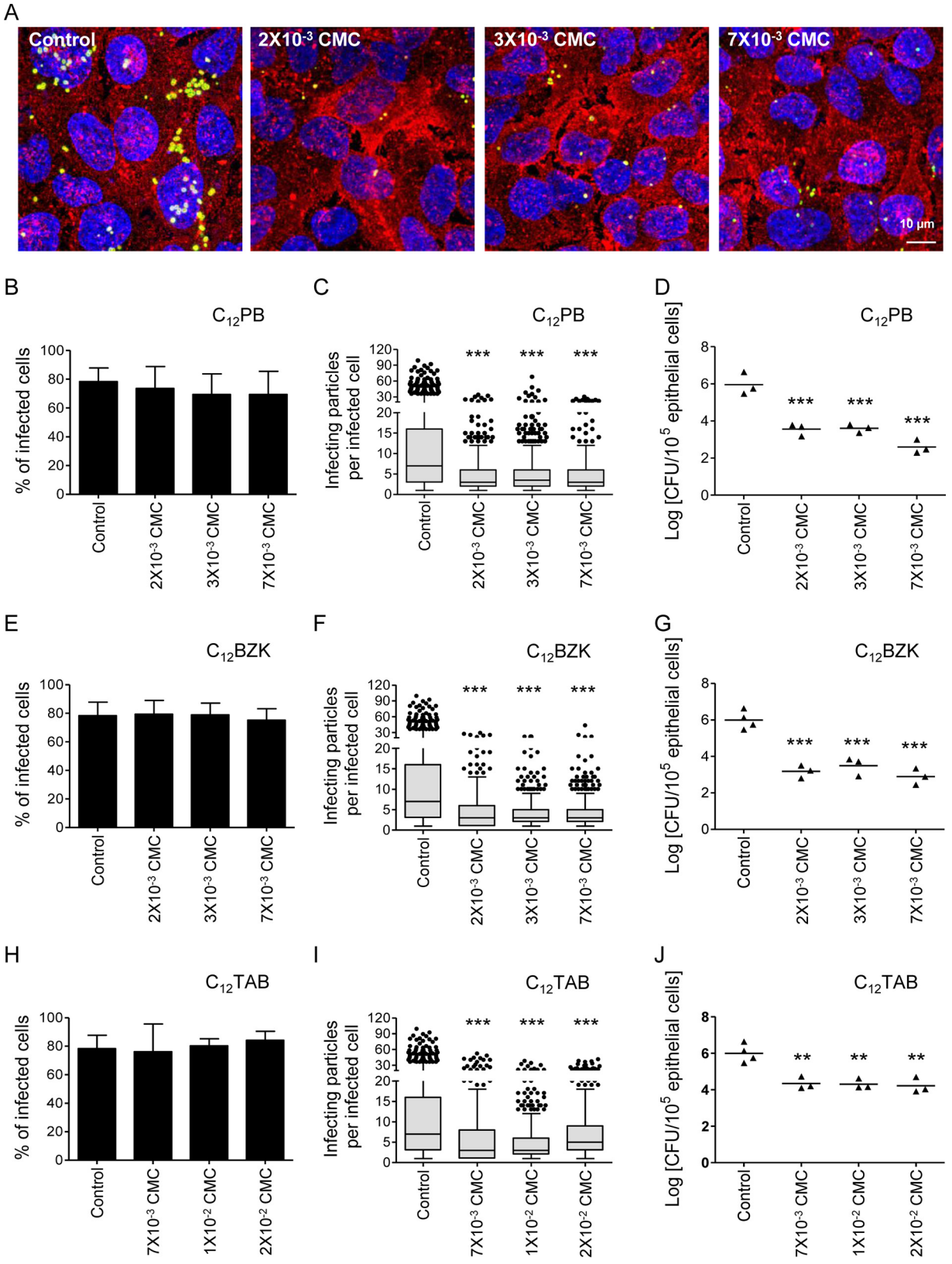

FIG 2 Effect of QAS on *N. gonorrhoeae* infectivity (concentrations are expressed as multiples of the QAS CMC. (A) Representative maximum fluorescence intensity projection images obtained by confocal microscopy of HeLa cells infected with *N. gonorrhoeae* (MOI, 100) for 1 h. C_{12}PB was added at the indicated concentrations during the infection. *N. gonorrhoeae* cells were visualized with an anti-*Neisseria* antibody (green), and the host cell cytoskeleton was visualized with an anti-α-actin antibody (red). HeLa cell nuclei were stained with Hoechst (blue). (B) The percentage of infected cells was determined from the immunofluorescence images, and data

RESULTS

Antimicrobial activity of short-chain *n*-alkyl QAS. In order to evaluate the antimicrobial activity of QAS against bacteria involved in STI and UGI, we determined the MICs of C_{12}PB, C_{12}BZK, and C_{12}TAB for several *Streptococcus agalactiae* (Gram-positive) and *N. gonorrhoeae* (Gram-negative) strains. The MICs for these compounds were also determined for some microorganisms of the commensal vaginal flora. In previous work (7, 8, 11), we have argued that the effective biocidal concentrations of QAS should be compared after normalization relative to their respective CMC. In what follows, we shall, therefore, compare the efficacy of different QAS using the CMC-normalized MIC (MIC/CMC) values. The rationale for reporting effective QAS concentrations as CMC-normalized values is explained in more detail in the supplemental text. CMC values for the QAS used have been previously reported by us (11). Using this definition, the relative antibacterial efficacy was C_{12}PB > C_{12}BZK > C_{12}TAB (Table 2). While the toxicity pattern was similar to that previously described for *Escherichia coli* (8), QAS were more effective against the pathogenic bacteria tested, regardless of being Gram-positive or Gram-negative. For the majority of the analyzed strains, the PB analogue was at least two times more effective in inhibiting bacterial growth than the BZK and TAB analogues. In contrast, the tested commensal *Lactobacillus* spp. were more resistant to QAS effects and no correlation between the antibacterial activity and QAS polar head structure was found (MIC/CMC ratios were the same or within a range of 1 dilution). The biocidal activity of QAS against the protozoan STI/UGI parasite *Trichomonas vaginalis* was also examined. In this case, the inhibitory effectiveness rank of the QAS examined was C_{12}PB > C_{12}BZK ≈ C_{12}TAB. The MIC/CMC values obtained, however, were two to four times higher for *T. vaginalis* than for the most resistant bacterium, *Lactobacillus jensenii*, suggesting that QAS would be less useful in the prevention of protozoan STI/UGI than of bacterial STI/UGI.

Toxicity of short-chain *n*-alkyl QAS to vaginal epithelial cells. As infections with *C. trachomatis* and *N. gonorrhoeae* are the most common bacterial STI and the most prevalent coinfections with HIV (1, 2), we studied the potential of QAS to inhibit these infections using an *in vitro* model. The primary target for bacterial and viral STI in women is the nonkeratinized squamous epithelium of the vagina and ectocervix, as well as the single-layer columnar epithelium of the endocervix. Thus, the End1 immortalized epithelial cell line from normal human endocervical tissue and HeLa epithelial cells originating from an adenosquamous carcinoma of the cervix were used as models of female lower genital tract epithelial cells. It has been shown that both cell lines support chlamydial growth (20, 21) as well as the attachment and intracellular uptake of piliated *N. gonorrhoeae* strains (17, 18).

In *in vitro* experiments that study the invasion of human host cells in culture by a pathogen, the concentration of the antiseptic agent used (in our case, QAS) must always be subtoxic to the host cells used, in our case End1 and HeLa cells. It was therefore important to determine the toxicity curves of QAS for End1 and HeLa cell cultures. This was done by evaluating the effects of QAS on the viability of End1 and HeLa cell lines using the MTT assay immediately or 24 h after exposure to increasing surfactant concentrations for 1 h (Fig. 1A to C). Consistent with our previous results (9, 11), exposure to QAS resulted in a persistent postexposure toxicity as can be seen by the low inhibitory concentrations 24 h postexposure compared to those observed immediately after the end of incubation. The QAS toxicity ranking for both cell lines was C_{12}PB ≈ C_{12}BZK > C_{12}TAB. Despite the fact that, contrary to HeLa cells, End1 cells can be fully polarized *in vitro*, resembling the morphological characteristics of epithelial cells of the vaginal mucosa (14), maturity of tight junctions is achieved only after 8 to 10 days in culture (22). Consequently, under our experimental conditions, End1 cells lack mature nonleaky tight junctions, justifying the similar IC_{50}s determined for End1 and HeLa cells. Since no significant differences in QAS susceptibility of these two cell lines were observed, further experiments were performed in HeLa cells as this cell line is easier to grow and maintain in culture. In previous work (8), we showed that the therapeutic indices (determined using a fully polarized columnar epithelial cell line) after 60 min of exposure to C_{12}PB were ~10 for a multidrug-resistant strain of *E. coli* and >20 for the most resistant strain of *N. gonorrhoeae* tested. Nonpolarized epithelial cells are more susceptible to QAS toxicity than fully polarized columnar epithelial cell lines, in which the tight junctions are well formed (11), so it is expected that the therapeutic indices are somewhat lower in the case of the nonpolarized cells. Nevertheless, they are useful practical models for infectivity. Therefore, in what follows, HeLa cells were used and the QAS concentrations were always lower than those that were toxic to HeLa cells.

Effect of QAS on *N. gonorrhoeae* infectivity. Invasion of HeLa cells by *N. gonorrhoeae* and its attenuation by C_{12}PB are shown in Fig. 2. Although C_{12}PB apparently had a very small effect on the percentage of infected cells at the concentrations tested (Fig. 2A and B), a significant reduction in gonococcal invasion was detected, as indicated by the lower numbers of internalized bacteria (Fig. 2A and C). In the absence of C_{12}PB (control) the average number of infecting particles per infected cell was 12.6, whereas in cells treated with C_{12}PB it was only 4.3 at a concentration of CMC/150 ($P < 0.001$). In order to evaluate the viability of the gonococci that were capable of infecting host cells, *N. gonorrhoeae*-infected HeLa cells were lysed 3 h postinfection and lysates were diluted and spread onto agar plates to estimate the number of recovered CFU per epithelial cell. As shown in Fig. 2D, a C_{12}PB concentration as low as CMC/500 reduced the number of CFU recovered by a factor of 1,000 and at CMC/150 by 10,000. The results obtained for C_{12}BZK were similar to those of C_{12}PB (Fig. 2E to G), whereas C_{12}TAB was less efficient in attenuating gonococcal infection,

are presented as means ± SD from 3 independent experiments. For each sample, at least 200 cells were analyzed. (C) Effects of C_{12}PB on the number of infecting particles per infected cell (box-and-whisker plot). For each sample, at least 30 infected cells were analyzed. Boxes indicate the median and the 25th to 75th percentiles for 3 independent experiments, while the whiskers represent the lowest and highest values (data range). Outliers are represented by the black circles. Kruskal-Wallis test (Dunn's posttest): ***, $P < 0.001$ (significantly different from control). (D) CFU recovered 3 h postinfection from 10^5 HeLa cells. Recoverable CFU/10^5 epithelial cell (*x* axis) values are logarithmically transformed. Each independent experiment is represented by a closed triangle. Lines indicate the means for 3 independent experiments. One-way analysis of variance (ANOVA) test (Bonferroni's posttest): **, $P < 0.01$; ***, $P < 0.001$ (both significantly different from control). (E to J) Effects of C_{12}BZK (E to G) and C_{12}TAB (H to J) on *N. gonorrhoeae* infectivity. Details are as indicated for C_{12}PB.

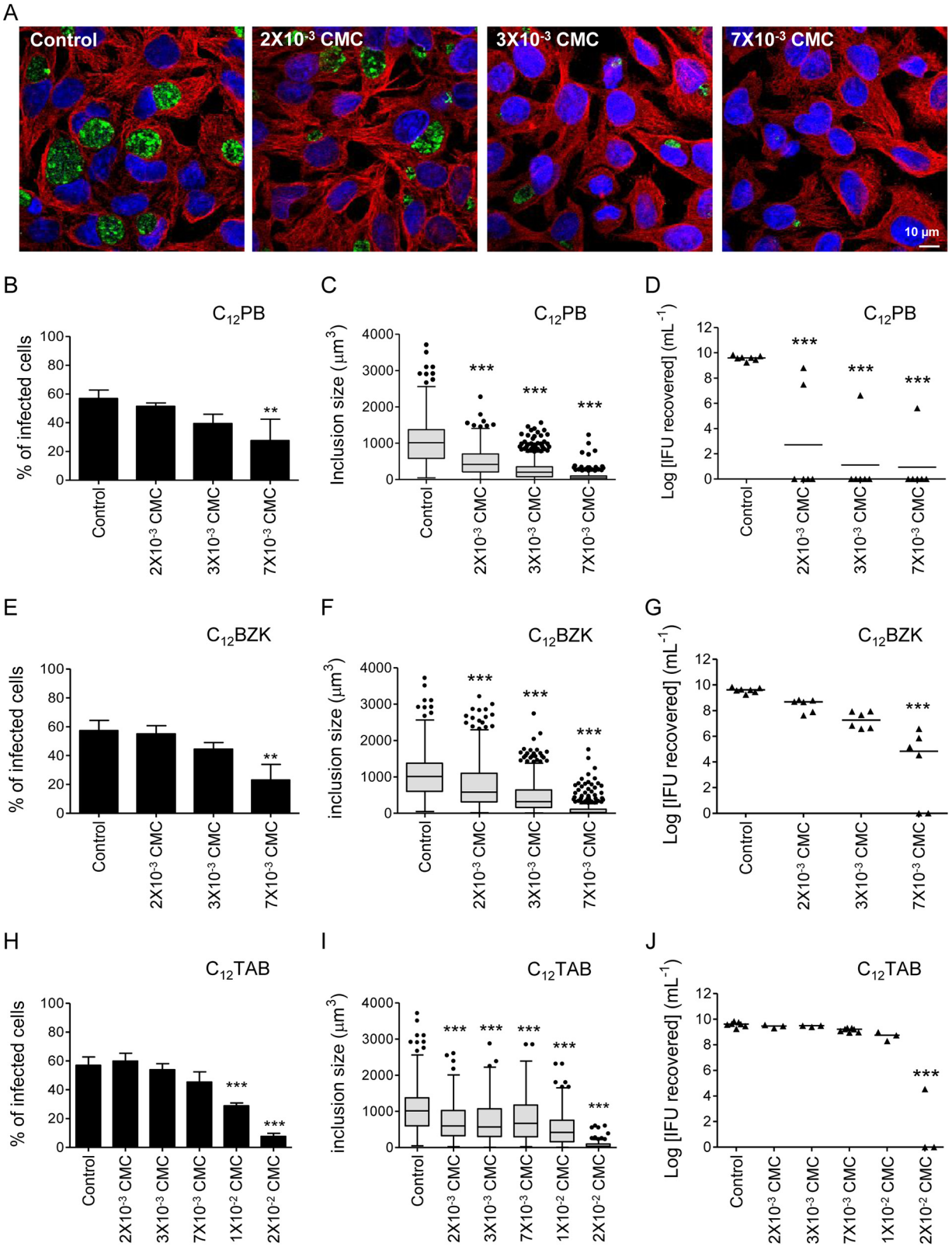

FIG 3 Effect of QAS on *C. trachomatis* infectivity (concentrations are expressed as multiples of the QAS CMC. (A) Representative fluorescence confocal images of HeLa cells infected by *C. trachomatis* (MOI, 5) for 24 h. C_{12}PB was added at the indicated concentrations for 1 h during the initial inoculation of the cells with *C. trachomatis*. After this initial incubation, C_{12}PB was washed out and the infection proceeded for the indicated times. Cells are costained with anti-MOMP (major outer membrane protein) of *C. trachomatis* (green) and β-tubulin (red) antibodies and 4′,6-diamidino-2-phenylindole (DAPI) (blue). (B) Immunofluorescence images were used to estimate the number of infected cells (i.e., cells showing inclusions) 24 h postinfection. For each sample, at least 450 cells were

higher concentrations being necessary to achieve comparable results (Fig. 2H to J).

Effect of QAS on *C. trachomatis* infectivity. *C. trachomatis* replicates exclusively within host cells in a membrane-bound vacuole, the inclusion, undergoing a developmental cycle whereby it alternates between the infectious, but nonreplicative, elementary bodies (EBs) and the noninfectious, but replicative, reticulate bodies (23). To understand if QAS interfere with the *Chlamydia* infectious cycle, HeLa cells were inoculated for 1 h with *C. trachomatis* strain L2/434, in the absence or presence of QAS concentrations that were previously determined to be subtoxic for the HeLa cells. The strain of *C. trachomatis* that we used is widely used in studies of *Chlamydia*-host cell interactions. Although infections caused by this serovar are less common than those caused by other strains and the pathogenicity profiles of the different serovars are distinct, they all share 98% identity at the nucleotide level. Hence, there is no reason to expect that the surfactants would behave differently toward distinct *C. trachomatis* serovars in a tissue culture model of infection.

Analysis of the number of infected cells 24 h postinfection showed that C_{12}PB inhibited the infection of HeLa cells by *C. trachomatis* in a dose-dependent manner, as seen by a decrease in the percentage of cells bearing visible inclusions, from 56.9% ± 5.9% under control conditions to 27.6% ± 14.9% at CMC/150 (Fig. 3A and B). Additionally, cultures that had been incubated with C_{12}PB resulted in inclusions with a much smaller size (Fig. 3A and C), strongly suggesting that C_{12}PB inhibits the intracellular growth of *C. trachomatis*. In fact, at CMC/150, inclusions are difficult to find due to their abnormally small size.

The effects of C_{12}PB on the formation of *C. trachomatis* infectious progeny (EBs) were also investigated. Cells infected in the absence or presence of QAS were lysed 44 h postinfection, and lysates were used to infect freshly seeded cells. Twenty-four hours after secondary infection, quantification of recovered IFUs was performed. As can be seen in Fig. 3D, C_{12}PB significantly decreased the formation of *C. trachomatis* infectious progeny, showing a substantial reduction in the recovered IFUs (in the worst case from six independent experiments, at least by a factor of 10 at CMC/500, while the mean value of the recovered IFUs showed a reduction of 7 orders of magnitude), a much stronger effect than that observed for the primary infection. At higher concentrations (i.e., CMC/300 and CMC/150), secondary infection was virtually abolished (for both concentrations in five of six independent experiments, no IFUs were recovered). In contrast, while the results obtained for C_{12}BZK regarding the percentage of infected cells were similar to those of C_{12}PB, C_{12}BZK was less effective in inhibiting the formation of *C. trachomatis* infectious progeny in the same concentration range (Fig. 3E to G). C_{12}TAB was also less effective in attenuating chlamydial infection, similar to what was observed for *N. gonorrhoeae* infectivity, and higher concentrations were necessary to achieve similar results (Fig. 3H to J). As discussed in some detail by us earlier (8), we believe the differences between the PB and the TAB/BZK derivatives to be a consequence of the delocalized positive charge in the polar head group of C_{12}PB that facilitates its translocation across the membranes of the host cells, exposing the invading infectious agents for an effectively longer time to this QAS.

DISCUSSION

The urgent need for woman-controlled, safe, and effective topical microbicides for the prevention of STI makes QAS-containing formulations an interesting option, since QAS have a well-known antimicrobial activity, combined with a low price and excellent chemical stability. Topical microbicides could also be a prophylactic solution for perinatal vertically transmitted UGI. Mother-to-child transmission of UGI can be prevented by screening pregnant women for these infections and providing antibiotic treatment to them before term or during childbirth. Yet, one major problem with these infections is their discontinuous nature: the mother can be infected at the time recommended for screening and no longer when going into labor or vice versa. In the first scenario, the canonical treatment and consequent protection will be provided, whereas in the second it will not. Additionally, when the canonical preterm protocols for pregnant women are not rigorously adhered to, a last-recourse prophylactic antiseptic treatment at term may be recommendable in order to avoid, or attenuate, the probability of vertical transmission. Potentially, the routine use of a microbicide gel immediately prior to term could dispense screening and unnecessary use of antibiotics while eliminating, or at least significantly attenuating, the risk of infection, but this will require further work *in vivo*. This aspect is of particular relevance in places where the standard procedures used in "developed" countries are either not available or not rigidly adhered to.

The results reported in the present paper show that the pathogenic bacteria analyzed were much more susceptible to QAS toxic effects than commensal *Lactobacillus* species. This difference in QAS susceptibility is of particular importance since lactobacilli are women's predominant members of the commensal vaginal flora (24), playing a central role in the maintenance of a low vaginal pH and contributing to avoid colonization of the urogenital tract by pathogenic bacteria (25, 26). An acidic vaginal pH has also been correlated with a decrease in HIV infection (27, 28). Recent studies employing cultivation-independent molecular approaches showed that the vaginal microbiome (VMB) is much more complex than previously appreciated and that conventional culture

analyzed. Data are presented as means ± SD for 6 independent experiments. One-way ANOVA test (Bonferroni's posttest): **, $P < 0.01$; ***, $P < 0.001$ (both significantly different from control). (C) Images obtained by combining a z series of 12 to 20 optical slices taken at 0.7-μm intervals were used to quantify the volume of inclusions 24 h postinfection (box-and-whisker plot). For each sample, at least 30 inclusions were analyzed. Boxes indicate medians and 25th to 75th percentiles for 4 independent experiments, while the whiskers represent the lowest and highest values (data range). Outliers are indicated by the black circles. Kruskal-Wallis test (Dunn's posttest): ***, $P < 0.001$, significantly different from control. (D) Effect of C_{12}PB treatment on the formation of infectious progeny of *C. trachomatis*. HeLa cells were infected by *C. trachomatis* L2/434 in the presence of the indicated concentrations of C_{12}PB, as described for panel A. After 44 h of infection, the cells were harvested, and the lysate was used to reinfect HeLa cells for 24 h. The number of inclusion-forming units (IFU) per milliliter of lysate was then determined after immunofluorescence microscopy of the infected cells, as detailed in Materials and Methods. IFU per milliliter (*x* axis) values are logarithmically transformed. Each independent experiment is represented by a closed triangle. Lines indicate the means for 6 independent experiments. One-way ANOVA test (Bonferroni's posttest): ***, $P < 0.001$, significantly different from control. (E to J) Effects of C_{12}BZK (E to G) and C_{12}TAB (H to J) on *C. trachomatis* infectivity. Details are as indicated for C_{12}PB.

techniques are limited, allowing the detection of only a small fraction of the microorganisms in the vaginal flora (29, 30). Therefore, detailed studies on the effects of QAS on the VMB, employing genetically controlled *ex vivo* models in combination with cultivation-independent methods (31, 32), should be performed in the future. This kind of approach will allow a more detailed analysis of the effects of QAS not only on healthy VMB but also on dysbiotic VMB collected from patients with bacterial vaginosis (32), the most common cause of vaginal infection in women of childbearing age, a main factor of increased susceptibility to infection by HIV and other STI (29). QAS, being neutral salts, will not alter vaginal pH directly, but indirect effects through alterations of the VMB cannot be excluded at this stage. Yet other important considerations are the effects of long-term exposure to QAS, their potential to act as inflammatory agents, and the likelihood of bacteria developing resistance (or coresistance to other antibiotics). While these aspects need to be assessed in future studies, the results reported in this paper suggest that short-chain QAS should be regarded as potentially valuable topical microbicides.

Of the QAS analyzed, C_{12}PB was found to be the most effective, which is in agreement with our previous results for *E. coli* (8). In addition, QAS were shown to attenuate *C. trachomatis* and *N. gonorrhoeae* infections at very low concentrations that do not affect the viability of nonpolarized host epithelial cells *in vitro*. More importantly, the decrease in pathogen infectivity is even greater when the secondary infectivity of these pathogens is considered. This suggests not only that a one-time application could reduce the risk of vertical transmission of infection but also that repeated topical application of C_{12}PB may have a curative potential for established infections by both *N. gonorrhoeae* and *C. trachomatis* without recourse to systemic antibiotic treatment.

ACKNOWLEDGMENTS

We thank Raina Fichorova (Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA) for the kind gift of the End1/E6E7 cell line.

We declare that we have no conflict of interest.

FUNDING INFORMATION

This work, including the efforts of Otilia V. Vieira, was funded by FCT (PTDC/BIA-BCM/112138/2009). This work, including the efforts of Otilia V. Vieira, was funded by FCT (HMSP-ICT/0024/2010). This work, including the efforts of Otilia V. Vieira, was funded by FCT (iNOVA4Health - UID/Multi/04462/2013).

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